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Title: Incremental metabolic benefits from cryoablation for paroxysmal atrial fibrillation: insights from metabolomic profiling

Brief Title: Metabolic benefits of cryoablation

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Abstract

Background: Cryoablation (CRYO) is a novel catheter ablation technique for atrial fibrillation (AF). However, uncertainty persists regarding the role of metabolic modifications associated with CRYO ablation. This study aimed to explore whether CRYO influences the metabolic signature, which has never been investigated before.

Methods: Paired serum samples of AF patients (n=10) were collected before and 24h after CRYO. Untargeted metabolomics analysis was conducted by using LC-MS system. Univariate and multivariate analyses were applied to identify differential metabolites. Pathway enrichment and Pearson correlation analysis were performed to reveal the disturbed metabolic pathways and potential interactions.

Results: Seventy-nine metabolites revealed a significant change from baseline to 24h after CRYO. Pathway analysis revealed that disturbed metabolites were enriched in unsaturated fatty acid biosynthesis, retrograde endocannabinoid signaling and neuroactive ligand-receptor interactions. Pearson correlation analysis showed a strong correlation between differential metabolites, biochemical markers, and clinical indicators.

Conclusions: Our results demonstrated that CRYO induces systemic changes in the serum metabolome of paroxysmal AF patients and provides potential metabolic benefits that could contribute to an enhanced understanding of the pathophysiology and metabolic mechanisms involved in catheter ablation.

Keywords: cryoablation, metabolomics, atrial fibrillation, polyunsaturated fatty acids, neuroactive ligand-receptor interaction pathways

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Significance Statement

This study is the first to demonstrate that cryoablation treatment may provide additional metabolic benefits to patients with paroxysmal AF by improving metabolite levels in addition to the clinical benefits obtained with pulmonary vein isolation. It compensates for the gap in the perception of cardiovascular benefits of ablations beyond the electrophysiological perspective.

Introduction

Atrial fibrillation (AF) is adults' most prevalent sustained cardiac arrhythmia, with 43.6 million cases worldwide and a preference for older age groups[1]. Pulmonary vein isolation (PVI) is a safe and effective treatment for AF patients. Cryoablation (CRYO) is a novel catheter ablation technique that has emerged as an effective and fundamental therapeutic strategy for PVI in patients with paroxysmal AF[2,3]. Recent studies have shown that initial treatment with cryoablation in patients with paroxysmal AF is more effective than drug therapy, with a lower incidence of atrial arrhythmias and lower AF burden than with initial use of antiarrhythmic drugs at 3-year follow-up[4]. The main principle of CRYO is to freezing of the ablation site by the endothermic process of evaporation of liquid nitrous oxide, thus damaging the tissue surrounding the pulmonary veins in contact with the balloon and forming a continuous ring of transmural injury to isolate the pulmonary veins[5].

The main metric of success with CRYO is the disappearance of the pulmonary vein potential immediately after freezing delivery. However, the other potential

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benefits of CRYO treatment beyond electrophysiological parameters are not fully understood. Extensive evidence indicates that cold exposure enhances brown adipose tissue (BAT) activity and that the high metabolic/thermogenic activity of brown fat can regulate lipid and glucose metabolism while consuming energy by accelerating the uptake and utilization of various metabolic substrates including glucose, fatty acids, succinate, or lactate[6,7]. In this case, metabolomic profiling seems very suitable to study the effects of CRYO treatment on cardiac metabolism. Specifically, understanding the metabolic traits of CRYO treatment permits inference into metabolic pathways associated with cardiovascular benefit. While previous metabolomic approaches in small sample studies of left atrial appendage closure for AF patients have yielded some important insights, studies on the metabolic signatures of CRYO have not been evaluated[8,9]. Accordingly, this study primarily aims to explore whether CRYO treatment in patients with paroxysmal AF may affect metabolic signature using untargeted metabolomics.

Methods

Study Population. An investigator-initiated, observational clinical trial was conducted at Wuhan University's Renmin Hospital for this study. Documented symptomatic paroxysmal AF patients were enrolled from October 2021 to January 2022. They then underwent a first catheter ablation procedure using CRYO. Before enrolling in the trial, patients with paroxysmal AF had at least one episode of AF documented by an electrocardiogram during the previous year. Before the procedure, the patient underwent medical history, physical examination, assessment of

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arrhythmia symptoms, medication review, 12-lead ECG, transthoracic echocardiography, and transesophageal echocardiogram. The two main requirements for inclusion were a left ventricular ejection fraction of ≥50% and an anteroposterior left atrium diameter of <5.0 cm. Detailed inclusion and exclusion criteria The exclusion criteria included persistent AF, secondary AF, cardiac procedures or implants, heart failure, coronary heart disease, structural heart disease, stroke or transient ischemic attack within 6 months, pericarditis or pericardial effusion, and malignant tumors. Patients signed informed consent forms before the procedure. The Renmin Hospital's Ethics Committee at Wuhan University approved this study (Approval No. WDRY2021-K147).

Cryoballoon ablation procedure. Subclavian vein and right femoral artery punctures, and the coronary sinus and ventricular electrodes were placed. Following atrial septal puncture, the 28-mm cryoballoon catheter (Arctic Front Advance, Medtronic, Minneapolis, USA) was advanced through the left atrium using the 15-Fr deflectable catheter over-the-wire. A spiral mapping catheter was placed in the targeted pulmonary vein, and the cryoballoon was placed at the pulmonary vein ostium. Under fluoroscopic guidance, the balloon was inflated, and venography was carried out to confirm pulmonary vein occlusion, which was identified as the endpoint of ablation **(Figure 1)**. Per pulmonary vein, each administered two freezes with targeted ablation times of 180 s. The superior vena cava carried out phrenic nerve pacing to ensure nerve integrity.

Blood sample preparation. All participants were required to fast for at least 8 h

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before sampling in both blood draws. Venous blood was collected from AF patients before CRYO and 24 hours after CRYO for further analysis. Venous blood samples were taken using heparinized tubes. Within 30 min, blood samples were inverted five to eight times, centrifuged (3000 rpm, 15 min at 4°C), and the serum was isolated and stored at −80°C until needed.

Serum nerve injury marker detection. Serum neurofilament light (NFL), glial fibrillary acidic protein (GFAP), S-100 protein beta chain (S100B), and brain fatty acid-binding protein (B-FABP) were measured using a commercial ELISA kit (CUSABIO BIOTECH CO., Ltd., Wuhan, China) according to the manufacturer's instructions.

Untargeted metabolomic profiling. Serum samples for metabolomic analysis were collected. 100 μL of blood was quenched in 300 μL of precooled methanol and acetonitrile (2:1, v/v). Each sample had internal standards added to it as a quality control measure. Samples were transferred for vacuum freeze-drying after 1 minute of vortexing. They were then resuspended in 150 μL of 50% methanol and centrifuged for 30 min at 4000 rpm to separate the supernatants, which were then used for injection into the liquid chromatography-mass spectrometry (LC-MS) system. To evaluate the reproducibility of the whole LC-MS analysis, a quality control (QC) sample was prepared by pooling the same volume of each sample.

The metabolites were separated and detected using a Waters 2D UPLC system (Waters, USA) coupled to a Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA). LC separation was performed on a Waters ACQUITY UPLC

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BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, USA). The binary gradient model of the high-performance liquid chromatography system was used to keep the column temperature at 45℃. Positive and negative ion modes of sample analysis were carried out with spray voltages of 3.8 kV and 3.2 kV, respectively. The mass scanning range was 70−1050 m/z with a resolution of 70000, and the automatic gain control target for MS acquisitions was set to 3e6 with a maximum ion injection time of 100 ms. The nitrogen sheath gas and nitrogen auxiliary gas flow rates were set to 40 L/min and 10 L/min, respectively. The pooled QC sample was initially injected five times to ensure system equilibrium. To further monitor the system's stability, QC samples were interspersed every ten samples.

LC-MS data processing. Compound Discoverer 3.0 software (Thermo Fisher Scientific, USA) was used to measure the peak characteristics of relevant metabolites. Peak extraction, peak grouping, retention time correction, peak alignment, and metabolite identification were all part of the data processing and analysis workflow. To ensure the quality of the data, QC sample analysis includes QC overlapping of base peak chromatogram (BPC), principal component analysis (PCA), peak lift quantity, and peak response intensity. By comparing the exact molecular mass data (m/z) of the samples with those from the database, the metabolites were identified using the online mzCloud and HMDB databases. To comprehend the functional properties of various metabolites and identify the primary biochemical metabolic pathways and signal transduction pathways involved in the metabolites, metabolic pathway enrichment analysis of differential metabolites was carried out using the

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Kyoto Encyclopedia of Genes and Genomics (KEGG) database.

Statistical Analysis. Categorical variables were presented as numbers and percentages, while continuous variables were presented as the mean ± standard deviation (SD). Kolmogorov-Smirnov or Shapiro-Wilk were used to test for normality. Depending on the degree of data normality, independent continuous variables were compared using either independent-sample parametric tests (paired Student's t-test) or non-parametric tests (paired Wilcoxon sighed-rank test). One-way analysis of variance with Tukey's multiple comparisons test was used to assess the significance of differences between multiple time points. To search for differential metabolites between groups, a combination of multivariate statistical analysis (PCA and Partial Least Squares-Discriminant Analysis (PLS-DA)), univariate analysis (Fold-Change [FC] and paired Wilcoxon sighed-rank test) was used. On the condition that $P < 0.05$, FC< 0.83 or >1.2, and VIP> 1 were met, significantly altered metabolites were acquired. After performing log2 -log conversion and pareto scaling with 7-fold cross-validation, the PLS-DA model was created. Heatmaps show standardized abundance data for each metabolite, and phenotype analysis was used to group significantly distributed metabolites. Pearson correlation analysis was implemented between significantly altered serum metabolites and clinical indicators. GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) or R (http://www.R-project.org, 4.2.2.) was used for statistical analyses. $P < 0.05$ was considered statistically significant.

Results

Baseline Patients' Characteristics and procedural data

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Patient baseline characteristics are shown in **Table S1**. The mean age was 60.0 ± 12.3 years, and 60% of patients were male. The mean left ventricular ejection fraction was 59.4 ± 1.8%, and the mean left atrial diameter was 3.9 ± 0.5 cm. **Table S2** provides an overview of the procedures' characteristics. Of the 10 patients, 100% (40 of 40 PVs) met the primary feasibility endpoint of complete acute PVI, and no adverse events were attributed to CRYO. The mean total procedure time was 68.0 ± 14.0 min, with a mean fluoroscopy time of 18.5 ± 5.6 min.

Metabolite changes associated with CRYO in patients with AF.

After sieving compounds with a relative peak area CV of 30% more in QC samples, 3755 and 1744 features in positive and negative modes, respectively, were found in the non-targeted metabolomic profiling **(Figure S1A)**. The PCA of the pooled QC samples clustered together, and the BPC of all QC samples overlapped well **(Figure S1B)**, demonstrating the authenticity and stability of LC-MS. **Figure 2A - B** illustrates how PLS-DA distinguished between the pre-and post-CRYO groups in both positive and negative modes. Serum levels of metabolites **(Figure 2C)** distinguished post-CRYO patients from pre-CRYO by statistical analysis. A total of 79 metabolites were identified referring to KEGG and HMDB databases **(Table S3)**. The heat map and hierarchical cluster analysis illustrate the different distribution patterns of differential metabolites between the two groups, as presented in **Figure 2D.** With an FC of 41.0491, Tyramine is one of the most significantly changed substances. Furthermore, we conducted correlation analysis with the corrplot package to reveal the potential interaction among the identified compounds, where metabolites were

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tended to cluster together in the correlation matrix **(Figure S2)**.

Differential metabolite identification and pathway analysis.

To explore the identities and functional enrichment of differential features, the above differential metabolites were entered as input data to perform the class identification and pathway enrichment analysis from the HMDB and KEGG. As shown in **Figure 3A - B**, the differential features were significantly involved in lipid metabolism, amino acid metabolism and signaling molecules and interaction and were mainly identified as fatty acyls, alpha-amino acids, peptides, as well as benzene and substituted derivatives. Pathway analysis was then performed to reveal detailed functional annotation through the clusterProfiler package, which showed the differentially expressed metabolites after CRYO were enriched in pathways including biosynthesis of unsaturated fatty acids, retrograde endocannabinoid signaling, neuroactive ligand-receptor interaction, thermogenesis and linoleic acid metabolism **(Figure 3C)**. To visualize the level of metabolites changed in these differential pathways, a functional annotation heatmap was then conducted as in **Figure 3D**. We discovered that all the altered metabolites (Arachidonic acid (AA), 8z, 11z, 14z-eicosatrienoic acid [Dihomo-gamma-linolenic acid DGLA], 11(z), 14(z)-eicosadienoic acid, 11(z)-eicosenoic acid, Cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), and linoleic acid) for the biosynthesis of the unsaturated fatty acid pathway were consistently increased. AA, DGLA, linoleic acid, as well as (±)12(13)-dihome **(Figure S3)** were participated in linoleic acid metabolism. Furthermore, there were 8 differential metabolites involved in the

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neuroactive ligand-receptor interaction pathway, among which Tyramine was presented as notably increased. Interestingly, gamma-aminobutyric acid (GABA), 2-arachidonoyl glycerol (2-AG), and anandamide (AEA) were also related to retrograde endocannabinoid signaling, together with glycerin, they were significantly increased after CRYO.

Relative changes in individual metabolites after CRYO.

To focus on the main disrupted pathways, we conducted further investigations into the abundance of metabolites involved in these pathways. The results showed significantly elevated levels of unsaturated fatty acids after CRYO treatment. Specifically, these detected metabolites were polyunsaturated fatty acids (PUFAs), which belong to the omega-6 and omega-3 series **(Figure 4)**. Retrograde signaling is the principal mode by which endocannabinoids (eCBs) mediate short- and long-term forms of plasticity at both excitatory and inhibitory synapses **(Figure 5A)**. After CRYO, the levels of the inhibitory neurotransmitter GABA were increased. Additionally, as the most relevant and prevalent regulators of synaptic function, AA and AEA were increased after CRYO. These two CBs were further degraded into AA and GC, both of which were consistently elevated in the post-CRYO group **(Figure 5C - 5G)**. Besides, a series of neuroactive ligands were differentially expressed in the neuroactive ligand-receptor interaction pathway **(Figure 5B)**, including decreased D-erythro-sphingosine1-phosphate (D-erythro-S1P) and Taurine, as well as increased GABA, 2-AG, Tyramine, AEA, Glycine and β-Alanine **(Figure 5E - L)**. Among these, Tyramine showed the most significant alteration. In this way, we hypothesized that

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CRYO could affect neural function. We tested this by analyzing the neural injury biomarkers across samples from AF patients pre- and post-CRYO. As expected, the level of serum NFL, GFAP, S100B, and B-FABP showed a significant increase immediately and 24h after the CRYO **(Figure S4)**. To gain insight into how these metabolites are involved in all the differential pathways, we further confirmed correlations among differential metabolites, neural injury biomarkers, and clinical indicators using Pearson's correlation coefficient r **(Figure 6)**. Within the subgroup, 7 differential PUFAs had high connections ($0.971 > r > 0.838$ and $P < 0.0001$). Metabolites related to retrograde endocannabinoid signaling (except GABA) indicated highly positive correlations ($0.911 > r > 0.51$ and $P < 0.05$). In addition, five metabolites referred to neuroactive ligand-receptor interaction such as Taurine, Tyramine, D-erythro-S1P, 2-arachidonoyl glycerol were clustered with high correlation ($0.898 > r > 0.715$ and $-0.692 < r < -0.509$, and $P < 0.05$). Interestingly, the above-mentioned metabolites strongly correlate with the seven PUFAs. D-erythro-sphingosine 1-phosphate was negatively correlated with FUFAs ($-0.645 < r < -0.41$), while Tyramine ($0.779 > r > 0.576$), 2-AG ($0.661 > r > 0.395$), AEA ($0.949 > r > 0.885$), and, glycerin ($0.958 > r > 0.849$), AA ($0.964 > r > 0.84$) were positively correlated. Moreover, neural injury biomarkers, especially GFAP and S100B in patients with AF were significantly positively correlated with Tyramine and PUFAs, while GFAP and B-FABP were negatively related to D-erythro-S1P. Among the echocardiographic parameters, there were only Glycine, 2-AG, and thromboxane b2 presented a slight correlation. In addition, the level of 2-AG after CRYO ($r = -0.679$, P

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= 0.031, **Figure S5**) and its relative alteration (r = -0.670, P = 0.034, **Figure S6**) showed negatively association with recurrent AF. These resulted indicated that the increase of 2-arachidonoyl glycerol after CRYO may affect the outcome of AF in a beneficial way.

Discussion

In this study, we show that the CRYO procedure is a cold-stress stimulus able to induce systemic changes in paroxysmal AF patients' serum metabolome, reflecting the whole-body acute response to cryo-balloon ablation in AF patients potentially providing them with metabolic benefits. The results showed significant alterations in pathways associated with the biosynthesis of unsaturated fatty acids, retrograde endocannabinoid signaling, neuroactive ligand-receptor interaction, thermogenesis, and linoleic acid metabolism after CRYO, which may indirectly reflect the effects of successful CRYO. These metabolic changes may be unique to cryoablation, which was not available in previous studies in regard to radiofrequency and other catheter ablation[10,11]. These may be worth investigating as candidate biomarkers for monitoring CRYO efficacy in future studies.

Among the known risk factors for AF, obesity and visceral fat accumulation play an important role in stimulating arrhythmogenic substrates and thus predisposing to AF. Numerous studies have shown that the amount of epicardial adipose tissue and peri-atrial adipose tissue is closely related to the development of AF[12,13]. Epicardial adipose tissue is predominantly white adipose tissue, and cold stimulus induces the browning of white adipose tissue and changes white adipose tissue gene expression

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and lipid metabolism to promote adaptive thermogenesis[14]. Cold stimulus promotes the secretion of the lipid mediator 12,13-dihome in BAT, thereby promoting the thermogenic activity of BAT, regulating lipid metabolism, and improving cardiac function[15,16]. In the present study, we demonstrated that CRYO, a cold stimulus, significantly increased 12,13- dihome levels after ablation, thereby activating BAT thermogenic lipokines, which may be a potential metabolic benefit from CRYO treatment.

12,13-dihome is an oxylipin. Oxylipins are bioactive lipids derived from the metabolism of PUFAs (omega-6 and omega-3) following the action of mono- or dioxygenases[17,18]. Omega-6 is a precursor of 12,13-dihome biosynthesis. Linoleic acid, an omega-6 PUFA, can be converted to linoleic epoxide through CYP450-dependent metabolism and finally metabolized to 12,13-dihome by soluble epoxide hydrolase[19]. We found that CRYO can significantly alter the metabolism of PUFAs, including the increasing of AA, DGLA, 11(z), 14(z)-eicosadienoic acid, 11(z)-eicosenoic acid, EPA, DHA and linoleic acid levels. Therefore, CRYO likely facilitates the stimulation of 12,13-dihome synthesis and thus enhances fatty acid uptake by BAT and stimulates the browning process in white adipose tissue.

PUFAs prevent cardiovascular diseases by regulating serum triglyceride levels, lipoprotein size, inflammation, plaque stability, and arrhythmia risk[20]. A Cochrane review revealed the effects of the increased omega-6 PUFAs on cardiovascular disease, which supported the benefit in people at high risk of myocardial infarction[21]. In 11 prospective cohort studies with omega-6 (mostly linoleic acid), higher linoleic

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acid concentrations were linked to a reduced risk of all cardiovascular disease outcomes, including cardiovascular disease mortality[22,23]. In addition, the reduced levels of DGLA, an omega-6 PUFA derived from linoleic acid, strongly correlate with the poor prognosis of cardiovascular disease[24]. In particular, recent studies showed higher circulating concentration and dietary intake of omega-6 fats were associated with lower risk of AF among middle-aged and older patients and multi-ethnic population[25,26]. In agreement with these, our metabolomic profile showed a significant increase of linoleic acid, DGLA, and 11(z),14(z)-eicosadienoic acid in omega-6 PUFAs by CRYO treatment, which could contribute to the cardioprotective effect. Notably, consumption of high proportions of dietary omega-6 compared to omega-3 can have deleterious effects, particularly on inflammatory states, yet this concern is not generally supported by research evidence[20].

We also found that CRYO treatment dramatically increased omega-3 PUFAs, especially increasing the levels of EPA and DHA. Omega-3 PUFAs are a component of the cardiomyocyte membrane, where they exert not only stabilizing effects but also direct electrophysiological effects[27]. Previous metabolomic analysis demonstrated significant decreasing of EPA in AF patients[10]. Supplementation of omega-3 has been reported to lower the incidence of AF in various conditions, such as post-cardiac surgery or following cardioversion of AF[28,29]. Omega-3 PUFA has been demonstrated to decrease both the inducibility of AF and the duration of pacing-induced AF episodes by inhibiting two major mechanisms that are responsible for maintaining AF: reentry and rapid focal ectopic firing[30]. Moreover, inflammation and the associated

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immune response contribute to the onset and maintenance of AF, as well as the electrical and structural atrial remodeling in AF[31]. Omega-3 PUFAs are precursors to resolvins, protective proteins, and other inflammation-resolving mediators, and new research suggests that these mediators may have strong anti-inflammatory qualities and contribute to the breakdown of inflammation[32].

In addition, aging increases the risk of AF, especially the associated atrial electrical and structural remodeling[33]. PUFAs such as linoleic acid, DGLA, EPA, and DHA activities decrease with age[34]. Studies reported that the level of PUFAs were inversely associated with incident AF among the elderly population who have the highest risk of AF[35]. Therefore, CRYO may effectively correct disorders of bioactive fatty acids (linoleic acid, DGLA, EPA, and DHA) to prevent AF and metabolic abnormalities associated with aging. Our findings imply that CRYO-induced changes in the biosynthesis of unsaturated fatty acids might reduce the negative effects of AF ablation. Sustained homeostasis of PUFAs may improve AF treatment and its long-term prognosis. However, this hypothesis needs to be verified through, additional cohort studies with longer follow-ups.

Recent studies suggest that the immune-modulatory lipid-signaling molecules eCBs may be the missing link between the beneficial effects of PUFAs and the management of cardiometabolic diseases[36]. The eCBs mediate the beneficial effects of omega-3 fatty acids on cardiometabolic disorders, and increased levels of 2-AG and oxylipin enhance the anti-inflammatory effects of Omega-3 fatty acids. Activation of the cannabinoid receptor has been shown to stimulate an anti-inflammatory state by

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increasing anti-inflammatory cytokines and also decreasing the levels of pro-inflammatory cytokines[37]. In addition, the ability of cannabinoid receptors to fine-tune and regulate GABAergic synaptic transmission through retrograde signaling can inhibit neuronal activity, and neuronal activation plays an important role in the development of AF[37]. Interestingly, our results likewise found that the eCBs-mediated retrograde signaling pathway was altered after CRYO, with 2-AG and AEA significantly elevated after ablation, which may contribute to anti-inflammation and enhance synaptic function and neurocognitive function.

Prior studies showed that the activation of the cardiac autonomic nervous system is often required for the triggering and possibly the maintenance of AF[38]. Especially considering the most recent study showing that extensive autonomic denervation alone without PVI was as successful as PVI at maintaining sinus rhythm at 1-year postablation[39]. In the present study, we found elevated serum nerve injury markers after CRYO treatment, suggesting that freezing may damage the ganglion plexus in the fat pad around the pulmonary vein, and KEGG analysis further discovered significant alterations in neuroactive ligand-receptor interaction pathways after CRYO treatment. Neuroactive ligands affect neuronal function by combining with intracellular receptors, which can bind transcription factors and regulate gene expressions[40]. Among them, tyramine is one of the most significant changed metabolites, which is related to tyrosine metabolism and further indicates that CRYO results in neuron injury and impacts cardiac sympathetic activity[41]. The study has reported that the cardioprotection effect of the neuroactive steroid is associated with

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neuroactive ligand-receptor interaction in patients treated with coronary artery bypass graft surgery[42]. In addition, another study also found that neuroactive ligand-receptor interaction was closely associated with arrhythmogenic right ventricular cardiomyopathy[43]. Based on these, we infer that CRYO might play a vital role in cardioprotection by affecting the neuroactive ligand-receptor interaction pathway.

This study has several limitations that should be noted. First, this study was cross-sectional with a small sample size, and a lack of control group without CRYO, which may lack of strong and reliable foundation for the conclusions, and randomized controlled trial studies with larger samples and deeper relevant mechanism exploration are required. Second, although untargeted platforms reveal more serum metabolites, their interpretability is limited, and they are frequently less quantifiable. Finally, our study is the first to explore the influence of successful CRYO on metabolic profiling and is therefore descriptive and hypothesis-generating.

Author contributions

MJX, FDG and JW: substantial contributions to data acquisition, or data analysis and writing; YJW, ZHL, JX, ZW, SYW: data collection and revision; LPZ, YYW: drafting the article or critically revising it for important intellectual content. LLY, HJ: conception, design, and supervision. All authors contributed to the article and approved the submitted version.

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Data availability statement

The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no competing interests.

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Figure Legends

Figure 1 Catheter ablation technologies. (A) The fluoroscopic image depicts the cryoballoon catheter positioned at the pulmonary vein ostium. **(B)** An illustration depicting the principal design of the cryoballoon.

Figure 2 Non-targeted metabolomics profiling analysis of human serum. (A, B) PLS-DA score plots from pre- and post-CRYO of AF patients. Validation plots 200 permutation tests in the positive mode (left) and the negative mode (right). **(C)** Volcano plot of differential metabolites. Red, blue, and grey indicate increased, decreased, and non-significant differential metabolites, respectively. VIP>1, FC < 0.83 or > 1.2, P< 0.05 were considered significant differences. **(D)** The hierarchical clustering heat map of the identified metabolites. Rows indicate metabolites; columns indicate sample group.

Figure 3 Differential metabolite identification and pathway analysis. (A, B) Differential metabolomics profiling annotation using KEGG and HMDB database searching and metabolic network analysis to explore the corresponding identification. **(C)** KEGG pathway enrichment analysis showing altered metabolic pathways of differential metabolites. Each circle represents a pathway, and the size and color of each circle indicate the enriched counts and significance of the pathway, with red being most significantly changed after ablation. **(D)** Functional annotation heatmap of the metabolites of the altered pathway. Rows indicate pathways; columns indicate

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metabolites. The color code indicates the FC in the changed compound. Black text shows FC of metabolites after log10-log conversion.

Figure 4 Differential metabolites in the biosynthesis of the unsaturated fatty acid. **(A)** Schematic representations of biosynthesis of the unsaturated fatty acid and significantly altered metabolites were shown in red text. **(B-H)** Box and dot plot profiles of the differential PUFAs. PUFA, polyunsaturated fatty acids.

Figure 5 Differential metabolites in the neuroactive ligand-receptor interaction and retrograde endocannabinoid signaling. (A) The illustration of the neuroactive ligand–receptor interaction pathway. Red and blue circles represent increasing and decreasing features, respectively. **(B)** The pattern of retrograde endocannabinoid signaling and metabolism. In brief, the postsynaptic activity leads to the production of eCBs, such as 2-AG and AEA, that moves backward across the synapse, bind presynaptic CBRs and suppresses the neurotransmitter release. And they were further degraded into AA and GC. GABA, gamma-aminobutyric acid; 2-AG, 2-aeachidonoyl glycerol; AEA, anadamide; AA, Arachidonic acid; GC, glycerin. **(C-L)** Box and dot plots of abundances of the metabolites involved in neuroactive ligand-receptor interaction and retrograde endocannabinoid signaling.

Figure 6 Correlation analysis among altered metabolites in pathway and clinical indicators.

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Correlation analysis among altered metabolites, classical neural injury biomarkers and echocardiography parameters. Pearson correlation coefficients between pairs of compounds are shown in the lower left corners of the panels. Six subgroups were shown with frames in different colors. In the upper right corners, the degree of correlation and p values are shown for each pair. Red and blue indicate negative and positive correlations, respectively. *$p < 0.05$; **$p < 0.01$.