

Dapagliflozin alleviates heart failure with preserved ejection fraction potentially by regulating the AIM2/caspase-1/GSDMD pathway and attenuating pyroptosis

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Abstract. Heart failure with preserved ejection fraction (HFpEF) is a common cardiovascular disorder characterized by a left ventricular ejection fraction of $\geq 45\%$, which is typically accompanied by diastolic dysfunction and symptoms of heart failure. Although dapagliflozin treatment may relieve symptoms in patients with HFpEF, the precise biological mechanisms underlying its therapeutic effects remain incompletely understood. Pyroptosis can induce inflammatory amplification and contribute to the development of cardiovascular disease. Nevertheless, the mechanism of dapagliflozin and pyroptosis in the pathogenesis and treatment of HFpEF remains largely unknown. The present study aimed to investigate the mechanism through which dapagliflozin regulates pyroptosis in HFpEF. First, a mouse model of HFpEF was established and dapagliflozin was administered to assess the phenotypes of HFpEF in mice and the level of pyroptosis in their myocardial tissues. Then, an HFpEF model of mouse cardiomyocytes was constructed, after which absent in melanoma 2 (AIM2) was knocked down and Caspase-1 was overexpressed to determine the AIM2/Caspase-1 signaling pathway in regulating pyroptosis under HFpEF conditions. Subsequently, cardiomyocytes were treated with dapagliflozin and AIM2 was overexpressed to investigate the mechanism by which dapagliflozin and AIM2 affect pyroptosis in HFpEF model cells. At the animal level, mice were treated with dapagliflozin and subjected to AIM2 overexpression to further explore the underlying mechanisms involved. Dapagliflozin alleviated the symptoms

of HFpEF in mice and decreased the level of pyroptosis in the myocardial tissue of HFpEF mice. Compared with the sham group, AIM2 protein levels in the myocardial tissue of HFpEF mice were elevated and dapagliflozin treatment decreased AIM2 protein levels. Changes in pyroptosis in the myocardial tissue of HFpEF mice were accompanied by fluctuations in AIM2 protein levels. At the cellular level, AIM2 down-regulation alleviated pyroptosis in mouse cardiomyocytes. Additionally, the data revealed a potential interaction between dapagliflozin and AIM2. Moreover, AIM2 regulated pyroptosis in mouse cardiomyocytes via the Caspase-1/Gasdermin D (GSDMD) axis. Lastly, it was demonstrated *in vitro* and *in vivo* that dapagliflozin may alleviate HFpEF symptoms in mice through mechanisms involving regulation of the AIM2/Caspase-1/GSDMD axis and attenuation of myocardial pyroptosis. In conclusion, dapagliflozin may alleviate HFpEF through mechanisms involving the AIM2/Caspase-1/GSDMD axis to attenuate pyroptosis, suggesting a potential therapeutic approach for the treatment of HFpEF.

Introduction

Heart failure (HF) with preserved ejection fraction (HFpEF) is currently the predominant form of HF and represents a major public health challenge (1). The incidence and prevalence of HFpEF continue to rise with increasing age and the increasing burden of obesity and sedentary and cardiometabolic disease (2). The diagnosis of HFpEF involves the assessment of cardiac dysfunction based on imaging techniques and assessment of increased left ventricular filling pressure (3). Although HFpEF accounts for $>50\%$ of all HF cases, the underlying mechanisms driving its pathogenesis remain unclear, thereby impeding the development and translation of mechanism-based therapies (4). Current treatments for HFpEF are largely restricted to alleviating congestion through diuretics, encouraging a healthy active lifestyle and managing comorbidities, as there are few effective treatments (5). Therefore, further exploration of the pathogenic mechanisms underlying HFpEF and the development of novel, effective therapeutic approaches are key.

Pyroptosis is an inflammatory form of programmed cell death mediated by Caspase-1, which differs markedly

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from classical apoptosis in both morphological and functional characteristics (6). Pathogen infection can trigger macrophages to undergo Caspase-1-mediated cell lysis, releasing pro-inflammatory cytokines (such as IL-1 β) and thereby initiating the host immune response. Shao (7) first identified Gasdermin D (GSDMD) as a substrate of inflammatory Caspases (Caspase-1/4/5/11) and the Gasdermin D N-terminal fragment (GSDMD-N) forms pores in the cell membrane, thereby triggering pyroptosis. Pyroptosis drives inflammatory amplification, increases the size of myocardial infarction and accelerates the occurrence of cardiovascular diseases, while it also represents a promising therapeutic target for cardiovascular disorders via the modulation of cardiomyocyte pyroptosis (8). The absent in melanoma 2 (AIM2) inflammasome is an intracellular protein platform with extensive pro-inflammatory and pro-pyroptotic properties (9). Hornung *et al* first identified AIM2 as a sensor for cytosolic double-stranded DNA (dsDNA), demonstrating that AIM2 binds to dsDNA via its HIN domain and recruits apoptosis-associated Speck-like protein containing a CARD and caspase-1 to form the AIM2 inflammasome complex (10). This study also showed that the activation of the AIM2 inflammasome can trigger Caspase-1-dependent pyroptosis and the release of IL-1 β /IL-18, contributing to host defense against intracellular pathogens such as DNA viruses and bacteria. Jin *et al* (11) further elucidated that AIM2 binds to dsDNA via its HIN domain, activates inflammasome assembly and promotes Caspase-1-dependent IL-1 β secretion and pyroptosis. Mechanistically, the AIM2 inflammasome activates the innate immune response by cleaving pro-Caspase-1 and processing IL-1 β and IL-18 into mature forms, while simultaneously promoting pyroptosis by generating the GSDMD-N fragment from GSDMD (12). AIM2 expression is elevated in patients with HF irrespective of the cause (that is, ischemic or dilated cardiomyopathy) (13,14). Therefore, the AIM2 inflammasome may serve as a novel biomarker and therapeutic target for HF. In addition, in murine HFpEF models, myocardial levels of the pro-inflammatory cytokines TNF- α and IL-1 β are found to be elevated, accompanied by increased inflammatory cell infiltration in cardiac tissue. Moreover, the inflammasome-mediated pyroptosis pathway is activated in epicardial adipose tissue and inhibition of GSDMD has been shown to reduce cardiomyocyte inflammation and autophagy (15). However, despite these findings, the mechanism of the AIM2/Caspase-1/GSDMD axis in HFpEF remains unclear.

Dapagliflozin is an inhibitor of sodium-glucose cotransporter type 2 (SGLT2) (16). Dapagliflozin can alleviate renal pyroptosis by regulating the HO-1/NOD-like receptor family pyrin domain containing 3 axis (17). Additionally, dapagliflozin has been shown to suppress pyroptosis mediated by the NLRP3/Caspase-1 pathway in vascular smooth muscle cells by repressing CTSB (18). In patients with HFpEF, 12 weeks of treatment with dapagliflozin was shown to improve symptoms, physical limitations and motor function in patients with HFpEF and was well tolerated in patients with chronic HFpEF (19). However, the molecular mechanism underlying the interplay between dapagliflozin and pyroptosis in HFpEF remains largely unclear. The present study hypothesized that dapagliflozin could alleviate pyroptosis and improve HFpEF by inhibiting the AIM2/Caspase-1/GSDMD

pathway. To investigate this hypothesis, an HFpEF model was constructed and dapagliflozin administered simultaneously to examine its effects on cardiac function and inflammatory responses, thereby evaluating the intervention effect of dapagliflozin during the development of HFpEF. Furthermore, the present study assessed pyroptosis in mouse cardiomyocytes by establishing a cell model combined with the interference of AIM2 to explore the potential mechanism through which dapagliflozin alleviates HFpEF.

Materials and methods

Animals. A total of 60 SPF-grade male C57 mice (8 weeks old, weight, 20–24 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. The animals were housed under controlled conditions (temperature, 22 $\pm$ 2°C; humidity, 50 $\pm$ 10%; 12-h light/dark cycle) with free access to standard chow and water. The animals underwent one week of adaptive feeding before formal experimental procedures were initiated. Prior to the start of the experiment, all mice were randomly assigned to different experimental groups. There were 5 mice in each group. During the experiment, the researchers were blinded to the group allocation information, ensuring that they were unaware of the specific group assignments during experimental operations and data collection.

After the experiment was completed, another researcher who did not participate in the experimental operations unblinded and analyzed the data, thereby ensuring the objectivity and accuracy of data processing.

Preparation of lentivirus. A third-generation lentiviral packaging system was used. 293T cells (cat. no. AW-CN086; Changsha Abiwei Biotechnology Co., Ltd.) were co-transfected with the transfer plasmid (oe-AIM2 or oe-NC), the packaging plasmid psPAX2, and the envelope plasmid pMD2.G. In total, 5 μ g transfer plasmid, 3.75 μ g of psPAX2, and 1.25 μ g of pMD2.G (mass ratio 4:3:1) were mixed and transfected for 48 h at room temperature using 25 μ l Lipofectamine 2000 (cat. no. 11668019; Invitrogen). Six h post-transfection, the medium was replaced with fresh complete medium containing 5% fetal bovine serum (cat. no. 10099141, Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin. At 48 h after transfection, the viral supernatant was collected. The supernatant was centrifuged at 4,000 $\times$ g for 10 min at 4°C to remove cell debris, followed by filtration through a 0.45 μ m filter. The filtrate was subjected to ultracentrifugation at 4,450 $\times$ g for 2 h at 4°C. After carefully discarding the supernatant, the viral pellet was resuspended in 1 ml PBS or cell culture medium, then centrifuged at 1,780 $\times$ g for 5 min at 4°C. The final titer was adjusted to 1 $\times$ 10⁷ TU/ml. Furthermore, successful lentiviral transduction *in vivo* was confirmed by western blot analysis of protein expression in mouse myocardial tissues at the end of the experiment. The lentivirus was injected into the myocardium, and 7 days after injection, model treatment and simultaneous intervention with dapagliflozin for 6 weeks were performed.

Group allocation and animal treatments. Experiment 1 was divided into four groups: Sham, Sham + Dapagliflozin, HFpEF and HFpEF + Dapagliflozin, with five animals in each

group. After the pH of N-omega-nitro-L-arginine methyl ester (L-NAME; 0.5 g/l; cat. no. BD3941-25 g; Bide Pharmatech Ltd.) was adjusted to 7.4, it was added to the drinking water, followed by the start of high-fat feeding and the HFpEF animal model was induced for 6 weeks (20).

Following the 6-week induction period and before the intervention with dapagliflozin, the successful establishment of the HFpEF model in mice treated with L-NAME combined with a high-fat diet was confirmed, in strict accordance with the diagnostic criteria for HFpEF recommended by the 2022 ACC/AHA/HFSA Heart Failure Guideline (21) and classic published studies in this field (22,23). Conventional echocardiography and Doppler imaging were performed to obtain representative left ventricular M-mode echocardiographs. The key echocardiographic parameters were measured and calculated, including left ventricular ejection fraction (LVEF), global longitudinal strain (GLS) and the ratio of mitral E wave to E' wave (E/E').

Heart weight to tibia length (HW/TL) was measured as an auxiliary evaluation index. In addition, systolic blood pressure (SBP) and diastolic blood pressure (DBP) of conscious mice were measured by the noninvasive tail-cuff method (BP-98A) (24). The mice in the HFpEF group met the diagnostic criteria for HFpEF, with preserved LVEF, typical diastolic dysfunction (increased E/E' ratio, decreased GLS) and abnormal blood pressure (elevated SBP and DBP), as well as obvious myocardial hypertrophy and myocardial fibrosis. Mice in the HFpEF + Dapagliflozin group received L-NAME and were intragastrically administered dapagliflozin (1 mg/kg; 1 ml/100 g; cat. no. BD161909-1g; Bide Pharmatech Ltd.) daily for 6 weeks (25). Dapagliflozin was administered concurrently with the induction of the HFpEF model. Sham group received an equal amount of normal saline and had unrestricted access to food (normal feed) and water.

Experiment 2 was divided into the following groups: Sham, oe-NC and oe-AIM2. The oe-NC and AIM2-overexpressing lentiviruses (oe-AIM2) were injected into the mouse myocardium at a viral concentration of 1×10^7 TU/ml with an injection volume of 30 μ l. Lentiviral transduction performed for 7 days following intramyocardial injection (25). Experiment 3 was divided into the following groups: HFpEF, HFpEF + dapagliflozin; HFpEF + dapagliflozin + oe-NC and HFpEF + dapagliflozin + oe-AIM2. In the HFpEF + dapagliflozin + oe-NC and HFpEF + dapagliflozin + oe-AIM2 groups, oe-NC and oe-AIM2 lentiviruses were injected into the mouse myocardium 7 days prior to modeling and dapagliflozin treatment, using the same viral concentration and an injection volume of 30 μ l (26). On the 8th day, HFpEF induction was started and dapagliflozin treatment was initiated simultaneously and continued for 6 weeks to evaluate the interventional efficacy of dapagliflozin during HFpEF progression. During the experimental period, the body weight of all mice were measured weekly and the changes recorded. After the experiment, the mouse myocardial tissues were collected for pathological analysis.

Anesthesia was performed when mice required invasive operations, including intramyocardial lentivirus injection, echocardiographic detection (for more accurate parameter measurement) and myocardial tissue collection. The anesthetic used was 1% pentobarbital sodium (CAS: 57-33-0;

cat. no. BD081201-10g; Bide Pharmatech Ltd.) and the administration method and dose were strictly standardized to ensure anesthesia safety and effectiveness. Mice were anesthetized by intraperitoneal injection at a dose of 50 mg/kg body weight (injection volume: 0.1 ml/10 g body weight). After injection, the mice were placed in a warm environment ($25 \pm 1^\circ\text{C}$) and observed continuously until the righting reflex disappeared (5-8 min), indicating that sufficient anesthesia was achieved. During the entire invasive operation, the mouse's respiratory rate and skin color were monitored to maintain the depth of anesthesia; if the mouse showed signs of waking (such as limb twitching, increased respiratory rate), supplementary anesthesia was performed at 1/3 of the initial dose to ensure the smooth progress of the operation.

Following the completion of all experimental interventions (6 weeks of intervention), all mice were subjected to humane sacrifice to collect myocardial tissues for subsequent experimental detection. The process was divided into two steps to ensure the mice suffered no pain: i) Pre-anesthesia: The same anesthetic as used in invasive operations was adopted, namely 1% pentobarbital sodium (CAS: 57-33-0; cat. no. BD081201-10g; Bide Pharmatech Ltd.). Mice were anesthetized by intraperitoneal injection at a dose of 100 mg/kg body weight (injection volume: 0.2 ml/10 g body weight), which was twice the dose used for surgical anesthesia. This high dose was designed to ensure deep anesthesia, so that the mice would not feel pain during the subsequent euthanasia process. ii) Sacrifice: After confirming that the mice had achieved deep anesthesia (loss of righting reflex and pain response), cervical dislocation was performed to ensure rapid and painless death of the mice. This method is a standard humane method for mice, which can cause immediate cardiac and respiratory arrest without causing unnecessary pain to the animals. To avoid false death and ensure the rigor of the experiment, three key indicators were comprehensively used to confirm the death of the mice after euthanasia and only when all three indicators were met was the mouse considered dead: i) Respiratory arrest: The chest movement of the mice observed continuously for >30 sec; no obvious chest undulation was observed, indicating that respiratory arrest has occurred. ii) Cardiac arrest: The chest of the mice was gently pressed with fingers for more than 1 min; no obvious heart-beat was felt, confirming cardiac arrest. iii) Pupil response: The pupils of the mice were observed under light stimulation; the pupils were dilated and unresponsive to light, indicating that the central nervous system has stopped functioning. All experimental protocols were approved by the Animal Ethics Committee of Changsha Hospital of Traditional Chinese Medicine (approval no. 2022111003).

Conventional echocardiography and doppler imaging. A previous study (26) conducted transthoracic echocardiography using a Vevo 2100 system equipped with an MS400 transducer (VisualSonics, Inc.). LVEF and other systolic function indices were obtained from short-axis M-mode scans at the mid-ventricular level in conscious mice that were gently restrained, with the papillary muscles visible in the imaging plane. Diastolic function measurements were collected from apical 4-chamber views of anesthetized mice using pulsed-wave Doppler and tissue Doppler techniques at the level

of the mitral valve. Representative left ventricular M-mode echocardiographic images were obtained. The percentage of LVEF and GLS, the ratio of the mitral E/E' and the HW/TL were quantified. Each parameter was measured ≥ 3 times.

Exercise exhaustion test. After 3 days of treadmill exercise, the mice were subjected to an exhaustion test. The mice were initially set to run uphill on a treadmill at a 20° incline, starting with a warm-up speed of 5 m/min for 4 min. The speed was subsequently increased to 14 m/min and the mice continued to run at this speed for an additional 2 min. Subsequently, the speed was incrementally increased by 2 m/min every 2 min until the mice reached exhaustion. Exhaustion was defined as the animal's failure to resume running within 10 sec after making direct contact with the electrically stimulated grid. Running duration was recorded and running distance was further calculated (26).

Tail cuff blood pressure recordings. SBP and DBP were assessed in conscious mice using the noninvasive tail-cuff method (BP-98A) (24). Each mouse was placed in an individual holder on a heated platform maintained at 37°C. Measurements were performed once the mice reached a stable state. Prior to the actual measurements, the mice underwent a training period to acclimate them to brief periods of restraint.

Intraperitoneal glucose tolerance test (ipGTT). ipGTT was conducted by administering glucose (2 g/kg in saline) following a 6-h fast (24,27). Tail vein blood glucose concentrations were quantified using a glucometer at baseline (0 min) and at 15, 30, 60, 90 and 120 min following glucose administration.

Flow cytometry. The degree of pyroptosis in myocardial tissue was assessed by flow cytometry. Myocardial tissues were cut into ~0.5 mm pieces using scissors and then homogenized. The resulting cell suspension was gently dispersed by repeated aspiration with a syringe. The cell suspension was filtered through a 100 μ m filter and the filtrate was collected and centrifuged at 178 x g for 5 min at room temperature. For each sample, 300 μ l of washing buffer was mixed with 2 μ l of FAM-YVAD-FMK (cat. no. ab219935; Abcam) and the solution was protected from light. Each sample was incubated with 300 μ l of the prepared dye solution at 37°C for 1 h in the dark for 1 h and then centrifuged at 178 x g for 5 min at room temperature. Subsequently, the samples were washed twice with 200 μ l of washing buffer. The samples were then stained with the PI working solution at 37°C for 10 min. Finally, pyroptosis was quantified as the percentage of cells that were double-positive for active caspase-1 (FAM-YVAD-FMK⁺) and PI⁺ using a flow cytometer (A00-1-1102, Beckman). Data acquisition and analysis were performed using FlowJo™ v10.8 Software (BD Life Sciences).

For cellular pyroptosis detection, the dye solution was prepared in the same manner. The number of samples was calculated first and a concentrated dye solution was prepared. For each sample, 300 μ l of washing buffer was mixed with 2 μ l of FAM-YVAD-FMK and the solution was protected from light. The remaining experimental steps were consistent with those for myocardial tissue detection. A detailed gating strategy is presented in Fig. S1.

Western blotting. Western blotting was performed to determine the levels of pro-Caspase-1, Caspase-1, GSDMD-N, GSDMD, pro-IL-1 β , IL-1 β , pro-IL-18, IL-18 and AIM2 in myocardial tissue or cells. Initially, the tissues or cell samples were lysed in RIPA buffer (cat. no. AWB0136; Changsha Abiwei Biotechnology Co., Ltd.) to extract total protein. Protein concentration was determined using a bicinchoninic acid assay. Equal amounts of protein (30 μ g/lane) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1.5 h at room temperature and subsequently incubated with primary antibodies overnight at 4°C. The primary antibodies used were anti-Caspase-1 (cat. no. ab207802, 1:1000; Abcam), anti-GSDMD (cat. no. 20770-1-AP; 1:5,000; Proteintech Group, Inc.), anti-GSDMD-N (cat. no. ab215203; 1:1,000; Abcam), anti-NLRP3 (cat. no. ab263899; 1:1,000; Abcam), anti-ASC (cat. no. 10500-1-AP; 1:15,000; Proteintech Group, Inc.), anti-pro-IL-1 β (16806-1-AP; 1:5,000; Proteintech Group, Inc.), anti-pro-IL-18 (cat. no. 10663-1-AP; 1:10,000; Proteintech Group, Inc.), anti-IL-1 β (cat. no. ab254360; 1:1,000; Abcam), anti-IL-18 (cat. no. ab191860; 1:1,000; Abcam), anti-AIM2 (cat. no. AWA00390; 1:1,000; Changsha Abiwei Biotechnology Co., Ltd.) and anti-GAPDH (cat. no. 10494-1-AP; 1:5,000; Proteintech Group, Inc.) antibodies. The membrane was then incubated with an HRP-conjugated goat anti-mouse secondary antibody (cat. no. SA00001-1, 1:5,000) or anti-rabbit secondary antibody (cat. no. SA00001-2, 1:6,000; both Proteintech Group, Inc.) for 1.5 h at room temperature. After incubation, the membrane was treated with enhanced chemiluminescence reagent (cat. no. AWA0005; Changsha Abiwei Biotechnology Co., Ltd.) for 1 min and imaged using an imaging system. GAPDH was used as the internal reference protein and protein band intensities were quantified using Quantity One 4.6.6 (Bio-Rad Laboratories, Inc.).

For subcellular fractionation analysis, membrane and cytoplasmic fractions were isolated separately prior to Western blot. Western blot analysis was then performed to detect the levels of GSDMD-N in the membrane and cytoplasmic fractions. The blots were probed with anti-sodium/potassium-transporting ATPase subunit α 1 (ATP1a; cat. no. ab76020, 1:10,000; Abcam) and anti-GAPDH antibodies. ATP1a was used as the membrane marker and GAPDH was used as the cytoplasmic marker and loading control, respectively. Band quantification was conducted as aforementioned.

Biochemical detection. The concentrations of lactate dehydrogenase (LDH), IL-1 β and IL-18 in mouse myocardial tissues or cell supernatants were quantified using biochemical assays. Specifically, commercial kits for LDH (cat. no. A020-1-2; Nanjing Jiancheng Bioengineering Institute), IL-1 β (cat. no. CSB-E08054m; Cusabio Technology, LLC) and IL-18 (cat. no. CSB-E04609m; Cusabio Technology, LLC) were used following the manufacturers' instructions to quantify the respective levels of these biomarkers.

Immunofluorescence (IF) staining. The levels of GSDMD and Caspase-1 in mouse myocardial tissue were assessed via IF staining. Tissue sections were first incubated at 60°C for 12 h. The sections were then deparaffinized and rehydrated,

followed by heat-induced antigen retrieval. Sections were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by washing with PBS. Sections were blocked with 5% BSA for 60 min at 37°C, followed by three washes with PBS. Primary antibody against GSDMD (cat. no. 20770-1-AP; 1:100; Proteintech Group, Inc.) was added and the sections were incubated overnight at 4°C, followed by three washes with PBS. The sections were incubated with HRP-conjugated secondary antibody from the immunohistochemistry secondary antibody kit (Mouse/Rabbit Ultra-Sensitive Polymer Detection System, cat. no. AWI0629; Changsha Abiwei Biotechnology Co., Ltd.; ready-to-use working solution without further dilution, for 30 min at 37°C, followed by three washes with PBS and then incubated with TYP-520 fluorescent dye at 37°C for 5-10 min in the dark, followed by rinsing with PBS three times for 5 min each. Heat-induced antigen retrieval was then performed again. The sections were blocked with 0.3% hydrogen peroxide for 15 min and rinsed with PBS three times for 5 min each. The sections were blocked with 10% normal goat serum (cat. no. 005-000-121; Jackson ImmunoResearch Laboratories) or 5% BSA for 60 min, followed by three 3-min washes with PBS. Appropriately diluted primary antibody against Caspase-1 (cat. no. AWA44822; 1:100; Changsha Abiwei Biotechnology Co., Ltd.) was added dropwise and the sections were incubated overnight at 4°C. After rinsing with PBS three times for 5 min each, the sections were incubated with an HRP-conjugated secondary antibody (cat. no. AWI0629; Changsha Abiwei Biotechnology Co., Ltd.) at 37°C for 31 min, followed by three washes with PBS. The sections were incubated with TYP-570 fluorescent dye at 37°C for 5 min in the dark, followed by rinsing with PBS three times for 5 min each. Heat-induced antigen retrieval was performed again and the sections were blocked with 0.3% hydrogen peroxide for 15 min before rinsing with PBS three times for 5 min each. The sections were blocked with 10% normal serum or 5% BSA for 60 min, followed by three 3-min washes with PBS. Appropriately diluted primary antibody against cTnT (cat. no. 15513-1-AP; 1:100; Proteintech Group, Inc.) was added dropwise and the sections were incubated overnight at 4°C. The sections were rinsed with PBS three times for 5 min each, incubated with an HRP-conjugated secondary antibody (cat. no. AWI0629; Changsha Abiwei Biotechnology Co., Ltd.) at 37°C for 31 min and rinsed again with PBS three times for 5 min each. The sections were incubated with TYP-620 fluorescent dye at 37°C for 5 min in the dark, followed by rinsing with PBS three times for 5 min each. DAPI (cat. no. AWC0293a; Changsha Abiwei Biotechnology Co., Ltd.) was applied to stain the nuclei at 37°C for 10 min. Finally, the sections were mounted with buffered glycerin and stored in a light-protected environment or examined under a fluorescence microscope.

The levels of Caspase-1 in mouse cardiomyocytes were assessed via IF staining. The coverslips were removed, washed with PBS 2-3 times and then in 4% paraformaldehyde for 30 min at room temperature, followed by six washes with PBS. Subsequently, the cells were permeabilized with 0.3% Triton X-100 for 30 min at 37°C. After three 3-min washes with PBS, the samples were blocked with 5% BSA for 60 min at 37°C, followed by three 3-min washes with PBS. Appropriate dilutions of the primary antibody against

Caspase-1 (cat. no. 22915-1-AP; 1:50; Proteintech Group, Inc.) were added and the samples were incubated at 4°C overnight, followed by three 5-min washes with PBS. The secondary antibody, CoraLite488-conjugated Goat Anti-Rabbit IgG (H + L) (cat. no. SA00013-2; 1:200; Proteintech Group, Inc.), was added and incubated for 90 min at 37°C, followed by three 5-min washes with PBS. For nuclear staining, the samples were incubated with DAPI for 10 min at 37°C. The samples were mounted with buffered glycerin and protected from light or viewed under a fluorescence microscope.

Wheat germ agglutinin (WGA) staining. Morphological changes in the myocardial tissue cell membrane were evaluated using WGA staining. Briefly, the tissue sections were incubated at 60°C for 12 h, then dewaxed to water and the antigens underwent heat-induced retrieval. The sections were placed in sodium borohydride solution for 30 min and then transferred to 75% ethanol solution for 5 min at room temperature. The WGA staining solution was prepared as follows: Particulate matter was diluted with 0.01 M PBS (pH 7.2-7.6) at a ratio of 1 mg/ml. The prepared WGA working solution was applied to the sections, which were then incubated in a humidified incubator at 37°C for 30 min under strict protection from light. For nuclear staining, the sections were stained with DAPI for 10 min at 37°C. The sections were then sealed with buffered glycerin and stored in a light-protected environment or examined under a fluorescence microscope.

Hematoxylin-eosin (H&E) staining. Pathological alterations in myocardial tissue were assessed using H&E staining. The sections were heated at 60°C for 12 h. Sections were placed in xylene (cat. no. 10023418; Shanghai Sinopharm Group Co., Ltd.) and rehydrated in descending ethanol. The sections were stained with hematoxylin (cat. no. AWI0001a; Changsha Abiwei Biotechnology Co., Ltd.) for 1-10 min, rinsed with distilled water and then counterstained using blue with PBS. Subsequently, the sections were dyed with eosin (cat. no. AWI0029a; Changsha Abiwei Biotechnology Co., Ltd.) for 1-5 min and rinsed with distilled water. The sections were dehydrated in a graded series of alcohol solutions (95-100%) for 5 min at each step or until they were completely dry. Finally, the sections were transferred to xylene (cat. no. 10023418; Shanghai Sinopharm Group Co., Ltd.) for 10 min, mounted with neutral gum (cat. no. AWI0238a; Changsha Abiwei Biotechnology Co., Ltd.) and examined under a microscope.

Masson staining. A Masson staining kit (cat. no. AWI0253a; Changsha Abiwei Biotechnology Co., Ltd.) was employed to evaluate collagen fiber deposition in the myocardial tissue of the mice. The tissue sections were incubated at 60°C for >12 h. Sections were placed in xylene and rehydrated in descending ethanol. A total of 2 ml nuclear dye was gently applied for 10 sec at room temperature. The staining solution was then rinsed thoroughly with tap water, followed by immersion of the sections in distilled water. The sections were placed in a weak alkaline solution, such as PBS (pH 7.2-7.6) or ammonia, for 5 min to ensure that the nuclei returned to a blue color. Subsequently, an appropriate volume of staining solution was added to cover the entire tissue section and allowed to stain for

5 min at room temperature. The sections were then thoroughly washed with the rinsing solution to remove excess stain. Subsequently, the sections were treated with a color separation solution for ~30 sec. Thereafter, an appropriate volume of restaining solution was added to completely cover the tissue sections, which were stained for ~1 min at room temperature and then rinsed with anhydrous ethanol. The sections were dried with cold air from a hair dryer, cleared with xylene and finally sealed.

Cell culture and treatment. Mouse cardiomyocytes were obtained from Changsha Abiwei Biotechnology Co., Ltd. (cat. no. AW-CNM080) and cultured in MEM supplemented with 10% high-quality fetal bovine serum (cat. no. 10099141, Gibco, Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. The mouse cardiomyocytes are immortalized cardiomyocytes (HL-1 cell line). Dapagliflozin (0, 0.25, 2.5, 5, 10, 20, 50, 100 and 200 $\mu\text{mol/l}$, 461432-26-8, MCE) was used to treat mouse cardiomyocytes for 24 h and the maximum concentration that did not affect cell viability was identified (25).

To investigate the role of AIM2 in pyroptosis in HFpEF, AIM2 was knocked down and the following groups were established: Control, short hairpin RNA-negative control (sh-NC), sh-AIM2#1, sh-AIM2#2 and sh-AIM2#3. After identifying the most effective interference fragment, the following groups were established: Control (mouse cardiomyocytes cultured normally for 72 h), Model group (mouse cardiomyocytes cultured normally for 48 h and then treated with 1 $\mu\text{g/ml}$ tunicamycin (11089-65-9, MCE) for 24 h), Model + sh-NC group (mouse cardiomyocytes transfected with sh-NC for 48 h, then treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h) and Model + sh-AIM2 group (mouse cardiomyocytes transfected with sh-AIM2 for 48 h and then treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h). In addition, to explore the role of Caspase-1 in pyroptosis in HFpEF, Caspase-1 was overexpressed and the following groups were established: Control, overexpression-negative control (oe-NC) and oe-Caspase-1. The cardiomyocytes were subsequently grouped as follows: Control group (mouse cardiomyocytes cultured normally for 72 h), Model group (mouse cardiomyocytes cultured normally for 48 h and treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h), sh-NC + oe-NC group (mouse cardiomyocytes transfected with sh-NC and oe-NC for 48 h and then treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h), sh-AIM2 + oe-NC group (mouse cardiomyocytes co-transfected with sh-AIM2 and oe-NC for 48 h and then treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h) and sh-AIM2 + oe-Caspase-1 group (mouse cardiomyocytes transfected with sh-AIM2 and oe-Caspase-1 for 48 h and then treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h).

In addition, the cells were divided into three groups: Control group (untreated normal control), oe-NC group (transfected with empty oe-NC vector) and oe-AIM2 group (transfected with AIM2 overexpression vector oe-AIM2). To investigate the mechanism of dapagliflozin and AIM2 in pyroptosis in HFpEF, the cells were grouped as follows: Control group (mouse cardiomyocytes cultured normally for 72 h), Model group (mouse cardiomyocytes cultured normally for 48 h and then treated with 1 $\mu\text{g/ml}$ tunicamycin for 24 h), Model +

for 48 h and then treated simultaneously with dapagliflozin and 1 $\mu\text{g/ml}$ tunicamycin for 24 h), Model + dapagliflozin + oe-NC group (mouse cardiomyocytes transfected with oe-NC for 48 h and then treated simultaneously with dapagliflozin and 1 $\mu\text{g/ml}$ tunicamycin for 24 h) and Model + Dapagliflozin + oe-AIM2 group (mouse cardiomyocytes transfected with oe-AIM2 for 48 h and treated simultaneously with dapagliflozin and 1 $\mu\text{g/ml}$ tunicamycin for 24 h).

Transfection was performed using Lipofectamine 3000 (cat. no. L3000015, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For knockdown, sh-AIM2 fragments (cat. no. HG-Mi1013779) or sh-NC (both HonorGene) were used at a final concentration of 100 nM. For overexpression, oe-AIM2 (HG-MO1013779, HonorGene), oe-Caspase-1 (HG-MO009807; both HonorGene) or oe-NC were used at 3 $\mu\text{g/well}$. Cells were incubated with the transfection mixture for 48 h at 37°C. The time interval between transfection and subsequent experimentation was 48 h. Untransfected control cells were maintained under identical culture conditions. The sequences used were as follows: sh-NC: 5'-TGC GGGCAAGTCCACCACCA-3'; sh-AIM2#1: 5'-GACCAC ATCACGGAGGAAGAACTGAA-3'; sh-AIM2#2: 5'-GCC CATTCTGTGAATAATACAAA-3'; and sh-AIM2#3: 5'-GAA ACTCCCAGGATTAGTAACTGAAG-3'.

Reverse transcription-quantitative (RT-q) PCR. The mRNA levels of AIM2 and Caspase-1 were quantified via RT-qPCR. First, mouse cardiomyocytes were seeded at a density of 5×10^5 cells/well in a 6-well plate and cultured until 80-90% confluence. Total RNA was isolated from samples using a TRIzol® total RNA extraction kit (cat. no. 15596026, Thermo Fisher Scientific, Inc.) and its concentration and purity were determined. The extracted total RNA was then reverse-transcribed into complementary DNA using an mRNA reverse transcription kit (cat. no. CW2569; CWBio) according to the manufacturer's protocols. Subsequently, relative gene expression levels were analyzed with Ultra SYBR Mixture (cat. no. CW2601; CWBio) on an ABI 7900 Real-Time PCR System (Applied Biosystems). The thermocycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 sec. A melt curve analysis was performed to confirm product specificity. Gene expression levels were calculated with the $2^{-\Delta\Delta C_q}$ method (28), with GAPDH as the internal reference gene. All experiments were performed in triplicate. The primers used were as follows: AIM2-F: 5'-CTGTCTGCCGCCATGCTTC-3'; AIM2-R: 5'-CATGAATATACCAGCAGTCCAGT-3'; Caspase-1-F: 5'-ACAAGGCACGGGACCTATG-3'; Caspase-1-R: 5'-TCC CAGTCAGTCCTGGAAATG-3'; GAPDH-F: 5'-GCGACT TCAACAGCAACTCCC-3'; and GAPDH-R: 5'-CACCT GTTGCTGTAGCCGTA-3'.

Cell Counting Kit 8 (CCK-8) assay. Cell viability was evaluated via a CCK-8 assay. Cells were trypsinized and seeded into a 24-well plate at a density of 5×10^3 cells per well, with 300 μl of culture medium added to each well. Triplicate wells were established for each group. After cell adhesion, 10 $\mu\text{l/well}$ of CCK-8 reagent (cat. no. NU679; Dojindo Laboratories, Inc.) was added at the corresponding time points following treatment

as aforementioned. After incubation at 37°C in a humidified atmosphere containing 5% CO₂ for 4 h, the absorbance was measured at a wavelength of 450 nm using a microplate reader.

Transmission electron microscopy (TEM). TEM experiment was only employed to evaluate mitochondrial damage, not to assess plasma membrane integrity or GSDMD pore formation. Briefly, the cells were fixed with 2.5% glutaraldehyde at 4°C for 6–12 h. After removal of the fixative, the cells were rinsed with PBS buffer. Then, 1% osmium tetroxide was added and the cells were fixed for 1–2 h. The cells were then dehydrated, embedded in pure epoxy resin and cured in an oven at 40°C for 12 h. The samples were further heated at 60°C for 48 h. After the embedded blocks were removed and trimmed, ultrathin sections (70–90 nm in thickness) were cut, mounted on copper grids and stained with electron-dense dyes (lead and uranium stains) at room temperature. Finally, the sections were examined using a transmission electron microscope (JEM 1400; JEOL, Ltd.).

Hoechst 33342/PI fluorescence staining. Pyroptosis was evaluated using Hoechst 33342/PI fluorescence staining. The excess medium was aspirated and 2,000 µl of dye working solution was added (1 mg/ml Hoechst storage solution prepared with DMSO and 1 mg/ml PI stock solution prepared with DDH₂O). The storage solution was diluted with preheated serum-free culture medium or PBS to obtain final concentrations of 10 µg/ml Hoechst working solution and 20 µg/ml propidium iodide working solution. The dye working solution was gently mixed with the cells to ensure complete coverage, followed by incubation for 5 min at room temperature. Subsequently, the dye working solution was removed and the cells were washed twice with serum-free culture medium for 5 min each time before observation under a fluorescence microscope.

Molecular docking. Molecular docking was performed to investigate the binding interaction between dapagliflozin and AIM2. VINA 1.1.2 software (vina.scripps.edu/) was used to perform docking simulations between compounds and proteins, employing semiempirical free energy fields to predict receptor-ligand binding energies. After docking simulations were completed, PyMOL software (version 2.6.2; pymol.org/) was used for structural visualization and chemical analysis to evaluate the stable binding conformation of dapagliflozin within the protein binding pocket and its intermolecular interactions with adjacent amino acid residues.

Drug affinity responsive target stability (DARTS) assay. DARTS was performed as previously described (29). The recombinant AIM2 protein (0.5 µg/ml) was incubated with different concentrations of dapagliflozin (0–50 µM) at room temperature for 1 h with gentle shaking. Pronase (5 µg/ml) was added and the mixture was incubated for 15 min at room temperature. The reaction was then terminated by adding loading buffer. AIM2 protein levels were subsequently detected by western blotting.

Cellular thermal shift assay (CETSA). For the living cell CETSA, cells were treated with or without dapagliflozin for 3 h. The cells were then collected and equally divided into

12 portions in 0.2% NP40-PBS solution, heated at specific temperatures (37–72°C) and subsequently cooled for 3 min. The samples were lysed by three freeze-thaw cycles using liquid nitrogen. After ultracentrifugation at 20,000 x g for 20 min at 4°C, the supernatant was collected and subjected to Western blot analysis (30). For concentration-dependent CETSA, the samples were treated with vehicle control or dapagliflozin (2, 5, 10, 20, 50, 100, 200, 300 or 500 µM), followed by heating for 3 min and cooling for 3 min. High-speed centrifugation at 20,000 x g for 20 min at 4°C was performed and the supernatant was collected for western blot analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 software (Dotmatics). Measurement data are presented as the mean ± standard deviation. The normality of data distribution was assessed using the Shapiro-Wilk test (31). For comparisons between two groups, the F-test associated with unpaired Student's t-test was used to assess homogeneity of variance (31). The Brown-Forsythe test was applied to examine homogeneity of variance across multiple groups (32). If both normality and homogeneity of variance were confirmed, differences between two groups were analyzed using an unpaired Student's t-test (31). When normality was confirmed but homogeneity of variance was not satisfied, differences between two groups were analyzed using the Mann-Whitney U test (33,34). For multiple-group comparisons of normally distributed data, one-way analysis of variance (ANOVA) was applied (35), followed by Tukey-Kramer post hoc tests when the ANOVA results were significant (36). In the Shapiro-Wilk test, P>0.1 indicated that the data followed a normal distribution. For the F-test and Brown-Forsythe test, P>0.05 indicated homogeneity of variance. For the Mann-Whitney U test, unpaired Student's t-test or one-way ANOVA, P<0.05 was considered to indicate a statistically significant difference. No data were excluded and all analyses were conducted in a blinded manner. For post-hoc power analysis, G*Power 3.1.9.2 software (Heinrich Heine University Düsseldorf) was employed with parameters including the actual effect size (Cohen's d), α=0.05, n=5 per group and the corresponding number of experimental groups. The results demonstrated that the power values of all primary endpoint indicators were greater than 0.8, confirming that a sample size of n=5 per group was sufficient to meet the detection requirements. Pearson correlation analysis was used to evaluate the linear relationship between two continuous variables (37). Before performing Pearson correlation analysis, normality tests were conducted to confirm that the data followed a normal distribution. The correlations between AIM2 protein levels and pyroptosis-related indices (IL-18, IL-1β, GSDMD-N and Caspase-1), as well as myocardial function indicators (LVEF, GLS and E/E'), were determined using Pearson correlation coefficients.

Results

Dapagliflozin improves HFpEF symptoms in mice. The present study constructed an HFpEF model and administered dapagliflozin simultaneously to evaluate the interventional effect of dapagliflozin during the development of HFpEF. Fig. 1A illustrates the overall experimental workflow from

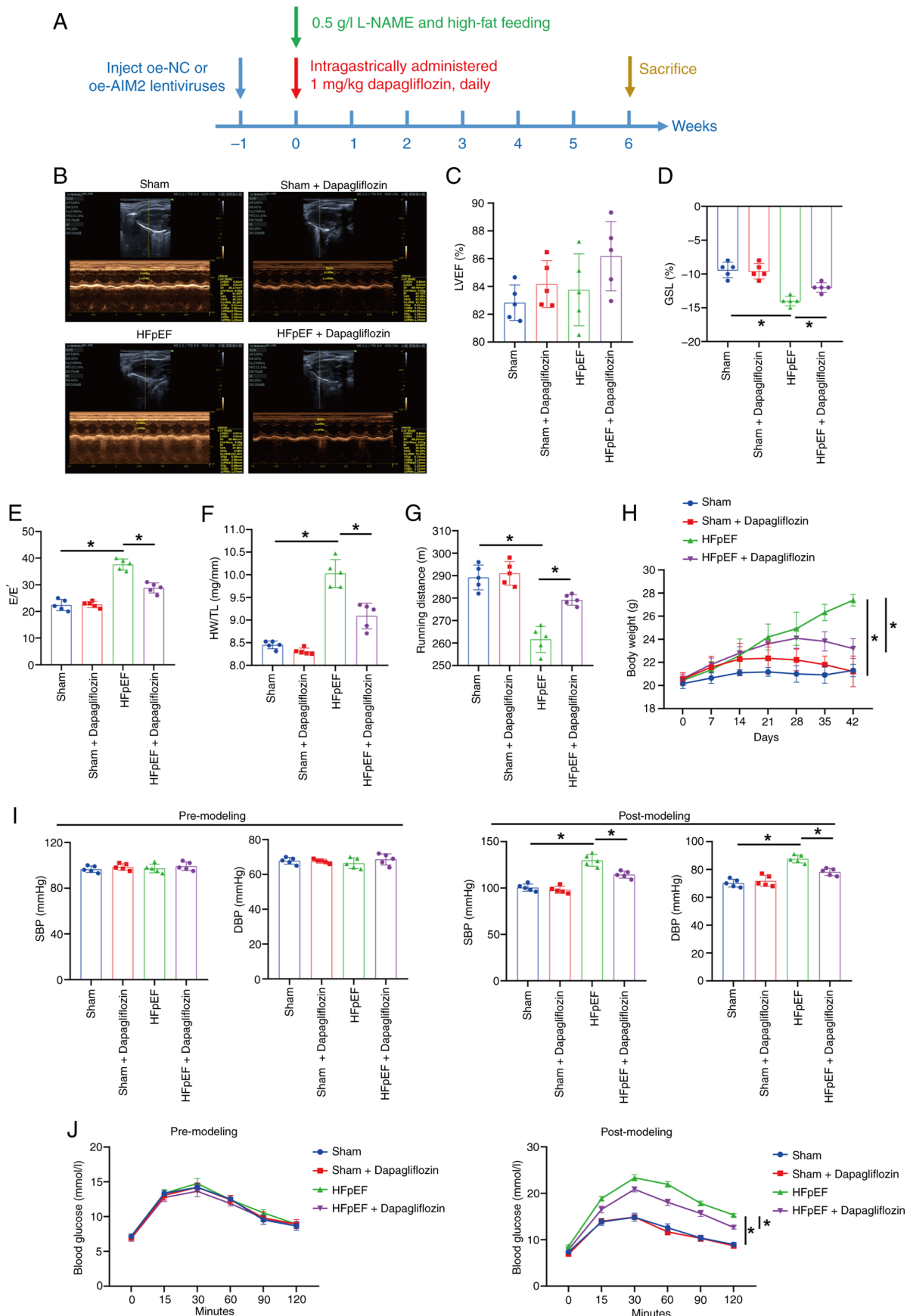


Figure 1. Dapagliflozin improves HFpEF symptoms in mice. (A) Schematic of the animal experimental protocol. (B) Representative left ventricular M-mode echocardiograph. (C) The percentage of the LVEF. (D) The percentage of GLS. (E) The ratio of the mitral E wave to the E' wave. (F) The ratio of heart weight to tibia length. (G) Exercise exhaustion test of running distance. (H) The weekly body weight measurements of the mice. (I) The SBP and DBP of conscious mice measured non-invasively using the tail-cuff method. (J) The blood glucose levels of mice detected using ipGTT. * $P < 0.05$. HFpEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; GLS, global longitudinal strain; E/E', ratio of the mitral E wave to the E' wave; HW/TL, ratio of heart weight to tibia length; SBP, systolic blood pressure; DBP, diastolic blood pressure; ipGTT, intraperitoneal glucose tolerance test.

lentiviral injection to the end of the study. Briefly, mice first received intramyocardial lentiviral injection for 7 days. On day 8 following lentiviral injection, HFpEF model induction was initiated and dapagliflozin intervention was started concurrently and continued for 6 weeks. Fig. 1B shows representative left ventricular M-mode echocardiographic images. The percentage of LVEF was essentially comparable among all groups (Fig. 1C). By contrast, compared with the Sham group, the percentage of GLS was lower in the HFpEF group, whereas dapagliflozin treatment was associated with an increase in GLS (Fig. 1D). Furthermore, compared with the Sham group, the E/E' ratio and heart weight to tibia length (HW/TL) ratio were higher in the HFpEF group, while the running distance in the exercise exhaustion test was reduced. Following dapagliflozin treatment, the E/E' and HW/TL ratios decreased, whereas the running distance increased (Fig. 1E-G). The present study also monitored body weight, blood pressure and blood glucose in mice. Over the 6-week intervention, mice in the HFpEF group exhibited increased body weight compared with the Sham group, whereas dapagliflozin treatment was associated with reduced body weight (Fig. 1H). Following model induction, SBP and DBP were markedly increased in the HFpEF group compared with the Sham group and blood glucose levels were also elevated. Following dapagliflozin administration, both SBP and DBP were markedly reduced, accompanied by a significant decrease in blood glucose (Fig. 1I and J). Collectively, these results demonstrated that dapagliflozin alleviated HFpEF-related phenotypes in mice. Given that dapagliflozin had no effect on the cardiac function of Sham mice, further exploration was conducted on the remaining three groups (Sham, HFpEF and HFpEF + Dapagliflozin groups).

Dapagliflozin reduces pyroptosis in the myocardial tissue of HFpEF mice. Next, the effect of dapagliflozin on the pyroptosis levels in the myocardial tissue of HFpEF mice were investigated. Compared with the Sham group, myocardial pyroptosis was markedly elevated in the HFpEF group, whereas this increase was markedly suppressed following dapagliflozin treatment (Fig. 2A). Additionally, the levels of pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β , IL-18 and LDH in myocardial tissue were increased in the HFpEF group compared with the Sham group; however, these elevations were reduced after dapagliflozin administration (Fig. 2B-E). WGA staining revealed that myocardial hypertrophy was more pronounced in the HFpEF group than in the Sham group and dapagliflozin treatment reduced myocardial hypertrophy to some extent (Fig. 2F). Moreover, compared with the Sham group, the HFpEF group exhibited increased myocardial inflammatory infiltration and fibrosis. After dapagliflozin treatment, inflammatory infiltration in myocardial tissue was reduced and fibrosis was alleviated (Fig. 2G and H). Together, these results indicate that dapagliflozin inhibited pyroptosis in the myocardial tissue of HFpEF mice.

Changes in pyroptosis levels in HFpEF mice are accompanied by fluctuations in AIM2 protein levels. AIM2 is elevated in human HF regardless of the cause (ischemic or dilated cardiomyopathy) (13) and may represent a novel biomarker

and therapeutic target for HF. Therefore, the role of AIM2 in HFpEF was next explored. The present study first assessed AIM2 protein levels in mouse myocardial tissue and found that, compared with the Sham group, AIM2 expression was increased in the HFpEF group, whereas AIM2 expression decreased after dapagliflozin treatment (Fig. 3A). Pearson correlation analysis further showed that AIM2 levels were positively associated with IL-1 β , IL-18, Caspase-1 and GSDMD-N (Fig. 3B). In addition, AIM2 levels were not markedly associated with LVEF; however, AIM2 levels were markedly negatively associated with GLS and markedly positively associated with E/E' (Fig. 3C), which further supports a potential role for AIM2 in HFpEF.

To determine whether AIM2 overexpression alone was sufficient to activate the Caspase-1/GSDMD axis, promote pyroptosis and exacerbate inflammatory injury, AIM2 expression was upregulated in mouse myocardial tissue and in cultured cardiomyocytes to investigate its functional role in HFpEF pathogenesis. *In vivo*, AIM2 protein expression was markedly higher in the oe-AIM2 group compared with the oe-NC group, confirming successful AIM2 overexpression in mouse myocardial tissue (Fig. 4A). Moreover, the protein levels of NLRP3, ASC, pro-Caspase-1, Caspase-1 (p20), GSDMD, GSDMD-N, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 were markedly elevated in the oe-AIM2 group compared with the oe-NC group, indicating that AIM2 overexpression robustly activated the Caspase-1/GSDMD pathway and promoted inflammatory factor release (Fig. 4B). Consistently, the levels of LDH, IL-1 β and IL-18 were markedly higher in the oe-AIM2 group than in the oe-NC group, suggesting that AIM2 overexpression exacerbated tissue injury and inflammatory responses (Fig. 4C and D). *In vitro*, cell viability was markedly lower in the oe-AIM2 group than in the oe-NC group, indicating that AIM2 overexpression reduced cardiomyocyte viability (Fig. 4E). Flow cytometric analysis further revealed that the proportion of pyroptotic (Caspase-1 positive) cells was markedly higher in the oe-AIM2 group than in the oe-NC group, suggesting that AIM2 overexpression markedly promoted cardiomyocyte pyroptosis (Fig. 4F). In line with these findings, the expression levels of AIM2, NLRP3, ASC, pro-Caspase-1, Caspase-1 (p20), GSDMD, GSDMD-N, pro-IL-1 β and pro-IL-18 were markedly increased in the oe-AIM2 group relative to the oe-NC group, further confirming activation of the Caspase-1/GSDMD pathway and enhanced inflammatory factor release following AIM2 overexpression (Fig. 4G). Additionally, LDH, IL-1 β and IL-18 levels in the cell supernatant were markedly higher in the oe-AIM2 group than in the oe-NC group, indicating aggravated cellular injury and inflammation upon AIM2 overexpression (Fig. 4H). To investigate whether AIM2 overexpression affects the subcellular localization of GSDMD-N, western blot analysis was performed on membrane and cytoplasmic fractions. As shown in the Figure 4I, compared with the oe-NC group, AIM2 overexpression markedly increased GSDMD-N expression in the membrane fraction (relative to the membrane marker ATP1a), while cytoplasmic GSDMD-N levels were also markedly elevated (relative to the cytoplasmic marker GAPDH). These results suggested that AIM2 may enhance pyroptosis execution by promoting GSDMD cleavage activation and its translocation to the cell membrane. Collectively, these results

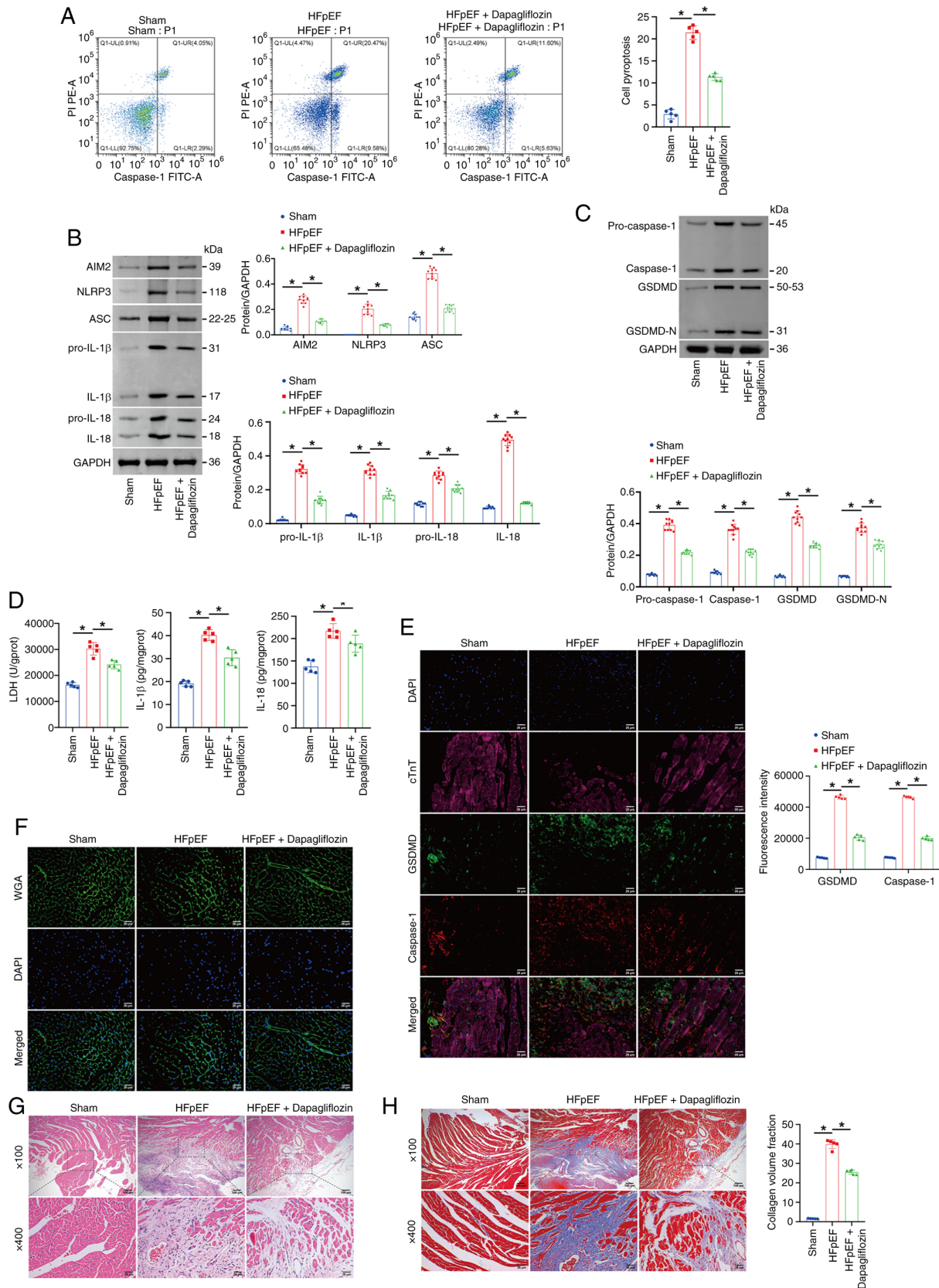


Figure 2. Dapagliflozin reduces pyroptosis in the myocardial tissue of HFpEF mice. (A) Flow cytometry analysis of myocardial tissue pyroptosis (Caspase-1). (B and C) Western blot analysis of pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in myocardial tissue. (D) LDH, IL-1 β and IL-18 levels in myocardial tissue were determined using commercial kits. (E) IF staining of GSDMD and Caspase-1. (F) WGA staining of morphological alterations in the myocardial tissue cell membrane in mice. (G) Pathological changes in myocardial tissues assessed via H&E staining. (H) Masson staining of collagen fiber deposition in the myocardial tissue of the mice. Scale bars, 100 μ m, magnification, x100; scale bars, 25 μ m, magnification, x400. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. * P <0.05. HFpEF, heart failure with preserved ejection fraction; GSDMD, Gasdermin D; GSDMD-N, Gasdermin D N-terminal fragment; AIM2, absent in melanoma 2; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis-associated Speck-like protein containing a CARD; LDH, lactate dehydrogenase; IF, immunofluorescence; WGA, wheat germ agglutinin; H&E, hematoxylin-eosin.

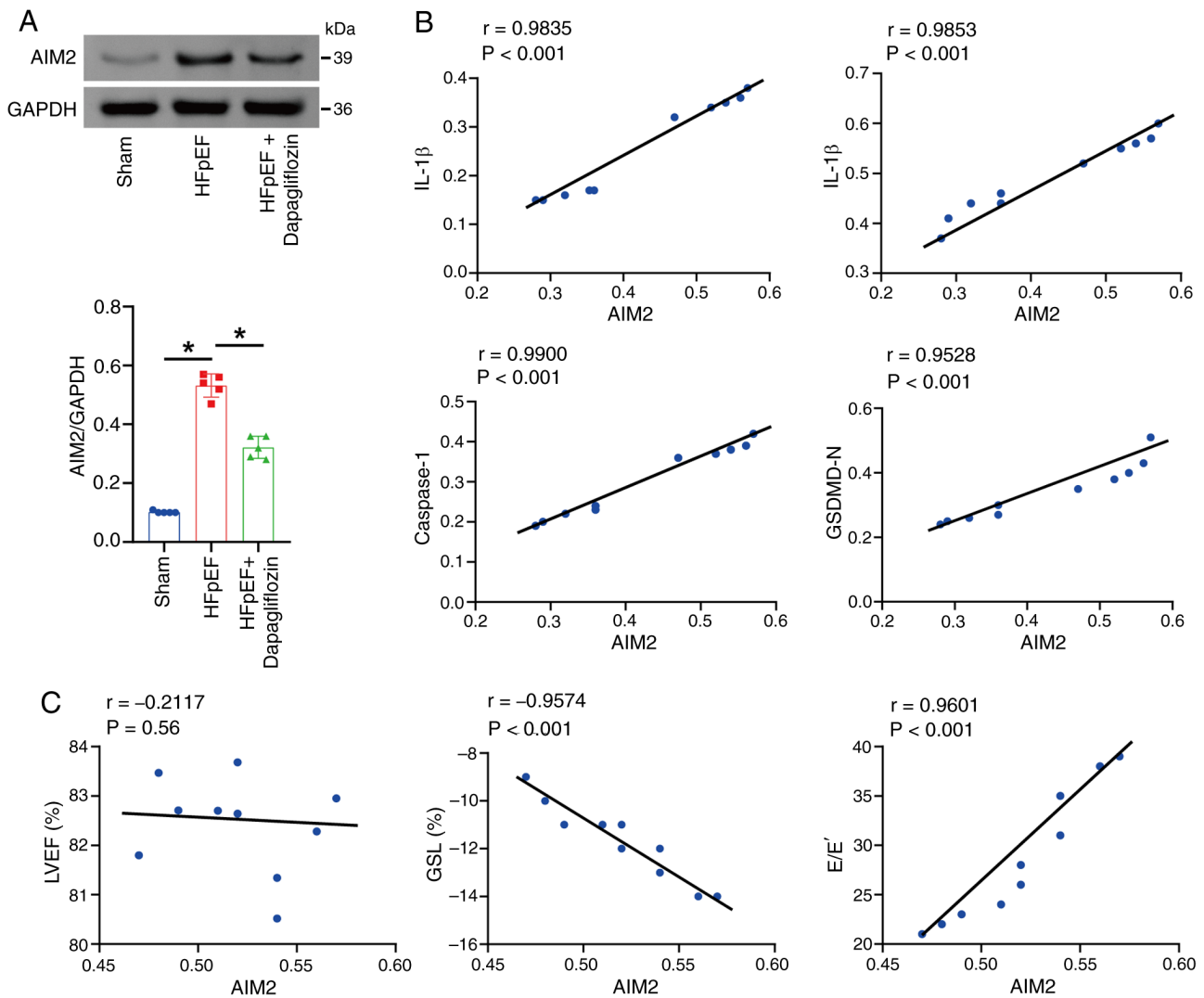


Figure 3. AIM2 expression is correlated with pyroptosis and cardiac function in mouse myocardial tissue. (A) Western blot analysis of AIM2 protein levels in the myocardial tissue of mice. (B) The Pearson correlation coefficient was used to determine the correlation between the AIM2 protein level and the pyroptosis indices IL-1 β , IL-18, Caspase-1 and GSDMD-N. (C) The correlation between AIM2 protein levels and myocardial function indicators (LVEF, GLS and E/E') was determined using the Pearson correlation coefficient. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. *P<0.05. AIM2, absent in melanoma 2; GSDMD-N, Gasdermin D N-terminal fragment; LVEF, left ventricular ejection fraction; GLS, global longitudinal strain; E/E', mitral E wave to the E' wave.

indicate that changes in pyroptosis levels in HFpEF mice were accompanied by corresponding fluctuations in AIM2 protein levels.

AIM2 knockdown alleviates pyroptosis in mouse cardiomyocytes. To investigate the role of AIM2 in mouse cardiomyocyte pyroptosis, the present study first knocked down AIM2. Compared with the sh-NC group, AIM2 expression was markedly downregulated in the sh-AIM2#1, sh-AIM2#2 and sh-AIM2#3 groups, with the most pronounced decrease in the sh-AIM2#3 group. Therefore, sh-AIM2#3 was selected for subsequent experiments (Fig. 5A). Next, mouse cardiomyocytes were treated with 1 μ g/ml tunicamycin to construct a cellular model. Compared with the Control group, cardiomyocyte viability was decreased, pyroptosis was increased and mitochondrial damage was aggravated in the Model group. Following AIM2 knockdown, the viability of mouse cardiomyocytes increased, pyroptosis decreased

and mitochondrial damage was alleviated (Fig. 5B-D). Furthermore, the levels of AIM2, pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β , IL-18 and LDH were markedly increased in the Model group relative to the Control group. Following AIM2 knockdown, the levels of these factors were decreased in mouse cardiomyocytes (Fig. 5E and F). Moreover, compared with the Control group, the percentage of PI-positive cardiomyocytes in the Model group was greater, whereas AIM2 interference reduced the proportion of PI-positive mouse cardiomyocytes (Fig. 5G). To investigate whether AIM2 regulates the subcellular localization of GSDMD-N, western blot analysis was performed on membrane and cytoplasmic fractions. As shown in the Fig. 5H, compared with the Control group, the Model group exhibited markedly elevated GSDMD-N protein expression in the membrane fraction, along with markedly increased GSDMD-N levels in the cytoplasmic fraction, indicating substantial GSDMD activation and membrane

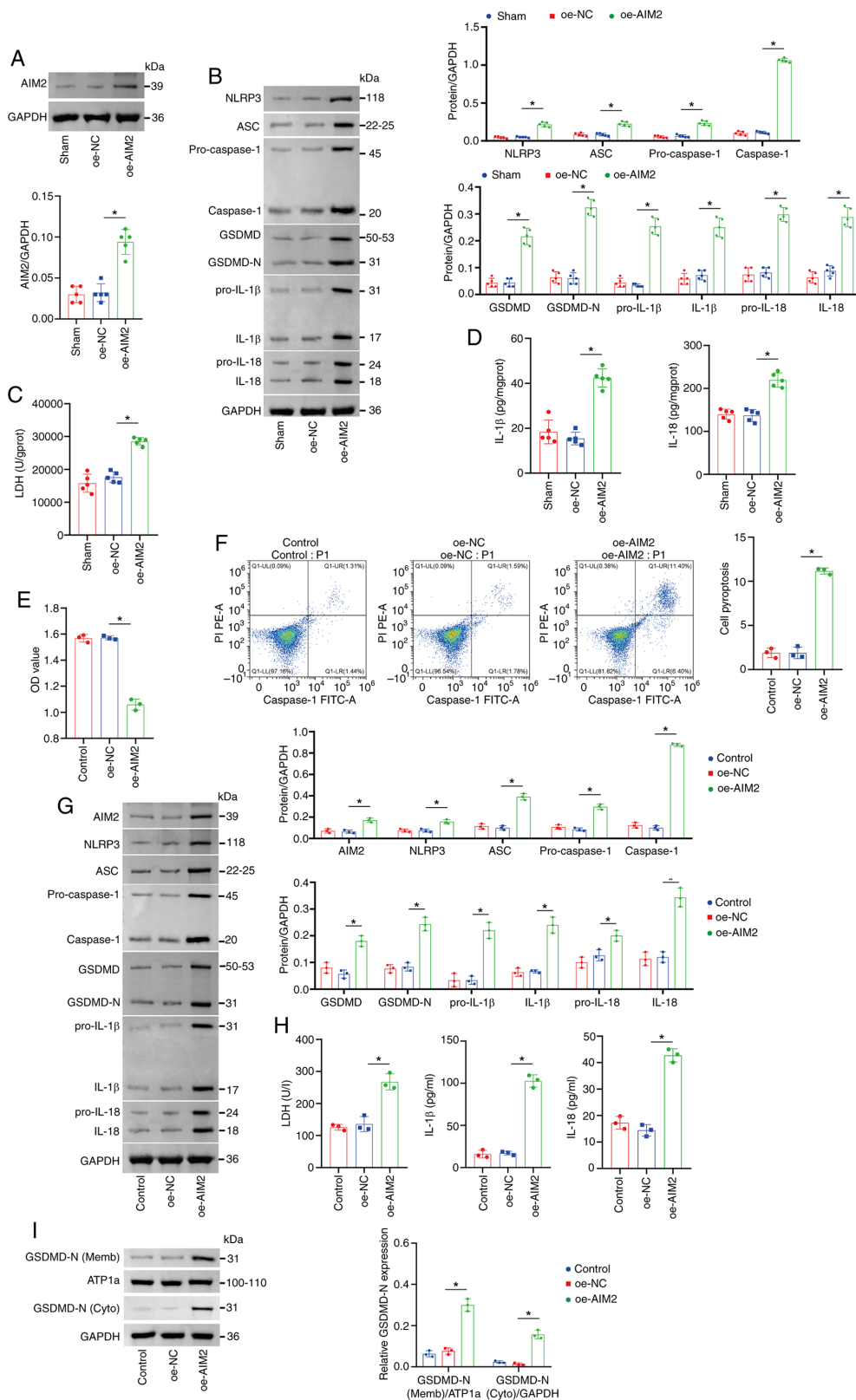


Figure 4. AIM2 promoted pyroptosis in mouse myocardial tissue and cardiomyocytes. (A) Western blot analysis of AIM2 protein expression in mouse myocardial tissue. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (B) Western blot analysis of the expression of NLRP3, ASC, pro-Caspase-1, Caspase-1 (p20), GSDMD, GSDMD-N, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 in myocardial tissue. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (C) Measurement of myocardial LDH and (D) Measurement of myocardial IL-1 β and IL-18 levels. (E) Cell viability detected by CCK-8 assay. (F) Cardiomyocyte pyroptosis rate (Caspase-1) measured by flow cytometry. (G) Western blot analysis of AIM2, NLRP3, ASC, pro-Caspase-1, Caspase-1 (p20), GSDMD, GSDMD-N, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in cell lysates. GAPDH was used as a loading control. (H) Measurement of LDH, IL-1 β and IL-18 levels in cell supernatant using commercial assay kits. (I) Western blot analysis of the levels of GSDMD-N in the membrane and cytoplasm. The blot was probed with anti-ATP1a/GAPDH antibody. ATP1a (membrane marker) and GAPDH (cytoplasmic marker) were used as a loading control. * $P < 0.05$. AIM2, absent in melanoma 2; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis-associated Speck-like protein containing a CARD; GSDMD, Gasdermin D; GSDMD-N, Gasdermin D N-terminal fragment; LDH, lactate dehydrogenase; oe, overexpressed; NC, negative control.

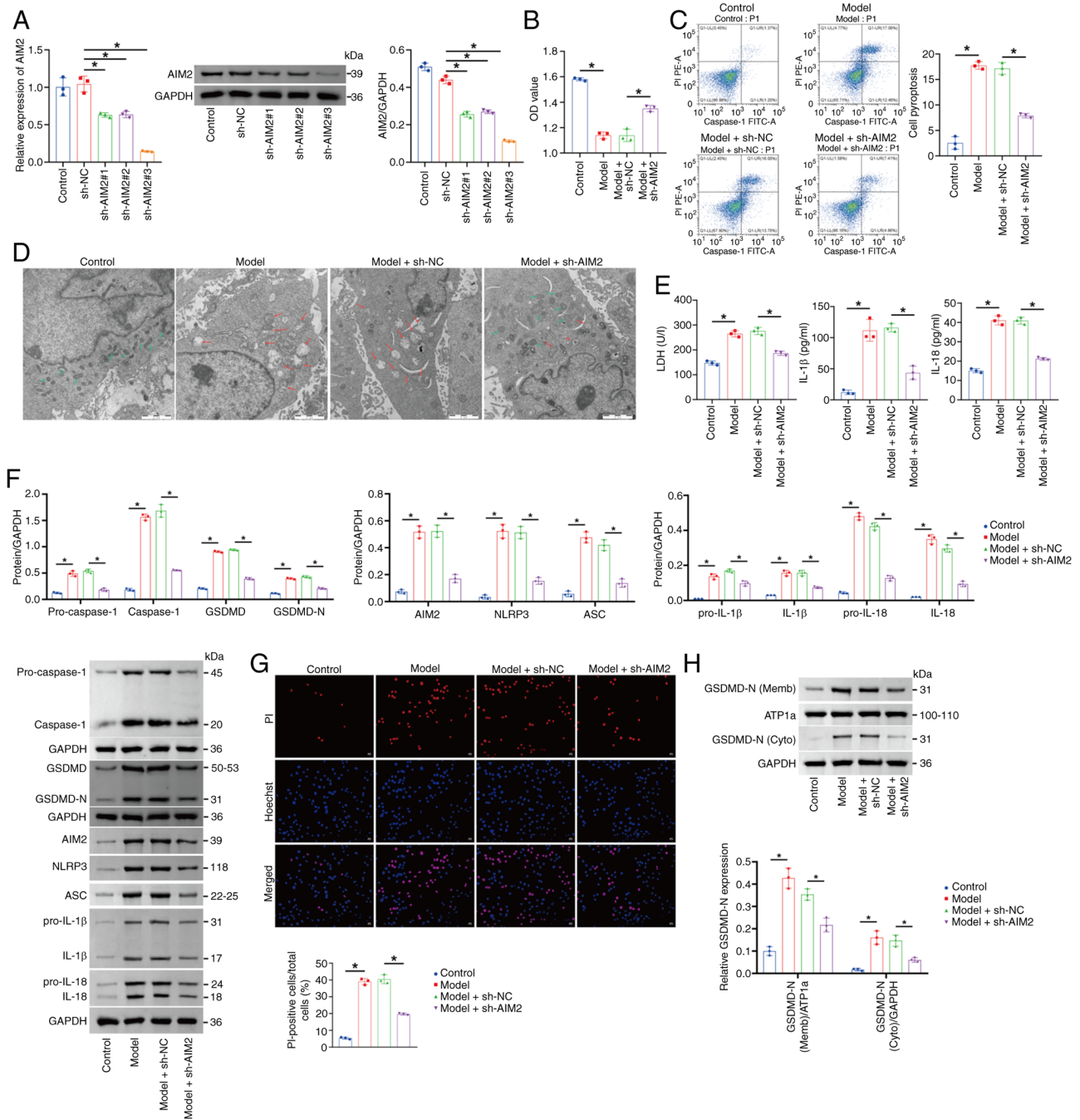


Figure 5. AIM2 knockdown alleviates pyroptosis in mouse cardiomyocytes. (A) AIM2 levels assessed by reverse transcription-quantitative PCR and western blotting. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (B) CCK-8 assay measurement of cell viability. (C) Flow cytometry analysis of pyroptosis (Caspase-1). (D) Transmission electron microscopy analysis of mitochondrial damage. Scale bar, 2 μ m. (E) LDH, IL-1 β and IL-18 levels were determined. (F) Western blot analysis of AIM2, Pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in the cell lysate. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (G) Hoechst 33342/PI fluorescence staining showing the percentage of PI-positive mouse cardiomyocytes. Scale bar, 50 μ m, magnification, x200. (H) Western blot analysis of the levels of GSDMD-N in the membrane and cytoplasm. The blot was probed with anti-ATP1a/GAPDH antibody. ATP1a (membrane marker) and GAPDH (cytoplasmic marker) were used as a loading control. * P <0.05. AIM2, absent in melanoma 2; GSDMD, Gasdermin D; GSDMD-N, Gasdermin D N-terminal fragment; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis-associated Speck-like protein containing a CARD; LDH, lactate dehydrogenase; sh, short hairpin; NC, negative control.

translocation in the model group. Transfection with sh-AIM2 markedly suppressed GSDMD-N protein levels in both the cell membrane and cytoplasm. ATP1a (membrane internal reference) and GAPDH (cytoplasmic internal reference) showed stable expression across all groups, confirming the reliability of subcellular fractionation and equal loading. These results

demonstrated that knockdown of AIM2 could alleviate pyroptosis in mouse cardiomyocytes.

AIM2 regulated pyroptosis in mouse cardiomyocytes via the Caspase-1/GSDMD axis. The AIM2/Caspase-1/GSDMD signaling pathway has been reported to mediate renal cell

pyroptosis in chronic kidney disease (38). However, the mechanism through which the AIM2/Caspase-1/GSDMD axis functions in HFpEF remains unclear. Therefore, Caspase-1 was overexpressed in mouse cardiomyocytes to explore the role of Caspase-1 in HFpEF-related pyroptosis. Compared with the oe-NC group, the levels of pro-Caspase-1 and Caspase-1 were markedly increased in the oe-Caspase-1 group (Fig. 6A and B). Compared with the Control group, cardiomyocyte viability was decreased, pyroptosis was increased and mitochondrial damage was aggravated in the Model group. Caspase-1 overexpression reversed the increased viability, reduced pyroptosis and alleviated mitochondrial damage in mouse cardiomyocytes caused by AIM2 knockdown (Fig. 6C-F). Furthermore, compared with the Control group, the levels of LDH, pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in the Model group were increased. However, the overexpression of Caspase-1 could reverse the decreased expression of LDH, pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 in mouse cardiomyocytes caused by the knockdown of AIM2 (Fig. 6G-J). Furthermore, compared with the Control group, the percentage of PI-positive cardiomyocytes in the Model group was greater. Caspase-1 overexpression reversed the decrease in PI-positive mouse cardiomyocytes caused by AIM2 knockdown (Fig. 6K and L). To investigate whether AIM2 regulates GSDMD-N membrane translocation through Caspase-1, western blot analysis was performed on membrane and cytoplasmic fractions. As shown in the Fig. 6M, compared with the Control group, the Model group displayed markedly increased relative expression levels of GSDMD-N protein in the cell membrane fraction, while GSDMD-N protein levels in the cytoplasmic fraction were also markedly upregulated, suggesting significant GSDMD activation and membrane translocation in the Model group. Compared with the sh-NC + oe-NC group, membrane GSDMD-N expression in the sh-AIM2 + oe-NC group was markedly decreased, with cytoplasmic GSDMD-N levels also decreasing synchronously; however, upon overexpressing Caspase-1 on the basis of sh-AIM2 (sh-AIM2 + oe-Caspase-1 group), GSDMD-N expression in both the cell membrane and cytoplasm was markedly rescued, indicating that Caspase-1 overexpression can reverse the inhibitory effect of AIM2 knockdown on GSDMD activation. ATP1a (membrane internal reference) and GAPDH (cytoplasmic internal reference) maintained stable expression across all groups, validating loading uniformity. Collectively, these results indicated that AIM2 regulates pyroptosis in mouse cardiomyocytes through the Caspase-1/GSDMD axis.

Dapagliflozin inhibits the AIM2/Caspase-1/GSDMD axis to alleviate pyroptosis in mouse cardiomyocytes. Dapagliflozin has been reported to improve symptoms, physical limitations and motor function in patients with HFpEF (19). However, the mechanism by which dapagliflozin and AIM2 regulate pyroptosis in HFpEF remains unclear. To explore whether dapagliflozin could bind to AIM2, the present study first performed molecular docking analysis. The molecular docking results showed that the binding energy between dapagliflozin and AIM2 was -5.6 kcal/mol. As the binding energy was lower than -5 kcal/mol, this suggested that the compound could form

relatively stable and favorable interactions with the protein (Fig. 7A). The results of the DARTS assay showed that dapagliflozin protected the AIM2 protein from pronase digestion in a dose-dependent manner, indicating a targeting association between dapagliflozin and the AIM2 protein (Fig. 7B). CETSA results revealed that specific binding of dapagliflozin to its target protein AIM2 markedly altered the thermal stability of AIM2 compared with the free protein, as reflected by a marked downward shift in its melting temperature. In living cells, dapagliflozin nevertheless enhanced AIM2 stability. At a fixed temperature of 65°C , the amount of soluble AIM2 increased in a concentration-dependent manner with increasing concentrations of dapagliflozin (Fig. 7C). Next, mouse cardiomyocytes were treated with different concentrations of dapagliflozin (0, 0.25, 2.5, 5, 10, 20, 50, 100 and $200\text{ }\mu\text{mol/l}$) to determine the maximum concentration that had no effect on cell viability. The results showed that treatment with $50\text{ }\mu\text{mol/l}$ dapagliflozin had no significant effect on cardiomyocyte viability (Fig. 7D). Therefore, this concentration was selected for subsequent experiments. Compared with the Control group, cardiomyocyte viability was lower, pyroptosis was greater and mitochondrial damage was more pronounced in the Model group. Following dapagliflozin treatment, cardiomyocyte viability increased, while pyroptosis and mitochondrial damage were decreased. However, overexpression of AIM2 reversed these effects (Fig. 7E-G). Moreover, compared with the Control group, LDH, pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in the Model group were elevated. After dapagliflozin treatment, LDH, pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in mouse cardiomyocytes decreased, while the overexpression of AIM2 reversed this phenomenon (Figs. 7H and 8A-C). Furthermore, compared with the Control group, the percentage of PI-positive cardiomyocytes was greater in the Model group. After dapagliflozin treatment, the percentage of PI-positive mouse cardiomyocytes decreased, whereas AIM2 overexpression increased the proportion of PI-positive cardiomyocytes (Fig. 8D). To investigate whether dapagliflozin regulates GSDMD-N membrane translocation through AIM2, western blot analysis was performed on membrane and cytoplasmic fractions. As shown in the Fig. 8E, compared with the Control group, the Model group exhibited markedly elevated relative expression levels of GSDMD-N protein in the cell membrane fraction, while cytoplasmic GSDMD-N levels were also markedly upregulated, indicating that the model successfully induced GSDMD cleavage activation and membrane translocation. Compared with the Model group, both membrane and cytoplasmic GSDMD-N expression were markedly suppressed in the Model + Dapagliflozin group; however, upon overexpressing AIM2 on the basis of dapagliflozin intervention (Model + Dapagliflozin + oe-AIM2 group), GSDMD-N membrane translocation and cytoplasmic expression levels were markedly rescued, demonstrating that AIM2 overexpression can reverse the inhibitory effect of dapagliflozin on GSDMD activation. ATP1a (membrane internal reference) and GAPDH (cytoplasmic internal reference) showed stable expression across all groups, validating the uniformity and reliability of subcellular fractionation and loading. Taken together, these results suggested that

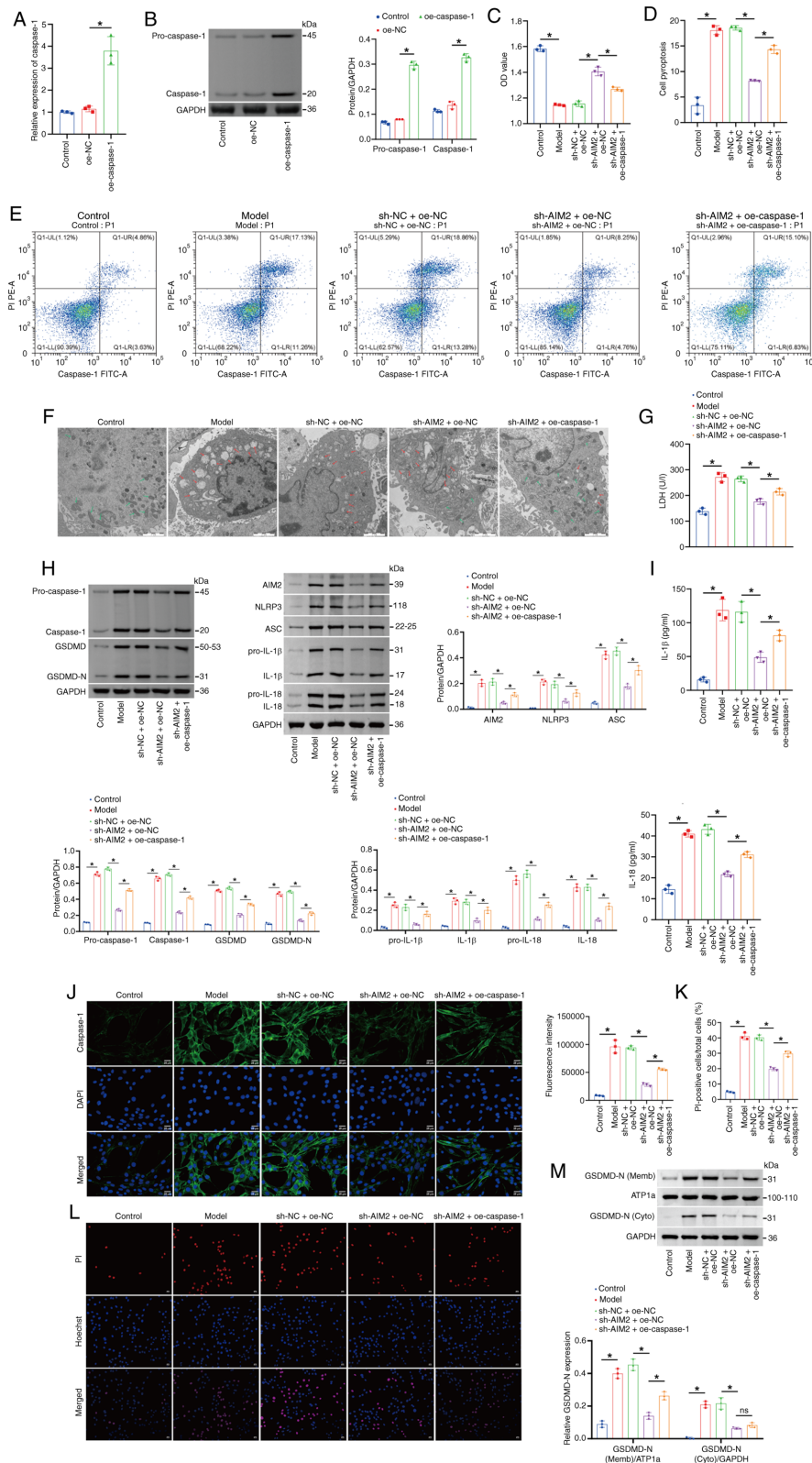


Figure 6. AIM2 regulates pyroptosis in cardiomyocytes via the Caspase-1/GSDMD axis. (A) Caspase-1 levels evaluated by reverse transcription-quantitative PCR. (B) Western blot analysis of pro-Caspase-1 and Caspase-1 levels. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (C) CCK-8 assay of cell viability. (D and E) Flow cytometry analysis of pyroptosis (Caspase-1). (F) TEM analysis of mitochondrial damage. Scale bar, 2 μ m. (G) LDH content in the cell supernatant. (H) Western blot assessment of pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in the cell lysate. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (I) IL-1 β and IL-18 levels in the cell supernatant. (J) IF staining of Caspase-1 in cells. (K and L) Hoechst 33342/PI fluorescence staining showing the percentage of PI-positive mouse cardiomyocytes. Scale bar, 50 μ m, magnification, x200; scale bar, 25 μ m, magnification, x400. (M) Western blot analysis of the levels of GSDMD-N in the membrane and cytoplasm. The blot was probed with anti-ATP1a/GAPDH antibody. ATP1a (membrane marker) and GAPDH (cytoplasmic marker) were used as a loading control. *P<0.05. AIM2, absent in melanoma 2; GSDMD, Gasdermin D; TEM, transmission electron microscopy; LDH, lactate dehydrogenase; GSDMD-N, Gasdermin D N-terminal fragment; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis-associated Speck-like protein containing a CARD; IF, immunofluorescence; ATP1a, sodium/potassium-transporting ATPase subunit α 1; oe, overexpressed; NC, negative control; sh, short hairpin.

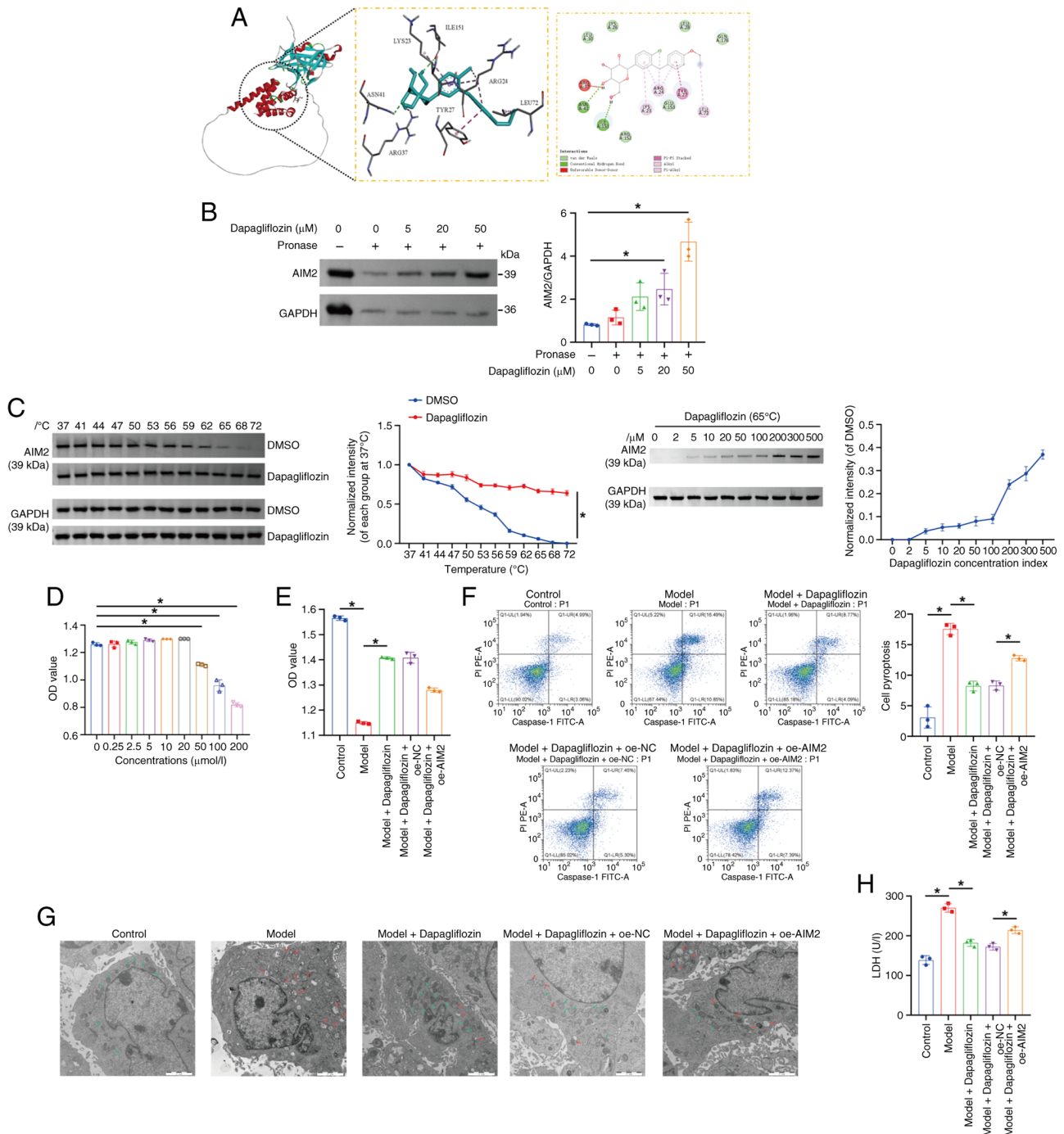


Figure 7. Dapagliflozin directly targets AIM2 and ameliorated cardiomyocyte pyroptosis and mitochondrial damage. (A) Molecular docking of dapagliflozin with AIM2. (B) DARTS assay verification of the binding of Dapagliflozin to AIM2. (C) CETSA was performed to measure the thermal stability of intracellular AIM2 protein following dapagliflozin treatment. (D) CCK-8 assay for determining the optimal concentration of dapagliflozin. (E) CCK-8 assay of cell viability. (F) Flow cytometry analysis of pyroptosis (Caspase-1). (G) TEM analysis of mitochondrial damage. Scale bar, 2 μm. (H) LDH content in the cell supernatant. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. * $P < 0.05$. AIM2, absent in melanoma 2; DARTS, drug affinity responsive target stability; CETSA, cellular thermal shift assay; TEM, transmission electron microscopy; LDH, lactate dehydrogenase; oe, overexpressed; NC, negative control; sh, short hairpin.

dapagliflozin inhibited the AIM2/Caspase-1/GSDMD axis and thereby alleviated pyroptosis in mouse cardiomyocytes.

Dapagliflozin inhibits the AIM2-Caspase-1/GSDMD axis to improve HFpEF symptoms in mice. Finally, AIM2 was overexpressed in combination with dapagliflozin treatment to explore further the underlying mechanisms at the animal level. Fig. 9A shows representative left ventricular M-mode

echocardiographic images. The LVEF percentage remained essentially consistent across all groups (Fig. 9B). Compared with the HFpEF group, the GLS percentage was higher in the HFpEF + dapagliflozin group, whereas AIM2 overexpression reduced the GLS percentage (Fig. 9C). Compared with the HFpEF group, the HFpEF + dapagliflozin group exhibited lower E/E' and HW/TL ratios and a longer running distance in the treadmill exhaustion test. Notably, AIM2 overexpression

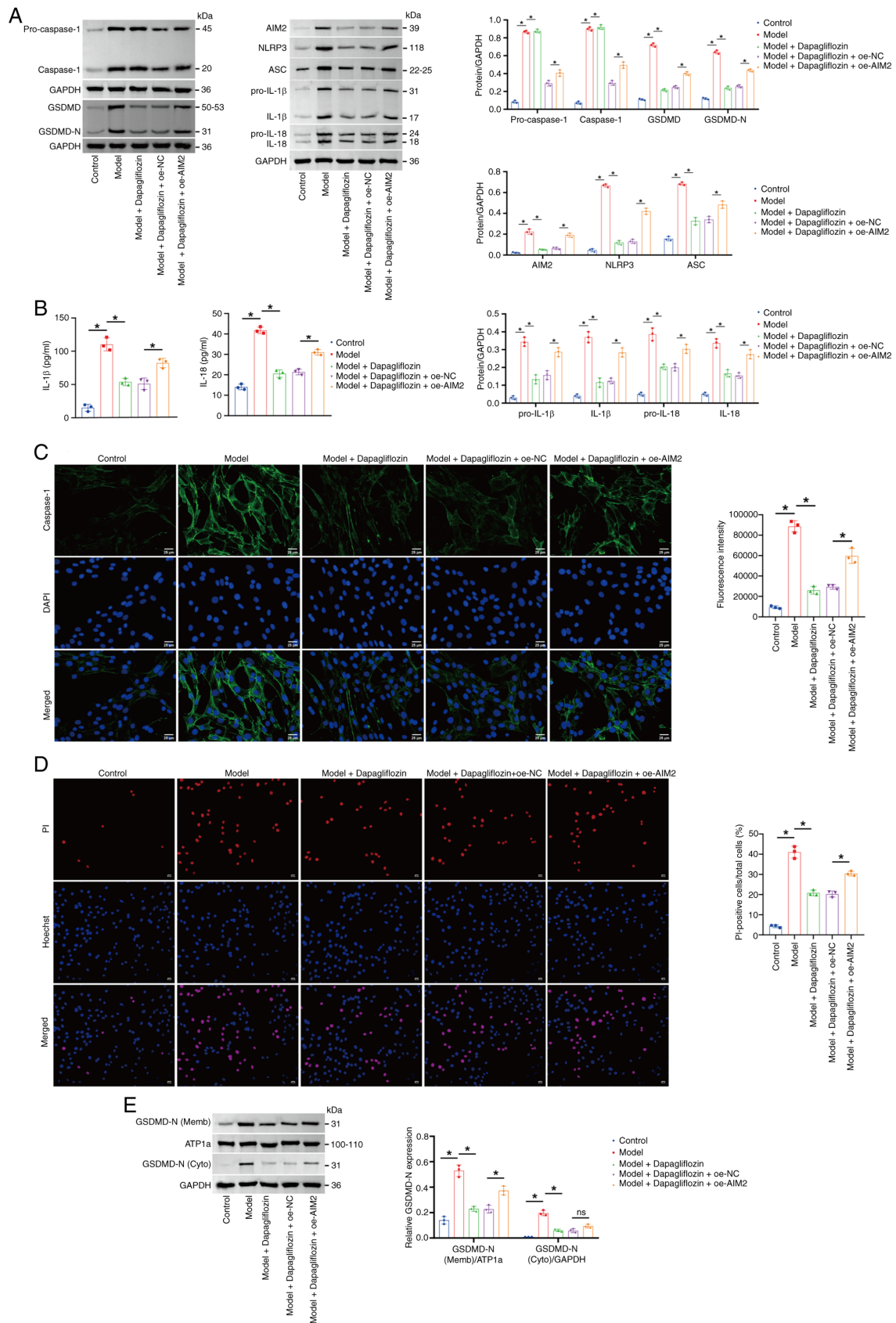


Figure 8. Dapagliflozin inhibits the AIM2/Caspase-1/GSDMD axis to alleviate pyroptosis in mouse cardiomyocytes. (A) Western blotting of pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in the cell lysate. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. (B) IL-1 β and IL-18 levels in the cell supernatant. (C) Immunofluorescence staining of Caspase-1 in cells. (D) Hoechst 33342/PI fluorescence staining showing the percentage of PI-positive mouse cardiomyocytes. Scale bar, 50 μ m, magnification, x200; scale bar, 25 μ m, magnification, x400. (E) Western blotting of the levels of GSDMD-N in the membrane and cytoplasm. The blot was probed with anti-ATP1a/GAPDH antibody. ATP1a (membrane marker) and GAPDH (cytoplasmic marker) were used as a loading control. *P<0.05. AIM2, absent in melanoma 2; GSDMD, Gasdermin D; GSDMD-N, Gasdermin D N-terminal fragment; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; ASC, apoptosis-associated Speck-like protein containing a CARD; ATP1a, sodium/potassium-transporting ATPase subunit α 1; oe, overexpressed; NC, negative control; sh, short hairpin.

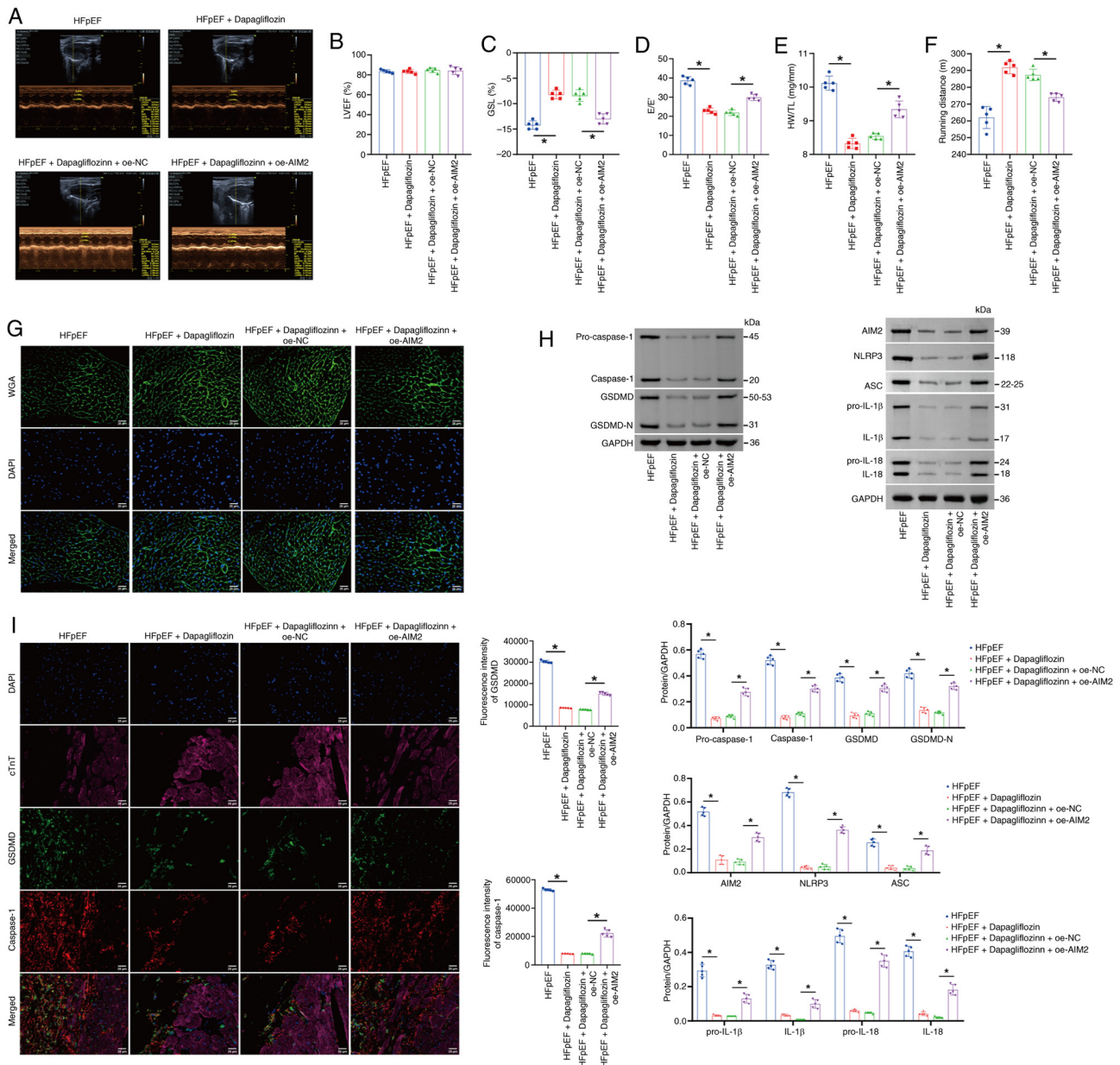


Figure 9. Dapagliflozin inhibits the AIM2-Caspase-1/GSDMD axis to improve HFpEF symptoms in mice. (A) Representative left ventricular M-mode echocardiography. (B) The percentage of the LVEF. (C) The percentage of GLS. (D) The E/E'. (E) HW/TL. (F) Exercise exhaustion test based on running distance. (G) WGA staining of the morphological alterations in the myocardial tissue cell membrane in the mice. (H) Western blot analysis of pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 levels in myocardial tissue. (I) Immunofluorescence staining showing the expression of GSDMD and Caspase-1. Scale bar, 25 μ m; magnification, x400. The blot was probed with anti-GAPDH antibody. GAPDH was used as a loading control. * P <0.05. AIM2, absent in melanoma 2; GSDMD, Gasdermin D; HFpEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; GLS, global longitudinal strain; E/E', ratio of the mitral E wave to the E' wave; HW/TL, ratio of heart weight to tibia length; WGA, wheat germ agglutinin; GSDMD-N, Gasdermin D N-terminal fragment; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; ASC, apoptosis-associated Speck-like protein containing a CARD; oe, overexpressed; NC, negative control; sh, short hairpin.

abolished these beneficial effects, leading to increased E/E' and HW/TL ratios and a decreased running distance (Fig. 9D-F). Additionally, myocardial hypertrophy was reduced in the HFpEF + dapagliflozin group compared with the HFpEF group, whereas AIM2 overexpression resulted in more severe myocardial hypertrophy (Fig. 9G). Moreover, compared with the HFpEF group, the levels of Caspase-1, GSDMD, GSDMD-N, AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 in myocardial tissue were decreased in the HFpEF + dapagliflozin group. After AIM2 overexpression, the levels of pro-Caspase-1, Caspase-1, GSDMD, GSDMD-N,

AIM2, NLRP3, ASC, pro-IL-1 β , pro-IL-18, IL-1 β and IL-18 increased in myocardial tissue (Fig. 9H and I). Collectively, these results suggested that dapagliflozin inhibited the AIM2-Caspase-1/GSDMD axis and thereby improved HFpEF-related phenotypes in mice.

Discussion

With an aging population and the increasing prevalence of obesity, diabetes and hypertension, HFpEF has become the main form of HF worldwide (39). However, effective treatments

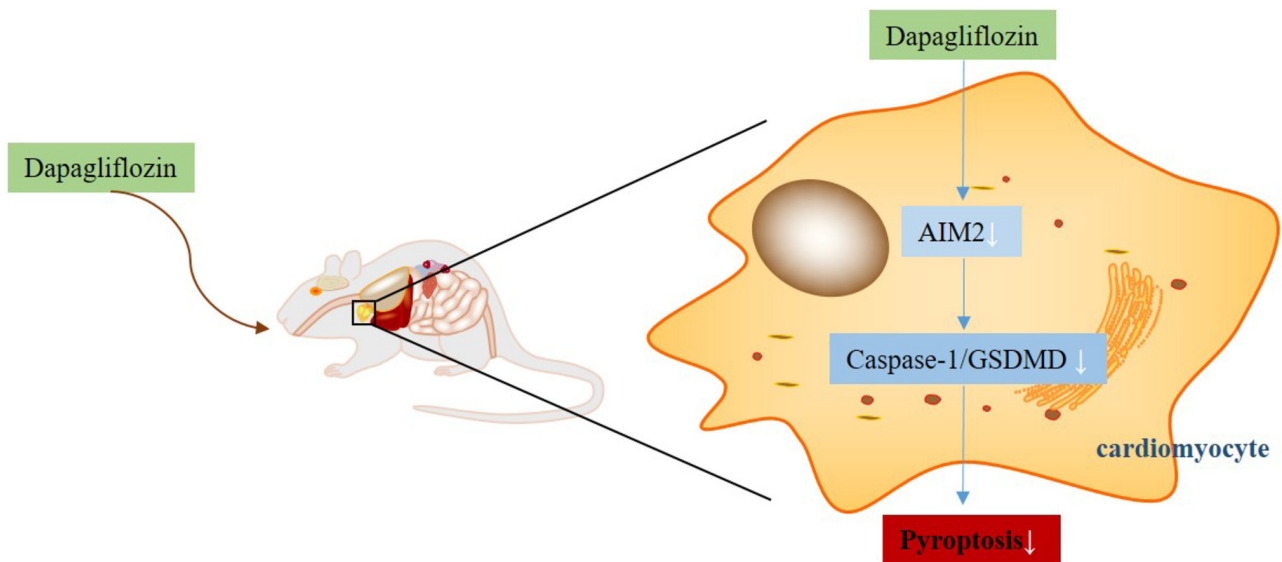


Figure 10. Dapagliflozin inhibition of the AIM2/Caspase-1/GSDMD axis attenuated myocardial tissue pyroptosis and improved HFpEF symptoms in mice. AIM2, absent in melanoma 2; GSDMD, Gasdermin D; HFpEF, heart failure with preserved ejection fraction.

for HFpEF remain limited. Therefore, elucidating the mechanisms underlying HFpEF may facilitate the identification of new therapeutic approaches. The present study investigated the mechanism by which dapagliflozin treats HFpEF, with a focus on pyroptosis, primarily at the animal and cellular levels. It was found that dapagliflozin may alleviate HFpEF through mechanisms involving the AIM2/Caspase-1/GSDMD axis to attenuate pyroptosis (Fig. 10). To the best of the authors' knowledge, this is the first study to link dapagliflozin to the regulation of the AIM2/Caspase-1/GSDMD axis in HFpEF.

SGLT2 inhibitors are used in the treatment of HF (40). SGLT2 inhibitor therapy can effectively alleviate symptoms and decrease overall hospitalization rates in patients with HF (41). Previous research has demonstrated that dapagliflozin, an SGLT2 inhibitor, can improve renal outcomes in patients with HF with reduced ejection fraction and reduce all-cause and cardiovascular mortality (42). In the EMPEROR-Preserved trial, dapagliflozin decreased the risk of cardiovascular death or hospitalization for HF and SGLT2 inhibitors may become a new standard of care for patients with HFpEF (43). In addition, dapagliflozin has been reported to prevent renal pyroptosis via regulation of the miR-155-5p/HO-1/NLRP3 axis (44). However, the mechanism by which dapagliflozin modulates pyroptosis in HFpEF remains poorly understood. The present study not only evaluated the effects of dapagliflozin on the pathological features of HFpEF but also comprehensively monitored changes in body weight, blood pressure and blood glucose levels. Mice receiving dapagliflozin exhibited significant improvements in body weight, blood pressure and blood glucose control. These findings suggested that, in addition to its direct myocardial effects, dapagliflozin may alleviate HFpEF-related pathological features by reducing body weight, improving glucose metabolism and lowering blood pressure. Importantly, these systemic effects may act in concert to contribute to HFpEF improvement rather than reflecting a single isolated mechanism. Furthermore, the present study demonstrated

that dapagliflozin alleviated HFpEF-related phenotypes and suppressed myocardial pyroptosis in HFpEF mice. Accordingly, the mechanism by which dapagliflozin regulates pyroptosis in HFpEF was further explored.

In recent years, pyroptosis, as a form of programmed cell death, has increasingly attracted attention because of its involvement in inflammatory responses and cardiovascular diseases. Pyroptosis can contribute to cardiac fibrosis, myocardial hypertrophy, cardiomyocyte death, myocardial dysfunction, excessive inflammation and cardiac remodeling (45,46). Accordingly, targeting pyroptosis has been considered a promising therapeutic strategy for HF treatment (47). The inflammasome is a multiprotein complex that plays a critical role in the innate immune response by promoting the cleavage and activation of IL-1 β and IL-18 (48). Among these complexes, the AIM2 inflammasome functions as a multiprotein platform that detects abnormal cytoplasmic dsDNA, thereby promoting cytokine maturation and release and ultimately triggering pyroptosis (49). Moreover, the AIM2 inflammasome is an important mediator of inflammatory signaling, as its activation induces the production of the proinflammatory cytokines IL-1 β and IL-18 and contributes to the initiation of the pyroptotic response involved in regulating excessive cell proliferation (50). In the present study, AIM2 protein levels in myocardial tissue were markedly elevated in the HFpEF group compared with the Sham group, whereas this increase was reversed following dapagliflozin treatment. Notably, changes in myocardial pyroptosis in HFpEF mice were accompanied by corresponding fluctuations in AIM2 protein levels. Pearson correlation analysis further showed that AIM2 levels were markedly negatively correlated with GLS and markedly positively associated with E/E', which supports a potential role for AIM2 in HFpEF. Collectively, these findings suggested that AIM2 may exert substantial effects on myocardial pyroptosis in HFpEF. From a mechanistic perspective, inhibition of AIM2 activity may help reduce myocardial injury and improve cardiac function; therefore, further investigations

were conducted to clarify the mechanisms linking AIM2 activation to pyroptosis in this context.

Pioneering research has clarified key molecular mechanisms of pyroptosis, particularly the central role of the Caspase-1/GSDMD pathway in this form of inflammatory cell death (7). AIM2, acting as a pattern-recognition receptor, can activate Caspase-1 by sensing cytosolic dsDNA, thereby initiating pyroptosis (10). Through its downstream effector caspase, Caspase-1, the AIM2 inflammasome exerts important biological functions, highlighting its potential involvement in cardio-cerebrovascular diseases and related metabolic disorders (51). In addition, activation of GSDMD, a prototypical member of the gasdermin family, is closely linked to inflammasome-dependent innate immune surveillance (52). Upon activation by inflammatory caspases such as Caspase-1 and Caspase-11, GSDMD is cleaved to generate the pore-forming N-terminal fragment, which disrupts plasma membrane integrity and ultimately drives pyroptosis (53). Research has indicated that, in chronic kidney disease, the AIM2-Caspase-1/GSDMD pathway is involved in mediating renal cell pyroptosis (38) and inhibition of AIM2 inflammasome activation, achieved by downregulating AIM2 and Caspase-1, has been shown to alleviate pyroptosis in early brain injury following subarachnoid hemorrhage induced by GSDMD (54). However, the functional mechanism of the AIM2/Caspase-1/GSDMD axis in HFpEF remains largely elusive. In the present study, the findings revealed that downregulation of AIM2 alleviated pyroptosis in mouse cardiomyocytes at the cellular level. Moreover, the data indicated that AIM2 regulated cardiomyocyte pyroptosis through the Caspase-1/GSDMD axis. Consistently, these results supported a crucial role for the AIM2/Caspase-1/GSDMD axis in mediating myocardial pyroptosis in HFpEF.

AIM2-associated pyroptosis has also been implicated in other disease settings. For example, sevoflurane has been shown to induce pyroptosis in mouse models of Alzheimer's disease by activating Caspase-1 through the NLRP3 and AIM2 pathways, while also facilitating GSDMD cleavage and the downstream production of IL-1 β and IL-18 (55). Additionally, *Talaromyces marneffe* has been reported to activate the AIM2-Caspase-1/-4/GSDMD pathway and thereby induce pyroptosis in hepatocytes (56). However, the mechanism by which dapagliflozin influences the AIM2/Caspase-1/GSDMD axis and pyroptosis in HFpEF remains unclear. The identification of AIM2 as a key regulator of pyroptosis in HFpEF therefore represents an important finding of the present study. In the context of HFpEF, upregulation of AIM2 may be associated with exacerbated inflammatory responses that contribute to cardiomyocyte injury and dysfunction. Mechanistically, AIM2 upregulation likely activates the Caspase-1/GSDMD axis to enhance proinflammatory cytokine release, which impairs the contractile and relaxational functions of cardiomyocytes. Moreover, AIM2-mediated pyroptosis may promote cardiomyocyte loss, further aggravating the pathological progression of HFpEF. Finally, the present study demonstrated both *in vitro* and *in vivo* that dapagliflozin-mediated inhibition of the AIM2/Caspase-1/GSDMD pathway attenuated myocardial pyroptosis and improved HFpEF-related phenotypes in mice.

AMPK plays an important role in energy metabolism and cardiovascular protection (57). SGLT2 inhibitors have been

shown to activate AMPK, thereby improving cardiomyocyte energy metabolism and function (58). Accordingly, AMPK activation may alleviate energy stress and injury in cardiomyocytes by promoting fatty acid oxidation and improving mitochondrial function. Mitochondrial dysfunction is an important pathophysiological characteristic of HFpEF (59) and SGLT2 inhibitors may enhance energy metabolism and antioxidant capacity in cardiomyocytes, at least in part, through improvements in mitochondrial function (60). In the present study, assessment of mitochondrial membrane potential showed that dapagliflozin treatment markedly increased mitochondrial membrane potential, indicating restoration of mitochondrial function. As improved mitochondrial function can enhance cellular energy metabolism and antioxidant capacity, it may consequently reduce cardiomyocyte injury and inflammatory responses (61). However, further studies are still required to clarify the specific mechanisms by which restored mitochondrial function contributes to dapagliflozin-mediated alleviation of HFpEF. Oxidative stress is also an important pathophysiological factor in HFpEF (62). Therefore, reducing oxidative stress may improve myocardial function by alleviating cardiomyocyte injury and limiting inflammatory responses (63). Nevertheless, future studies are needed to further define the contribution of oxidative stress reduction to the amelioration of HFpEF by dapagliflozin.

However, the present study has some limitations. While molecular docking, DARTS and CETSA analyses provide valuable information regarding the potential binding sites and interaction patterns between dapagliflozin and AIM2, they do not by themselves directly demonstrate the actual physical binding of the two molecules under physiological conditions. Further studies using techniques such as surface plasmon resonance or isothermal titration calorimetry will be required to definitively confirm direct binding and characterize the binding affinity. Although the HFpEF model induced by L-NAME plus a high-fat diet has been widely used to investigate the pathophysiological mechanisms of HFpEF (22,23) and successfully recapitulated key features of HFpEF in the present study, such as diastolic dysfunction and inflammatory responses, this model cannot fully recapitulate the complex pathophysiological processes of human HFpEF. Human HFpEF is a multifactorial disease whose pathogenesis involves multiple comorbidities (such as hypertension, diabetes, obesity and coronary heart disease) and the aging process (64). By contrast, the model induced by L-NAME and a high-fat diet mainly mimics the adverse effects of hypertension and metabolic disorders on the heart, but fails to fully replicate all the pathophysiological features of human HFpEF. Aging is one of the important risk factors for HFpEF and its effect on cardiac structure and function plays a critical role in human disease (65). However, the L-NAME and high-fat diet-induced model is usually established in young mice, which cannot fully mimic the cumulative aging-related damage to the heart. Aging-related changes, such as increased cardiomyocyte apoptosis, extracellular matrix remodeling and mitochondrial dysfunction, are not adequately represented in this model. Due to the aforementioned limitations of the L-NAME and high-fat diet-induced model, the translational relevance of the present study may be restricted to a certain extent. Although this model provides valuable insights into the pathophysiological

mechanisms of HFpEF, it does not fully accurately mimic the human disease. Therefore, caution is needed when translating these findings into clinical applications. Future studies are required to further explore animal models that more closely resemble the pathophysiological characteristics of human HFpEF, so as to improve the translational relevance of the research results. In addition, the main limitation of the present study is that all experiments were based on mouse models and *in vitro* cell lines, without validation using human myocardial samples. Although mouse models and cell lines provide valuable experimental platforms, they cannot fully simulate the complex pathophysiological processes of human HFpEF. Owing to the lack of validation in human samples, the translational potential of the present findings into clinical practice may be limited. While the results from mouse models and cell lines offer important insights into the mechanism of action of dapagliflozin, these findings need to be further validated in human samples to ensure their clinical relevance and reproducibility. Future studies should include more human myocardial samples to evaluate the potential role of dapagliflozin in the treatment of HFpEF. The present study focused on the therapeutic effects of 6-week dapagliflozin intervention in the HFpEF model. Although this short-term intervention has revealed the significant beneficial effects of dapagliflozin in improving cardiac function, inhibiting inflammatory responses and pyroptosis, considering that HFpEF is a chronic disease, the 6-week intervention period may be insufficient to comprehensively evaluate the long-term effects of dapagliflozin. Long-term intervention studies are essential for assessing the effects of dapagliflozin on cardiac remodeling, survival rate and sustained suppression of pyroptosis. Given the relatively short intervention period in the present study, it is acknowledged that this may limit the comprehensive evaluation of the long-term effects of dapagliflozin. Future studies need to design longer-term intervention experiments for improved understanding of the long-term mechanisms and clinical potential of dapagliflozin in the treatment of HFpEF. Due to equipment and experimental conditions, SEM images showing membrane blebbing or pore structures have not yet been obtained. Although the supplementary western blotting results confirmed GSDMD-N translocation to the plasma membrane (indirect evidence of pore formation), direct SEM evidence of plasma membrane pore structures is still lacking. SEM experiments will be further supplemented in future studies to provide more direct evidence for GSDMD-mediated plasma membrane damage.

Furthermore, the present study did not use pharmacological AIM2 inflammasome activators or inhibitors to verify the role of this pathway in mediating the therapeutic effect of dapagliflozin. This was mainly due to the lack of highly specific drugs targeting the AIM2 inflammasome and the constraints of experimental conditions. The existing non-specific inflammasome inhibitors (such as Shikonin) mostly act by interfering with the assembly process of the AIM2 inflammasome complex and directly inhibiting Caspase-1 activity, without changing the expression level of AIM2 protein itself. They cannot accurately simulate the protein level-dependent regulatory effect achieved by AIM2 knockdown/overexpression in the present study, making it difficult to accurately verify the core role of AIM2 protein level in the therapeutic mechanism

of dapagliflozin (66,67). At the same time, the screening of appropriate drug concentrations and administration methods requires a large number of preliminary pre-experiments, which cannot be completed in time for this revision due to the limitations of the current experimental cycle and resource conditions. Although the rescue experiments (AIM2 overexpression/knockdown) have provided indirect evidence for the involvement of the AIM2 pathway, there is still a lack of direct pharmacological verification and the potential interference of other inflammasome subtypes (such as NLRP3 and NLRC4) cannot be completely excluded, which also brings certain limitations to the conclusions of the present study. Moreover, the present study only focused on the regulatory role of the AIM2/Caspase-1/GSDMD axis and did not further explore the upstream molecular mechanism by which dapagliflozin regulates AIM2 expression, so it is impossible to clarify whether dapagliflozin indirectly regulates AIM2 level through other signaling pathways, which is also a direction that needs further improvement in future studies. Although the rescue experiments (AIM2 overexpression/knockdown) provided indirect evidence for the involvement of the AIM2 pathway, there is still a lack of direct pharmacological verification. Future studies will actively seek specific AIM2 inflammasome activators and inhibitors and clarify the upstream mechanism of dapagliflozin regulating AIM2 expression and perform relevant experiments to further confirm the causal relationship between the AIM2 inflammasome pathway and the therapeutic effect of dapagliflozin. At the same time, the sample size will be expanded, the experimental design optimized, potential interference factors reduced and the reliability and universality of the research conclusions improved.

In conclusion, the findings of the present study indicated that dapagliflozin may be beneficial for improving HFpEF. The present study established both *in vitro* and *in vivo* HFpEF models and administered dapagliflozin, thereby demonstrating that dapagliflozin may alleviate HFpEF through mechanisms involving the AIM2/Caspase-1/GSDMD axis to attenuate pyroptosis. Further clinical trials and experimental studies are needed to deepen our understanding of the regulatory mechanisms of dapagliflozin and to facilitate its broader clinical application in the management of HFpEF.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LX and XZ contributed to the study conception and design. Material preparation, data collection and analysis were

performed by SD, HY, TL and YL. The first draft of the manuscript was written by SD. LX and XZ confirm the authenticity of all the raw data. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Ethics Committee of Changsha Hospital of Traditional Chinese Medicine (approval no. 2022111003). The present study is in accordance with the ARRIVE guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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