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Metabolomic analysis reveals the role of gut microbiota metabolic disorders in heart failure due to congenital heart disease

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Abstract

Objective: This study aimed to explore the effects of gut microbiota metabolic disorders caused by gut microbiota dysbiosis on heart failure due to congenital heart disease (CHD) through metabolomic analysis. **Methods:** Patients with congestive heart failure caused by left-to-right shunt CHD were selected as the subjects. Thirty infants with heart failure due to CHD admitted to the Department of Cardiovascular Surgery of our hospital from April 2022 to August 2022 were included in this study. Thirty healthy infants of the same age and sex who visited our hospital during the same period were selected as the control group. Faecal samples were collected from each participant and subjected to metabolomic analysis. **Results:** Compared with those in the control group, the levels of 125 metabolites increased, whereas those of 147 metabolites decreased in the heart failure group. Compared

with those in the control group, the levels of indoxyl, arachidonic acid, erucic acid, and DL-glycerol 1-phosphate were significantly increased in the heart failure group, whereas the level of 1-aminocyclopropanecarboxylic acid was significantly decreased. Pathway analysis of differentially abundant metabolites revealed that, compared with those in the control group, the metabolic pathways of linoleic acid metabolism, PPAR signalling, and arachidonic acid metabolism were significantly upregulated in the heart failure group. The NT-BNP level was significantly positively correlated with indoxyl, arachidonic acid and erucic acid ($P<0.05$). There was a significant positive correlation between cardiac function scores and the levels of indoxyl and arachidonic acid ($P<0.05$). **Conclusion**: In this exploratory study, infants with congestive heart failure due to CHD exhibited significant changes in gut microbiota metabolites and metabolic pathways. The gut metabolites of indoxyl and arachidonic acid significantly were increased, and the metabolic pathways of linoleic acid metabolism, the PPAR signalling pathway, and arachidonic acid metabolism were significantly upregulated in the heart failure infants. Increased gut metabolites of indoxyl and arachidonic acid were positively correlated with the severity of heart failure.

Keywords: Gut microbiota, Metabolomics, Congenital heart

disease, Heart failure, Metabolic disorders

Background

Heart failure is a complex clinical syndrome characterised by abnormal changes in cardiac structure and function caused by any aetiology, and it represents the terminal stage of various cardiac diseases.[1,2] Currently, the activation of the neuroendocrine system leading to pathological myocardial remodelling is key to the occurrence and progression of heart failure.[3] Medications currently

used in modern medicine include angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, β-receptor blockers, mineralocorticoid receptor antagonists, and angiotensin receptor neprilysin inhibitors. However, current treatments target only a small fraction of presumed pathophysiological pathways, and effective therapies for most patients and for the prevention of heart failure are still lacking.[4] The gut is a complex microecosystem, and an increasing number of studies have indicated that the metabolites of the gut microbiota hold promise as important targets for intervention in heart failure.[5-9]

Numerous studies have shown that the gut microbiota and their metabolites play significant roles in the pathophysiology of heart failure.[10-13] Like endocrine organs, the gut microbiota produce bioactive metabolites that interact through various pathways, including the short-chain fatty acid pathway, the bile acid pathway, and the trimethylamine N-oxide/trimethylamine pathway, to cause physiological changes in the host. Their presence in the circulation can enhance the inflammatory milieu, thereby promoting the progression of heart failure.[14-19] Our team’s previous research revealed that heart failure in infants with congenital heart disease (CHD) caused intestinal microbiota disorders, which were characterised by an increase in pathogenic bacteria, a decrease in

beneficial bacteria, and a reduction in diversity and richness.[20] Huang revealed that gut microbiota dysbiosis was observed in neonates with critical CHD, characterised by the depletion of Bifidobacterium and the overgrowth of Enterococcus, which was highly correlated with metabolomic perturbations.[21] On the basis of these studies, we conducted a cohort study to explore the metabolomics of the gut microbiota in infants with CHD-related congestive heart failure and to analyse the effects of gut microbiota metabolites caused by microbial dysbiosis on heart failure.

Methods

Study design and setting

Thirty infants with heart failure due to CHD admitted to the cardiovascular surgery department of our hospital from April 2022 to August 2022 were included in this study. Thirty healthy infants of the same age and sex who visited our hospital during the same period were selected as the control group. All the patients in the heart failure group were preoperatively diagnosed with CHD through echocardiography.

Eligibility criteria

The inclusion criterion was heart failure caused by CHD. The exclusion criteria were as follows: 1. concurrent major organ diseases, 2. concurrent digestive tract diseases, 3. concurrent

infections or the use of antibiotics, and 4. refusal by the parents to participate in this study.

Faecal sample collection

Faecal samples were collected before surgery and drug treatment. We collected 2 ml faecal samples from each patient and immediately froze them in liquid nitrogen. The faecal samples were subsequently stored at -80 °C.

Experimental Methods[22-24]

Sample preparation

1. An appropriate amount of the sample was accurately weighed into a 2 mL centrifuge tube, 600 μL of MeOH (stored at -20 °C) (containing 2-amino-3-(2-chloro-phenyl)-propionic acid [4 ppm]) was added, and the sample was vortexed for 30 s.

2. Glass beads (100 mg) were added, and the samples were placed in a tissue grinder for 90 s at 60 Hz.

3. Room-temperature ultrasound was performed for 10 min.

4. The samples were centrifuged for 10 min at 12,000 rpm and 4 °C, and the supernatant was filtered through a 0.22-μm membrane and transferred into a detection bottle for liquid chromatography (LC)-mass spectrometry (MS) detection.

LC conditions

LC analysis was performed on an Ultimate 3000 UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC® HSS T3 column (150×2.1 mm, 1.8 µm) (Waters, Milford, MA, USA). The column was maintained at 4 °C. The flow rate and injection volume were set at 0.25 mL/min and 2 µL, respectively. For LC-ESI(+)-MS analysis, the mobile phases consisted of (C) 0.1% formic acid in acetonitrile (v/v) and (D) 0.1% formic acid in water (v/v). Separation was conducted under the following gradient: 0–1 min, 2% C; 1–9min, 2%–50% C; 9-12 min, 50%–98% C; 12–13.5 min, 98% C; 13.5–14 min, 98%–2% C; 14–20 min, 2% C. For LC-ESI(-)-MS analysis, the analytes were analysed with (A) acetonitrile and (B) ammonium formate (5 mM). Separation was conducted under the following gradient: 0–1 min, 2% A; 1–9 min, 2%–50% A; 9–12 min, 50%–98% A; 12–13.5 min, 98% A; 13.5–14 min, 98%–2% A; and 14–17 min, 2% A.

MS conditions

The MS detection of metabolites was performed on a Q Exactive system (Thermo Fisher Scientific, USA) with an ESI ion source. Simultaneous MS1 and MS/MS (full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltages, 3.50 kV and -2.50 kV for ESI(+) and ESI(-),

respectively; capillary temperature, 325 °C; MS1 range, m/z 100–1000; MS1 resolving power, 70000 FWHM; number of data-dependent scans per cycle, 10; MS/MS resolving power, 17500 FWHM; normalised collision energy, 30%; and automatic dynamic exclusion time.

Data processing and multivariate analysis

The raw data were first converted to the mzXML format by MSConvert in the ProteoWizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction, and alignment. The metabolites were identified by their accurate mass (<30 ppm) and MS/MS data, which were matched with the HMDB (http://www.hmdb.ca), MassBank (http://www.massbank.jp/), LipidMaps (http://www.lipidmaps.org), mzCloud (https://www.mzcloud.org), and KEGG (http://www.genome.jp/kegg/) databases. Robust LOESS signal correction was applied for data normalisation to correct for any systematic bias. After normalisation, only ion peaks with relative standard deviations of less than 30% in quality control (QC) were kept to ensure proper metabolite identification. Ropls software was used for all multivariate data analyses and modelling.

The Ropls software was used for all multivariate data analyses and modelings. After scaling data, models were built on principal

component analysis (PCA), orthogonal partial least square discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA). The metabolic profiles could be visualized as score plot, where each point represents a sample. The corresponding loading plot and S-plot were generated to provide information on the metabolites that influence clustering of the samples. All the models evaluated were tested for over fitting with methods of permutation tests. The descriptive performance of the models was determined by R2X(cumulative) (perfect model: R2X(cum) =1) and R2Y (cumulative) (perfect model: R2Y(cum) =1) values while their prediction performance was measured by Q2 (cumulative) (perfect model: Q2(cum) =1) and a permutation test. The permuted model should not be able to predict classes: R2 and Q2 values at the Y-axis intercept must be lower than those of Q2 and the R2 of the non-permuted model. OPLS-DA allowed the determination of discriminating metabolites using the variable importance on projection (VIP). The P value, Variable importance projection (VIP) produced by OPLS-DA, fold change (FC) was applied to discover the contributable-variable for classification. Finally, P value < 0.05 and VIP values > 1were considered to be statistically significant metabolites.

QC was carried out using quality control samples. The results revealed that the quality control samples were clustered, with good repeatability and reliable outcomes.

Pathway analysis

Differentially abundant metabolites were subjected to pathway analysis by MetaboAnalyst. The metabolites and corresponding pathways were visualised using the KEGG Mapper tool.[25]

Cardiac function scores of infants

The degree of heart failure was assessed using the modified Ross scale.[26] There were 6 score indicators: the participant's sweating position, frequency of rapid breathing, breathing condition, respiratory rate, heart rate, and liver size. Each item was scored as 0, 1, or 2 according to the severity of symptoms from mild to severe. A higher score indicated more severe heart failure. A total score of 0–2 indicated "no heart failure", a total score of 3-6 indicated "mild heart failure", a total score of 7-9 indicated "moderate heart failure", and a total score of 10 to 12 indicated "severe heart failure".

Statistical Analysis

We performed the statistical analysis via SPSS 25.0. Continuous data are presented as the medians and quartiles. Continuous variables were compared through the Mann–Whitney U test.

Comparisons between groups of categorical variables were performed using the chi-square test. A p value of <0.05 was considered to indicate statistical significance.

Results

Demographics

A total of 30 infants with congestive heart failure due to CHD were enrolled in this study as the heart failure group, including 26 cases of ventricular septal defects, 3 cases of patent ductus arteriosus, and 1 case of an aortopulmonary window. There were 17 males and 13 females in the heart failure group. Their median age was 1.7 (1.0, 3.2) months, their weight was 1.8 (1.1, 2.6) kg, their pulmonary artery pressure was 61 (48, 73) mmHg, their cardiac function score was 9 (5, 10), their left-to-right shunt opening size was 7.8 (6.8, 8.5) mm, and their NT-BNP concentration was 9786 (6130, 18961) pg/ml. With respect to feeding methods, 10 infants were breastfed, 12 infants received mixed feeding, and 8 infants received formula. Thirty age- and sex-matched infants without heart disease, including 20 males and 10 females, were included in the control group. Their median age was 1.8 (1.1, 2.6) months, and their weight was 4.8 (3.6, 5.4) kg. With respect to feeding methods, 15 infants were breastfed, 11 infants received mixed feeding, and 4 infants received formula.

There were no significant differences between the two groups in terms of sex, age, weight, or feeding method ($P < 0.05$) (Table 1, Table 2).

Differences in the metabolites of the gut microbiota between the two groups

A total of 272 metabolites differed between the heart failure group and the control group. Compared with those in the control group, the levels of 147 metabolites increased, and the levels of 125 metabolites decreased in the heart failure group.

A volcano plot of the differentially abundant metabolites revealed that the most significant differences were as follows: compared with those in the control group, the levels of indoxyl, arachidonic acid, erucic acid, and DL-glycerol 1-phosphate were significantly increased in the heart failure group, whereas the level of 1-aminocyclopropanecarboxylic acid was significantly decreased (Figure 1).

Differences in metabolic pathways between the two groups

Pathway analysis of the differentially abundant metabolites revealed that the significantly affected metabolic pathways included linoleic acid metabolism, the PPAR signalling pathway, and arachidonic acid metabolism (Figure 2).

Correlations between cardiac function parameters and the

intestinal metabolites indoxyl, arachidonic acid, and erucic acid

An analysis of the correlations between the NT-BNP level and the intestinal metabolites indoxyl, arachidonic acid, and erucic acid in patients with heart failure revealed significant positive correlations ($P < 0.05$) (Figure 3). Furthermore, there was a significant positive correlation between the cardiac function scores and the indoxyl and arachidonic acid levels in patients with heart failure ($P < 0.05$) (Figure 4).

Discussion

Gut microbiota metabolites play a significant role in the pathogenesis of heart failure. A recent study by Hayashi et al. revealed a correlation between amino acid metabolism disorders and gut microbiota dysbiosis in patients with heart failure.[27] A substantial body of evidence supports the protective effects of short-chain fatty acids against heart failure and their crucial role in maintaining the integrity of the intestinal barrier.[28-31] Askin et al. reported that the systemic and metabolic effects of propionic acidaemia can be detrimental to the heart.[32] There are several ways in which propionic acidaemia may cause cardiac disease. For instance, propionic acidaemia induces cardiomyopathy. Additionally, propionic acidaemia can lead to cardiac arrhythmias

owing to metabolic imbalances affecting the heart's electrical signalling, resulting in arrhythmias that impair pumping. Chen et al. reported that a diet high in saturated fat and sugar leads to elevated trimethylamine N-oxide levels, which can cause fibrosis, myocardial inflammation, and impaired diastolic function.[33] Gut microbiota dysbiosis in patients with heart failure is characterised by high circulating levels of trimethylamine N-oxide, which can promote cardiac remodelling through myocardial fibrosis and proinflammatory effects.[34,35] Elevated levels of trimethylamine N-oxide have been identified as a prognostic biomarker for both acute and chronic heart failure.[36,37] However, these findings have focused primarily on adult heart failure.

This study explored the metabolomic changes in the gut microbiota of infants with CHD-related congestive heart failure. The results revealed that, compared with those in the control group, the levels of indoxyl, arachidonic acid, and erucic acid were significantly greater in the heart failure group. The NT-BNP level was significantly positively correlated with indoxyl, arachidonic acid, and erucic acid. There was a significant positive correlation between the cardiac function scores and indoxyl and arachidonic acid levels. These results indicated that the upregulation of the metabolites indoxyl and arachidonic acid was related to heart

failure.

Indoxyl is a molecule produced by gut bacteria and is formed from tryptophan in the intestine.[38] The majority of indole is converted into indoxyl sulphate by colonic epithelial cells and the liver. Both indole and indoxyl sulphate are considered to have biological effects and are associated with the heart and kidney.[39,40] Indoxyl sulphate can activate the renin receptor and lead to the activation of angiotensin receptors, which may promote myocardial hypertrophy and fibrosis.[41] Indoxyl sulphate also reduces the activity of nitric oxide synthase, which may play a significant role in the development of chronic heart failure according to experimental and clinical studies.[42] Indoxyl sulphate increases oxidative stress, thereby exacerbating the pathophysiological processes of heart failure. These factors may explain the correlation observed in this study between the levels of indole and indoxyl sulphate and heart damage.

Arachidonic acid is present in all mammalian cells and is among the most abundant polyunsaturated fatty acids. Arachidonic acid and its metabolites play important roles in the occurrence, progression, and prognosis of heart failure. They are involved in several pathological processes of heart failure, including lipid metabolism, the inflammatory response, oxidative stress, and

cardiomyocyte apoptosis.[43-45] Metabolites such as leukotrienes and certain prostaglandins have strong proinflammatory effects. The metabolism of arachidonic acid may generate reactive oxygen species, leading to oxidative stress. Oxidative stress can damage the DNA, proteins, and lipids of cardiomyocytes, thereby affecting their normal function. Moreover, oxidative stress can also activate multiple signalling pathways, promoting cardiomyocyte apoptosis and fibrosis. Dysregulation of arachidonic acid metabolism may lead to an imbalance in vasodilation and vasoconstriction, thereby affecting cardiac blood supply and cardiac function.

Pathway analysis of the differentially abundant metabolites in our study revealed that the metabolic pathways of linoleic acid metabolism, the PPAR signalling pathway, and arachidonic acid metabolism were significantly upregulated in the heart failure group. Disruption of arachidonic acid metabolism may lead to exacerbated inflammatory responses, increased oxidative stress, and vascular dysfunction, thereby worsening the symptoms of heart failure. Changes in the levels of arachidonic acid metabolites can serve as biomarkers for the prognosis of heart failure, and modulation of the arachidonic acid metabolic pathway may provide novel therapeutic targets for the treatment of heart failure.[43,46]

Linoleic acid is a polyunsaturated fatty acid that is an essential

component of cell membranes and is involved in the regulation of cell signalling, inflammatory responses, and lipid metabolism. Linoleic acid is the principal fatty acid component of cardiolipin, which is a core component involved in mitochondrial oxidative phosphorylation.[47] In recent years, the relationship between linoleic acid metabolism and cardiovascular disease has attracted considerable attention, especially its role in heart failure.[48] Studies have shown that a high-fat diet rich in linoleic acid can lead to myocardial hypertrophy and left ventricular dilation, thereby slowing the progression of chronic heart failure.[49] Genes and proteins associated with linoleic acid are closely related to energy metabolism and inflammation. Linoleic acid metabolites, such as 9-HODE and 13-HODE, have strong proinflammatory effects and can induce the release of proinflammatory cytokines (e.g., interleukin-1β) from macrophages, thereby further exacerbating inflammatory responses.[50]

The PPAR signalling pathway involves a class of transcription factors belonging to the nuclear receptor superfamily. It participates in physiological processes such as lipid metabolism, glucose homeostasis, inflammatory responses, and cell differentiation by regulating gene expression. The PPAR signalling pathway plays a crucial role in the occurrence and development of

heart failure, primarily by modulating metabolism, inflammatory responses, and oxidative stress to influence cardiac function.[51,52] Abnormal activation or inhibition of the PPAR signalling pathway can lead to metabolic disorders, affect the energy supply to the heart, and result in excessive activation of inflammatory responses and oxidative stress, thereby impacting cardiac function.

Excessive inflammatory and oxidative stress responses are closely related to the occurrence and development of heart failure. The upregulation of metabolic pathways involved in linoleic acid metabolism, PPAR signalling, and arachidonic acid metabolism was related to the intensified inflammatory response and oxidative stress response. These findings may explain the correlation observed in this study between gut microbiota metabolic disorders and heart failure.

Breast milk is the gold standard for infant nutrition. However, when breast milk is unavailable or insufficient to meet the infant’s needs, formula milk is considered an effective alternative. De Bernardo et al. reported good similarity in the faecal metabolome between breast milk and formula milk, which confirmed the efficacy of formula preparations as substitutes for breast milk.[53] Wang et al. demonstrated that the α and β diversity and metabolic functions of the faecal microbiome differed between breastfed

infants and formula-fed or mixed-fed infants.[54] Sillner et al. reported that unsaturated fatty acids and human milk oligosaccharides were increased in breastfed children, whereas Maillard products were detected in the faeces of formula-fed children. Elevated levels of sulphated bile acids were detected in the stool samples of breastfed infants, whereas secondary bile acids were increased in formula-fed infants.[55] The main food for infants is milk. To minimise the influence of food on the intestinal flora and metabolism as much as possible, this study selected infants as the research subjects, and there was no difference in the feeding methods between the heart failure group and the control group. However, since this study was a single-centre study with a small sample size, it was impossible to conduct a matching analysis. There would still be interference from food on the research results. This is a limitation of the study. In the future, we need to increase the sample size to ensure that the food of the enrolled infants is the same to eliminate the influence of food on the results.

Limitations

As a preliminary exploratory study, this study had several limitations. First, this research was a preliminary exploratory study and was conducted as a single-centre study with a small sample size. This resulted in limited statistical power and was prone to

more confounding factors. Second, CHD patients without heart failure were not included. Infants without heart disease comprised the control group, and CHD was a confounding factor. In future studies, we will increase the sample size and add a control group of infants with CHD without heart failure. A three-group (CHD with heart failure, CHD without heart failure, and healthy controls) study would be more reasonable. Third, the gut microbiota and its metabolism are susceptible to many factors; therefore, fully controlling for confounding factors was important in this study. Great efforts were made to control for confounding factors, such as choosing infants as subjects; ensuring the absence of differences in age, weight, and feeding patterns between the two groups; and collecting specimens from children with heart failure before treatment. However, some confounding factors could not be controlled for, which may have affected the results, such as the different feeding patterns within the groups and the differences in body weight and nutritional status between the two groups. In future research, we will learn from past experience, adopt a more rigorous design, and control and eliminate the influence of these confounding factors. Fourth, this study was an observational study that merely included a description of correlations. This study mainly reports associations between metabolites and heart failure

severity but does not provide mechanistic validation. No microbial group sequencing was performed to link bacteria with metabolites, and no experimental verification or functional pathway confirmation was performed. Conclusions concerning the causal role of the gut microbiota remain speculative. Future research should explore and verify the specific mechanisms through which the microbiota or metabolites influence the progression of heart failure.

Conclusion

As an exploratory study, this study revealed that infants with congestive heart failure due to CHD have significant changes in gut microbiota metabolites and metabolic pathways. The gut metabolites of indoxyl and arachidonic acid significantly were increased, and the metabolic pathways of linoleic acid metabolism, the PPAR signalling pathway, and arachidonic acid metabolism were significantly upregulated in the heart failure infants. Increased gut metabolites of indoxyl and arachidonic acid were positively correlated with the severity of heart failure. Increased secretion of these metabolites and up-regulation of these metabolic pathways may lead to increased inflammatory response and oxidative stress. These processes may associated with the occurrence and development of heart failure.

Abbreviation

CHD: Congenital heart disease

LC: liquid chromatography

MS: mass spectrometry

QC: quality control

PCA: principal component analysis

PLS-DA: partial least square discriminant analysis

VIP: variable importance on projection

FC: fold change

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Not applicable.

Author Contributions

ZQL, WZC, OQX and WY designed the experiments, performed the statistical analysis, and drafted the manuscript. LYN and ZYT collected the data. CQ supervised the study. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Fujian Children's Hospital (2022ETKLR10079) and strictly adhered to the tenets of the Declaration of Helsinki. The parents or guardians of the patients gave written informed consent for their respective minors to participate in the study.

Consent for publication

Not applicable.

Competing Interests

All authors declare that they have no competing interests.

Clinical trial number

Not applicable

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Figure legends

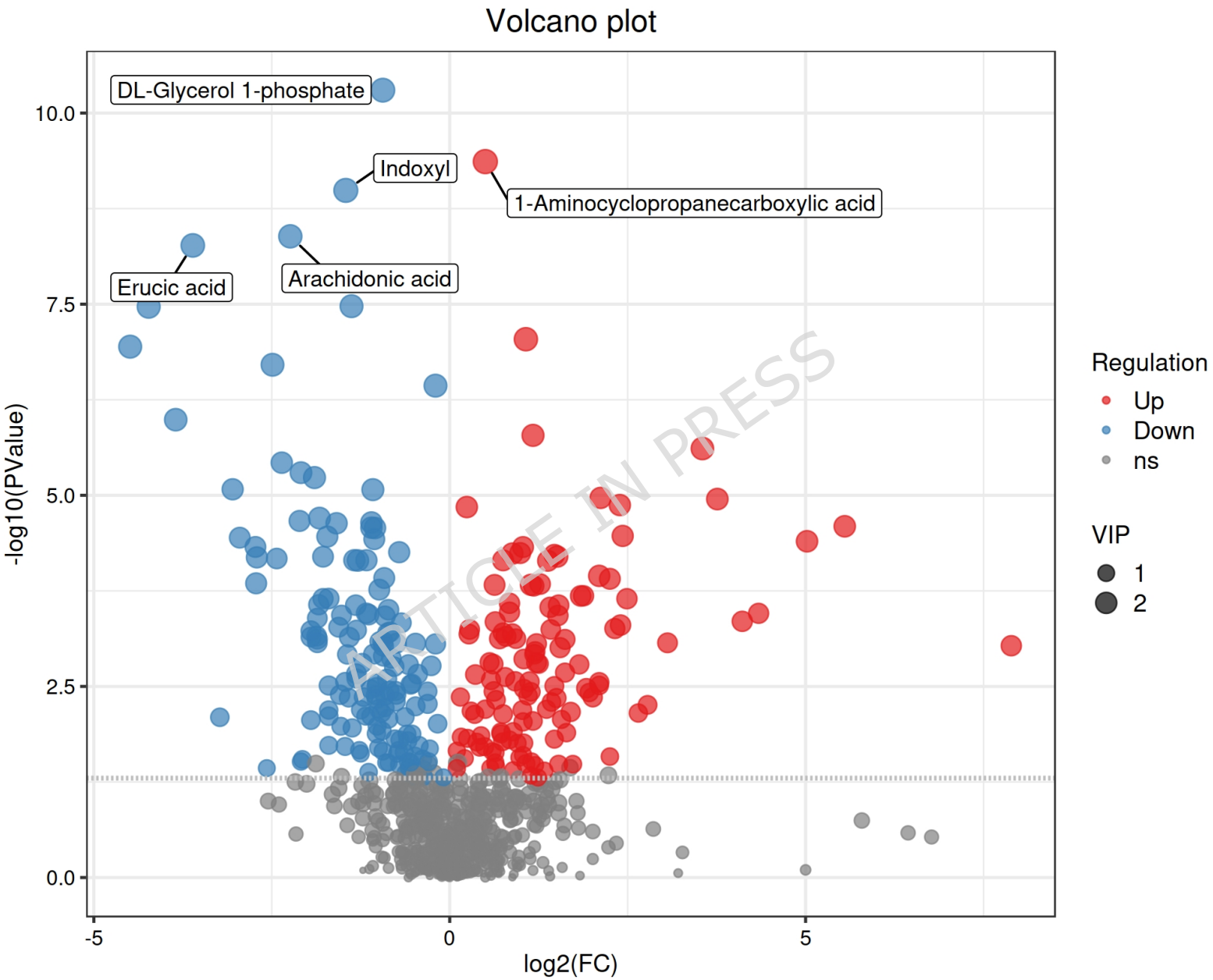
Figure 1: Compared with the control group, the Indoxyl, Arachidonic acid, Erucic acid, and DL-Glycerol 1-phosphate were significantly up regulated in the heart failure group, while the 1-Aminocyclopropanecarboxylic acid was significantly down regulated.

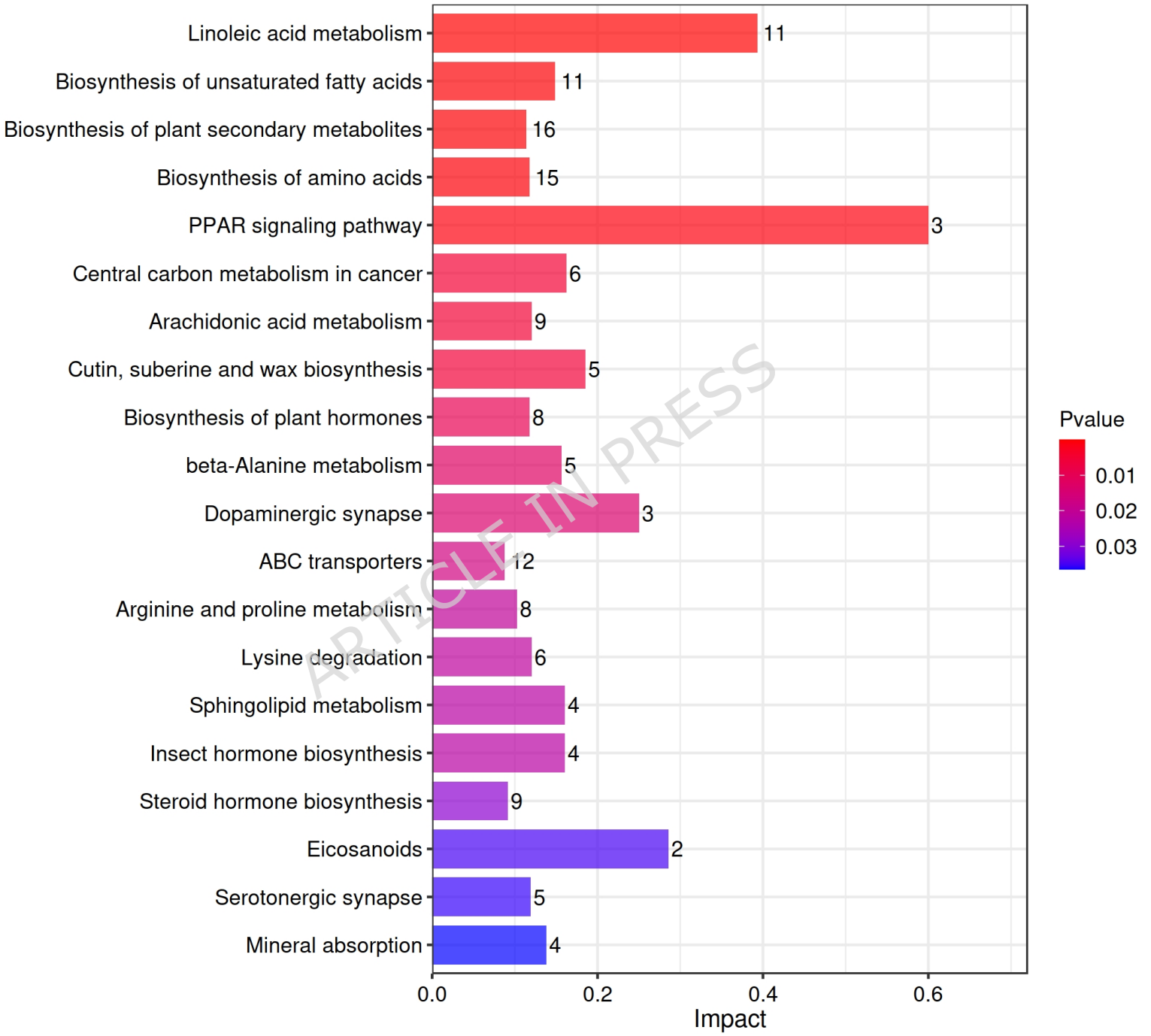
Figure 2: Compared with the control group, the metabolic pathways of Linoleic acid metabolism, PPAR signaling pathway, and Arachidonic acid metabolism were significantly up-regulated in the heart failure group.

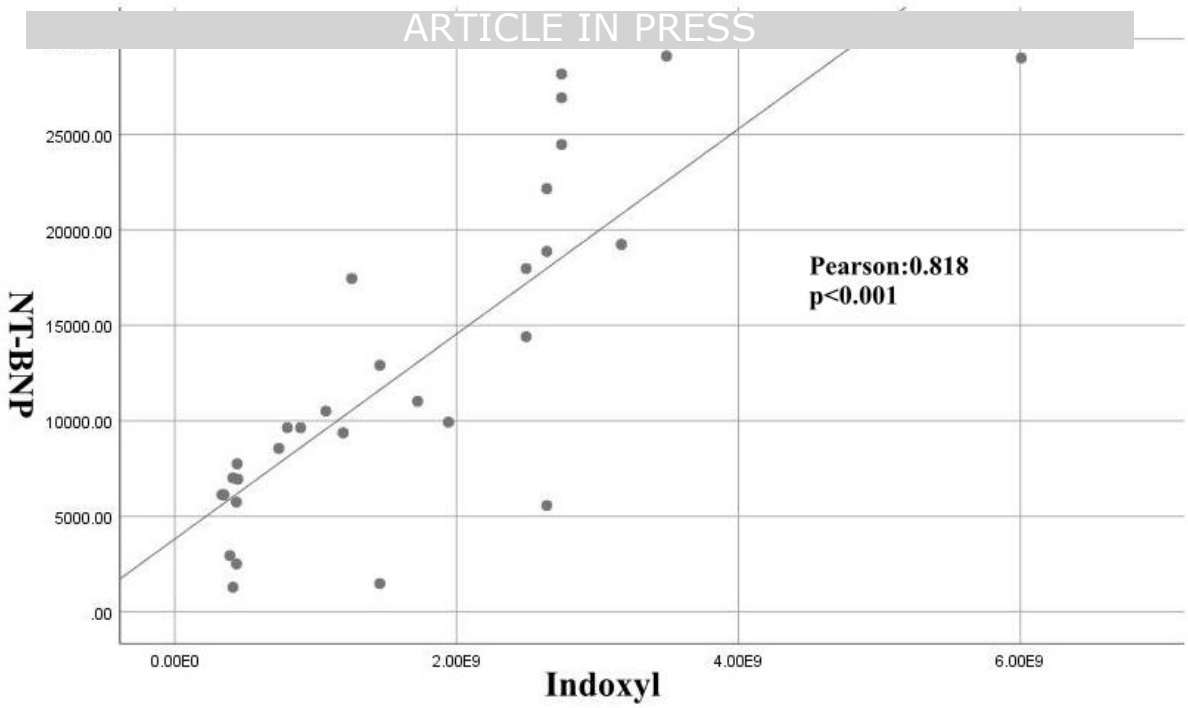
Figure 3: There was a significant positive correlation between

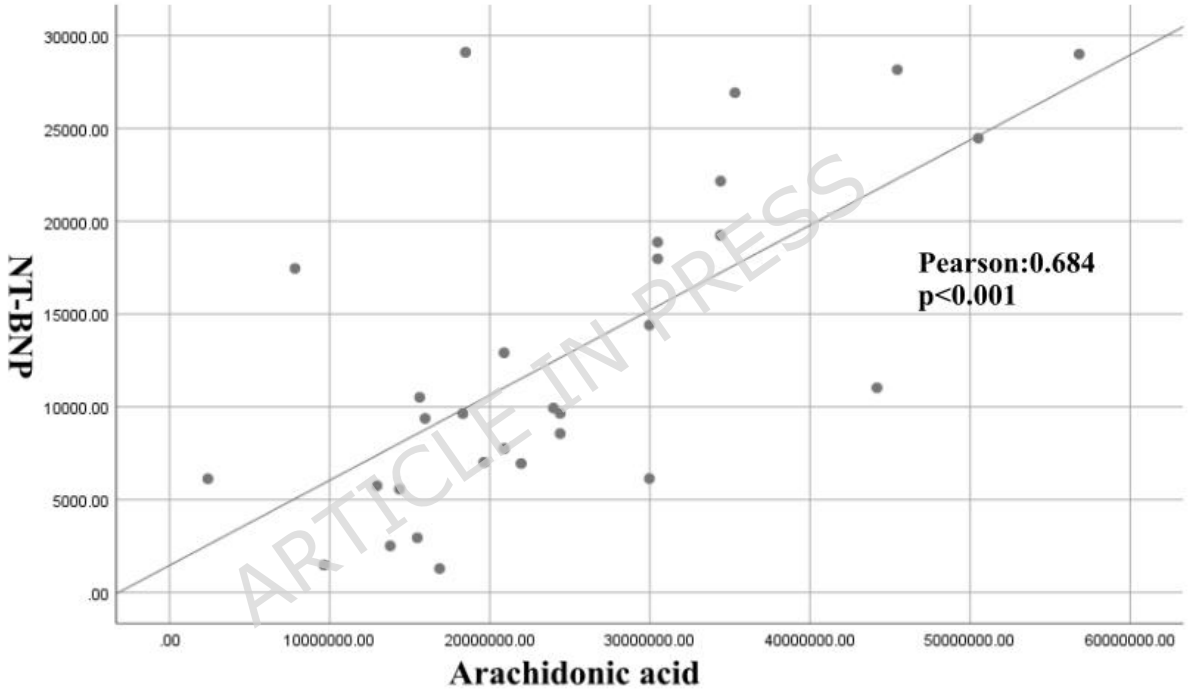
NT-BNP and Indoxyl, Arachidonic acid and Erucic acid.

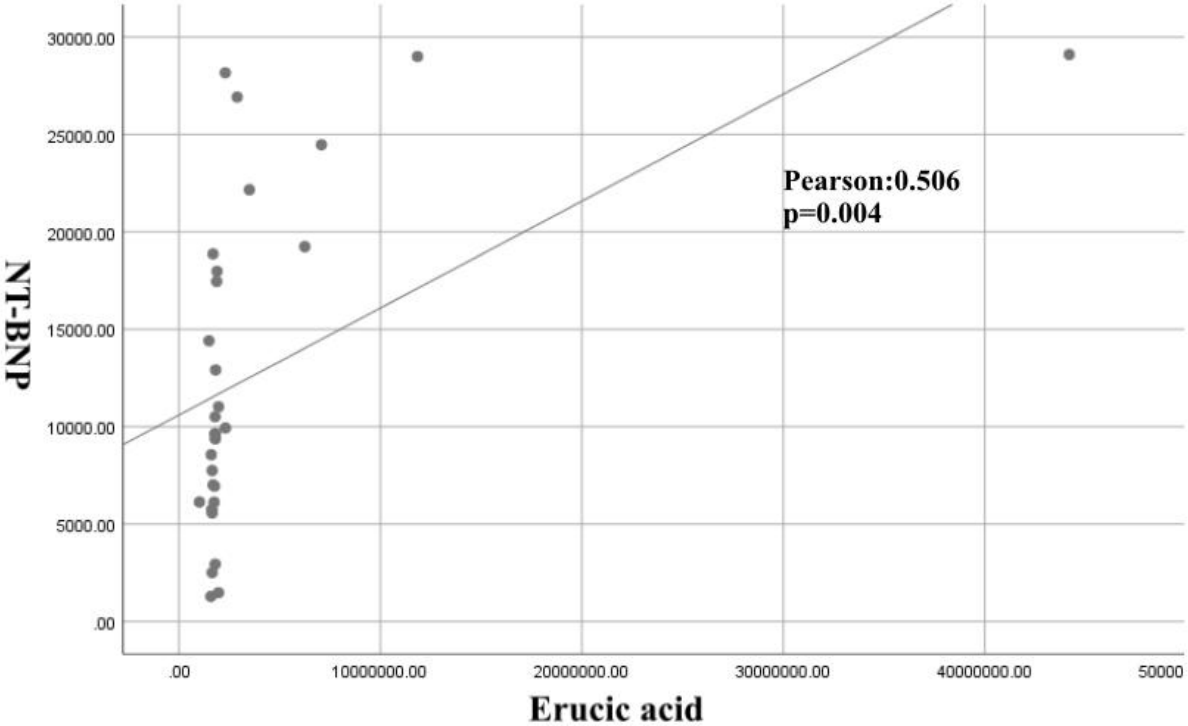
Figure 4: There was a significant positive correlation between cardiac function scores and Indoxyl and Arachidonic acid.

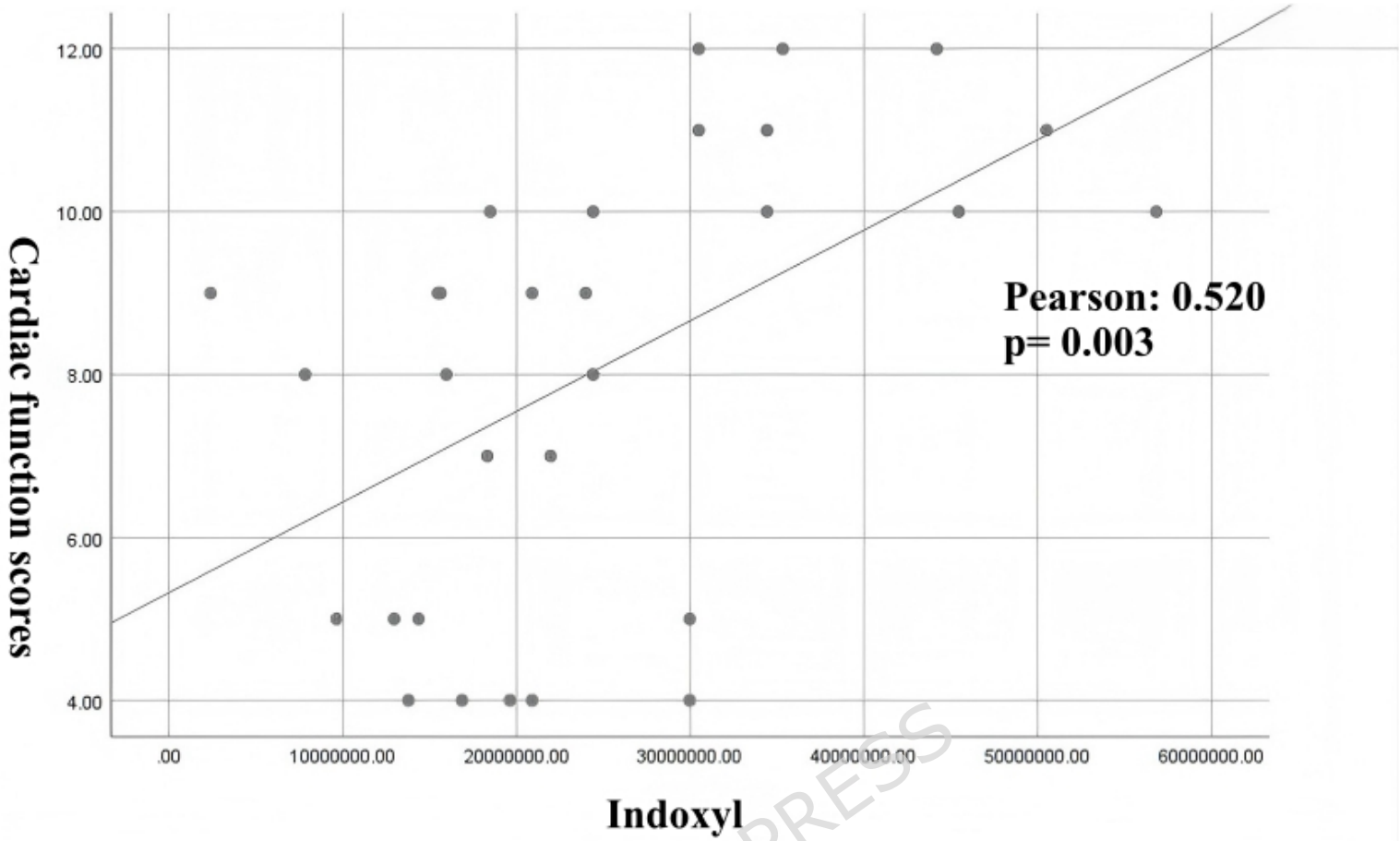












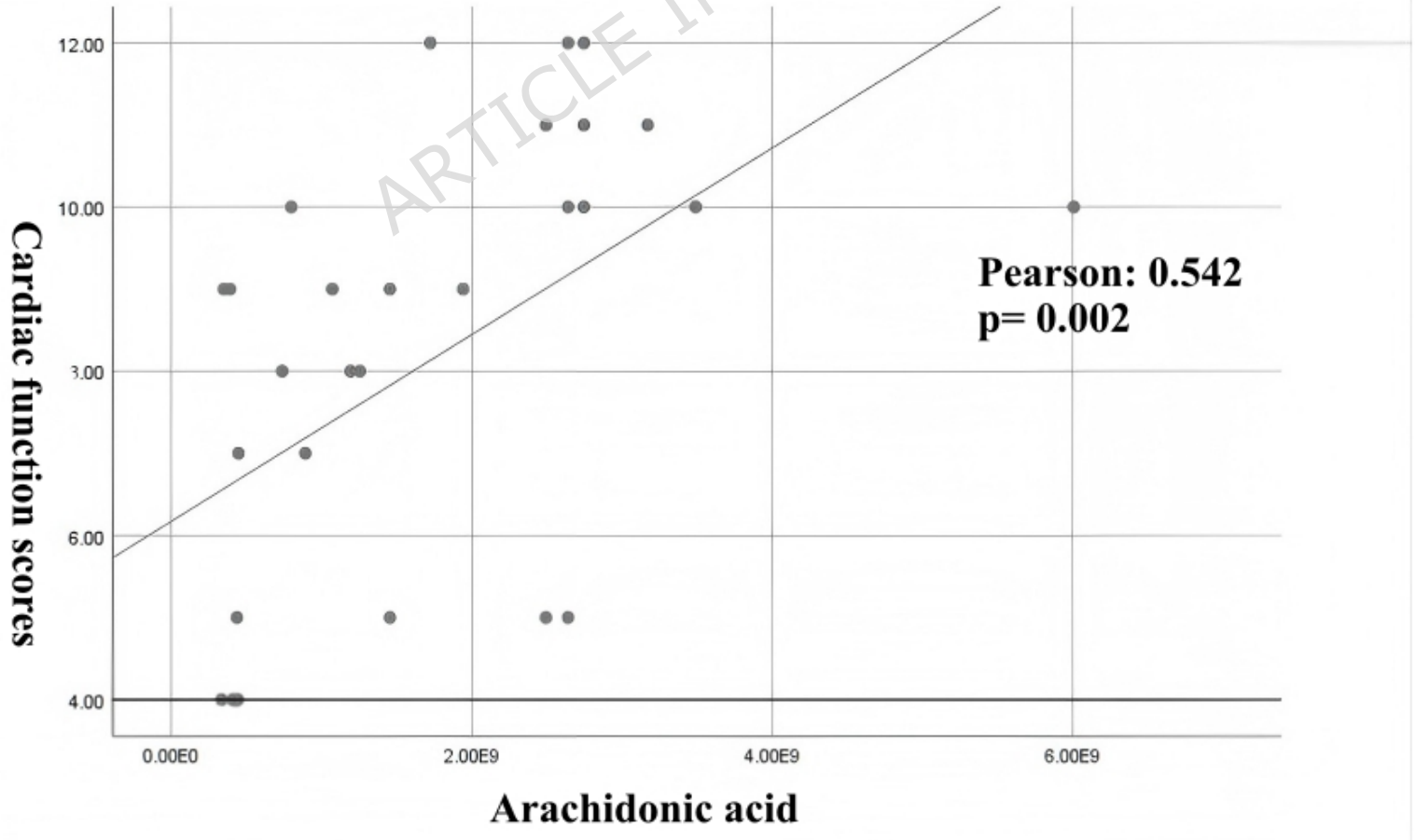


Table 1. Comparison of the general data between the two groups

	Heart failure group	Control group	P value
Age	1.7 (1.0, 3.2)month	1.8 (1.1, 2.6)month	0.953
Gender			
Male	17	20	0.426
Female	13	10	
Weight	4.3 (3.5, 5.2)kg	4.8 (3.6, 5.4)kg	0.609
Feeding methods			
Breast feeding	10	15	0.305
Formula feeding	8	4	
Mixed feeding	12	11	

Table 2. General clinical data of the heart failure group

Number of patients with various diseases	
Ventricular septal defect	26
Patent ductus arteriosus	3
Aortopulmonary window	1
NT-BNP	9786 (6130, 18961)pg/ml
Cardiac function scores	9 (5, 10)
Pulmonary artery pressure	61 (48, 73)mmHg
Size of the left-to-right shunt opening	7.8 (6.8, 8.5) mm
Left ventricular ejection fraction	63.2 (61.9, 64.9)%
Left ventricular end-diastolic diameter	23 (21.8, 24.6)mm