Biomarkers and key pathways in atrial fibrillation associated with mitral valve disease identified by multi-omics study

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Background: Mitral valve disease (MVD)-associated atrial fibrillation (AF) is one of the most common arrhythmias with an increased risk of thromboembolic events. This study aimed to identify the molecular mechanisms and possible biomarkers for chronic AF in MVD by using multi-omics methods.

Methods: This prospective study enrolled patients with MVD (n=100) undergoing mitral valve replacement surgery. The patients were allocated into chronic AF and sinus rhythm (SR) groups. Plasma samples were collected preoperatively. Proteomics was performed with isobaric tags for relative and absolute quantitation (iTRAQ) to identify differential proteins (DPs) between the two groups. The selected DPs were then validated in a new cohort of patients by enzyme-linked immunosorbent assay (ELISA). A gas chromatography-mass spectrometer was used in the metabolomics study to identify differential metabolites (DMs). Bioinformatics analyses were performed to analyze the results.

Results: Among the 447 plasma proteins and 322 metabolites detected, 57 proteins and 55 metabolites, including apolipoprotein A-I (ApoA-I), apolipoprotein A-II (ApoA-II), LIM domain only protein 7 (LMO7), and vitronectin (VN) were differentially expressed between AF and SR patients. Bioinformatics analyses identified enriched pathways related to AF, including peroxisome proliferator-activated receptor alpha (PPARα), the renin angiotensin aldosterone system (RAAS), galactose, biosynthesis of unsaturated fatty acids, and linoleic acid metabolism.

Conclusions: Using integrated multi-omics technologies in MVD-associated AF patients, the present study, for the first time, revealed important signaling pathways, such as PPARα, as well as possible roles of other signaling pathways, including the RAAS and galactose metabolism to understand the molecular mechanism of MVD-associated AF. It also identified a large number of DPs and DMs. Some identified proteins and metabolites, such as ApoA-I, ApoA-II, LMO7, and VN, may be further developed as biomarkers for MVD-associated AF.

Keywords: Atrial fibrillation (AF); mitral valve disease (MVD); proteomics; metabolomics

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Introduction

Atrial fibrillation (AF) is one of the most common arrhythmias (1,2). It is known that electrical, contractile, and structural remodeling play important roles in the initiation and maintenance of AF (3). Patients with mitral valve disease (MVD) and AF are associated with an increased risk of thromboembolic events. A recent study (4) reported that the prevalence of persistent or permanent AF in MVD is as high as 87.1% in mitral regurgitation and 86.6% in aortic regurgitation, highlighting the importance of AF in MVD. Despite efforts to discover plasma biomarkers in MVD (5) and to identify the role of ion channels (6), the molecular mechanism of AF in MVD remains unclear. Recently, proteomics studies were performed to explore the mechanisms of AF (6-10). These studies identified the differential proteins (DPs) in the right and left atrial appendage of rheumatic MVD patients (6,7,9), or pathways including ketone body (8) or glutathione (10) metabolism and the peroxisome proliferator-activated receptor (PPAR) pathway (9,10), which are related to postoperative AF (8-10).

We recently performed multi-omics studies in MVD (5,6,9), congenital heart disease (11-14), and coronary artery disease (15). In human atrial tissues from patients with rheumatic MVD in persistent AF compared to sinus rhythm (SR) using a combined transcriptomic and proteomic approach, we found that intracellular chloride channels (6) as well as PPARα, β, and γ pathways play an important role in the development of AF (9). However, for persistent and chronic AF in MVD, no combined proteomics and metabolomics studies have been reported. In this study, we used multi-omics methods to investigate plasma samples from patients with MVD in AF or in SR undergoing heart valve replacement surgery to identify pathways and biomarkers associated with AF. We present the following article following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting checklist (available at http://dx.doi.org/10.21037/atm-20-3767).

Methods

Patient recruitment and sample collection

From January 2013 to December 2017, 100 consecutive patients with MVD who underwent mitral valve replacement surgery at TEDA International Cardiovascular Hospital were enrolled and grouped into either chronic AF (n=70) or SR (n=30). AF was diagnosed by history, physical examination, and a 12-lead electrocardiogram. For comparison, patients in SR were screened to ensure that they had no AF by direct questioning about symptoms suggestive of AF and by analysis of 12-lead electrocardiograms. Patients with infective endocarditis, sick sinus syndrome, familial paroxysmal AF (lone AF/familial AF), pulmonary heart disease, cardiomyopathy, hyperthyroidism, chronic kidney diseases, coronary artery disease, and those under the age of 18 years in both groups were also excluded to reduce prevalence-incidence bias. The demographic characteristics of patients are shown in Tables S1,S2.

This study was approved by the Ethics Committee of the TEDA International Cardiovascular Hospital {No. [2019]-0315-1}. Written informed consent was obtained from all patients enrolled in the study and conforms to the principles outlined in the Declaration of Helsinki (as revised in 2013). Blood samples were collected from all patients the day before surgery and were then centrifuged at 3,500 rpm for 10 min to separate the plasma from blood cells.

Proteomics analysis

A discovery-validation workflow was utilized in the proteomics analysis, and isobaric tags for relative and absolute quantitation (iTRAQ) were used for the proteomics study. As eight isobaric tags (m/z 113, 114, 115, 116, 117, 118, 119, and 121) were applied in the study, the plasma samples from 24 patients were divided into six groups (three AF groups and three SR groups). Each of these groups contained the plasma samples from four patients. Also, plasma samples from 12 AF or 12 SR patients were used as controls (Figure S1). Thus, the eight isobaric tags were concomitantly labeled in the plasma samples for the iTRAQ study (16).

In the validation phase, DPs identified by the above iTRAQ study were validated in plasma samples taken from a new cohort of patients using enzyme-linked immunosorbent assays (ELISA).

Metabolomics analysis

Plasma samples were thawed at room temperature and were then prepared for gas chromatography-mass spectrometry (GC-MS) analysis (Figure S1). ChromaTOF software (v4.34, LECO, St. Joseph, MI, USA) and the Fiehn database were used to process the raw data and to provide qualitative information about the metabolites. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database was used to generate significant pathways in the metabolomics dataset (17).

Creation of pathway maps

All of the omics data were used to compare proteins/enzymes with metabolic pathways. Quantitative changes of individual metabolites and proteins were calculated and graphed using the KEGG mapping tools. By combining proteomics and metabolomics data, we were able to identify the common pathways involved in AF.

Statistical analysis

SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad 6.0c software (GraphPad Software, La Jolla, CA, USA) were used for statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). Comparisons of statistical data were performed using either the independent samples t-test or Chi-square tests. A P value of <0.05 was considered statistically significant.

More detailed methods are presented in Appendix 1.

Results

Patient characteristics

The clinical characteristics of the study cohorts are summarized in Tables S1,S2. Patients in the AF group presented with larger left atrium-systolic diameters (LA-Ds), right ventricular-diastolic diameters (RV-Dd), right atrium-systolic diameters (RA-Ds), and lower left ventricular ejection fraction (LVEF) than those in the SR group in both iTRAQ (Table S1) and ELISA validation (Table S2).

Proteomics

A large number of unique peptides were detected by iTRAQ. By matching this data with the theoretical spectrum, 447 reliable proteins were identified. Under the condition of fold change (FC) >1.2 or <0.83 and P value ≤0.05, the number of differentially expressed proteins in the AF group was 57 (Table S3, *Figure 1A*), including 33 up-regulated and 24 down-regulated (https://cdn.amegroups.cn/static/public/atm-20-3767-1.xlsx).
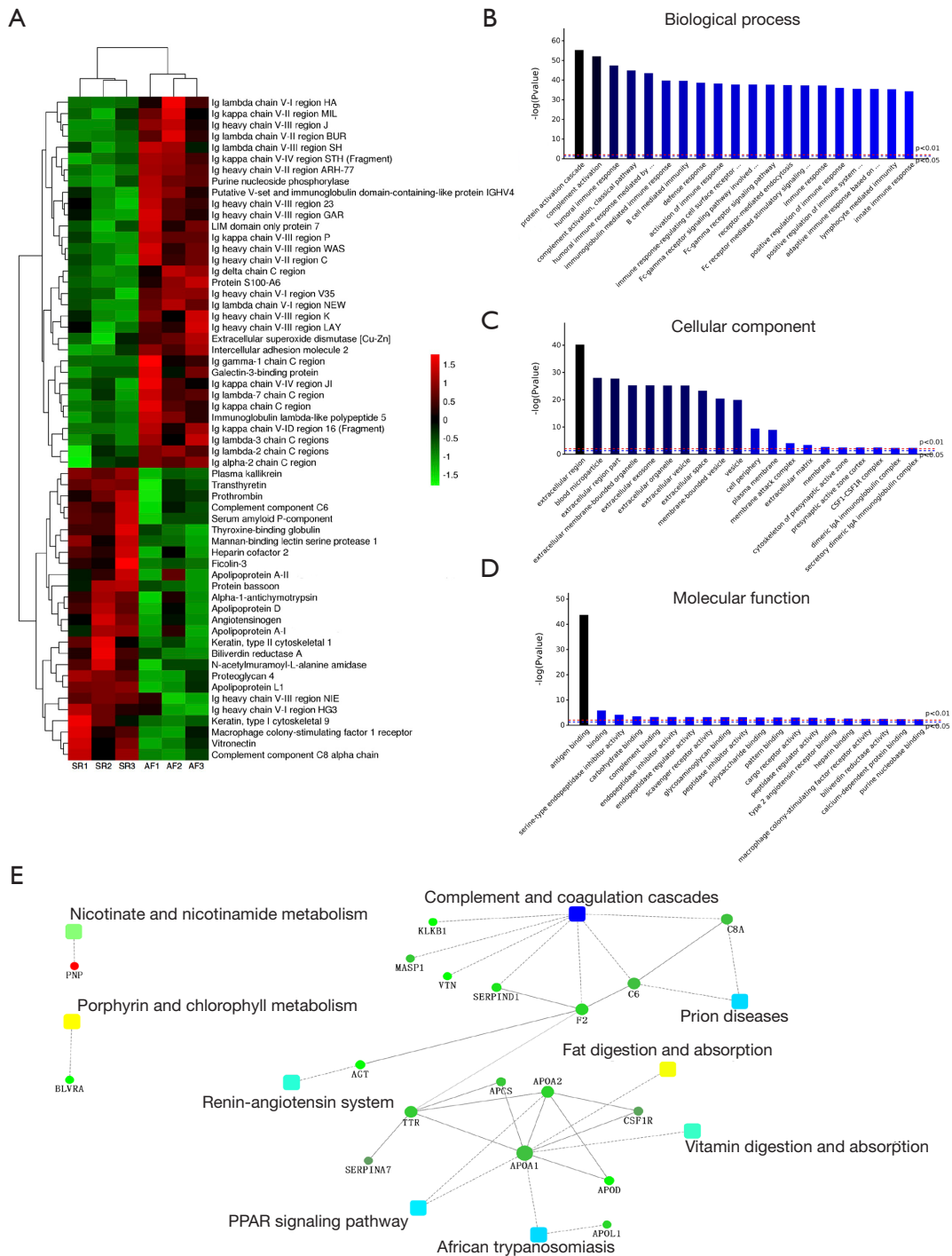
Bioinformatics analyses including Gene Ontology (GO) function and enrichment, KEGG pathway, and protein-protein interaction (PPI) were also performed (https://cdn.amegroups.cn/static/public/atm-20-3767-1.xlsx), and the results are shown in *Figure 1B,C,D,E*. Biological processes such as immune response (72.7%), defense response (74.5%), receptor-mediated endocytosis (50.9%), protein activation cascade (56.3%), and complement activation (52.7%) were enriched. Cellular component enrichment analysis revealed that most of the differentially expressed proteins were from the extracellular region (94.7%) and that binding (96.3%) was their main molecular function. Furthermore, KEGG pathway analysis demonstrated several pathways, such as the PPAR signaling pathway, renin-angiotensin system, complement and coagulation cascades, cell adhesion molecules, and adherent junction, were enriched. The PPI is shown in *Figure 1E*.

Protein validation by ELISA

Ten proteins were selected to validate the proteomics result by ELISA: apolipoprotein A-I (ApoA-I), apolipoprotein A-II (ApoA-II), LIM domain only protein 7 (LMO7), prothrombin fragment 1+2, vitronectin (VN), angiotensinogen, ficolin-3, serum amyloid P-component (SAP), macrophage colony-stimulating factor 1 receptor (CSF-1R), and purine nucleoside phosphorylase (PNP) (*Figure 2*). Six were successfully validated. Of these, the concentration of ApoA-I, angiotensinogen, CSF-1R, and VN 2 was lower in the AF group than those in the SR group, whereas the concentration of LMO7 and prothrombin fragment 1+2 was higher.

Metabolomics analysis in chronic AF

In the comparison of the plasma metabolite profile between 30 AF patients and 26 SR patients, 322 metabolites were discovered, including 55 differential metabolites (DMs) (Table S4) between AF and SR patients under the condition of variable importance in projection (VIP) >1 in the partial least-squares discriminant analysis (PLS-DA) model and P values <0.05 (https://cdn.amegroups.cn/static/public/atm-20-3767-2.xlsx). The heatmap of these metabolites is

Page 4 of 11

Li et al. Biomarkers/pathways in AF with MV disease

Figure 1 Analysis of proteomics in patients with AF compared to SR. (A) Unsupervised hierarchical clustering heatmap of proteins identified from the iTRAQ study (12 patients of AF and 12 patients of SR). The functional cluster analysis of DPs (left of heatmap) and the protein expression values (red: up-regulation, green: down-regulation) are demonstrated. (B,C,D) Classification of identified DPs based on their functional annotations using GO function and enrichment analysis: (B) biological process, (C) cell component, and (D) molecular function. (E) PPI network. Circular and square nodes represent the identified proteins and KEGG pathway terms, respectively. AF, atrial fibrillation; SR, sinus rhythm; iTRAQ, isobaric tags for relative and absolute quantitation; DPs, differential proteins; GO, Gene Ontology; PPI, protein-protein interaction; KEGG, Kyoto Encyclopedia of Genes and Genomes.
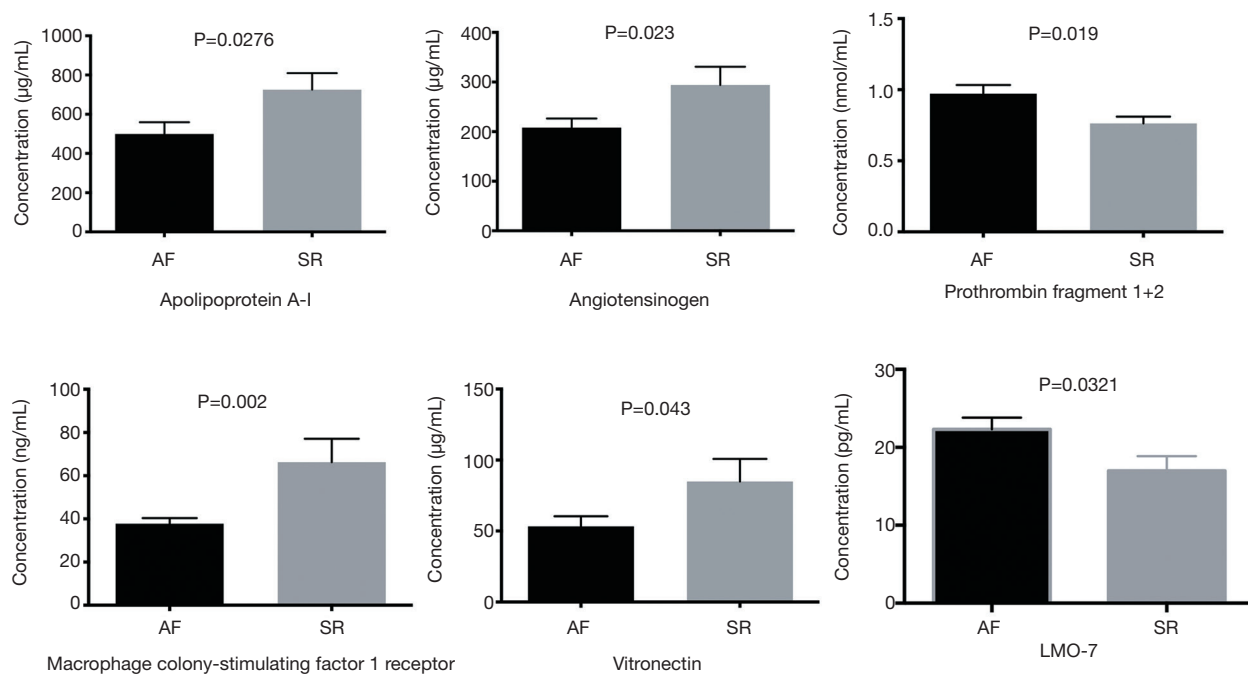
Figure 2 Validation of DPs identified from proteomics study. ELISA validation of DPs in AF patients (n=50) and SR patients (n=30). The figure shows that plasma levels of ApoA-I, angiotensinogen, CSF-1R, and VN were lower in AF patients compared to SR patients. Furthermore, the plasma level of LMO7 and prothrombin fragment 1+2 was higher in AF patients compared to SR patients. Data are shown as mean ± SEM. DPs, differential proteins; ELISA, enzyme-linked immunosorbent assay; AF, atrial fibrillation; SR, sinus rhythm; ApoA-I, apolipoprotein A-I; CSF-1R, colony-stimulating factor 1 receptor; VN, vitronectin; LMO7, LIM domain only protein 7; SEM, standard error of the mean.

displayed in *Figure 3*. The sucrose concentration, cellobiose, lactose, raffinose, and galactinol was elevated in AF, along with several unsaturated fatty acids (such as arachidonic acid, palmitic acid, and linoleic acid). Furthermore, bioinformatics analysis was performed, and metabolic pathways including galactose metabolism, biosynthesis of unsaturated fatty acids, linoleic acid metabolism, ATP-binding cassette (ABC) transporters, and starch and sucrose metabolism were enriched, demonstrating a close connection between AF and these pathways.

Combined proteomics and metabolomics analysis in chronic AF

We integrated the proteomics and metabolomics datasets to generate a global view of AF patients' plasma signatures. The global cross-correlation analysis of identified metabolites and proteins demonstrated the recapitulation of known metabolome-proteome reaction models. As a result, the connection between identified proteins, metabolites, and

several known enzymatic reaction models were established in *Figure 4*. On the other hand, KEGG mapping tools were used to integrate the above proteomics and metabolomics datasets into the human metabolic reference map (hsa01100) to provide a unified metabolic signature. As shown in *Figure 4*, each node represents a metabolite, the concentration of which was altered between the two groups. There were concurrent changes in both metabolites and proteins in the PPARα pathway and nucleotide metabolism. Notably, ELISA validation of PNP, an enzyme that regulates several purine metabolism reactions, revealed that the concentration of PNP was not significantly different between the two groups. Interestingly, proteomics and metabolomics analyses revealed that two proteins in the PPARα pathway, ApoA-I, and ApoA-II, were down-regulated in the AF patients.

Discussion

In the present combined study of proteomics and metabolomics on the plasma of patients with heart valve

Page 6 of 11

Li et al. Biomarkers/pathways in AF with MV disease
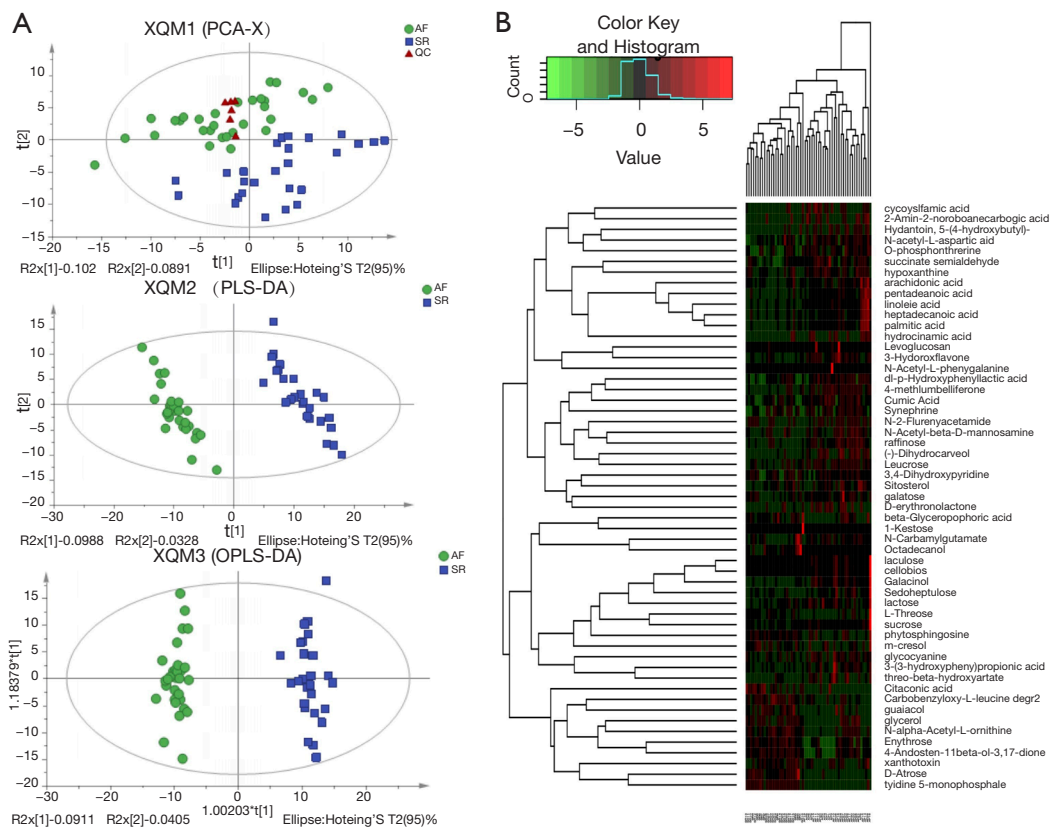
Figure 3 Metabolomics analysis in patients with AF compared to SR. Patients of AF (n=30) or SR (n=26) were analyzed by GC-MS. (A) PCA, PLS-DA, and OPLS-DA of the data in this study. PCA scores plot displays the variability among AF, SR, and QC samples; PLS-DA plot and OPLS-DA plot compare the variability between AF and SR samples. The quality of the models is described by the the proportion of variance in the data explained by the X and Y models and indicates goodness of fit (R2X and R2Y) values. (B) Heatmap of the metabolites in the GC-MS study. Fifty-five metabolites (rows) distinguished AF patients from SR patients (columns) under the condition of VIP >1 in the PLS-DA model and P values <0.05 in the Student's t-test. Unsupervised hierarchical clustering was performed using the R Programming Language (version 3.3.1). AF, atrial fibrillation; SR, sinus rhythm; GC-MS, gas chromatography-mass spectrometry; PCA, principal component analysis; PLS-DA, partial least-squares discriminant analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis; QC, quality control; VIP, variable importance in projection.

disease, we found that: (I) among the 447 proteins and 322 metabolites detected, 57 proteins and 55 metabolites were differentially expressed between AF patients and SR patients; (II) by using bioinformatics tools, several pathways, particularly the PPARα pathway, were enriched; (III) other enriched pathways included the metabolic pathway, renin angiotensin aldosterone system (RAAS), galactose metabolism, biosynthesis of unsaturated fatty acid, and linoleic acid metabolism; and (IV) several DPs and metabolites, such as ApoA-I, ApoA-II, and LMO7, may be further developed as biomarkers for AF in patients with MVD in order to monitor disease progress. The results

from our integrated omics study in the plasma of MVD patients with AF compared to MVD patients in SR have not been previously reported (see Table S5 for published studies). *Figure 5* is a schema that briefly demonstrates our findings. The findings of this study complement the results of other studies regarding AF (18,19). However, our study provides important insights into multi-omics changes in MVD-associated AF.

In this study, down-regulation of ApoA-I was observed in the AF patients. In comparison, the change of ApoA-II was only found in the proteomics study. ApoA-I and ApoA-II are the major components of high-density lipoprotein
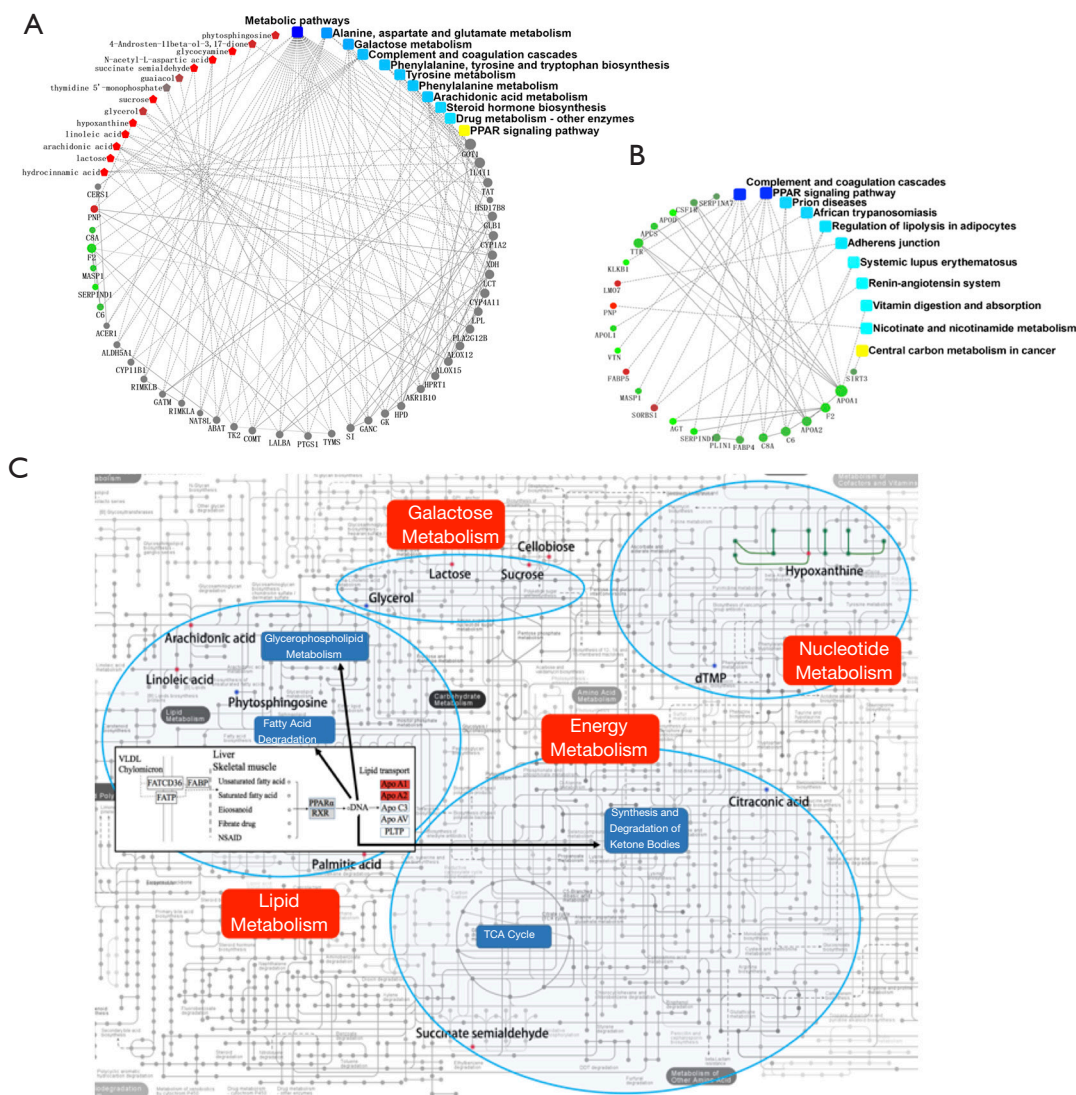
Figure 4 Integrated "-omics" analysis reveals global metabolic changes. (A) Cross-correlation analysis of identified proteins and metabolites from plasma samples. (B) PPI analysis of plasma proteomics findings. Note that PPAR pathway is now listed as one of the top pathways. Circular nodes represent the identified up- (red) and down-regulated (green) proteins, square nodes represent the KEGG pathway terms, and pentagonal nodes represent the identified metabolites. Solid or dashed grey lines between the molecules indicate the interaction with bigger and smaller confidence scores, respectively. (C) KEGG mapping tools were used to integrate the proteomics and metabolomics data sets into the human metabolic reference map (hsa01100). Red and blue represent up- and down-regulated, respectively. Lines represent reactions regulated by differentially expressed proteins and dots represent metabolites. Blue shading over lipid metabolism, galactose metabolism, nucleotide metabolism, and energy metabolism serve to highlight pathways that are highly represented. PPI, protein-protein interaction; PPAR, peroxisome proliferator-activated receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes.

cholesterol (HDL-C), and the genes encoding these two proteins are activated by PPARα via direct transcriptional control (20). PPAR is a major transcriptional regulator of fatty acid metabolism (21). The PPAR pathway regulates the expression of various genes, including proteins involved in fatty acid degradation, glycerophospholipid metabolism, synthesis and degradation of ketone bodies, etc. (16).

Moreover, PPARα is a member of the ligand-activated nuclear receptor superfamily (22), and the dimer binds

Page 8 of 11

Li et al. Biomarkers/pathways in AF with MV disease

Discovery of biomarkers and key pathways at the protein and metabolite levels in atrial fibrillation (AF) associated with mitral valve disease
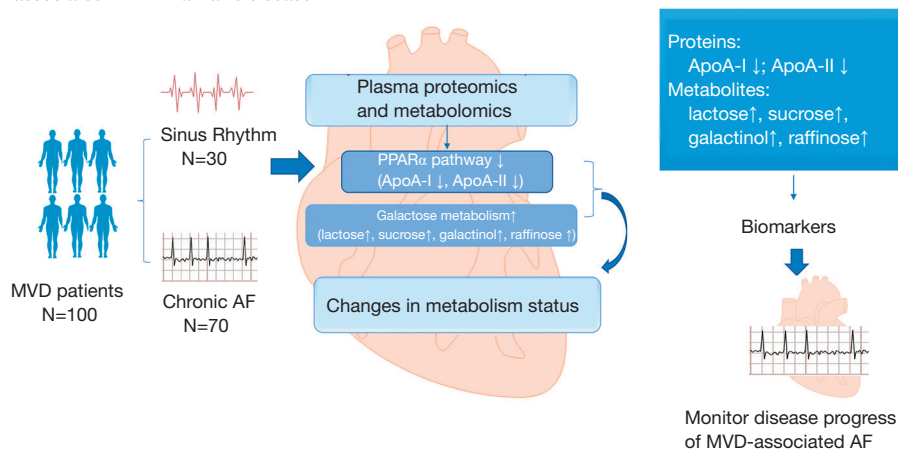
Figure 5 Schema of discovery of biomarkers and key pathways at the protein and metabolite levels in AF associated with MVD. The potential biomarkers and key pathways were investigated in the plasma of MVD patients with chronic AF by using multi-omics technologies. Dysregulation of the PPARα pathway (lower ApoA-I and ApoA-II) and upregulation of the galactose metabolism (higher lactose, sucrose, galactinol, and raffinose) indicates changes in the metabolism status in patients with MVD-associated AF. These proteins/metabolites may be developed as biomarkers to monitor the disease progress of MVD-associated AF. AF, atrial fibrillation; MVD, mitral valve disease; PPARα, peroxisome proliferators-activated receptor alpha; ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II.

to PPAR response element (PPRE) on the promoter region of target genes and activates their expression (23). PPARα also serves as a xenobiotic and lipid sensor to regulate fatty acid oxidation, fatty acid storage, lipid uptake, triacylglycerol (TAG) synthesis, glucose oxidation, lipoprotein synthesis, and inflammatory responses, and thus plays a key role in energy combustion (24). The down-regulation of PPARα is most likely attributable to the down-regulation of ApoA-I and ApoA-II. As a result of PPARα down-regulation, lipid metabolism is suppressed, and glucose oxidation is activated to force the heart to utilize more glucose instead of fatty acids. This notion is supported by the present study's metabolomic findings, as the concentration of plasma unsaturated fatty acids increased, indicating the accumulation of fatty acids in the circulation. Considering these findings, we hypothesize that AF is associated with the heart's altered metabolic status and PPARα plays an important role in this alternation (*Figure 6*). Our study suggests that AF may impair the heart's energy metabolism, and thus, the balance between lipid uptake and utilization is compromised. As a result, metabolic remodeling exists in the heart and is involved in the development of AF.

Several other proteins were also validated in the plasma of AF patients. Angiotensinogen is an essential component of the RAAS. It has recently been established that angiotensinogen and RAAS are closely associated with AF initiation and maintenance (25). It was reported that angiotensinogen gene polymorphisms and several other RAAS-related gene polymorphisms were associated with non-familial structural AF (26). The present study demonstrated that angiotensinogen concentration decreased in MVD patients with AF compared to those with SR. However, further exploration is required to understand the role of RAAS in AF better.

LMO7 is a member of the LIM domain-containing protein family and has been linked to the actin cytoskeleton organization. Interestingly, it has been reported that LMO7 is functionally involved in mammalian heart development, especially the development of the conduction system, and that knockdown of the LMO7 gene in zebrafish model may lead to arrhythmia, bradycardia, and delocalization of the heart (27). The present study showed up-regulation of LMO7 in the AF patients, suggesting that LMO7 plays a pathophysiological role in AF development. To our knowledge, this study is the first to reveal the alternation of the LMO7 protein in MVD-associated AF patients. These results suggest that the plasma level of LMO7 may be developed as a biomarker of AF.

VN is a glycoprotein found in serum and tissues, and
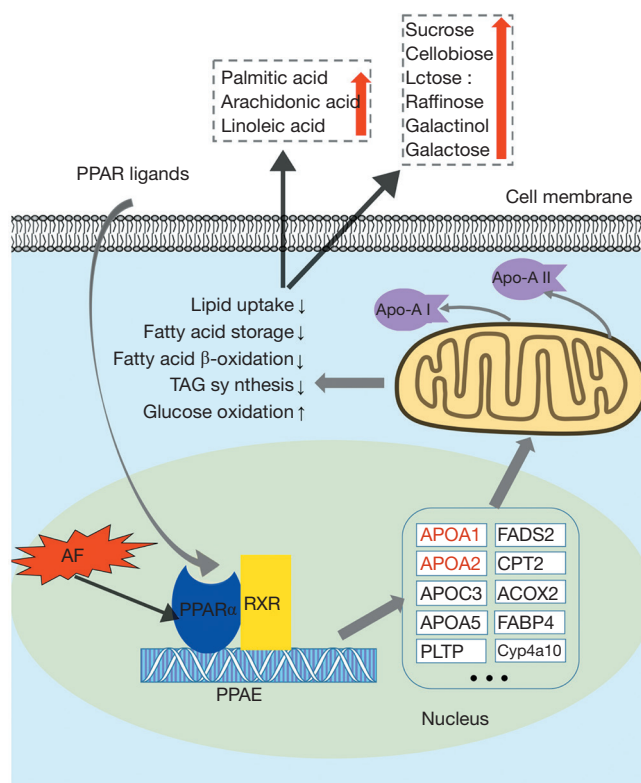
Figure 6 PPARα plays a crucial role in the lipid metabolism in the process of AF. PPARα heterodimerizes in the nucleus with RXR, and then the dimer binds to the PPRE on the promoter region of target genes to activate their expression. Down-regulation of PPARα in the process of AF inhibits the expression of PPARα targeted genes, resulting in down-regulation of lipid uptake, fatty acid storage, fatty acid β-oxidation and TAG synthesis, as well as up-regulation of glucose oxidation. Therefore, the concentration of circulating unsaturated fatty acids (palmitic acid, arachidonic acid, linoleic acid) is increased due to decreased consumption, while the concentration of carbohydrates (sucrose, cellobiose, lactose, *et al*.) is increased due to complexed compensate mechanisms to fulfill the energy demands of the heart. PPARα, peroxisome proliferators-activated receptor alpha; AF, atrial fibrillation; RXR, retinoid X receptors; PPRE, PPAR response element; TAG, tricarboxylic acid cycle.

serves as a cell-to-substrate adhesion molecule. Biological processes, including immune response, complement activation, and vascular endothelial growth factor receptor signaling pathway regulation, are also related to VN (28). VN inhibits the membrane-damaging effect of the terminal cytolytic complement pathway (28). In this study, we observed a down-regulation of VN concentration in AF patients, suggesting that the complement system is activated in MVD-associated AF, and that the plasma level of VN may be a potential biomarker of AF.

In this study, AF's development was inevitably correlated to some clinical variables, such as left atrium (LA) size (see Tables S1,S2). Nevertheless, our study aimed to identify biomarkers of AF. Therefore, we included all patients who satisfied the study's selection criteria and divided them into

two groups according to the heart rhythm (either sinus or AF). The differences in the protein and metabolite levels between the two groups were then investigated. The above DPs and metabolites were successfully identified as potential biomarkers for AF.

Study limitations

This study has some limitations that should be noted. Both proteomics and metabolomics studies reveal abundant unnamed proteins and metabolites. In order to perform bioinformatics analyses, the corresponding identification (ID) in certain databases is required. In fact, some proteins or metabolites failed to be recognized by these databases, and therefore the data were "invalid" and were unable to be

Page 10 of 11

Li et al. Biomarkers/pathways in AF with MV disease

analyzed at this stage. With future advances in technology, more information may be provided.

Conclusions

In conclusion, the present study, for the first time, identified a large number of DPs and metabolites by using multi-omics technologies in MVD-associated AF patients. Our study provides an integrative view with the combined analyses of proteomics and metabolomics, and reveals that the PPAR signaling pathway, and particularly PPARα pathway, plays an important role in AF. Other pathways involved in MVD-associated AF are the RAAS, galactose metabolism, biosynthesis of unsaturated fatty acids, and linoleic acid metabolism. Some identified DPs and metabolites, such as ApoA-I, ApoA-II, LMO7, and VN, may be further developed as biomarkers for MVD-associated AF patients to monitor disease progress.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-3767

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-3767

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-3767). All authors report that they have a patent pending for a diagnostic kit of multiple protein biomarkers and its application in atrial fibrillation. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of the TEDA International Cardiovascular Hospital {No. [2019]-0315-1} and conforms to the principles outlined in the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from each patient before being included in the study.

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Materials and methods

Patient recruitment and sample collection

One hundred patients with HVD who came to TEDA-International Cardiovascular Hospital, Tianjin, China, for surgery from January 2013 to December 2017 were enrolled in this study. All patients underwent mitral valve replacement. Patients with chronic AF or SR were assigned to the AF group (n=70) or SR group (n=30), respectively. A diagnosis of AF was made based on the medical history, physical examination, and a 12-lead electrocardiogram before and after admission for the valve surgery. For comparison, patients in SR were screened to ensure that they had no AF by direct questioning about symptoms suggestive of AF and by the analysis of all 12-lead electrocardiograms before and after admission for valve surgery. Patients with infective endocarditis, sick sinus syndrome, familial paroxysmal AF (lone AF/familial AF), pulmonary heart disease, cardiomyopathy, hyperthyroidism, and chronic kidney diseases were excluded from this study. Patients who were diagnosed with coronary atherosclerotic heart disease or under the age of 18 were also excluded. The demographic characteristics of all patients enrolled in this study are shown in Table S1.

The study was approved by the Ethics Committee of TEDA-international cardiovascular hospital and written informed consent was obtained from all patients enrolled in the study.

Blood samples were collected in EDTA tubes from patients the day before surgery and the plasma was then centrifuged at 3,500 rpm for 10 min to separate from blood cells.

Proteomics analysis

A discovery-validation workflow was used in the proteomics analysis. iTRAQ technology was used for the proteomics study. Because there are eight Isobaric tags (m/z 113, 114, 115, 116, 117, 118, 119 and 121), the plasma samples from 24 patients were divided into six groups (three AF group and three SR group), each of these groups contained the plasma from four patients. In addition, plasma samples from 12 AF or 12 SR patients were used as control groups, respectively (Figure S1). Thus, the eight Isobaric tags were concomitantly labeled in the plasma samples for the iTRAQ study.

In the validation phase, identified DPs were validated in a larger study population using ELISA in plasma samples.

Plasma high abundance protein depletion, protein extraction and quantification

The ProteoExtractTM Albumin/IgG Removal Kit (CALBIOCHEM, USA) was used to remove more than 80% of the top-two (serum albumin and IgG) high abundance proteins from plasma samples according to manufacturer's instructions. Total protein from sample was extracted by ProteoPrep® Total Extraction Sample Kit (Sigma-Aldrich, USA). The eluted samples were mixed in 5 volumes of cold acetone and stored at –20 ℃ for 1 hour, followed by centrifugation at 5,000 g for 30 minutes. The deposit was dried and dissolved in lysis solution for 1 hour and the solution was again centrifuged at 15,000 g for 15 minutes. The supernatant was collected and Bradford method was used to determine the concentration of the protein extracts.

Protein digestion and iTRAQ labeling

Protein (100 µg) from each sample was precipitated by cold acetone at –20 ℃ for 1 hour and then centrifuged at 12,000 rpm for 15 minutes, after which the deposit was collected and dissolved with 50 µL dissolution buffer. Proteins in the solution were reduced (4 µL reducing reagent, 60 ℃ for 1 hour), alkylated (2 µL cycsteine-blocking reagents, room temperature for 10 minutes), digested [50 µL sequencing-grade trypsin (50 ng/µL), 37 ℃ for 12 hours] and centrifuged at 12,000 rpm for 20 minutes. Following digestion with trypsin, iTRAQ regent (AB SCIEX, Framingham, MA, USA) was added and each sample was labeled with a unique iTRAQ tag (m/z 113, 114, 115, 116, 117, 118, 119 and 121).

2D-LC-MS/MS analysis

The peptides were dried in a vacuum freeze dryer and re-suspended in 100 µL of buffer A. Reversed-phase liquid chromatography (RPLC) was used to fractionate sample on the Agilent 1200 HPLC system (Agilent, USA) equipped with HPLC column (Narrow-Bore 2.1×150 mm, 5 µm, Agilent, USA). A total of 10 segments were collected at a rate of 0.3 mL/min using a nonlinear binary gradient starting with buffer A and transitioning to buffer B. The fractions were dried in a vacuum freeze dryer and re-dissolved in nano-RPLC buffer A. Each segment was loaded on a Eksigent nanoLC-UltraTM 2D System (AB SCIEX, USA) mounted with a C18 nanoLC trap column (100 µm

× 3 cm, C18, 3 µm, 150 Å) and separated over a 70 min acetonitrile gradient from 5% to 35% in 0.1% formic acid combined with a Triple TOF 5600 System (AB SCIEX, USA) fitted with a Nanospray III source (AB SCIEX, USA). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30PSI, nebulizer gas of 5PSI, and an interface heater temperature of 150 ℃.

iTRAQ data analysis and bioinformatics analysis

After data pre-processing, standardization, and quality control, the original data were screened for reliable peptides and proteins. ID of peptides from tandem mass spectrometry (MS) was achieved using the Protein Pilot Software v.5.0 (AB SCIEX, USA) against Uniprot_Homo Sapiens Database to match the theory data in order to obtain result of protein ID. The criteria for DPs were established as: (I) unique peptide ≥1, apart from invalid values and anti-library data; (II) FC >1.2 or <0.83; and (III) P value <0.05 (t-test for repeated data for over three times). Proteins fulfilling these criteria were considered to have significant difference in expression between the two groups. A list of DPs identified was implemented on the on-line software DAVID Bioinformatics Resources 6.8 (NIH) for GO (including biological process, molecular function and cellular component) function and enrichment analysis. KEGG pathway database (18) and STRING database were used to perform pathway analysis and PPI analysis.

ELISA

Following the success of the iTRAQ labeling and 2D-LC-MS/MS screening for changes in protein abundance, ELISA was employed to validate the results of the proteomics analysis. Selection of candidate proteins for ELISA validation was based on (I) expressed differentially in AF patient group and SR patient group; (II) potential functional or pathological significance in AF; (III) more than 1 peptide was identified by LC-MS/MS; and (IV) not been reported before in AF patients at the protein level.

In new group of patients, the further validation of candidate proteins was performed by using human ELISA kits (CUSABIO Biotech Co., Ltd., Wuhan, China). By the selection criteria mentioned above, there were two proteins, carbamoyl-phosphate synthetase I and complement factor H-related protein 2, that were further validated in the plasma. The methods followed the manufacturer's instructions.

Metabolomics analysis

Plasma samples were thawed at room temperature and 50 µL of the sample was mixed with 10 µL of internal standard (2-chloro-l-phenylalanine, 0.3 mg/mL, dissolved in methanol) and then vortexed for 10 s. Subsequently, 150 µL of ice-cold mixture of methanol and acetonitrile (2/1, vol/vol) were added and then vortexed for 1 min and ultrasonicated for 5 min. The sample was then placed at –20 ℃ for 10 min and centrifuged at 15,000 rpm for 10 min. The samples were then prepared for GC-MS analysis.

GC-MS analysis

The samples destined for GC/MS analysis were dried in a freeze concentration centrifugal dryer and mixed with 80 µL of 15 mg/mL methoxylamine hydrochloride in pyridine prior to being derivatized using BSTFA. After vortex (2 min) and incubation (37 ℃, 90min), 80 µL of BSTFA (with 1% TMCS) and 20 µL n-hexane were added to each sample and the mixture was again vortexed (2 min) and then derivatized (70 ℃, 60 min). A mixture of aliquots of all samples were used as QC controls and they were injected at regular intervals (every ten samples) throughout the analytical run. Samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977A MSD system (Agilent, CA, USA). Samples were injected (1 µL, splitless mode, 260 ℃) onto a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J & W Scientific, Folsom, CA, USA) and eluted (1.0 mL/min He, initial temperature 50 ℃, 15 ℃/min to 125 ℃, 5 ℃/min to 210 ℃, 10 ℃/min to 270 ℃, 20 ℃/min to 305 ℃, 5 min at 305 ℃), ionized (EI, 70 eV), and scanned from 50–450 m/z after a solvent delay of 5 min (source 230 ℃, quad at 150 ℃). Data was analyzed using ChromaTOF software (v4.34, LECO, St. Joseph, MI, USA).

Data processing and bioinformatics analysis

ChromaTOF software and Fiehn database were used to process the raw data and provide qualitative information about the metabolites. Data were normalized by total peak area of each sample using Excel 2007 and imported into SIMCA-P software (version 14.0, Umetrics, Umeå, Sweden) for multivariate statistical analysis. Principal component analysis (PCA) was used to visualize the general distribution of samples and the stability of the whole analysis process. PLS-DA and orthogonal PLS-DA (OPLS-DA) were

used to distinguish the differences of general metabolic profiling between different patient groups. To avoid model overfitting, a default of seven rounds of cross-validation in SIMCA-P software was applied. Moreover, OPLS-DA models were validated by a permutation analysis with 200 times. DMs were selected according to VIP values >1 in OPLS-DA model as well as P values <0.05 in Student's test (Figure S1). KEGG pathway database was used to generate significant pathways in metabolomics dataset.

Creation of pathway maps

All the omics data including transcriptomics, proteomics, and metabolomics data were used to compare proteins/enzymes with metabolic pathways. Quantitative changes of individual metabolites and proteins as compared with control samples were calculated and graphed by using the KEGG mapping tools. In particular, by combining proteomics with metabolomics data, we were able to identify the common pathways involved in AF.

Statistical analysis

SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad 6.0c software (GraphPad Software, La Jolla, CA, USA) were used for statistical analysis. Data are expressed as mean ± SEM. Comparisons of statistical data were performed using either the independent samples t-test or Chi-square tests. A P value of <0.05 was considered statistically significant.
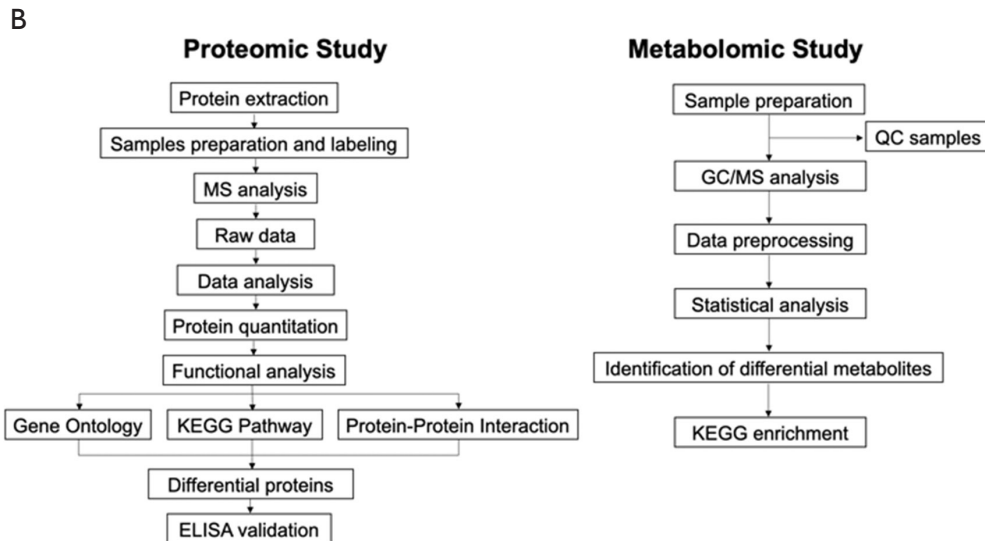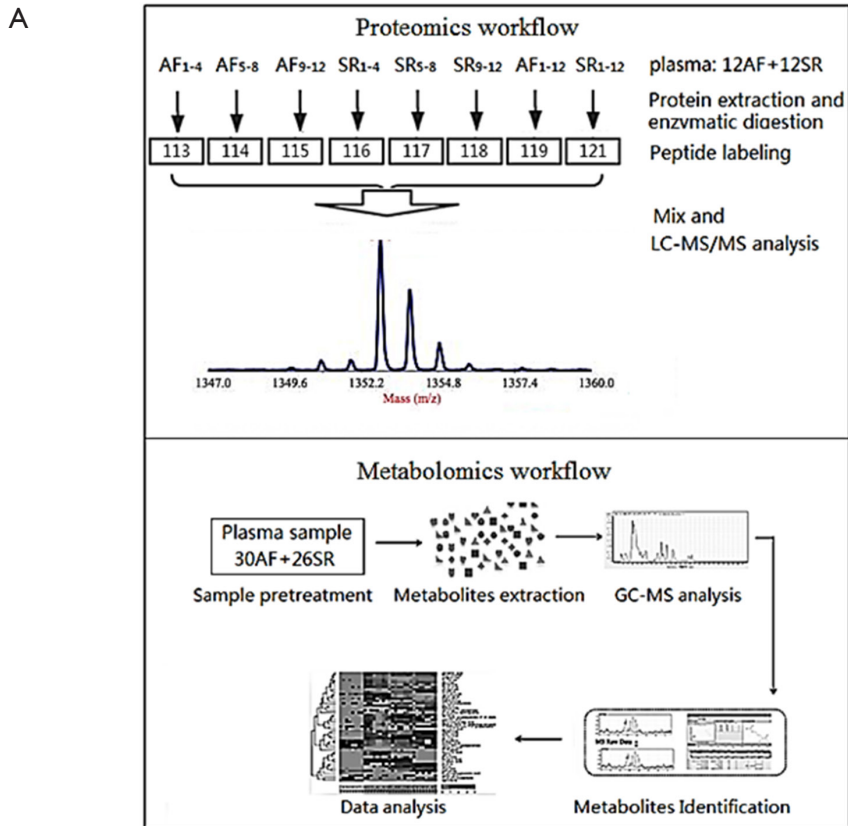
Figure S1 Proteomics (iTRAQ) and metabolomics workflow of plasma samples in the study. (A) Workflow of proteomics (iTRAQ) and metabolomics in plasma samples. AF, atrial fibrillation samples; SR, sinus rhythm samples; AF1–12, plasma from AF patients; SR1–12, plasma from SR patients. (B) Flowchart of the proteomics (iTRAQ) and metabolomics procedures. iTRAQ, isobaric tags for relative and absolute quantitation.

Table S1 Demographics of study population in the study of iTRAQ and GC-MS

Characteristics	AF (n=30)	SR (n=26)	P value
Male, n (%)	15 (50.0)	13 (50.0)	1.00
Age, year	50.2±8.3	49.5±11.5	0.79
BMI	23.9±2.6	23.0±3.3	0.28
Smoking, n (%)	14 (46.7)	9 (34.6)	0.36
Drinking, n (%)	9 (30.0)	6 (23.1)	0.56
Hypertension, n (%)	2 (6.7)	3 (11.5)	0.87
Hyperlipemia, n (%)	3 (10.0)	0 (0.0)	0.29
Diabetes mellitus, n (%)	2 (6.7)	1 (3.8)	1.00
LV-Dd, mm	49.9±7.6	51.3±9.6	0.55
LA-Ds$_{(A-P)}$, mm	60.1±9.8	50.5±8.5	0.000
RV-Dd$_{(L-R)}$, mm	36.3±5.9	32.0±4.8	0.005
RA-Ds$_{(L-R)}$, mm	41.3±8.9	33.5±4.9	0.000
LVEF	54.3±11.6	61.5±4.6	0.006
Pulmonary hypertension, n (%)	25 (83.3)	17 (65.4)	0.12
TCHOL, mmol/L	4.2±1.0	4.5±0.9	0.23
TG, mmol/L	1.5±1.1	1.1±0.4	0.048
HDL-C, mmol/L	1.0±0.2	1.2±0.3	0.03
LDL-C, mmol/L	2.6±0.8	2.9±0.8	0.27

All variables displayed as mean ± SEM. iTRAQ, isobaric tags for relative and absolute quantification; GC-MS, gas chromatography-mass spectrometer; AF, atrial fibrillation; SR, sinus rhythm; BMI, body mass index; LV-Dd, left ventricular-diastolic diameter; LA-Ds(A-P), left atrium-systolic diameter (anterior-posterior); RV-Dd(L-R), right ventricular-diastolic diameter (left-right); RA-Ds(L-R), right atrium-systolic diameter (left-right); LVEF, left ventricular ejection fraction; TCHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SEM, standard error of the mean.

Table S2 Demographics of study population in the study of ELISA

Characteristics	AF (n=50)	SR (n=30)	P value
Male, n (%)	14 (28.0)	14 (46.7)	0.09
Age, year	55.8±8.1	49.6±10.8	0.005
BMI	23.9±3.4	22.8±3.1	0.13
Smoking, n (%)	14 (28.0)	11 (36.7)	0.42
Drinking, n (%)	10 (20.0)	7 (23.3)	0.72
Hypertension, n (%)	4 (8.0)	2 (6.9)	1.00
Hyperlipemia, n (%)	2 (4.0)	0 (0.0)	0.71
Diabetes mellitus, n (%)	4 (8.0)	1 (3.3)	0.72
LV-Dd, mm	49.0±7.0	50.7±9.1	0.37
LA-Ds$_{(A-P)}$, mm	57.0±9.1	50.0±8.1	0.001
RV-Dd$_{(L-R)}$, mm	35.2±6.2	31.8±4.5	0.013
RA-Ds$_{(L-R)}$, mm	38.9±8.2	33.8±4.9	0.004
LVEF	58.7±5.2	61.9±4.5	0.009
Pulmonary hypertension, n (%)	37 (74.0)	19 (63.3)	0.31
TCHOL, mmol/L	4.3±1.0	4.4±0.9	0.66
TG, mmol/L	1.3±0.9	1.1±0.4	0.20
HDL-C, mmol/L	1.2±0.3	1.2±0.3	0.86
LDL-C, mmol/L	2.6±0.8	2.8±0.8	0.41

All variables displayed as mean ± SEM. ELISA, enzyme-linked immunosorbent assay; AF, atrial fibrillation; SR, sinus rhythm; BMI, body mass index; LV-Dd, left ventricular-diastolic diameter; LA-Ds(A-P), left atrium-systolic diameter (anterior-posterior); RV-Dd(L-R), right ventricular-diastolic diameter (left-right); RA-Ds(L-R), right atrium-systolic diameter (left-right); LVEF, left ventricular ejection fraction; TCHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SEM, standard error of the mean.

Table S3 DPs identified by iTRAQ in plasma samples

Symbol	FC (AF/SR)	Symbol	FC (AF/SR)
IGHG1	25.833	THRB	0.543
LG3BP	1.991	CO6	0.637
IGKC	3.616	KLKB1	0.411
LAC2	1.325	AACT	0.660
IGHD	2.570	VTNC	0.412
IGHA2	1.478	HEP2	0.492
IGLL5	3.091	K2C1	0.427
KV403	1.683	CO8A	0.627
SODE	1.776	TTHY	0.588
HV103	1.647	THBG	0.811
HV303	1.615	PGRP2	0.510
HV315	2.125	K1C9	0.600
ICAM2	1.410	PRG4	0.557
HV322	1.223	ANGT	0.420
LV301	1.781	MASP1	0.592
LAC7	2.318	APOL1	0.541
HV311	2.320	FCN3	0.696
KV109	1.888	SAMP	0.601
KV203	2.534	APOD	0.429
LAC3	1.270	HV309	0.772
HV209	1.947	HV102	0.743
HV314	1.496	BSN	0.301
KV405	2.856	CSF1R	0.770
HV319	1.581	BIEA	0.407
KV306	1.321		
HV202	1.554		
LV103	1.252		
IV4F8	2.258		
LV102	1.526		
S10A6	1.237		
LMO7	1.287		
PNPH	1.475		
LV205	1.250		

DPs, differential proteins; iTRAQ, isobaric tags for relative and absolute quantification; GC-MS, gas chromatography-mass spectrometer; FC, fold change; AF, atrial fibrillation; SR, sinus rhythm.

Table S4 DMs distinguished by GC-MS in AF and SR patients in plasma samples

Metabolites	FC (AF/SR)	VIP	P value
Thymidine 5'-monophosphate	0.055960169	4.35825	4.50226E–20
D-Altrose	0.072301309	4.1636	6.61704E–15
Galactinol	8.526085448	3.54995	2.54838E–10
Linoleic acid	5.133578375	1.42721	5.05711E–09
Palmitic acid	2.92289767	1.13529	3.271E–07
Raffinose	3.672639357	2.78736	4.40291E–07
Citraconic acid	0.152894906	2.69137	5.25766E–07
Phytosphingosine	0.587703735	2.80858	3.61056E–06
N-alpha-Acetyl-L-ornithine	0.386690837	3.02793	3.98051E–06
Pentadecanoic acid	5.032190986	2.67135	5.42421E–06
(–)-Dihydrocarveol	9.794830761	2.4979	2.76024E–05
beta-Glycerophosphoric acid	0.553812067	2.82388	7.41314E–05
Glycerol	0.463294272	2.57287	0.000117695
4-Androsten-11beta-ol-3,17-dione	0.546169665	2.22298	0.000132546
Guaiacol	0.368431764	2.42314	0.000134285
Erythrose	0.625372928	2.50641	0.000198983
Carbobenzyloxy-L-leucine degr2	0.358601984	2.30824	0.000365596
O-phosphonothreonine	1.996050482	1.97919	0.0003823
Hydantoin, 5-(4-hydroxybutyl)-	3.783275513	2.34158	0.000426659
Hypoxanthine	2.119196166	2.09096	0.000636647
Sucrose	3.436732319	1.5977	0.000790514
N-Acetyl-beta-D-mannosamine	2.48042303	2.09165	0.000957718
Sedoheptulose	4.021531542	2.15246	0.00113941
3-Hydroxyflavone	2.8151879	1.78086	0.00166879
M-cresol	0.717116571	1.79058	0.002835953
Hydrocinnamic acid	4.384060772	1.70452	0.003039648
Cyclohexylsulfamic acid	3.739043866	1.85271	0.003040377
Galactose	1.649779532	1.644	0.003634949
Leucrose	2.231222736	1.76262	0.003793272
L-Threose	0.860696309	1.80378	0.004950915
2-Amino-2-norbornanecarboxylic acid	3.813652323	1.56531	0.005543599
Succinate semialdehyde	1.67042504	1.46701	0.005713745
Cumic acid	1.697941629	1.51777	0.006084477
Cellobiose	11.99964756	1.8388	0.007029194
Arachidonic acid	2.428534575	1.39718	0.009960894
Threo-beta-hydroxyaspartate	7.939586292	1.67525	0.010931149
dl-p-Hydroxyphenyllactic acid	1.774520697	1.72497	0.011137399
1-Kestose	0.705017583	1.44337	0.011995735
3-(3-Hydroxyphenyl)propionic acid	3.788173168	1.54174	0.012404505
4-Methylumbelliferone	2.234238851	1.52814	0.013906513
Xanthotoxin	0.638548088	1.71556	0.014839106
Lactose	3.171432565	1.591	0.0150684
Octadecanol	0.564233061	1.28234	0.015256356
N-Acetyl-L-phenylalanine	16.52219043	1.65853	0.016071066
Synephrine	1.371721751	1.55285	0.016552426
D-erythronolactone	3.265839367	1.49591	0.018409016
Levoglucosan	6.85372796	1.80614	0.019807515
Sitosterol	1.760365011	1.60135	0.020508144
N-2-Fluorenylacetamide	1.770829226	1.49582	0.021659028
N-Carbamylglutamate	1.073073155	1.11081	0.024175094
Heptadecanoic acid	2.634785578	1.13994	0.027701354
N-Acetyl-L-aspartic acid	1.496477986	1.28475	0.035626135
Lactulose	9.597210611	1.64151	0.03598997
Glycocyamine	1.904311519	1.50353	0.037441883
3,4-Dihydroxypyridine	1.081370306	1.43717	0.041316434

DMs, differential metabolites; GC-MS, gas chromatography-mass spectrometer; FC, fold change; AF, atrial fibrillation; SR, sinus rhythm; VIP, variable importance in projection.

Table S5 Omics studies in AF

Author/reference	Materials (human or animal)	Omics study
Liu *et al.*, (7)	Left and right atrial appendages (human)	Proteomics
Tu *et al.*, (29)	Left and right atrial appendage (human)	Proteomics
Zhang *et al.*, (30)	Left and right atrial appendages (human)	Proteomics
Modrego *et al.*, (31)	Left and right atrial appendage (human)	Proteomics
Mayr *et al.*, (8)	Atrial appendage tissues (human)	Metabolomics & proteomics
Alonso *et al.*, (32)	Serum (human)	Metabolomics
Jiang *et al.*, (6)	Atrial tissues (human)	Transcriptomics & proteomics
Gao *et al.*, (5)	Plasma (human)	Proteomics
Barallobre-Barreiro *et al.*, (33)	Atrial specimens (human)	Glycoproteomics
Huang *et al.*, (34)	Atrial appendage tissues (human)	Proteomics
De Souza *et al.*, (35)	Left-atrial cardiomyocytes and tissues (dogs)	Proteomics & metabolomics
Ko *et al.*, (36)	Fasting plasma (human)	Metabolomics

AF, atrial fibrillation.

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