

## EFFECT OF CARDIOPULMONARY BYPASS ON ANNEXIN A1 EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF CHILDREN WITH CONGENITAL HEART DISEASE

UTICAJ KARDIOPULMONARNOG BAJPASA NA EKSPRESIJU ANEKSINA A1 U MONONUKLEARNIM ČELIJAMA PERIFERNE KRVI DECE SA UROĐENIM SRČANIM OBOLJENJIMA

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**Summary:** This study aimed to investigate the effect of cardiopulmonary bypass (CPB) on Annexin A1 expression in the peripheral blood mononuclear cells (PBMCs) of children with congenital heart disease (CHD). A total of 30 children receiving CPB for interventricular septal defect were included. Peripheral blood was collected before and after CPB. PBMCs were collected by density gradient centrifugation. Protein extraction was performed by lysis and subjected to 2D-QUANT for protein quantitation. Isoelectric focusing electrophoresis (IEF) was carried out followed by gel image analysis. Protein spots with a difference in expression of >1.5 fold were collected as candidate proteins which were subjected to mass spectrometry for the identification of differentially expressed proteins. Western blot assay was employed to confirm the expressions of target proteins. Peripheral blood collected at two time points was subjected to two-dimensional electrophoresis, and a total of 12 differentially expressed proteins were identified. Of them, 5 proteins had decreased expression before CPB (T0) but their expressions increased after CPB (T1); the remaining 7 proteins had increased expressions before CPB but their expressions reduced after CPB. One of these differentially expressed proteins was Annexin A1. Western blot assay confirmed that Annexin A1 expression began to increase at 0.5 h after CPB, and the increase of Annexin A1 was more obvious after CPB. Our findings primarily indicate the potential mechanism underlying the role of PBMC in inflammatory response following CPB, and provide a target for the prevention and control of post-CPB systemic inflammatory response syndrome (SIRS).

**Keywords:** annexin A1, cardiopulmonary bypass, peripheral blood mononuclear cells, congenital heart disease

**Kratka sadržaj:** Svrha studije bila je da se istraži uticaj kardiopulmonarnog bajpasa (KPB) na ekspresiju aneksina A1 u mononuklearnim ćelijama periferne krvi (MČPK) dece sa urođenim srčanim oboljenjima (SO). Istraživanjem je obuhvaćeno ukupno 30 dece kod koje je zbog interventrikularnog septalnog defekta izveden kardiopulmonarni bajpas. Uzorci periferne krvi uzeti su pre i posle zahvata. MČPK su sakupljene centrifugiranjem u gradijentu gustine. Proteini su ekstrahovani lizom a kvantitacija proteina obavljena je metodom 2D-QUANT. Izvršena je elektroforeza izoelektričnim fokusiranjem (EIF) a potom analiza *gel image*. Proteinske tačke sa razlikom u ekspresiji >1,5 puta uzete su kao proteini kandidati, koji su podvrgnuti masenoj spektrometriji radi identifikovanja različite ekspresije proteina. Za potvrđivanje ekspresija ciljnih proteina upotrebljen je test *Western blot*. Uzorci periferne krvi uzeti su dva puta u određenom vremenskom razmaku i podvrgnuti dvodimenzionalnoj elektroforezi te je identifikovano ukupno 12 diferencijalno izraženih proteina. Od toga je 5 proteina imalo smanjenu ekspresiju pre KPB (T0), ali se njihova ekspresija posle KPB povećala (T1); preostalih 7 proteina imalo je povećanu ekspresiju pre KPB, ali se njihova ekspresija smanjila posle KPB. Jedan od ovih diferencijalno izraženih proteina bio je aneksin A1. Testom *Western blot* potvrđeno je da je ekspresija aneksina A1 počela da raste 0,5 h posle KPB, dok je porast aneksina A1 posle KPB bio upadljiviji. Naši nalazi prevashodno ukazuju na potencijalni mehanizam uloge MČPK u inflamatornom odgovoru posle KPB i pružaju metu za prevenciju i kontrolu sindroma post-KPB sistemskog inflamatornog odgovora (SSIO).

**Ključne reči:** aneksin A1, kardiopulmonarni bajpas, mononuklearne ćelije periferne krvi, urođeno srčano oboljenje

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### Introduction

Surgery is the most effective strategy for the treatment of congenital heart diseases (CHD) in children for which cardiopulmonary bypass (CPB) pro-

vides favorable conditions. However, CPB may also activate complements, cause release of endotoxin and granulocyte activation and induce the expressions of adhesion molecules and proinflammatory factors, resulting in a systemic, non-specific inflammatory reaction which is the main cause of post-operative complications, including the compromised functions of the heart, lung and kidney, post-perfusion lung, respiratory failure, liver and kidney failure, gastrointestinal edema and cerebral injury. These complications finally attribute to the high morbidity and mortality (1–3). There is evidence showing that the peripheral blood mononuclear cells (PBMC) secrete a series of cytokines involved in the inflammatory reaction following CPB, but the exact molecular mechanism is largely unclear (4).

To investigate the specific mechanism of the involvement of PBMC in the post-CPB inflammatory reaction, proteomics was employed in the present study to observe the proteomic profiling of PBMC following CPB in children with CHD. Our results may provide markers to early predict organ dysfunction after CPB and present a target for drug development for these patients to improve their prognosis.

## Patients and Methods

### *Main reagents and drugs*

Propofol injection (AstraZeneca, UK), cisatracurium besylate (Jiangsu Hengrui Co., Ltd), fentanyl citrate (Yichang Renfu Pharmaceutical), heparin sodium injection (Hebei Changshan Biochemical Pharmaceutical Co. Ltd.), protamine (Shanghai First Biochemical Pharmaceutical Co. Ltd.), separation medium for PBMC (10771, Sigma, USA), Anti-Annexin A1 polyclonal antibody (CST, USA), rabbit horseradish peroxidase conjugated secondary antibody (CST, USA), Chemiluminescence kit (Pierce, France) were all used in the present study.

### *Main equipment*

Drager PM 8060 multifunctional anesthesia unit, IntelliVue MP 70 multifunctional monitor, Vividi TEE (GE, USA), Orchestra workstation for intravenous infusion (Fresenius Kabi), BIS monitor (ASPECT, SN: C022331, USA), Medex pressure sensor (Smiths Medical, USA), Sungwon central venous catheter (triple lumen) (Sungwonmedical, Korea) and low-temperature centrifuge (Beckman, USA) were used in the study.

### *General information*

A total of 30 children receiving surgical intervention due to a ventricular septal defect, aged 5–10 years, were recruited from our hospital, and the ASA grade was II. Surgery was performed on a non-beating heart through a cardiopulmonary bypass, and these

patients had no history of other diseases. Oxygen breathing was done at 2–5 L/min through a nasal cannula. Five lead ECG, SpO<sub>2</sub>, NIBP and BIS were monitored. Fentanyl (4 µg/kg), propofol (2.0 mg/kg) and atracurium (0.15 mg/kg) were used for anesthesia induction. Following endotracheal intubation, arterial pressure was directly monitored by radial artery catheterization. Then, right internal jugular vein puncture was performed followed by catheterization with a triple lumen central venous catheter for the monitoring of CVP and administration of vasoactive drugs. During the surgery, sevoflurane, propofol and fentanyl were used for anesthesia maintenance. ECG, SpO<sub>2</sub>, radial arterial pressure, CVP, nasopharyngeal temperature and urine volume were monitored continuously and the blood gas analysis was performed at the designed time points.

### *Sample collection*

After anesthesia, 3–5 mL of heparin anti-coagulated arterial blood were collected before (T1) and after (T2) surgery of which 3 ml were used for the isolation of PBMC by density gradient centrifugation. Then, cell lysis was performed in lysis buffer of 4 fold volume of cell suspension at 4 °C for 30 min followed by centrifugation at 12000 rpm for 15 min. The supernatant was collected. Finally, the lysate in different groups was mixed and the proteins in each group were enriched. The protein concentration was determined by the 2D-QUANT method.

### *Two-dimensional electrophoresis*

The linear gel bands (24 cm; pH 3–10) were used. A total of 300 µg (about 20 µL) were mixed in loading buffer (7 mmol/L urea, 2 mol/L sulfourea, 4% CHAPS, 65 mmol/L DTT, 0.2% IEF buffer and bromophenol blue) at a final volume of 450 µL. The protein samples were added to the hydration plate in which IPG gel was put. One hour later, the plate was sealed by 2–3 mL of mineral oil followed by incubation for 16 at room temperature. Isoelectric focusing electrophoresis (IEF) was then performed. Subsequently, the gel was obtained and the mineral oil was removed with a filter followed by SDS-PAGE. The gel was collected and marked by cutting a corner. Analytical silver-staining was performed with mass spectrum compatible silver nitrate.

### *Gel image analysis*

Images were captured by using the UMAX PowerLook1100 projection scanner and then analyzed using PDQuest7.1.0 software. The detection of protein spots and their quantitation, background removal and spot matching were performed. Following automatic protein spot detection, the specific spot

was assigned an SSP number followed by spot editing to remove some confounding spots. In the spot matching, a reference gel was selected from T0 gel and some landmarks were established followed by matching other spots. The un-matching spots were automatically added to the reference gel. The quantity of a protein spot was defined as the sum of the intensities of all pixels. To more accurately reflect the changes in the quantity of protein spots, the content of each spot was expressed as a percentage (%vol) of the quantity of the specific spot to the quality of total spots, which was also known as the relative content of specific spot. Following comparisons, the protein spots with a difference in expression of >1.5 fold were selected as candidates for subsequent mass spectrometry.

#### *Detection of differentially expressed proteins by mass spectrometry*

The gels containing the above target protein spots were collected and then incubated in 100 mmol/L  $\text{NH}_4\text{HCO}_3$  containing 30% ACN for decoloration. Then, dehydration was performed with 100% ACN and subsequently drying. These gels were treated with 10  $\mu\text{L}$  of trypsin solution (12.5  $\mu\text{g}/\text{mL}$ ) at 4  $^\circ\text{C}$  for 1 h. After removal of the trypsin solution, these gels were incubated in 50 mmol/L  $\text{NH}_4\text{HCO}_3$  (30–50  $\mu\text{L}$ ) at 37  $^\circ\text{C}$  for 14–16 h. By using a vacuum dryer, the volume was reduced to 10  $\mu\text{L}$ , and ZipTip was used to remove the ions. Elution was performed with 1  $\mu\text{L}$  of 50% CAN (containing 0.1% TFA) to acquire the degraded peptide segments. The 1  $\mu\text{L}$  of CHCA (5 mg/mL) was added to the eluate and the mixture added to the stainless steel sample plate. Following crystallization, mass spectrometry was performed according to the instructions in 4000 Series Explorer Software. The MS/MS and MS spectra were collected and compared with those in the database to analyze the protein sequence. The phosphorylated proteins potentially involved in the post-CPB inflammatory reaction were identified.

#### *Confirmation by Western blot assay*

Proteins of equal quantity were mixed in 5  $\mu\text{L}$  of 4 $\times$  loading buffer followed by being heated at 95  $^\circ\text{C}$  for 5 min for protein denaturation. Then, 12% SDS-PAGE was performed and proteins were transferred onto a PVDF membrane which was then blocked in 5% non-fat milk in TBST (20 mmol/L Tris-base, 137 mmol/L NaCl, 0.1% Tween-20) at 4  $^\circ\text{C}$  overnight. After washing in TBST, the PVDF membrane was treated with mouse anti-Annexin A1 antibody (1:1000) at room temperature for 1 h followed by washing in TBST thrice. Subsequently, the membrane was treated with HRP conjugated anti-mouse secondary antibody (1:2000) at room temperature for 1 h. After washing in TBST thrice, visualization was performed

for 1 min and the representative photographs were captured and analyzed in a Kodak IS2000R image workstation.

#### *Statistical analysis*

Quantitative data were expressed as mean  $\pm$  standard deviation ( $\bar{x}\pm s$ ) and One-Way ANOVA Test was carried out for analysis. Statistical analysis was performed with SPSS version 13.0 and a value of  $P<0.05$  was considered statistically significant.

## **Results**

### *General information*

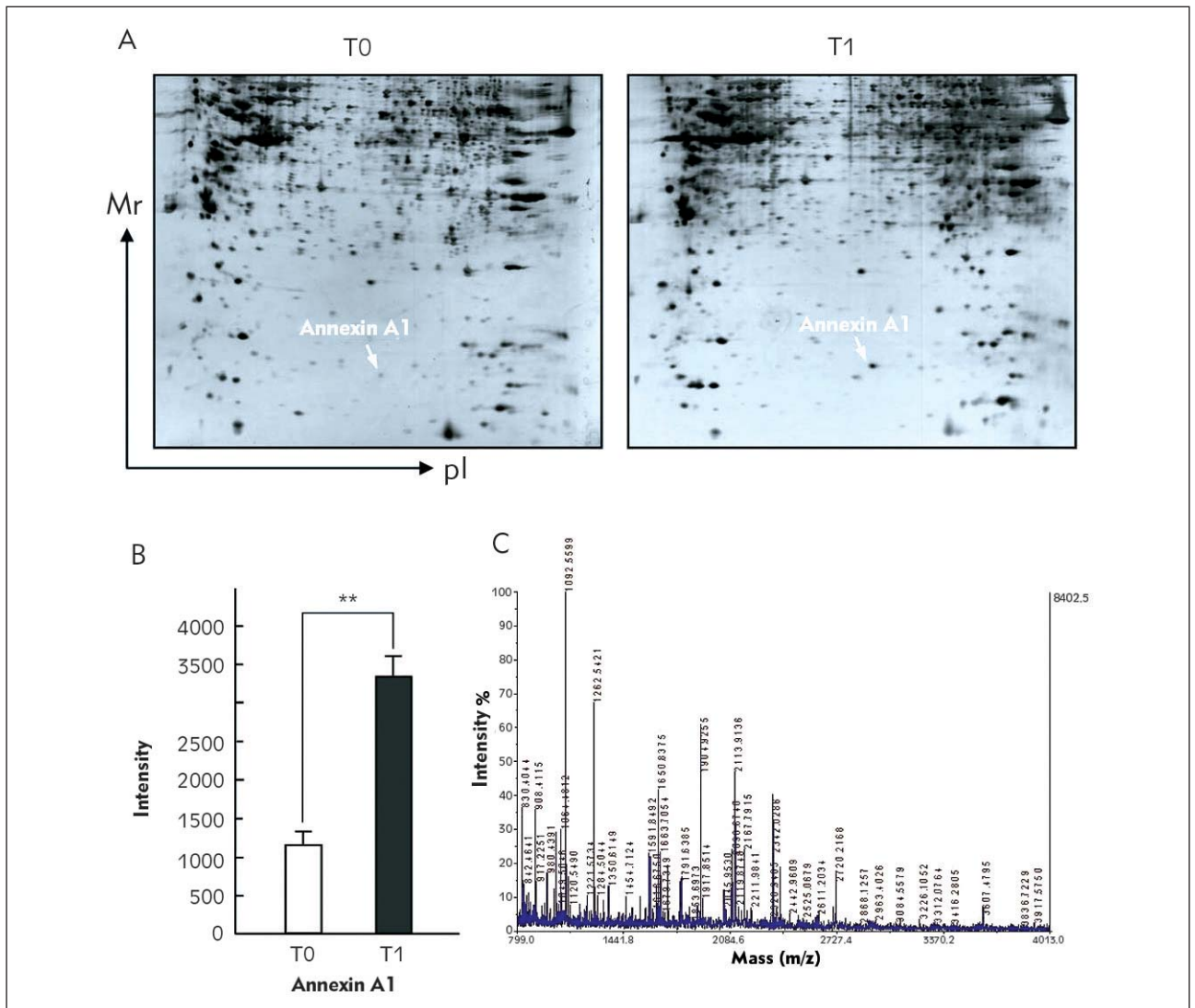
Surgery was performed successfully in all children, who recovered smoothly after intervention. Post-operative complications were not observed and the patients were discharged after recovery (Table I).

Two-dimensional electrophoresis was performed in PBMC which was collected before (T0) and after (T1) surgery. A total of 12 differentially expressed proteins were identified (Figure 1) of which 5 had down-regulation in T0 group but up-regulation in T1 group, and the remaining 7 had up-regulation in T0 group but down-regulation in T0 group. Mass spectrometry was performed in these 12 proteins and difference was found in only one protein (Annexin A1) (Figure 2).

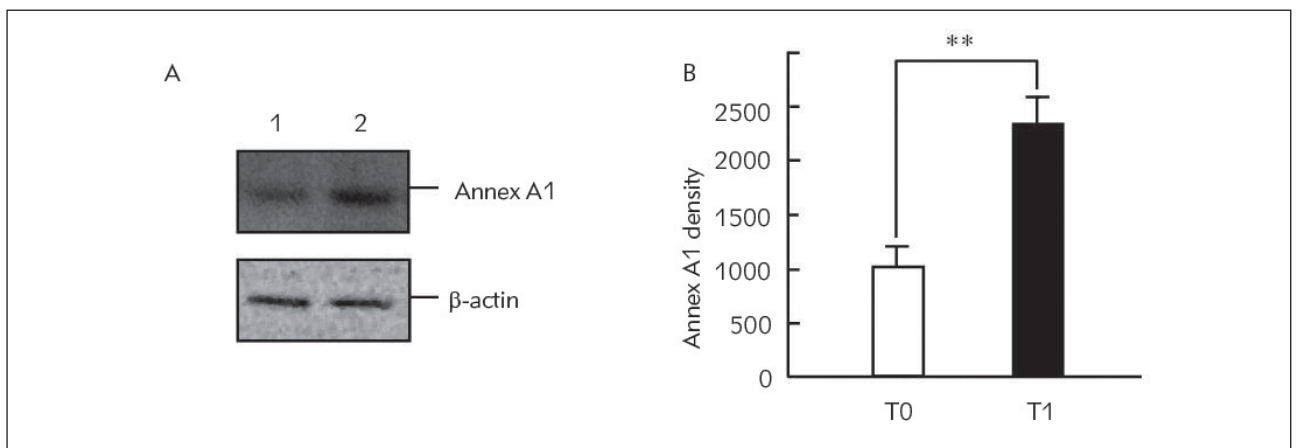
Annexin A1 is an important protein regulating inflammation and plays critical roles in the generation of inflammatory metabolites and the adhesion between neutrophils/monocytes and endothelial cells (5, 6). Thus, the Annexin A1 expression was further confirmed by the Western blot assay. Results showed the Annexin A1 expression began to increase at 0.5 h after CPB, and significant increase of Annexin A1 expression was observed at the end of surgery

**Table I** Peri-operative clinical information of patients.

Characteristics	mean $\pm$ standard deviation
Gender (M/F)	17/13
Time of CPB (min)	87.9 $\pm$ 33.4
Time of aortic occlusion (min)	58.8 $\pm$ 25.7
Volume of blood loss (mL)	245.5 $\pm$ 103.6
Time of endotracheal intubation (h)	17.7 $\pm$ 4.4
Days in ICU after surgery (d)	2.4 $\pm$ 0.5
Days of fever (d)	0.7 $\pm$ 1.0
Days of hospitalization after surgery (d)	14.7 $\pm$ 2.7



**Figure 1** Expression and identification of Annexin A1 in T0 group and T1 group  
 A. 2D electrophoresis of proteins in PMBC in T0 group and T1 group; B. Expression of Annexin A1 in T0 group and T1 group (\*\*P<0.001, n=6); C. Identification of Annex A1 by mass spectrometry.



**Figure 2** Confirmation of Annexin A1 expression in PBMC in T0 group and T1 group by Western Blot assay.



( $P=0.000$ ). This finding suggested that CPB promoted the Annexin A1 expression in the PBMC, which was consistent with the results in 2D electrophoresis.

## Discussion

The CPB induced complex inflammatory reaction is currently regarded as the pathological basis of a series of subsequent complications (7). After CPB, post-perfusion lung and respiratory failure, liver and kidney dysfunction, gastrointestinal edema and cerebral injury may occur as primary or secondary sequences of the inflammation cascade (8, 9). CPB can activate the endothelial cells and induce the release of cytokines affecting the prognosis of patients undergoing CPB (10). In children with CHD, surgery is frequently performed in the presence of CPB. In the childhood, the functions of a majority of organs (such as pulmonary circulation) are immature and thus children are susceptible to severe complications of CPB (11). In the CPB induced systemic inflammatory response syndrome (SIRS), the circulating PBMC are not only the effector cells but the cells regulating the inflammatory reaction (12). Thus, investigating the effect of CPB on the proteomic profiling in PBMC is helpful to elucidate the role of PBMC in the CPB induced SIRS and to identify the inflammation related signal transduction proteins involved in the SIRS.

In the present study, the two-dimensional electrophoresis of proteins was employed to separate the proteins in PBMC before and after surgery. To clarify the role of PBMC in the CPB induced inflammation, we compared the two-dimensional map at two time points, and the standard was set for the identification of differentially expressed proteins: a difference in expression of  $>2.5$  fold. The aim was to find the proteins the expressions of which were significantly changed in PBMC following CPB, and these proteins would be the potential participants in CPB induced SIRS. In our study, a total of 10 differentially expressed proteins were identified. Among them, 4 proteins had increased expression before surgery and the remaining 6 had elevated expression after surgery. Subsequently, mass spectroscopy was performed to detect these 10 proteins, one of which was identified as Annexin A1. The Annexin A1 expression after surgery was markedly increased when compared with that before surgery.

Annexin A1 is one member of the structurally related calcium-dependent phospholipid binding proteins, and widely expressed in humans (13). Annexin A1 has been found to be involved in many physiological processes including cellular secretion, signal transduction, inflammatory reaction and apoptosis (14, 15). In the inflammatory reaction, Annexin A1 is an important inflammation regulating protein and plays crucial roles in the generation of inflammatory metabolites and adhesion between neutrophils/monocytes and endothelial cells (16, 17). Studies have shown that Annexin A1 can inhibit the activities of cytoplas-

mic phospholipase A2, inducible nitric oxide synthase and inducible cyclooxygenase (COX-2) in microglia, and suppress the release of IL-1, IL-6 and TNF- $\alpha$ , which prevent the excessive inflammatory response (18–20). Thus, the Annexin A1 expression in PBMC was further confirmed. Following anesthesia, the Annexin A1 expression in PBMC was relatively low, but dramatically increased after surgery. This indicates that CPB can induce the Annexin A1 expression in children which then inhibit the excessive inflammatory response. To confirm the findings in two-dimensional electrophoresis, another 16 children receiving surgical intervention due to a ventricular septal defect in the presence of CPB were recruited and the PBMC were also collected at corresponding time points. Following protein extraction, the Western blot assay was performed to detect the Annexin A1 expression. The results were consistent with those in two-dimensional electrophoresis. That is, the Annexin A1 was significantly increased following surgery in the presence of CPB when compared with that before surgery. The mechanism of up-regulated Annexin A1 expression is still unclear. We speculate that the interaction between blood and artificial materials, destruction of cellular components and the surgical wound may induce the SIRS. As a negative feedback, some antiinflammatory factors (including IL-10) are also activated to prevent the excessive inflammatory response. Annexin A1, as an important antiinflammatory protein, is also activated and subsequently inhibits the excessive inflammatory response to a certain extent, producing a protective effect (21). However, whether Annexin A1 is activated by CPB directly or by other inflammatory mediators generated following CPB, the relationship between the CPB time and the degree of Annexin A1 activation and the correlation between the extent of Annexin A1 activation and prognosis are largely unknown and require to be further studied.

Taken together, we employed the proteomics technique to investigate the influence of CPB on the proteomic profiling in PBMC, and the differentially expressed proteins were further analyzed by using bioinformatics. A total of 11 differentially expressed proteins were successfully identified and the Annexin A1 expression was further confirmed by the Western blot assay. Our results primarily disclose the potential molecular mechanism of CPB induced inflammatory reaction and provide evidence for future studies. Moreover, our findings provide a theoretical basis for the prevention and control of post-CPB SIRS. However, how the differentially expressed proteins including Annexin A1 are activated in the CPB induced SIRS and which signaling pathways are involved in the post-CPB SIRS are questions that remain to be answered.

## Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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