Protocol to study the effects of AMPK-mTOR/PINK-Parkin dual signaling pathways on the formation of coronary heart disease showing blood stasis symptom pattern based on traditional Chinese medicine theory of “heart governing blood and vessels”

An-Ni Chen1, Man-Li Zhou1, Yun-Feng Yu1, 2, Kang-Yan Tang1, Lin-Juan Yang1, Mo-Fei Shi1, Wei-Xiong Jian1. 2*

1College of Traditional Chinese Medicine, Hunan University of Chinese Medicine, Changsha 410208, China. 2Department of Spleen-stomach, The First Affiliated Hospital of Hunan University of Chinese Medicine, Changsha 410007, China. '*'National Key Discipline of Traditional Chinese Medicine Diagnostics, Hunan Provincial Key Laboratory, Hunan University of Chinese Medicine, Changsha 410208, China.

*Corresponding to: Wei-Xiong Jian, College of Traditional Chinese Medicine, Hunan University of Chinese Medicine, No. 300 Xuexi Road, Hanpu Science Education Park, Yuele District, Changsha 410208, China. E-mail: daxiong20001977@163.com.

Author contributions
An-Ni Chen and Wei-Xiong Jian conceived and designed this study. An-Ni Chen, Man-Li Zhou and Yun-Feng Yu wrote the manuscript. Kang-Yan Tang, Lin-Juan Yang, Mo-Fei Shi and Wei-Xiong Jian refined the protocol. All authors contributed to the article and approved the submitted version.

Competing interests
The authors declare no conflicts of interest.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (No. 81973753 to Jian WX), Hunan Postgraduate Scientific Research Innovation Project (CX 2022T81) and Hunan University Students’ Innovation and Entrepreneurship Training Program (2022J205116).

Peer review information
Aging Communications thanks Yang Yang and other anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
CHD, coronary heart disease; AS, atherosclerosis; TCM, traditional Chinese medicine; FG, fibrinogen; AMPK, adenosine monophosphate-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; VIP, variable importance in projection.

Citation

Abstract
In this study, we aim to combine gene transfection techniques with the modeling methods previously employed by the research group to deeply investigate the corresponding theories of traditional Chinese medicine regarding “myocardial energy metabolism” and “aortic thrombosis”. Our goal is to elucidate the biological mechanism underlying the occurrence and development of coronary heart disease with blood stasis syndrome from the perspectives of “heart and vessels” and “Qi” (in traditional Chinese medicine, it refers to the most fundamental and subtle substances that constitute the human body and maintain life activities. At the same time, it also has the meaning of physiological function. In terms of traditional Chinese medicine, Qi and different words are used together to express different meanings) and blood”. The research content is divided into four modules as follows: 1. establishment of an animal model of coronary heart disease with blood stasis syndrome through fibrinogen overexpression. 2. Investigation of the mitochondrial quality control system in coronary heart disease with blood stasis syndrome under fibrinogen overexpression. 3. Study of platelet autophagy in coronary heart disease with blood stasis syndrome under fibrinogen overexpression. 4. Examination of the relationship between the AMPK-mTOR pathway and metabolism in platelet autophagy of coronary heart disease with blood stasis syndrome under fibrinogen overexpression. Ninety-six Sprague Dawley rats will be randomly assigned to the following groups: control group, model group, fibrinogen group and adenov-associate virus group. All rats will undergo a 14-week model construction process, and modern molecular biology methods will be employed to evaluate the model and examine relevant research indicators. The obtained data will be analyzed according to a predefined statistical analysis plan.

Keywords: coronary heart disease with blood stasis syndrome; heart governing blood and vessels; AMPK-mTOR/PINK-Parkin; energy metabolism; platelet autophagy; study protocol
Background

Coronary heart disease (CHD) falls under the category of ischemic heart disease, which is the most common type of atherosclerosis (AS) leading to organ damage. The prevalence and fatality rate of CHD surpass those of tumors and other diseases, making it a significant global public health concern [1]. Since the 1990s, epidemiological studies worldwide have identified a range of “traditional risk factors” for CHD, including age, smoking and total cholesterol. With the advancement of evidence-based medicine, our understanding of CHD’s pathogenesis has evolved, with “metabolism-related factors” being recognized as “new risk factors” and employed in primary and secondary prevention of CHD [2]. Metabolic abnormalities play a role in the occurrence and progression of CHD through various mechanisms. Insufficient oxygen supply to the heart leading to abnormal myocardial energy metabolism, oxidative stress, pre-inflammatory state, and pre-thrombotic state induced by the release of metabolites such as reactive oxygen species, all contribute to the development of CHD [3].

Blood stasis syndrome is a commonly encountered clinical syndrome in CHD [4]. Traditional Chinese medicine states, “the Qi (in traditional Chinese medicine, it refers to the most fundamental and subtle substances that constitute the human body and maintain life activities. At the same time, it also has the meaning of physiological function. In terms of traditional Chinese medicine, Qi and different words are used together to express different meanings) of the human body swims in the blood, and the Qi is the commander of the blood, and the blood runs accordingly”. Many scholars believe that the normal circulation of blood in the human body relies on the abundance of heart Yang (the exciting, driving, warm side of the heart), the adequacy of blood filling, and the smooth flow of pulse channels. The coordinated functioning of heart Yang, including warmth, propelling force, and stabilization, ensures the circulation of blood within the vessels and throughout the body. Under pathological conditions, the balance of heart Qi’s (the essence of the heart is manifested as the heart governing blood and vessels, functional activity of the main divine mind) role in promoting blood circulation is disrupted, leading to poor blood circulation, stagnation in the pulse channels, formation of blood clots, and CHD with blood stasis syndrome occurs after the formation of static blood.

Some scholars have proposed that “Qi is functional, and the metabolism of Qi is related to energy metabolism” [5]. Adenosine triphosphate and “Qi” share a common material basis, and to some extent, have a common connotation [6]. Professor Jie Wang has also pointed out that “Qi” is the necessary material basis for maintaining normal physiological functions in the human body, and the theory of “benefiting Qi” is largely associated with correcting energy metabolism disorders [7]. In traditional Chinese medicine (TCM), there is a significant similarity in the physiological and pathological understanding of blood stasis syndrome and thrombosis [8]. Thrombus formation is often accompanied by changes in platelets and other related factors. Thromboembolism resulting from thrombus formation exacerbates energy metabolism disorders caused by ischemia and hypoxia. It has been reported that energy deficiency leads to the accumulation of reactive oxygen species, which subsequently activates adenosine monophosphate-activated protein kinase (AMPK) [9]. AMPK is known as the intracellular energy sensor. Activation of AMPK up-regulates catabolism, regulates mitochondrial energy metabolism, and participates in platelet autophagy through phosphorylation of downstream proteins [10]. AMPK is closely associated with mitochondrial biogenesis and mitophagy. Activation of the AMPK signaling pathway triggers the translocation of PGC-1α from the cytoplasm to the nucleus and up-regulates the expression level of the mitophagy-related protein PARKIN [10, 11]. Platelet dysfunction plays a key role in the formation of AS, promoting not only thrombosis but also serving as an important link between inflammation and AS [12]. mTOR is situated downstream of AMPK, and there exists an antagonistic negative feedback relationship between mTOR and AMPK. When the body is under energy stress, the activation of mTOR is inhibited while autophagy is stimulated. There is a molecular basis for the occurrence of autophagy in platelets. The AMPK-mTOR signaling pathway regulates the induction of platelet autophagy, which takes place during the process of platelet activation [13]. However, the mechanism behind this process requires further clarification and improvement. Fibrinogen (FG) is a significant factor that contributes to increased blood viscosity [14]. It functions both as a coagulation factor and an inflammatory factor, playing a role in the early inflammatory response as well as the subsequent thrombosis of CHD [15]. Lee et al. employed metabolomics analysis techniques to determine the metabolic changes between resting and H2O2-treated human platelets [13]. Nevertheless, experimental research on how platelet metabolic changes participate in the formation of a rat model of CHD with blood stasis syndrome featuring FG overexpression has not been conducted thus far.

In this study, our focus will be on the rat model of CHD with blood stasis syndrome featuring FG overexpression. We aim to describe the scientific significance of “heart governing blood and vessels (the heart Qi drives the blood to run in the veins and flow throughout the body, circulating endlessly, playing a nutritive and moisturizing role)” from the perspectives of “myocardial energy metabolism” and “platelet autophagy”. Our approach will be based on modern research findings and will utilize the AMPK-mTOR/PINK1-Parkin dual signaling pathways. By exploring the pathological mechanisms underlying the occurrence and progression of CHD with blood stasis syndrome, we anticipate contributing to the future development of targeted treatments in clinical practice. The study assumption is depicted in Figure 1, as shown below.

Methods and design

Trial design

Based on the overexpression of FG and the TCM theory of “heart governing blood and vessels”, this study aims to investigate the connections between myocardial energy metabolism and platelet autophagy. To achieve this, four interconnected studies were conducted. The study procedures are depicted in Figure 2, as shown below.

Establishment of animal model of CHD with blood stasis syndrome in FG overexpression. After one week of adaptive feeding, a total of 96 Sprague Dawley rats were randomly divided into four groups for the experiment: the control group, model group, FG group and adeno-associated virus group. Except for the control group, the other three groups were fed a high-lipid diet. The construction of the

Figure 1 Modern scientific connotation mechanism of the theory of “heart governing blood and vessels”
rat model of CHD with blood stasis syndrome began by completing the transfection of the fibrinogen gene. The animal model was constructed following the previous modeling method established by our research group [16]. The virus used for transfection had a titer of $1.7 \times 10^{13}$ vg/mL, with a dosage of 200 μL per rat injection. The virus was constructed by Hancheng Biotechnological Company (Shanghai, China). After 7 days of adaptable feeding with a high-lipid diet, the rats were injected with vitamin D3 at a dose of 500,000 IU/kg. Following 14 days of high-fat feeding, a second injection of vitamin D3 at a dose of 200,000 IU/kg was administered. After 10 weeks of continuous modeling, the rats were subcutaneously injected with isopropenol at a dose of 5 mg/kg for three consecutive days. One week later, a standard II lead electrocardiogram was recorded. The successful modeling was determined by the persistent abnormality of blood lipid levels, hemorheology-related indicators, the formation of rat aortic artery plaque, and the elevation or significant depression ($\geq 0.1$ mV) of the ST segment in the electrocardiogram. The research involving experimental animals was approved by the Research Ethics Committee of Experimental Animal Center and Science and Technology Innovation Center of Hunan University of Chinese Medicine (ID: LL2022061601). The research group provided the application form for ethical review of animal experiment welfare for this study.

Study on mitochondrial quality control in the myocardium of CHD with blood stasis syndrome in FG overexpression. 1. The mitochondrial morphology of myocardial tissue in each group was observed using a transmission electron microscope. 2. Changes in myocardial enzyme indexes in each group were detected using enzyme-linked immunosorbent assay (ELISA). 3. The target protein of myocardial tissue in each group was analyzed using Western blot. 4. Changes in mitochondrial membrane potential of myocardial tissue in each group were detected using flow cytometry (FCM).

Study on platelet autophagy with blood stasis syndrome in FG overexpression. 1. FCM is used to detect changes in membrane surface proteins during platelet activation in each group. 2. The interaction between FG and platelet surface activating protein in each group is detected by the proximity ligation assay. 3. Transmission electron microscopy is used to observe changes in autophagy-related proteins in each group. 4. Western blot is used for the semi-quantitative analysis of platelet autophagy-related proteins in each group. 5. The degree of deposition of platelet aggregation-associated proteins in the arterial wall is detected by Immunofluorescence. 6. ELISA is used to detect changes in serum inflammatory indexes in each group.

Association of metabolites and AMPK-mTOR metabolic pathway regulating platelet autophagy. Autophagy plays a crucial role in regulating cellular metabolic capacity. Nonetheless, the impact of platelet autophagy-mediated metabolism on platelet function in the FG overexpression model of CHD with blood stasis syndrome is still unclear. In this study, we employed metabolomics analysis to investigate the metabolic alterations in platelets among each group. We identified metabolites that exhibited significant changes ($P < 0.05$, variable importance in projection (VIP) > 1.2) and conducted pathway analysis to further elucidate these findings.

### Criteria for successful construction of CHD with blood stasis syndrome in FG overexpression

To evaluate the successful construction of the animal model of CHD with blood stasis syndrome in FG overexpression, the following four indicators are utilized: 1. blood lipid and hemorheology tests: the normal value is determined based on the 95% reference value range of the control group. In comparison to the control group, the blood lipid levels and at least one parameter of blood rheology (plasma viscosity or whole blood viscosity) in the other groups should be higher than the normal value. 2. Hematoxylin-eosin staining: hematoxylin-eosin staining is performed on the aorta to assess the formation of aortic atheromatous plaques. 3. Electrocardiogram: the electrocardiogram of rats in the control group is used as a reference. ST segment elevation or significant depression ($\geq 0.1$ mV) is considered indicative of the syndrome. 4. Detection of fibrinogen overexpression effect: Western blot analysis is employed to determine FG protein expression levels, while quantitative polymerase chain reaction is used to assess FG mRNA expression levels. Significant differences, in comparison to the non-transfection group, indicate successful transfection. These indicators are crucial for evaluating the successful establishment of the FG overexpression model of CHD with blood stasis syndrome in animals.

### Evaluating indicators

Study on mitochondrial quality control in the myocardium of CHD with blood stasis syndrome in FG overexpression. 1. Mitochondrial morphology and numbers are observed under transmission electron microscope. 2. The contents of adenosine monophosphate-activated protein kinase.

Submit a manuscript: https://www.tmrjournals.com/aging
Study on platelet autophagy of CHD with blood stasis syndrome in FG overexpression. 1. The changes of membrane surface protein αIIbβ3 and P-selectin during platelet activation were detected by FCM. 2. The binding of FG to αIIbβ3 on platelets is confirmed by proximity ligation assay. 3. Transmission electron microscopy is used to observe the changes of autophagolysosome of platelets. 4. Semi-quantitative analysis of platelet autophagy related proteins (PINK1, Parkin, P62, ub-Mfn2, LC3II/LC3I, ATG5). 5. The degree of deposition of αIIbβ3 and FG in arterial wall is detected. 6. Serum TNF-α and IL-1β are determined by ELISA.

Association of metabolites and AMPK-mTOR metabolic pathway regulating platelet autophagy. Metabolic analysis is conducted to examine the metabolic alterations occurring in platelets within each group. Metabolites with significant changes (P < 0.05, VIP > 1.2) are selected. Pathway analysis will be performed to gain insights into the affected metabolic pathways and their potential implications in the context of the study.

Statistical analysis plans
The data will be organized using Excel, and statistical analysis will be conducted using SPSS version 21.0. The measurement data will be presented as mean ± standard deviation (X ± s). Normality and homogeneity of variance tests will be performed to assess the data. For example, comparisons between multiple groups with normality and homogeneity of variance, the least-significant difference test in one-way ANOVA will be employed. In cases where there is non-homogeneity of variance, Tamhane’s T2 test will be used. For independent samples that do not conform to normality, the non-parametric Kruskal-Wallis H test will be applied, and the measurement data will be represented by the median (M). A significance level of P < 0.05 will be considered statistically significant.

The metabolites identified through Progenesis QI V2.3 are subjected to screening based on specific criteria. This includes assigning 20 points for accurate molecular weight in primary mass spectrometry, 20 points for fragment identification in secondary mass spectrometry, and 20 points for isotope distribution. The total score should be equal to or greater than 50 points. These screening criteria generate a two-dimensional data matrix. For qualitative analysis of the data, multivariate statistical methods are employed using SIMCA 14.1 software. This includes principal component analysis and orthogonal partial least squares analysis. Differential metabolites are determined based on the VIP (VIP > 1.2) value of the first principal component in the orthogonal partial least squares analysis model, combined with the P value from a student’s t-test (P < 0.05). The names of the identified differential metabolites are then entered into the metaboanalyst 5.0 website (https://www metaboanalyst.ca/) for metabolic pathway analysis. A screening standard of P < 0.05 is applied to identify potential key metabolic pathways.

Discussion
The heart plays a pivotal role among the five zang (a collective name for the five organs of the heart, liver, spleen, lungs, and kidneys) viscera. In TCM, the theory of “heart governing blood and vessels” is a crucial concept within the framework of “visceral manifestations”. This theory effectively summarizes the primary physiological functions of the heart [17]. The normal circulation of blood in the human body relies on the abundance of heart yang, the adequacy of blood supply, and the unobstructed flow of pulse channels. When the heart Qi is insufficient, the function of “heart governing blood and vessels” becomes impaired, resulting in poor blood flow and stagnation within the veins, leading to the formation of “static blood (blood stagnation or clotting in the body)”. Over time, static blood accumulates and gives rise to blood stasis syndrome, which can be understood as a pathological condition in modern medicine [18]. With the continuous advancements in preclinical medicine, researchers have discovered a certain correlation between the concept of “Qi” in TCM and the notion of “mitochondria” in modern medicine [19]. Moreover, there are significant similarities in the pathological understanding of blood stasis syndrome in TCM and thrombosis in Western medicine [20].

Our previous research has demonstrated the critical role of dynamic balance between mitochondrial fusion and fission in myocardial cells for maintaining normal mitochondrial population and function. The dynamics of mitochondria undergo changes in response to variations in energy demand within myocardial cells. Mitochondrial fusion protein Mfn2 mainly has fusion effect in the early stage of the formation of CHD with blood stasis syndrome. Overexpression of Mfn2 can prevent mitochondrial fragmentation, increased membrane permeability, and apoptosis induced by ischemia and hypoxia in the formation of CHD with blood stasis syndrome. The expression level of Mfn2 remains high in cases of blood stasis syndrome, which may represent a compensatory mechanism to limit mitochondrial fragmentation. As the disease progresses, Mfn2 begins to mediate the process of mitochondrial autophagy, and it is ubiquitinated in a Parkin-dependent manner. This adjustment regulates the number of mitochondria to adapt to the cellular environment and avoids unnecessary division and fusion of mitochondria. OPA1, on the other hand, not only participates in inner membrane fusion but also maintains the integrity of the cristae. The decrease in OPA1 expression is closely associated with the accelerated development of CHD with blood stasis syndrome. Mitochondrial outer membrane protein Dp1 and receptor Fis1 play a splitting role in mitochondrial fission, particularly in conditions of sustained energy crisis. They separate damaged mitochondria from healthy ones, preparing them for mitochondrial autophagy, while simultaneously alleviating the energy demand-supply imbalance in the body.

The development of CHD is a complex and progressive pathological process, wherein mitochondrial dynamics-related proteins play a significant role. Platelets, as the primary responders in vascular circulation, contribute to arterial thrombosis through aggregation and adhesion [21]. So far, although numerous experimental studies have individually examined the AMPK-mTOR/PINK-Parkin signaling pathways, exploring the dual signaling pathways guided by the theory of “heart governing blood and vessels” is still in its early stages. Building upon previous research, this paper focuses on the theoretical discussion from two perspectives: “Qi” and “blood”, encompassing the main aspects of “energy metabolism” and “autophagy”. “Heart governing blood and vessel” is a crucial concept within the framework of “visceral manifestations” in TCM. Considering the rapid advancements in preclinical medicine and the modernization of TCM, it is more advantageous to promote precise treatments for CHD with blood stasis syndrome by integrating TCM with Western medicine. This approach allows for a comprehensive exploration of the scientific significance of “heart governing blood and vessel” in the language of modern medicine, particularly from a cellular biology perspective. Investigating the patterns of myocardial energy metabolism and the coordination of platelet autophagy phenotypes during the progression of CHD with blood stasis syndrome will contribute to future clinical developments in targeted treatments.

References
3. He H, Chen YQ. Analysis of research progress on different
metabolic factors and the occurrence and development of coronary heart disease. Prev Treat Cardiovas Dis 2020;10(02):94–96. (Chinese) Available at: https://lns.cnki.net/kcms2/article/abstract?v=3u0qgG8C44YOTIOATRk9bYV5YSj7sIoR1PA7RkjuxjAk4dHxokyBDxoxFPy4nsAAMaA7MIhapQ6G0dyq7BaHiZ3p0&uniplatform=ZKPT


5. Yan WM, Jiang YP. Discussion on the essence of "Qi" in traditional Chinese medicine. J Hunan Univ Med Chin 1980(01):46–52 + 54. (Chinese) Available at: https://lns.cnki.net/kcms2/article/abstract?v=3u0qghG8C44YOTIOATRk9bYV5YSj7sIoR1PA7RkjuxjAk4dHxokyBDxoxFPy4nsAAMaA7MIhapQ6G0dyq7BaHiZ3p0&uniplatform=ZKPT

6. Chen WW. Probe into the essence of "Qi" in traditional Chinese medicine from Bioenergy. J Beijing Univ Tradit Chin Med 1994;02. (Chinese) Available at: https://lns.cnki.net/kcms2/article/abstract?v=3u0qghG8C44YOTIOATRk9bYV5YSj7sIoR1PA7RkjuxjAk4dHxokyBDxoxFPy4nsAAMaA7MIhapQ6G0dyq7BaHiZ3p0&uniplatform=ZKPT

7. Jiang WR, Wang J. Relationship between pectoral qi and myocardial ischemia in patients with chronic heart failure. China J Tradit Chin Med Pharm 2017;32(05):2084–2086. (Chinese) Available at: https://lns.cnki.net/kcms2/article/abstract?v=3u0qghG8C44YOTIOATRk9bYV5YSj7sIoR1PA7RkjuxjAk4dHxokyBDxoxFPy4nsAAMaA7MIhapQ6G0dyq7BaHiZ3p0&uniplatform=ZKPT

8. Meng F, Wang XH, Lu HR. Textural analysis on Blood Stasis Syndrome origin. J Basic Chin Med 2020(05):569–570 + 574. (Chinese) Available at: https://lns.cnki.net/kcms2/article/abstract?v=3u0qghG8C44YOTIOATRk9bYV5YSj7sIoR1PA7RkjuxjAk4dHxokyBDxoxFPy4nsAAMaA7MIhapQ6G0dyq7BaHiZ3p0&uniplatform=ZKPT


10. Cao ST. AMPK-PINK1/Parkin Mediated Mitophagy on Restoration of Intestinal Barrier Damaged by Oxidative Stress in Piglets and Curcumin’s Regulation Mechanism. Zhejiang University; 2020. (Chinese) Available at: https://id.wanshangdata.com.cn/biexiy/Y3873169


20. Huang Y, Yin HJ, Chen KJ. Heart Dominating Blood Circulation and Vessels and Prethrombotic State. China J Tradit Chin Med Pharm 2011;26(04):633-636. (Chinese) Available at: https://lns.cnki.net/kcms2/article/abstract?v=3u0qghG8C44YOTIOATRk9bYV5YSj7sIoR1PA7RkjuxjAk4dHxokyBDxoxFPy4nsAAMaA7MIhapQ6G0dyq7BaHiZ3p0&uniplatform=ZKPT


Submit a manuscript: https://www.tmrjournals.com/aging