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

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× 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

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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.

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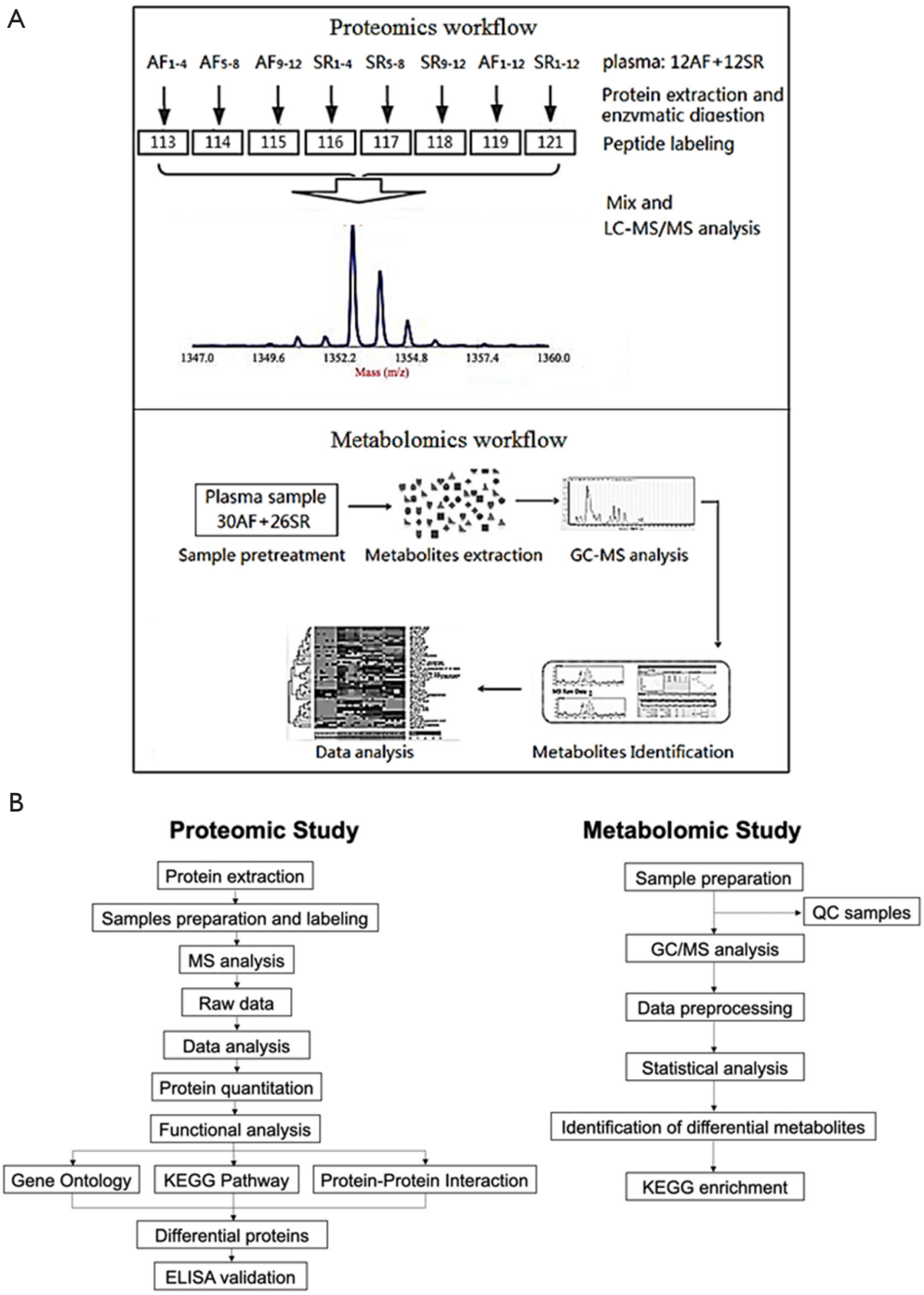

Figure S1 Proteomics (iTRAQ) and metabolomics workflow of plasma samples in the study. (A) Workflow of proteomics (iTRAQ) and metabolomics in plasma samples. AF, atrial fibrillation samples; SR, sinus rhythm samples; AF1–12, plasma from AF patients; SR1–12, plasma from SR patients. (B) Flowchart of the proteomics (iTRAQ) and metabolomics procedures. iTRAQ, isobaric tags for relative and absolute quantitation.

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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.

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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
$LA\text{-}Ds_{(A\text{-}P)}$, mm	57.0±9.1	50.0±8.1	0.001
$RV\text{-}Dd_{(L\text{-}R).}$, mm	35.2±6.2	31.8±4.5	0.013
$RA\text{-}Ds_{(L\text{-}R)}$, 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.

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Table S3 DPs identified by *i*TRAQ 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.

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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.

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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.

References

29. Tu T, Zhou S, Liu Z, et al. Quantitative proteomics of changes in energy metabolism-related proteins in atrial tissue from valvular disease patients with permanent atrial fibrillation. Circ J 2014;78:993-1001.
30. Zhang P, Wang W, Wang X, et al. Protein analysis of atrial fibrosis via label-free proteomics in chronic atrial fibrillation patients with mitral valve disease. PLoS One 2013;8:e60210.
31. Modrego J, Maroto L, Tamargo J, et al. Comparative expression of proteins in left and right atrial appendages from patients with mitral valve disease at sinus rhythm and atrial fibrillation. J Cardiovasc Electrophysiol 2010;21:859-68.
32. Alonso A, Yu B, Qureshi WT, et al. Metabolomics and incidence of atrial fibrillation in African Americans: the Atherosclerosis Risk in Communities (ARIC) study. PLoS One 2015;10:e0142610.
33. Barallobre-Barreiro J, Gupta SK, Zoccarato A, et al. Glycoproteomics reveals decorin peptides with anti-myostatin activity in human atrial fibrillation. Circulation 2016;134:817-32.
34. Huang WJ, Zhou R, Zeng XR, et al. Comparative proteomic analysis of atrial appendages from rheumatic heart disease patients with sinus rhythm and atrial fibrillation. Mol Med Rep 2011;4:655-61.
35. De Souza AI, Cardin S, Wait R, et al. Proteomic and metabolomic analysis of atrial profibrillatory remodelling in congestive heart failure. J Mol Cell Cardiol 2010;49:851-63.
36. Ko D, Riles EM, Marcos EG, et al. Metabolomic profiling in relation to new-onset atrial fibrillation (from the Framingham Heart Study). Am J Cardiol 2016;118:1493-6. Erratum in: Am J Cardiol 2017;120:339.

http://dx.doi.org/10.21037/atm-20-3767