



Article

# PCSK9 Inhibitor Alirocumab Improves Diabetic Cardiomyopathy Through the ERK/p38 MAPK Signaling Pathway

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## Abstract

PCSK9 is a gene associated with familial hypercholesterolemia and is involved in other biological processes such as apoptosis, autophagy, and inflammatory responses. This study aims to further validate whether PCSK9 inhibitors can improve diabetic cardiomyopathy and elucidate their mechanisms of action. This study utilized H9c2 cells and C57BL/6J mice to validate the efficacy of the PCSK9 inhibitor alirocumab through in vivo and in vitro experiments. In vitro, alirocumab was shown to enhance cell viability and reduce oxidative stress in H9c2 cells under high glucose stress. It can also decrease the expression levels of inflammatory reaction and mitochondrial apoptosis-related proteins. Through in vivo experiments, we demonstrated that alirocumab can reduce myocardial hypertrophy and improve cardiac function in diabetic cardiomyopathy mice. Meanwhile, alirocumab treatment increased mitochondrial size and quantity in the hearts of diabetic cardiomyopathy mice, promoted mitochondrial fusion, and reduced the number of damaged mitochondria. Alirocumab could also reduce the percentage of myocardial fibrosis and oxidative stress in mice. Finally, we found that alirocumab can improve cardiac function in diabetic cardiomyopathy through the ERK/p38 MAPK pathway. Our data demonstrate that the PCSK9 inhibitor alirocumab provides protective effects against diabetic cardiomyopathy, offering fundamental experimental support for its clinical application in this condition.

**Keywords:** diabetic cardiomyopathy; PCSK9 inhibitor; MAPK signaling pathway

## 1. Introduction

Diabetes (DM) is one of the risk factors for cardiovascular disease, and the International Diabetes Federation estimates that there will be nearly 500 million people who are overweight and develop insulin resistance, and 642 million people will be diagnosed with type 2 diabetes by 2040 [1]. The chronic complications of T2DM frequently involve the heart, brain, kidneys and other vital organs. Among them, diabetic cardiomyopathy (DCM) is an important cause of heart failure in diabetic patients and significantly affects the quality of life. Diabetic patients with heart failure can be diagnosed with DCM after excluding other clear causes such as hypertension, structural heart disease and ischemic heart disease. Diabetic cardiomyopathy was first described in 1972 in four diabetic patients who presented with symptoms of heart failure. The early stage of DCM is characterized by metabolic disturbances that promote adaptive changes in cardiac structure and function, leading to cardiac remodeling, fibrosis, and diastolic dysfunction, and ultimately to reduced left ventricular ejection fraction [2].



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Current research has identified the following pathological mechanisms involved in diabetic cardiomyopathy: (1) abnormal insulin signaling pathways; (2) glycototoxicity; (3) cardiac steatosis; (4) mitochondrial dysfunction and oxidative stress; (5) endoplasmic reticulum (ER) stress; (6) impaired calcium regulation; (7) cardiomyocyte programmed death (e.g., apoptosis, pyroptosis, and autophagy); (8) unopposed activation of the renin–angiotensin–aldosterone system (RAAS); (9) non-adaptive immune regulation; (10) microvascular and coronary artery endothelial dysfunction; and (11) exosomal dysregulation [3]. Currently, the commonly used therapeutic drugs in clinical practice include angiotensin system blockers (ACE inhibitors, ARBs, and ARNIs),  $\beta$ -receptor blockers, mineralocorticoid receptor antagonists (MRAs), and sodium–glucose cotransporter 2 (SGLT2) inhibitors. In addition, basic research has demonstrated that statins, a class of lipid-lowering drugs, can ameliorate the endothelial damage of DCM and reduce myocardial fibrosis and dysfunction in experimental DCM by reducing myocardial oxidative stress and upregulating angiogenic factors [4].

Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) is a gene associated with familial hypercholesterolemia and was first identified in 2003. Genetic studies have confirmed that individuals with a functionally acquired PCSK9 mutation exhibit severe familial hypercholesterolemia characterized by increased PCSK9 activity and elevated low-density lipoprotein cholesterol (LDL-C) levels. In patients with reduced PCSK9 due to mutation, LDL-C levels are lower and the risk of coronary heart disease is also reduced [5].

The mechanism of PCSK9 is primarily related to the regulation of low-density lipoprotein receptor (LDLR). After synthesis, PCSK9 is transported primarily through the endoplasmic reticulum (ER) and trans-Golgi network (TGN). Sortilin binds to PCSK9 and mediates its secretion into the plasma. The secreted PCSK9 binds to LDLR on the cell surface and prevents LDLR from taking up LDL [6].

The discovery of the PCSK9-related mechanism has led to the development of various therapeutic strategies to reduce circulating PCSK9 levels. Strategies targeting PCSK9 inhibition include monoclonal antibodies, antisense oligonucleotides (ASOs), small interfering RNA (siRNA), small molecule inhibitors, peptide mimetics, CRISPR gene editing and peptide vaccines. Among them, monoclonal antibodies, including alirocumab, evolocumab, and the small interfering RNA inclisiran, have been approved for marketing in China. They are widely used in lipid-lowering therapy for hyperlipidemia and coronary atherosclerotic cardiovascular disease [7].

In addition to regulating lipid metabolism, PCSK9 may also be involved in other biological processes, such as apoptosis, autophagy, pyroptosis, ferroptosis, inflammation, energy metabolism and tumor immunity, and has been associated with diabetes and neurodegenerative diseases. The BIOSAT-CHF cohort study further demonstrated that circulating PCSK9 levels were significantly elevated in patients with heart failure and were associated with the prognosis of heart failure. More significantly, PCSK9 also plays a pathogenic role in the pathogenesis of diabetes complicated by cardiovascular disease. In human and mouse heart tissue following acute myocardial infarction, PCSK9 was shown to be strongly expressed in the border region of the infarcted area. At the same time, the upregulation of PCSK9—both endogenous and that secreted by cardiomyocytes—was positively correlated with increased infarct size and decreased contractile function. Conversely, inhibition of PCSK9 has been shown to reduce myocardial infarct size [8]. Increased PCSK9 expression in ischemic hearts induces autophagy in cardiomyocytes and activates the nuclear factor kappa-B (NF- $\kappa$ B) signaling pathway, stimulating the secretion of pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ) [9]. In chronic myocardial ischemia, increased expression of PCSK9 induces mitochondrial DNA damage and activates the NLRP3 inflammasome to induce cell

pyroptosis [10]. In addition, PCSK9 expression has been shown to be positively correlated with elevated levels of apoptosis markers such as BAX, caspase-3 and caspase-9 [11]. PCSK9 is also closely associated with inflammatory responses. In macrophages, the overexpression of PCSK9 mediates the increased expression of pro-inflammatory cytokines by activating the TLR4/NF- $\kappa$ B signaling pathway [12].

In view of the high prevalence of diabetes and the adverse effects of diabetic cardiomyopathy on patient prognosis, the exploration of its treatment methods has important clinical application value. Although statins are currently considered first-line agents for reducing mortality and major cardiovascular events in diabetic patients without established coronary heart disease, and studies have confirmed that statins can improve cardiac function in diabetic cardiomyopathy by inhibiting apoptosis and oxidative stress, their use is not without limitations. In pancreatic  $\beta$ -cells, proper membrane structure is essential for glucose-mediated insulin secretion. Changes in cellular cholesterol levels may negatively affect this process, leading to  $\beta$ -cell dysfunction. Accordingly, real-world studies have demonstrated that statin use is associated with deterioration of glucose homeostasis and an increased risk of new-onset T2DM in a dose-dependent manner [13,14]. These adverse effects, to some extent, limit the therapeutic benefit of statins in the management of diabetic cardiomyopathy.

However, PCSK9 inhibitors, as a novel class of lipid-lowering agents, have little effect on blood glucose in real world studies. In the ODYSSEY phase 3 trial, alirocumab significantly reduced LDL-C and other atherogenic lipid parameters, and was generally well tolerated in DM and ASCVD patients without increasing the incidence of new-onset diabetes in patients with prediabetes or normal blood glucose [15]. Furthermore, basic research has shown that the lack of PCSK9 in the liver does not significantly alter the plasma or pancreatic insulin levels in mice, indicating that circulating PCSK9 (mostly from the liver) does not significantly affect LDL-R expression in the pancreas [16,17], which may partially explain real-world statistical findings. Monoclonal antibodies targeting PCSK9 specifically bind to circulating PCSK9 and are therefore unlikely to significantly impact LDL-R expression in pancreatic  $\beta$ -cells. In addition, a synthesis of the existing literature reveals that the mechanism of PCSK9 for heart failure and the possible mechanism characteristics of diabetic cardiomyopathy have many overlapping points. To date, most studies on PCSK9 inhibitors have focused on myocardial infarction and diabetes-related interventional therapy. High-quality evidence confirming the efficacy and mechanistic role of PCSK9 inhibitors in the context of diabetic cardiomyopathy as an independent pathological entity remains lacking, thus leaving a substantial gap for further investigation.

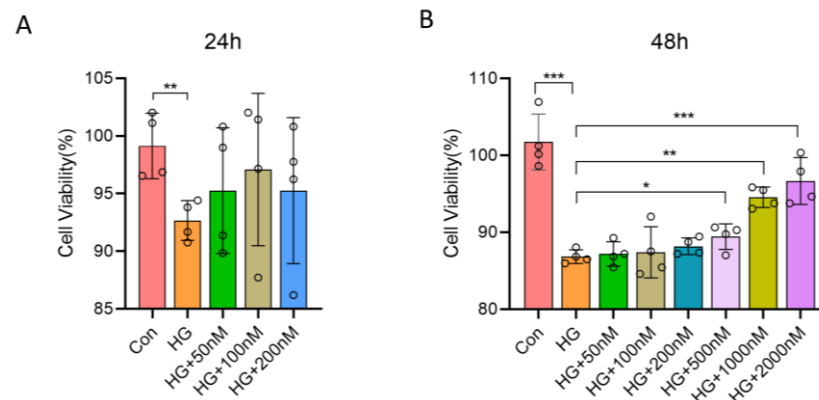
Accordingly, the present study proposes the hypothesis that PCSK9 inhibitors may serve as a viable and potentially superior therapeutic option for diabetic cardiomyopathy. The cardioprotective effects of PCSK9 inhibition were further evaluated in high glucose-damaged cardiomyocytes and in a mouse model of diabetic cardiomyopathy, and the underlying mechanisms were explored.

## 2. Results

### 2.1. Alirocumab Was Able to Improve the Decreased Activity of H9c2 Cells Under High-Glucose Treatment

In order to verify whether PCSK9 inhibitors have a protective effect on high-sugar-induced myocardial cell injury, and to select the appropriate concentration of PCSK9 inhibitors for subsequent experiments, this experiment used the CCK8 kit to detect the cell activity of H9c2 cells treated with different concentrations of the PCSK9 inhibitor alirocumab. The results showed that, after 24 h and 48 h of 40 mM high-sugar treatment to H9c2 cardiomyocytes, the activity of cardiomyocytes decreased significantly (\*\*  $P_{24h} < 0.01$  and

\*\*\*  $P_{48h} < 0.001$ ). However, when H9c2 cells were treated with 50 nM, 100 nM and 200 nM alirocumab in 40 mM high-sugar medium for 24 h, the cell vitality was not significantly improved. After 48 h of treatment with 50/100/200/500/1000/2000 nM concentrations of alirocumab, it was observed that 500, 1000 and 2000 nM alirocumab could improve the decreased activity of H9c2 cells under high-sugar treatment (\*  $P_{500nM} < 0.05$ , \*\*  $P_{1000nM} < 0.01$ , and \*\*\*  $P_{2000nM} < 0.001$ ) (Figure 1). In subsequent cell experiments, the PCSK9 inhibitor alirocumab was used at 500 nM, 1000 nM and 2000 nM concentrations as the low concentration, medium concentration and high concentration groups respectively.

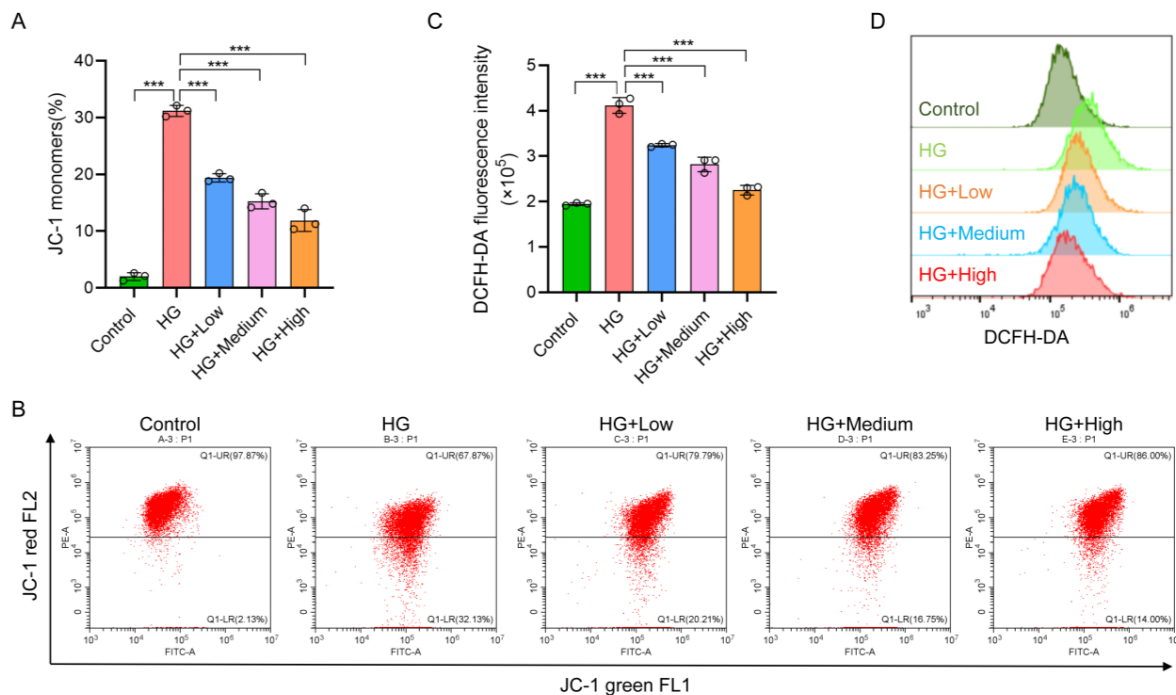


**Figure 1.** Effects of PCSK9 inhibitor alirocumab on the activity of cardiomyocytes damaged by high sugar. (A). H9c2 cells were treated with 50 nM, 100 nM and 200 nM of the PCSK9 inhibitor alirocumab for 24 h in a 40 mM high-sugar-culture medium. The activity of cardiomyocytes was detected by the CCK8 kit. (B). H9c2 cells were treated with 50 nM, 100 nM, 200 nM, 500 nM, 1000 nM and 2000 nM of the PCSK9 inhibitor alirocumab for 48 h in 40 mM high-sugar-culture medium. The activity of cardiomyocytes was detected by the CCK8 kit ( $n = 4$  for each group; \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

## 2.2. Alirocumab Can Reduce ROS Production and Mitochondrial Membrane Potential of Cardiomyocytes in Rats with High-Sugar Injury

Mitochondria play a pivotal role in the process of apoptosis. The decrease in mitochondrial transmembrane potential is considered to be the earliest event in the cascade reaction of apoptosis. In addition, reactive oxygen species (ROS) are involved in intracellular signal transduction and regulation, and play an important role in cell cycle, gene expression and the maintenance of homeostasis. When the body is stimulated by external stimuli, the level of ROS rises sharply, exceeding the body's own clearance capacity, leading to the imbalance of oxidative–antioxidant balance and the occurrence of oxidative stress. Diabetic myocardial injury usually leads to increased mitochondrial ROS and decreased membrane potential, promoting mitochondrial division or inhibiting mitochondrial fusion [18,19]. To further verify the effects of PCSK9 inhibitors on oxidation and apoptosis in cardiomyocytes under high-glucose injury, JC-1 and ROS flow cytometry were performed. The JC-1 flow cytometry analysis demonstrated that the concentration of JC-1 haploid in rat cardiomyocytes with hyperglycemic injury was significantly elevated compared to the normal control group (\*\*\*  $p < 0.001$ ), accompanied by decreased membrane potential indicating increased apoptosis. When treated with the PCSK9 inhibitor alirocumab at concentrations of 500 nM, 1000 nM, and 2000 nM, the JC-1 haploid concentration was markedly reduced (\*\*\*  $p < 0.001$ ), membrane potential increased, and apoptosis cells decreased. Meanwhile, ROS flow detection showed that the fluorescence intensity of DCFH-DA in myocardial cells of rats with high-glucose injury was significantly increased compared with normal control group, indicating that the level of reactive oxygen species increased. However, the addition of alirocumab at 500 nM, 1000 nM and 2000 nM concentrations significantly reduced the fluorescence intensity of DCFH-DA, indicating that alirocumab can also reduce

the production of reactive oxygen species and oxidative stress in cardiomyocytes of rats with high-glucose injury (Figure 2).



**Figure 2.** Effect of alirocumab on ROS and mitochondrial membrane potential in cardiomyocytes under high-glucose injury. (A). H9c2 cells were treated with the control group, high-sugar-treatment group (40 mM), and PCSK9 inhibitor alirocumab at 500 nM, 1000 nM, and 2000 nM concentrations for 48 h in 40 mM high-sugar medium. JC-1 flow cytometry was used to detect the haploid concentration (%). (B). JC-1 membrane potential detection. (C,D). DCFH-DA fluorescence intensity was detected by ROS flow and the statistical data ( $n = 3$  for each group; \*\*\*  $p < 0.001$ ).

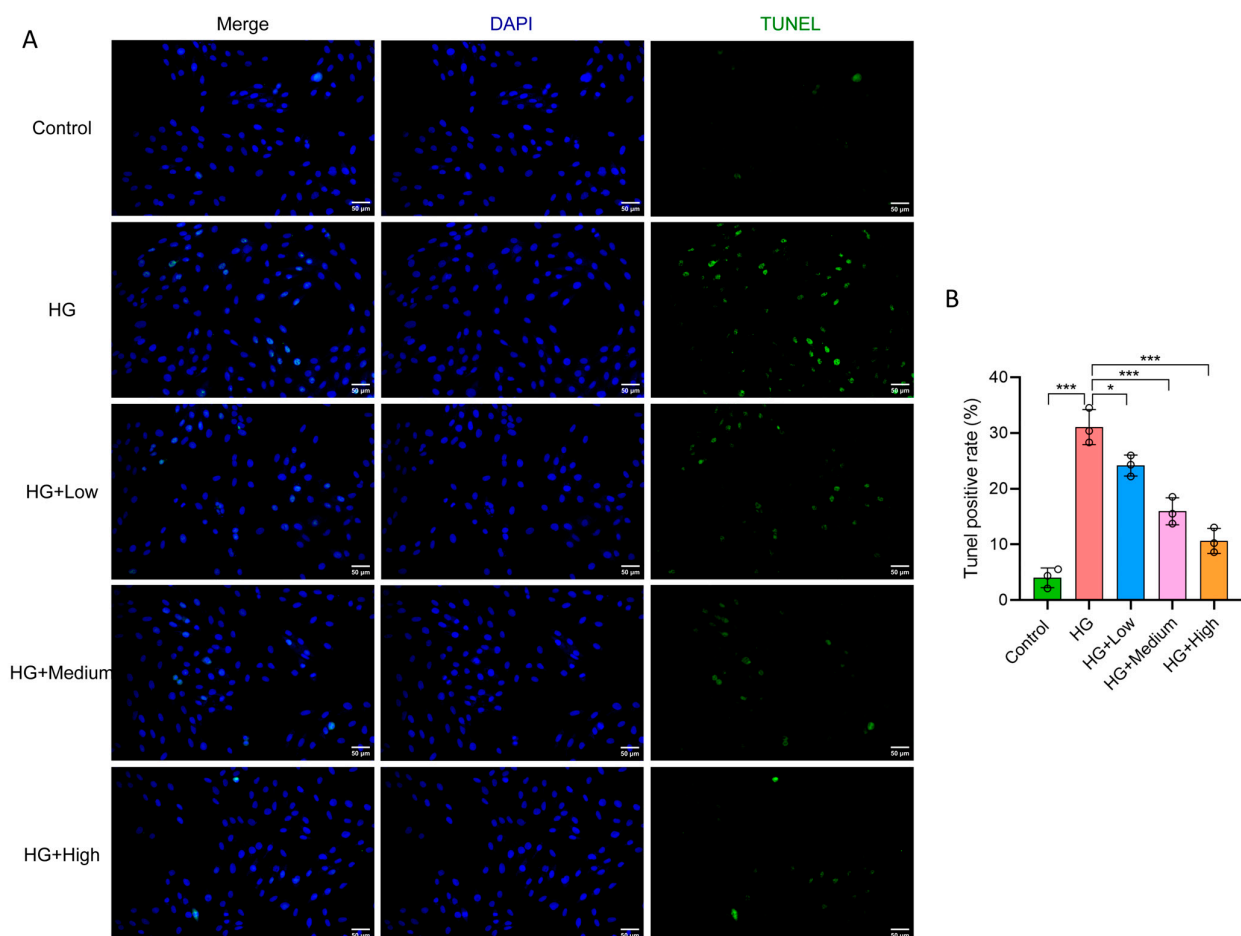
### 2.3. Alirocumab Can Reduce the Positive Rate of Tunnel Staining and Reduce the Number of Apoptotic Cells

In diabetic patients, hyperglycemia and insulin resistance induce endoplasmic reticulum (ER) stress, which involves impaired  $\text{Ca}^{2+}$  processing and accumulation of misfolded proteins. The interaction between oxidative stress, ER stress, and compromised  $\text{Ca}^{2+}$  processing promotes cardiomyocyte death by increasing apoptosis, necrosis, and autophagy. To further investigate the effects of PCSK9 inhibitor alirocumab on apoptosis in high-glucose-treated cardiomyocytes, we employed Tunnel fluorescence staining to assess cell death. The results demonstrated a significantly elevated positive rate of Tunnel staining in cardiomyocytes from rats subjected to high-glucose treatment (\*\*\*  $p < 0.001$ ), indicating increased apoptotic cells. Notably, alirocumab treatment effectively reduced the positive rate of Tunnel staining (\*  $p_{\text{low}} < 0.05$ , \*\*\*  $p_{\text{medium}} < 0.001$ , and \*\*\*  $p_{\text{high}} < 0.001$ ), thereby decreasing apoptotic cells (Figure 3).

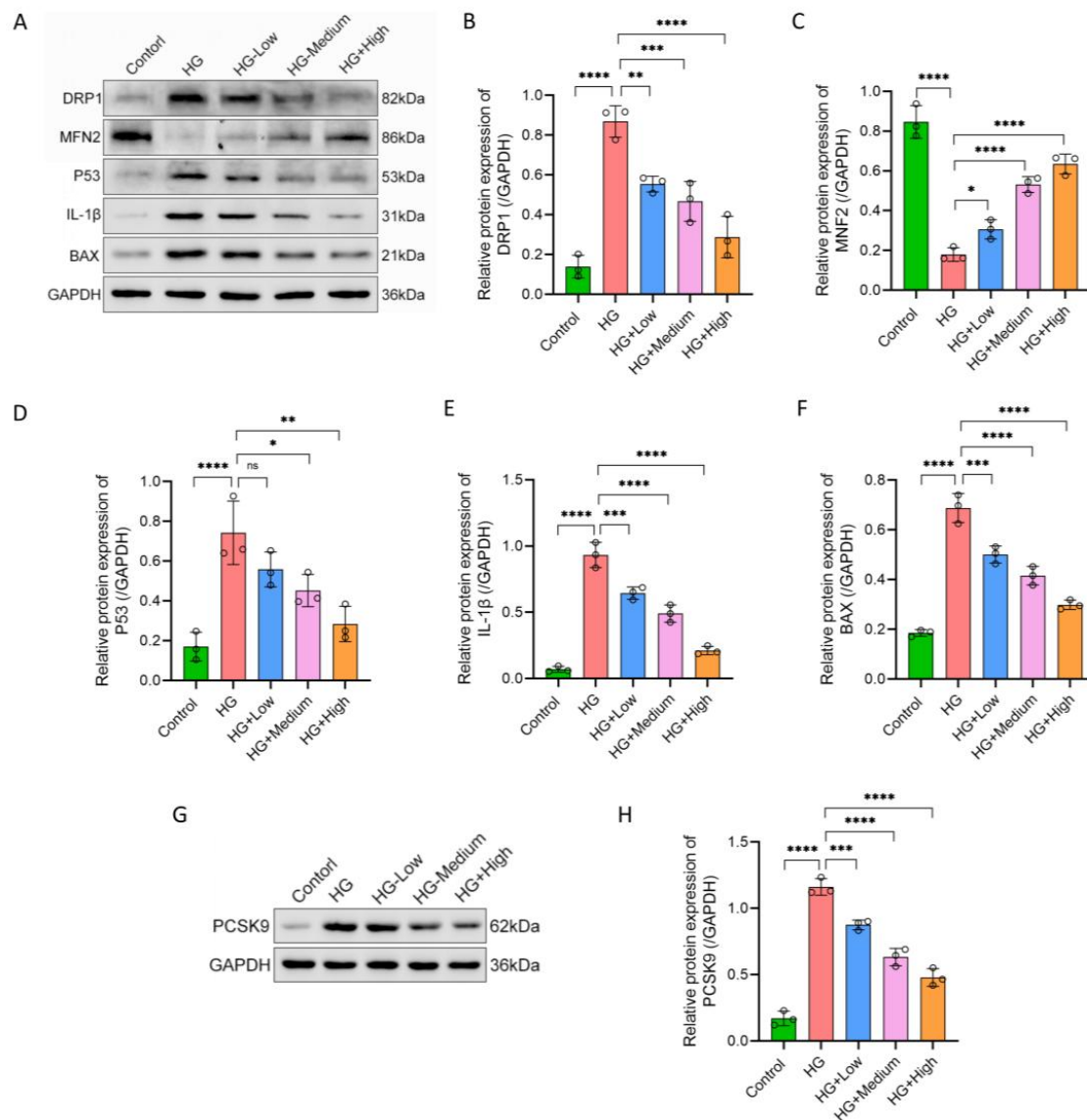
### 2.4. Alirocumab Can Reduce the Inflammatory Response and Mitochondrial Apoptosis-Related Protein Expression in Myocardial Cells Damaged by High Sugar

Mitochondrial apoptosis is one of the main pathways of cell apoptosis. The important executive molecule of mitochondrial division is Dynamin-related protein 1 (Drp1), which is mainly present in the cytoplasm and recruited to mitochondria by Fission 1 (Fis1) for fission reaction [20,21]. The main executive molecule of mitochondrial fusion is mitochondrial fusion protein 2 (Mfn2), which plays an important role in regulating mitochondrial morphology [22,23]. Furthermore, P53 and BAX exhibit mutual regulation and interaction that induces apoptosis and participates in DNA repair. IL-1 $\beta$ , a classic pro-inflammatory

cytokine, stimulates the release of inflammatory factors such as IL-6 and TNF- $\alpha$  while activating T-cells, thereby triggering both local and systemic inflammatory responses. And apoptosis and inflammatory response are important processes in diabetic myocardial injury. To further investigate protein changes in cardiomyocytes under hyperglycemic injury and determine whether PCSK9 expression increases in such conditions, Western blot experiments were conducted. The results demonstrated that hyperglycemic injury induces the upregulation of Drp1, P53, BAX, and IL-1 $\beta$  protein expressions in cardiomyocytes (\*\*\*\*  $p < 0.0001$ ), while significantly reducing Mfn2 protein expression (\*\*\*\*  $p < 0.0001$ ). Compared to the high-sugar-damage control group, the high concentration PCSK9 inhibitor alirocumab significantly reduced the expression of Drp1, P53, BAX, and IL-1 $\beta$  proteins in cells treated with high sugar (\*\*\*\*  $p_{\text{Drp1}} < 0.0001$ , \*\*  $p_{\text{P53}} < 0.01$ , \*\*\*\*  $p_{\text{IL-1}\beta} < 0.0001$ , and \*\*\*\*  $p_{\text{Bax}} < 0.0001$ ), while increasing Mfn2 protein expression (\*\*\*\*  $p_{\text{Mfn2}} < 0.0001$ ). The improvement effect was more pronounced compared to the medium-low concentration of alirocumab. Furthermore, existing studies have confirmed elevated levels of PCSK9 in diabetic patients' sera. To further investigate whether PCSK9 expression is altered in myocardial tissue, this study conducted additional analyses of PCSK9 protein levels. The results demonstrated that high-glucose damage can induce increased PCSK9 expression in cardiomyocytes (\*\*\*\*  $p < 0.0001$ ), while treatment with the PCSK9 inhibitor alirocumab reduces its protein expression (\*\*  $p_{\text{low}} < 0.001$ , \*\*\*\*  $p_{\text{medium}} < 0.0001$ , and \*\*\*\*  $p_{\text{high}} < 0.0001$ ) (Figure 4, detailed results are available in the Supplementary Materials).



**Figure 3.** Effect of alirocumab on apoptosis of cardiomyocytes in the presence of high glucose injury. (A). Representative images of cells in each group detected by TUNEL fluorescence staining. (B). Statistical data of positive rate of TUNEL fluorescent staining ( $n = 3$  for each group; \*  $p < 0.05$  and \*\*\*  $p < 0.001$ ).

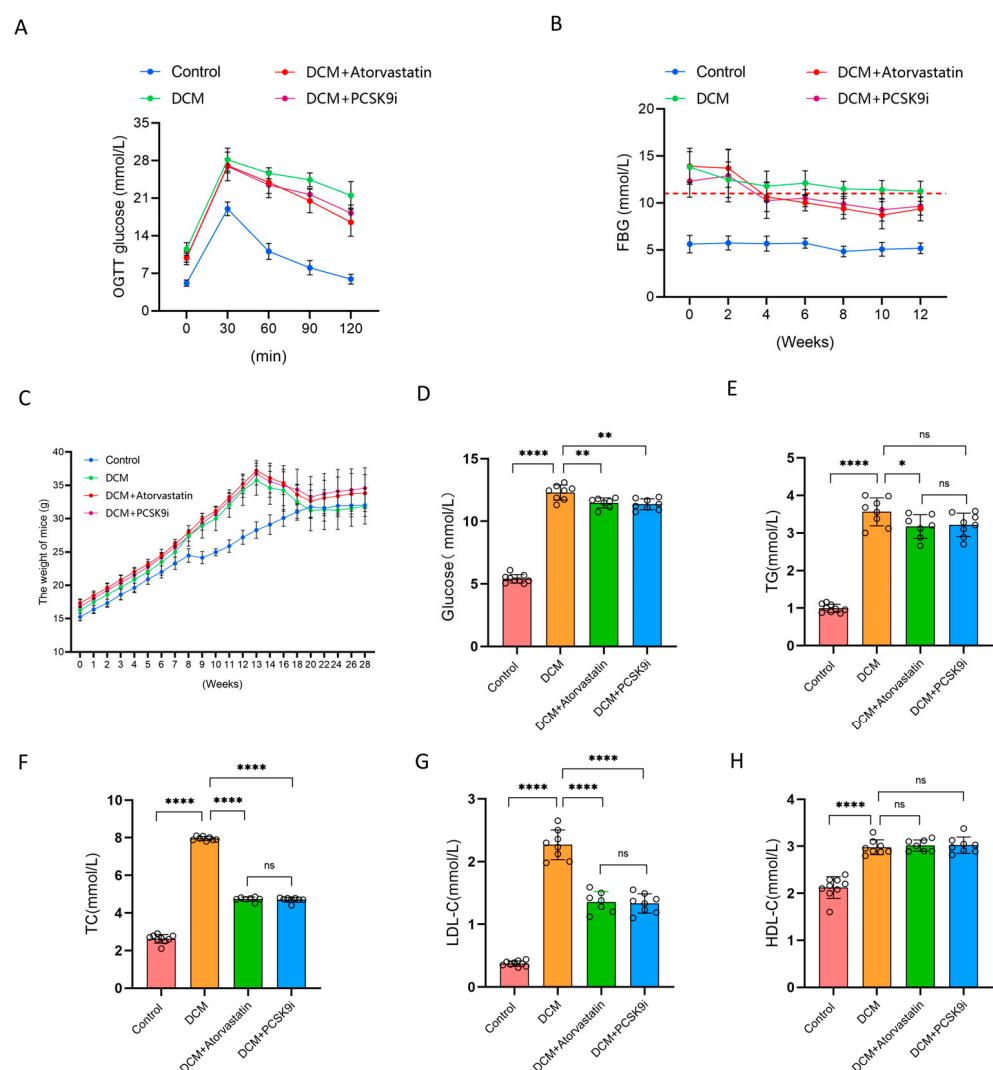


**Figure 4.** Effect of alirocumab on protein expression in cardiomyocytes damaged by high sugar. (A). H9c2 cardiomyocytes were treated with 40 mM high sugar combined with 500/1000/2000 nM alirocumab for 48 h. Representative pictures of protein expression of Drp1/Mnf2/P53/IL-1β/BAX in cells were detected by Western blot. (B–F). Statistical results of gray values of protein expression detection of Drp1/Mnf2/P53/IL-1β/BAX (ns: no statistical difference; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ ). (G). H9c2 cardiomyocytes were treated with 40 mM high sugar combined with 500/1000/2000 nM alirocumab for 48 h, and representative pictures of PCSK9 protein expression in cells were detected by Western blot. (H). Statistical results of detection of gray value of PCSK9 protein expression ( $n = 3$  for each group; \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ ).

### 2.5. Weight and Blood Glucose Monitoring During Mouse Rearing

During the feeding period, the body weight (g) of mice in each group was measured weekly and recorded. The results showed that the diabetic cardiomyopathy group had significantly increased body weight compared to the normal control group. The body weight of mice in all groups generally showed an upward trend, with the diabetic group reaching its peak at 13 weeks and the normal control group reaching its peak at 20 weeks. After that, the body weight of all groups showed a downward trend. After 16 weeks of feeding, the diabetic cardiomyopathy group had more weight loss than the treatment group. At the same time, the fasting blood glucose level of each group of mice was measured every 2 weeks during the feeding period. The results showed that the blood glucose level of the

diabetic cardiomyopathy group was significantly higher than that of the normal control group, while the blood glucose level of the PCSK9 inhibitor alirocumab treatment group decreased compared with that of the diabetic cardiomyopathy control group ( $** p < 0.01$ ). After 11 weeks of drug administration in mice, a glucose tolerance test was performed. The results showed that the normal control group could restore the blood glucose concentration to a normal level in about 2 h, while the diabetic cardiomyopathy group significantly delayed the time to restore the blood glucose concentration to a normal level, indicating impaired glucose tolerance. In addition, after 12 weeks of feeding, serum lipid levels were measured in mice across all groups. The results demonstrated that alirocumab treatment significantly reduced total cholesterol (TC) levels by 40%. Further lipid profiling revealed that this reduction was primarily attributed to a 45% decrease in LDL-cholesterol, while HDL-cholesterol levels remained unchanged. Furthermore, the results demonstrated that there was no statistically significant difference in lipid-lowering efficacy between atorvastatin and alirocumab (Figure 5).



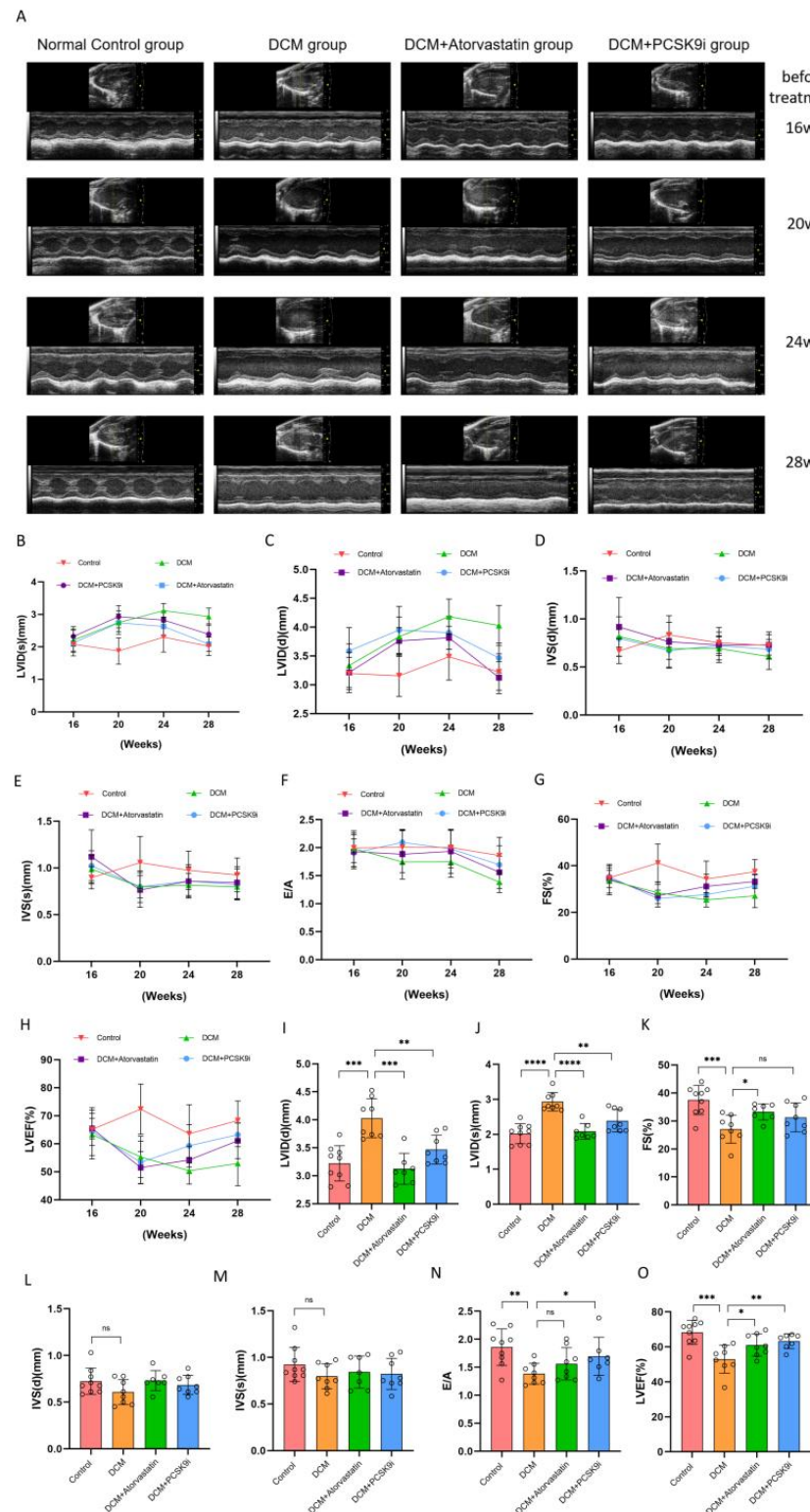
**Figure 5.** Statistics of blood glucose and body weight during mouse feeding. (A). The glucose tolerance test was performed 11 weeks after the start of administration in mice. After fasting overnight, mice were intraperitoneally injected with 40% glucose (2 g/kg) and blood glucose levels were measured 15, 30, 60, 90 and 120 min after glucose injection. (B). Fasting blood glucose statistics of each group during feeding. (C). Weight statistics of mice in each group. (D). Statistical difference in fasting blood glucose in each group. (E–H). Analysis of serum lipid levels in different groups ( $n = 7$ – $9$  mice for each group, ns: no significant difference;  $* p < 0.05$ ,  $** p < 0.01$ , and  $**** p < 0.0001$ ).

## 2.6. Alirocumab Can Improve Ventricular Contraction and Diastolic Function of Diabetic Cardiomyopathy Mice in Cardiac Ultrasound

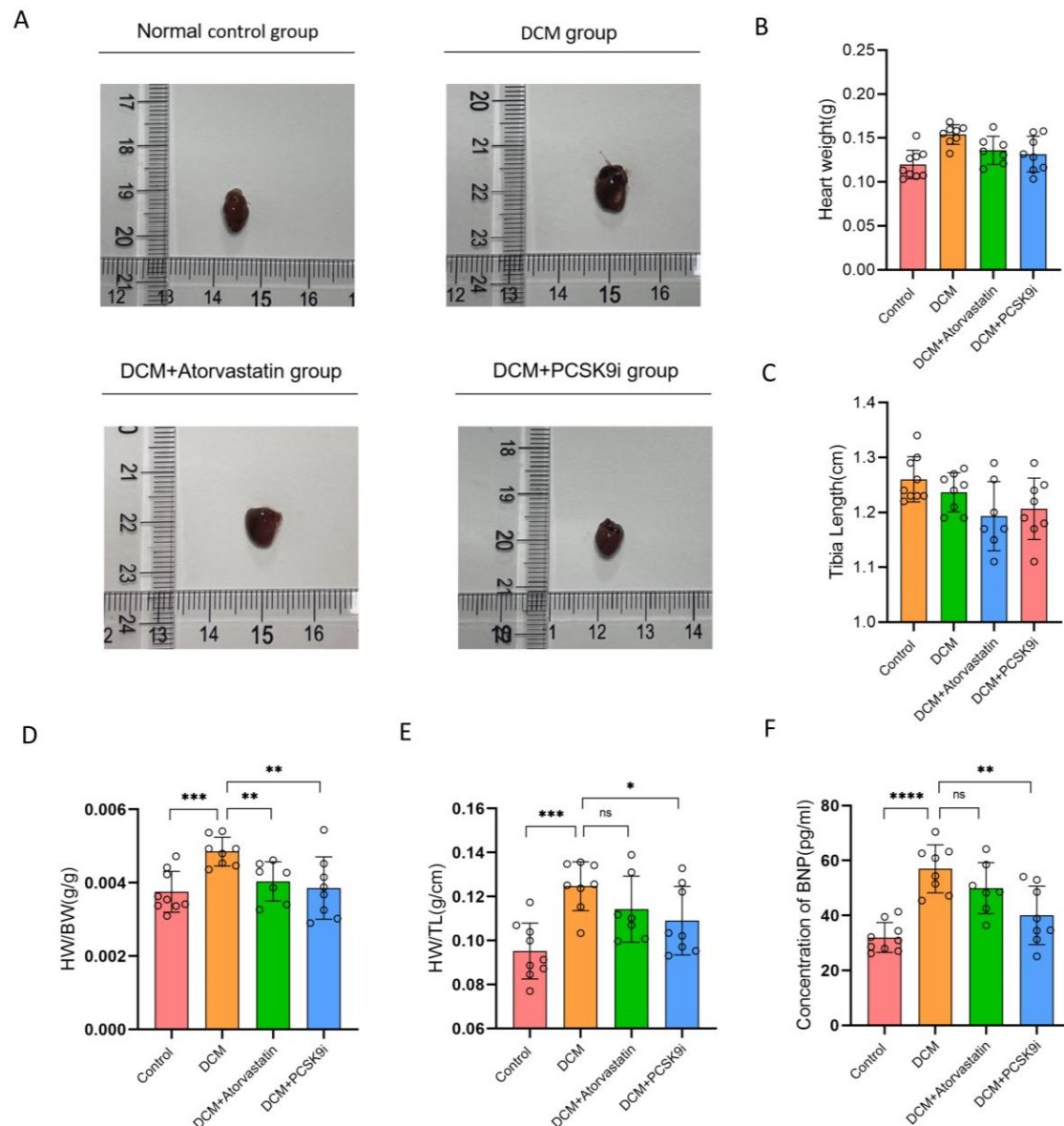
To further evaluate the effects of the PCSK9 inhibitor alirocumab on ventricular wall thickness and cardiac contractile/diastolic function, in vitro echocardiography was performed 1 day before drug administration in mice and every 4 weeks post-administration. The study captured longitudinal M-mode and B-mode cardiac images, with measurements of left ventricular ejection fraction percentage (LVEF%), fractional stroke volume percentage (FS%), left ventricular ejection volume at end-systole (LVIDs), left ventricular ejection volume at diastole (LVIDd), interventricular septum at diastole (IVSd), interventricular septum at systole (IVSs) and the E/A ratio. The results showed that the LVID (s) and LVID (d) in the diabetic cardiomyopathy group were significantly higher than those in the normal control group, while the FS%, LVEF% and E/A ratio were all markedly reduced. After treatment with the PCSK9 inhibitor alirocumab, the LVID (s) and LVID (d) showed significant decreases compared to the diabetic cardiomyopathy group ( $** p < 0.01$ ), while there was no statistically significant difference in FS%. However, the E/A ratio and LVEF% were increased compared with the diabetic cardiomyopathy group ( $* P_{E/A} < 0.05$  and  $** P_{LVEF\%} < 0.01$ ), and there was no significant statistical difference between the groups in IVS (d) and IVS (s) (Figure 6). The results showed that the PCSK9 inhibitor alirocumab reduced myocardial hypertrophy in diabetic cardiomyopathy and improved ventricular contraction and diastolic function, slowing the progression of heart failure.

## 2.7. Alirocumab Reduced Myocardial Hypertrophy and Serum BNP Levels in Mice with Diabetic Cardiomyopathy

In the late stage of diabetic cardiomyopathy, pathological changes can cause cardiac remodeling, fibrosis and diastolic dysfunction, and ultimately reduce left ventricular ejection fraction. Moreover, BNP is a biomarker of heart failure; when the heart is damaged or the heart load increases, ventricular cells release BNP to promote fluid balance and reduce the heart load. The elevation of the BNP level is of great value for the diagnosis of heart failure, and the level of BNP is closely related to the severity of heart failure. In this experiment, the heart was taken after 12 weeks of drug administration in mice, and the heart weight and tibia length of each group were measured. The ratio of heart weight to body weight (HW/BW) and heart weight to tibia length (HW/TL) were used to evaluate myocardial hypertrophy in mice [24,25]. Blood was taken from the eyes, and serum was separated for ELisa to detect the BNP concentration in each group. Statistical results showed that the heart weight/body weight ratio and heart weight/tibia length ratio were significantly increased in the diabetic cardiomyopathy group compared with the normal control group, indicating that the heart was hypertrophied in the diabetic cardiomyopathy group compared with the normal control group. PCSK9 inhibitor alirocumab treatment significantly reduced the HW/BW ratio ( $** p < 0.01$ ) and HW/TL ratio ( $* p < 0.05$ ). In contrast, the atorvastatin group showed improvement in the HW/BW ratio but no statistically significant reduction in the HW/TL ratio. At the same time, BNP serum ELisa test showed that the BNP concentration in diabetic cardiomyopathy group was significantly increased compared with normal control group, while the alirocumab treatment group could significantly reduce serum BNP concentration compared with diabetic cardiomyopathy group, and atorvastatin treatment group showed no significant difference in serum BNP concentration compared with diabetic cardiomyopathy group (Figure 7). The results indicate that the PCSK9 inhibitor alirocumab can reduce myocardial hypertrophy and improve cardiac function in diabetic cardiomyopathy.



**Figure 6.** Results of echocardiography evaluation in mice. (A). Representative images of M and B types of long axis cardiopulmonary ultrasound in each group of mice before treatment and after 4 w, 8 w and 12 w. (B–H). Summary of LVEF%, FS%, LVID (s), LVID (d), IVS (d), IVS (s), and E/A ratio data measured in each group of mice before treatment and after 4 w, 8 w, and 12 w of administration. (I–O). Statistical analysis of LVEF%, FS%, LVID (s), LVID (d), IVS (d), IVS (s), and E/A ratio differences among mouse groups at 12 weeks post-treatment ( $n = 7–9$  mice for each group, ns: no significant difference; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ ).

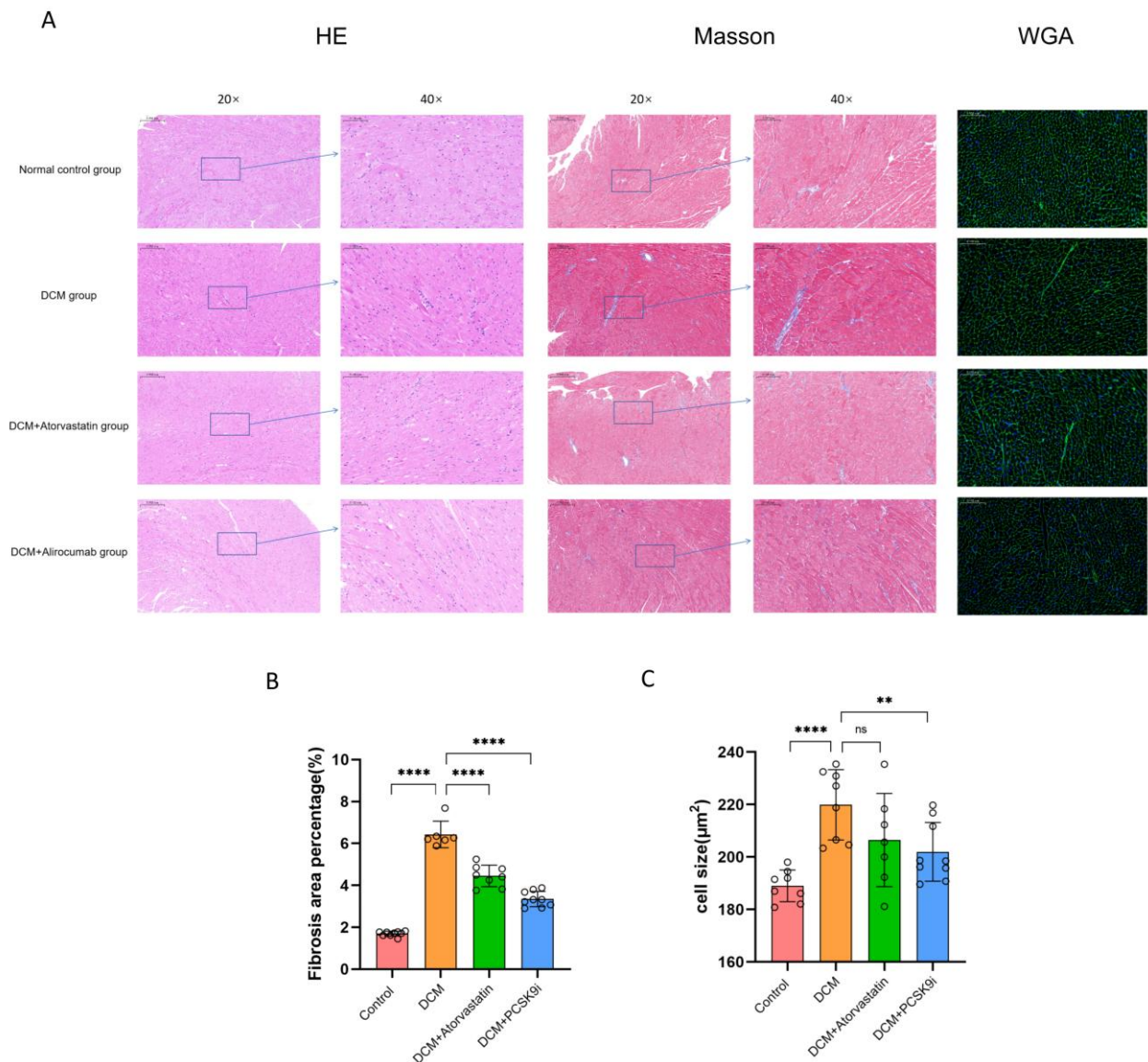


**Figure 7.** The weight of heart, length of tibia and serum BNP level were analyzed. (A). Photo of mouse heart specimen. (B,C). Statistical analysis of heart weight and tibia length in mice. (D). Statistical data of the heart weight (g)/body weight (g) ratio (HW/BW) in mice. (E). Statistical data of the heart weight/tibia length ratio (HW/TL) in mice (g/cm). (F). BNP serum concentration (pg/mL) of mice in each group ( $n = 7-9$  mice for each group, ns: no significant difference; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ ).

## 2.8. Alirocumab Effectively Inhibits Cardiomyocyte Enlargement and Reduces Myocardial Fibrosis in Diabetic Cardiomyopathy Mice

To further compare the size of the heart and the level of fibrosis between the treatment group and the control group, the tissue specimens of the heart were stained after fixation. First, HE staining revealed that the diabetic cardiomyopathy group exhibited cardiac enlargement compared to the normal control group, while the PCSK9 inhibitor treatment group showed mild reduction in cardiac enlargement in diabetic cardiomyopathy mice. Additionally, Masson staining was performed to further assess myocardial fibrosis levels. Using ImageJ 1.52a software, the fibrosis percentages of myocardial tissues across groups were statistically analyzed. Results showed that the diabetic cardiomyopathy group exhibited significantly higher myocardial fibrosis percentages compared to the normal control group. Both PCSK9 inhibitor alirocumab and Atorvastatin demonstrated significant

reduction in myocardial fibrosis percentages (\*\*\*\*  $p < 0.0001$ ). WGA staining results showed that the diabetic cardiomyopathy group exhibited significantly enlarged cardiomyocytes compared to the normal control group. While atorvastatin showed no marked improvement in cardiomyocyte size, the PCSK9 inhibitor alirocumab significantly but modestly reduced the cardiomyocyte area in diabetic cardiomyopathy mice (\*\*  $p < 0.01$ ) (Figure 8). The results demonstrate that the PCSK9 inhibitor alirocumab effectively inhibits ventricular remodeling in diabetic cardiomyopathy mice by reducing cardiomyocyte enlargement and decreasing myocardial fibrosis percentage.

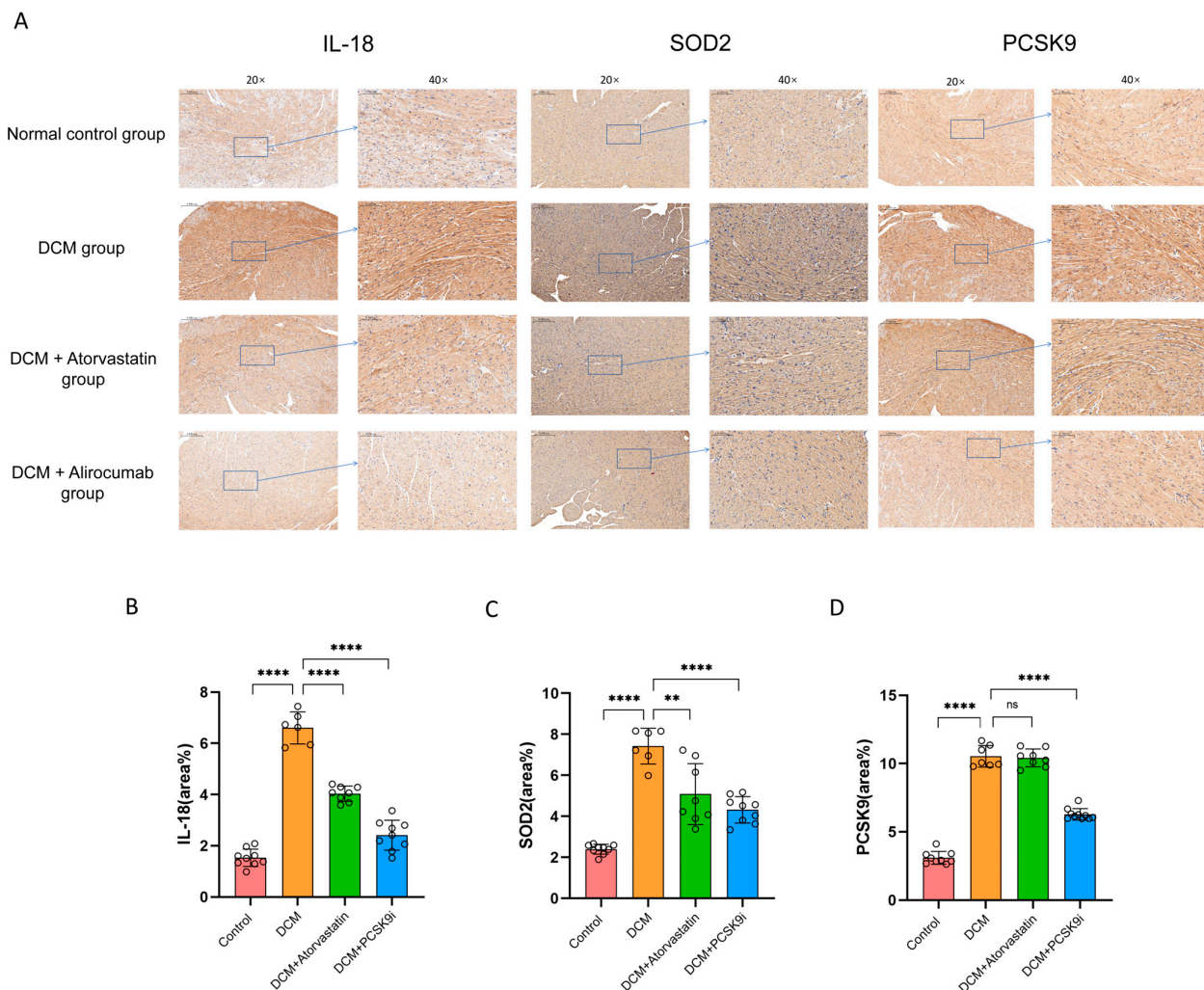


**Figure 8.** Coloring images and statistical analysis of mouse heart tissue. (A). Representative images of HE staining, Masson staining, and WGA staining in the hearts of mice from different groups (20× and 40× magnification). (B). The percentage (%) of cardiac fibrosis in each group of mice was statistically analyzed using Masson staining (\*\*\*\*  $p < 0.0001$ ). (C). Statistical analysis of WGA staining results showed the cardiac cell area (μm<sup>2</sup>) in each group ( $n = 6–9$  mice for each group, ns: no significant difference; \*\*  $p < 0.01$  and \*\*\*\*  $p < 0.0001$ ).

### 2.9. Alirocumab Effectively Reduces Inflammatory Responses and Oxidative Stress in Diabetic Cardiomyopathy, While Also Decreasing PCSK9 Expression in Myocardial Tissue

IL-18 plays a pivotal role in inflammatory responses by working synergistically with IL-12 to boost innate immune responses and stimulate the production of other cytokines

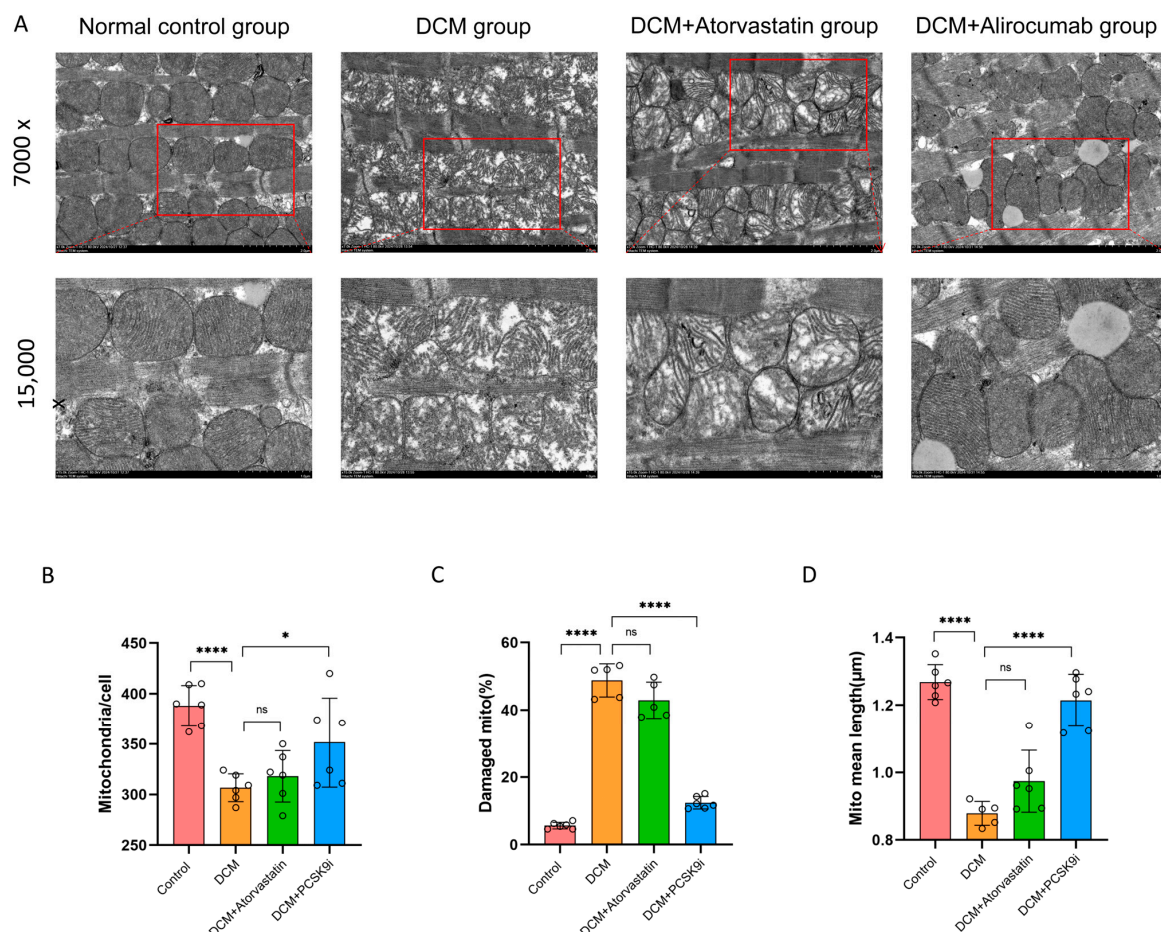
such as TNF- $\alpha$  and IL-1 $\beta$ . SOD2 is a key antioxidant enzyme found in the mitochondrial inner membrane. It converts superoxide anions into oxygen and hydrogen peroxide, effectively preventing oxidative damage caused by free radicals. Inflammatory response and oxidative stress are important pathological mechanisms of diabetic cardiomyopathy. To further investigate the effects of PCSK9 inhibitors on inflammatory responses and oxidative stress, and to determine their ability to suppress PCSK9 expression in myocardial tissue, immunohistochemical analyses of IL18, SOD2, and PCSK9 were conducted. The results showed significantly elevated levels of IL-18, SOD2, and PCSK9 in the diabetic cardiomyopathy group (\*\*\*\*  $p < 0.0001$ ). Atorvastatin significantly reduced IL-18 (\*\*\*\*  $P_{IL-18} < 0.0001$ ) and mildly decreased SOD2 (\*\*  $p_{SOD2} < 0.01$ ), but showed no significant effect on PCSK9 levels. The PCSK9 inhibitor treatment group showed significantly lower levels of IL-18, SOD2, and PCSK9 compared to the diabetic cardiomyopathy group (\*\*\*\*  $p < 0.0001$ ) (Figure 9). The analysis shows that alirocumab effectively reduces inflammatory responses and oxidative stress in diabetic cardiomyopathy. At the same time, alirocumab can reduce peripheral circulating PCSK9 levels and also partially decrease PCSK9 expression in myocardial tissue.



**Figure 9.** Immunohistochemical images and data analysis of mouse myocardial tissue. (A). Representative images of immunohistochemical staining for IL-18, SOD2 and PCSK9 in myocardial tissue from different mouse groups. (B). Percentage (%) of positive immunohistochemical staining area for IL-18 in mouse myocardial tissue. (C). Percentage (%) of positive immunohistochemical staining area for SOD2 in mouse myocardial tissue. (D). Percentage (%) of positive immunohistochemical staining area for PCSK9 in mouse myocardial tissue ( $n = 6-9$  mice for each group, ns: no statistically significant difference, \*\*  $p < 0.01$  and \*\*\*\*  $p < 0.0001$ ).

### 2.10. Alirocumab Promotes Mitochondrial Fusion in the Hearts of Diabetic Cardiomyopathy Mice, Inhibits the Reduction in Mitochondrial Cristae Density, and Reduces the Number of Damaged Mitochondria

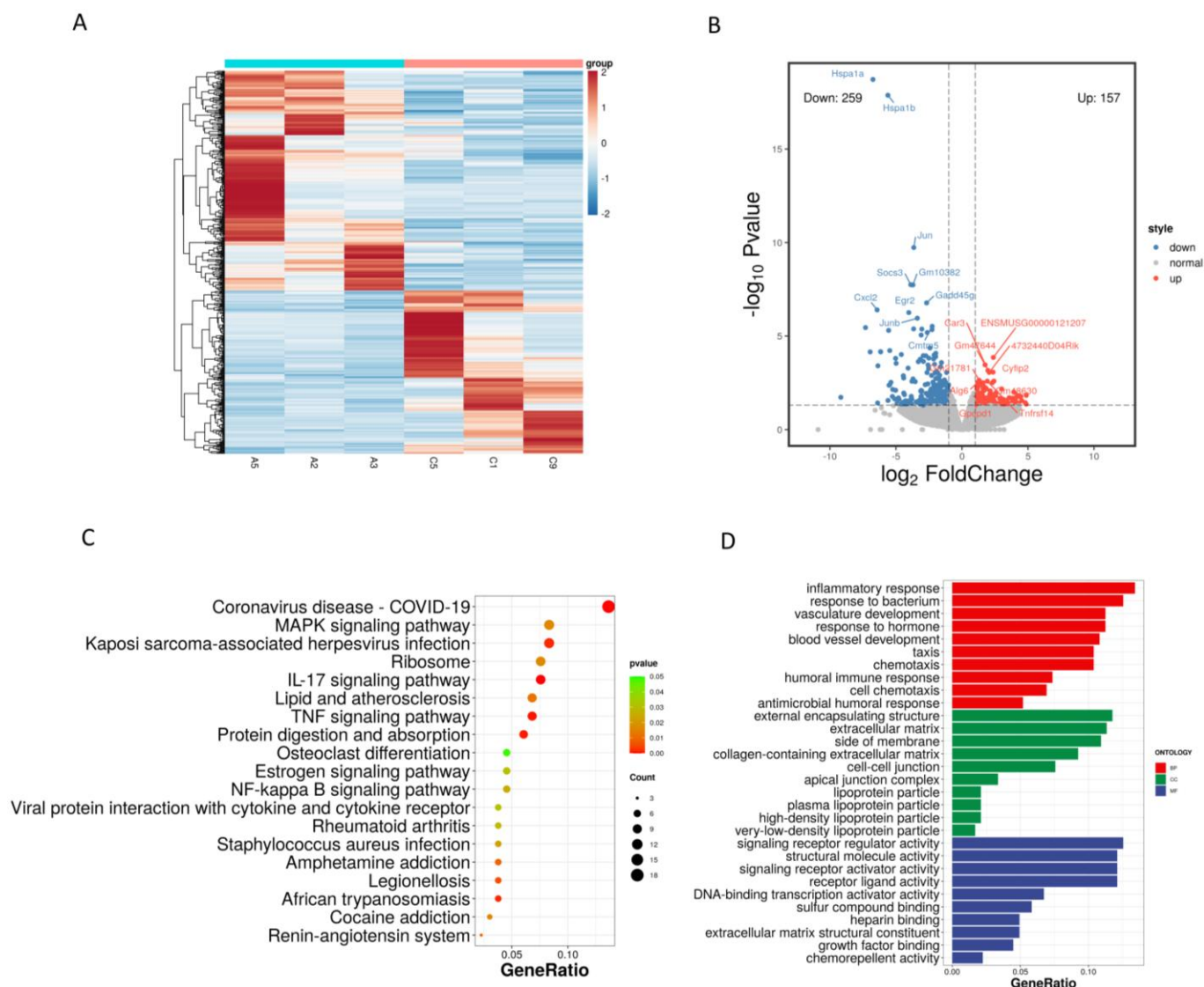
Research indicates that mitochondrial homeostasis is compromised in diabetic pathophysiology. Cardiac adipotoxicity inhibits mitochondrial fusion and promotes mitochondrial fission, resulting in mitochondrial energy metabolism abnormalities, myocardial apoptosis, and myocardial hypertrophy and fibrosis [26]. To further investigate whether PCSK9 inhibitors could modulate mitochondrial dynamics in diabetic cardiomyopathy mice, we performed electron microscopy analysis of myocardial tissue. By quantifying mitochondrial numbers and sizes, we evaluated mitochondrial fusion and fission, while also counting damaged mitochondria. The results showed that the number and size of mitochondria were significantly reduced in the diabetic cardiomyopathy group compared with the normal control group, and the mitochondrial crista was more destroyed. The PCSK9 inhibitor alirocumab treatment increased both the size ( $**** p < 0.0001$ ) and quantity ( $* p < 0.05$ ) of mitochondria in the hearts of diabetic cardiomyopathy mice, indicating that alirocumab promotes mitochondrial fusion in these hearts. Furthermore, compared with the diabetic cardiomyopathy control group, the PCSK9 inhibitor alirocumab significantly inhibited the reduction in mitochondrial cristae density in the hearts of diabetic cardiomyopathy mice, and decreasing the number of damaged mitochondria (Figure 10).



**Figure 10.** Electron microscopy and statistical analysis of mouse cardiac tissue. (A). Representative images of mitochondria in cardiac tissue of mice in each group under electron microscopy. (B). Statistical data of mitochondrial number in mouse cardiac tissue under electron microscopy. (C). Statistical data on the percentage (%) of damaged mitochondria in mouse cardiac tissue under electron microscopy. (D). Mitochondrial diameter (μm) in mouse cardiac tissue under electron microscopy ( $n = 5-6$  mice for each group; ns: no significant difference;  $* p < 0.05$  and  $**** p < 0.0001$ ).

### 2.11. The Therapeutic Effects of Alirocumab in Diabetic Cardiomyopathy Are Associated with the MAPK Signaling Pathway

To further investigate the mechanism of the PCSK9 inhibitor alirocumab in treating diabetic cardiomyopathy in mice, we employed transcriptome sequencing to compare intergroup differences. The results were visualized using volcano plots and cluster maps, and subjected to GO enrichment analysis and KEGG enrichment analysis. The effects of the PCSK9 inhibitor alirocumab may be associated with the MAPK signaling pathway, ribosome signaling pathway, interleukin-17 (IL-17) signaling pathway, and tumor necrosis factor (TNF) signaling pathway. It is also associated with lipid metabolism, atherosclerosis, inflammatory responses, vascular development, hormonal regulation, and chemotaxis. Notably, numerous genes are enriched in the MAPK signaling pathway and inflammatory responses, indicating strong biological connections (Figure 11).



**Figure 11.** Statistical analysis of transcriptome sequencing results and related enrichment analysis. (A,B). Volcano plots and cluster maps of transcriptome sequencing results. (C). KEGG enrichment analysis of PCSK9 inhibitor alirocumab in diabetes cardiomyopathy associated pathways. (D). GO enrichment analysis of PCSK9 inhibitor alirocumab in pathways associated with diabetic cardiomyopathy.

The MAPK signaling pathway is closely associated with diabetic cardiomyopathy, with key family members such as ERK, JNK, and p38 playing crucial roles in its pathogenesis. For example, the p38 MAPK pathway induces cardiomyocyte apoptosis, promotes

myocardial fibrosis, and inhibits cardiac contraction; the JNK MAPK pathway exacerbates insulin resistance, promotes cardiomyocyte apoptosis, and contributes to myocardial hypertrophy; the ERK MAPK pathway drives myocardial hypertrophy and promotes fibroblast proliferation. These pathological changes ultimately contribute to the structural and functional alterations of diabetic cardiomyopathy, leading to diastolic dysfunction and eventually progressing to systolic dysfunction. Therefore, this study will further investigate the mechanism of PCSK9 inhibitor alirocumab in treating diabetic cardiomyopathy and its correlation with the MAPK signaling pathway by detecting relevant signaling molecules through Western blot and qPCR.

## 2.12. Alirocumab Improves Diabetic Cardiomyopathy by Inhibiting the ERK/p38 MAPK Signaling Pathway and Reduces PCSK9 Expression in Myocardial Tissue

To investigate the mechanism of PCSK9 inhibitor alirocumab in treating diabetic cardiomyopathy, this study employed qPCR and Western blotting to analyze MAPK pathway-related gene and protein expression levels in cardiac tissues from different mouse groups based on transcriptome sequencing data. The results showed that the diabetic cardiomyopathy group exhibited significantly higher expression levels of TNF- $\alpha$ , TGF- $\beta$ , p-38, JNK, ERK1/2, c-Jun, c-Fos, and MMP1 genes compared to the normal control group. The alirocumab treatment effectively reduces the gene expression of TNF- $\alpha$ , TGF- $\beta$ , p-38, JNK, and ERK1/2 (\*\*\*\*  $p_{\text{TNF}\alpha} < 0.0001$ , \*\*\*\*  $p_{\text{TGF}\beta} < 0.0001$ , \*\*\*\*  $p_{\text{p-38}} < 0.0001$ , \*  $p_{\text{JNK}} < 0.05$ , \*\*\*\*  $p_{\text{ERK1}} < 0.0001$ , and \*\*\*\*  $p_{\text{ERK2}} < 0.0001$ ), thereby decreasing c-Jun and c-Fos expression (\*\*\*\*  $p < 0.0001$ ) and inhibiting AP-1 activity, which further reduces the gene expression level of its downstream signaling molecule MMP1 (\*\*\*\*  $p < 0.0001$ ). The atorvastatin treatment group showed no statistically significant difference in JNK, c-Fos, and MMP1 gene expression compared to the diabetic cardiomyopathy group (Figure 12).

Western blot analysis revealed that the phosphorylation levels of ERK and p38 were elevated in the diabetic cardiomyopathy group compared with those in the normal control group, and the protein expression levels of c-Fos, c-Jun, MMP1, and PCSK9 were also increased. Treatment with alirocumab reduced the phosphorylation levels of ERK (\*\*  $p < 0.01$ ) and p38 (\*\*\*  $p < 0.001$ ) compared with those in the diabetic cardiomyopathy group, further decreasing the protein expression levels of c-Fos, c-Jun, and MMP1 (\*\*\*  $p_{\text{c-fos}} < 0.001$ , \*\*\*\*  $p_{\text{c-jun}} < 0.0001$ , and \*\*\*  $p_{\text{MMP1}} < 0.001$ ). Alirocumab also inhibited the upregulation of PCSK9 protein expression in myocardial tissue (Figure 13). This study suggests that the therapeutic effect of the PCSK9 inhibitor alirocumab on diabetic cardiomyopathy may be mediated, at least in part, by molecules associated with the ERK/p38 MAPK signaling pathway. Furthermore, in addition to reducing circulating PCSK9 levels, alirocumab also decreased PCSK9 expression in myocardial tissue.

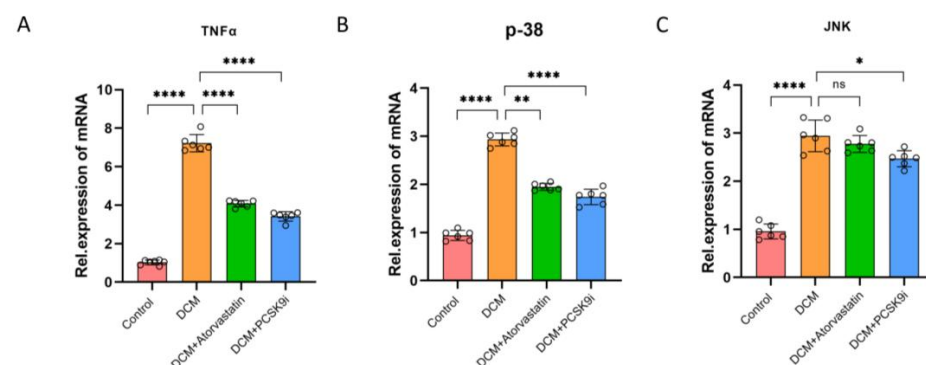
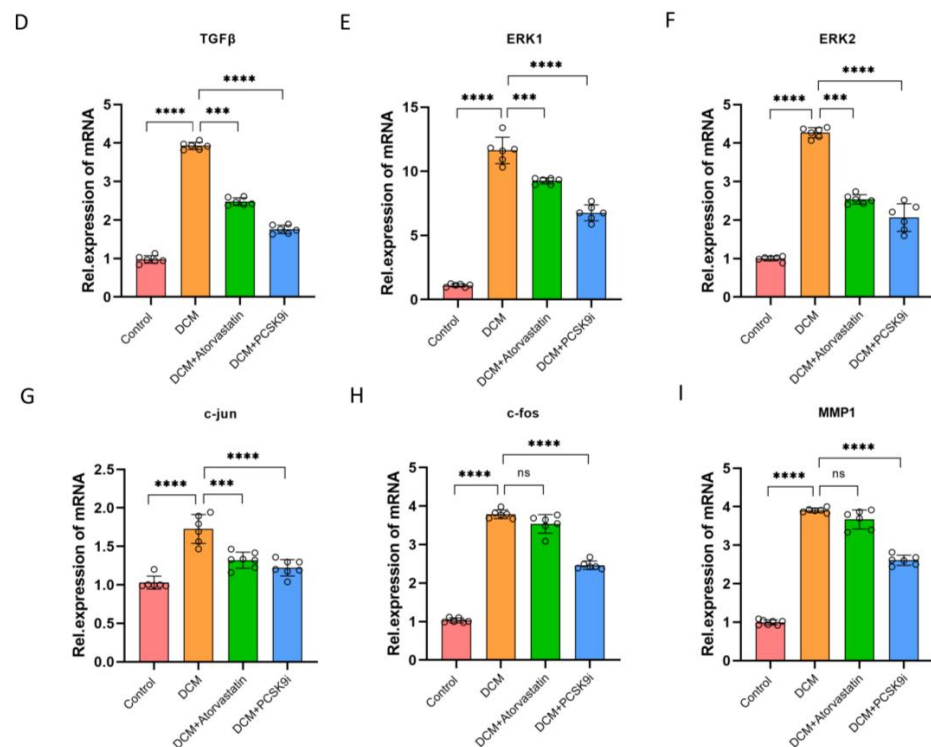
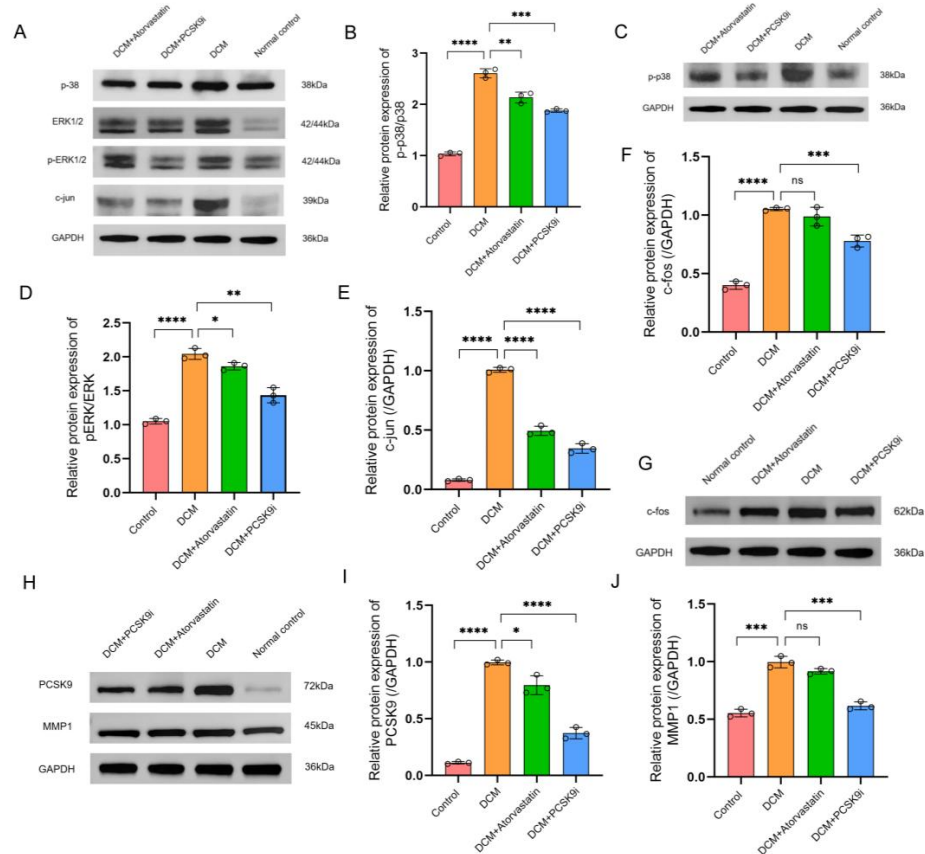


Figure 12. Cont.



**Figure 12.** Statistical chart of mRNA expression levels of ERK/p-38 MAPK pathway-associated genes in mouse cardiac tissue. (A–I). Statistical data on mRNA expression levels of the genes TNF- $\alpha$ , p-38, JNK, TGR $\beta$ , ERK1, ERK2, c-Jun, c-Fos, and MMP1 ( $n = 6$ –7 for each group, normalized to 18S expression; ns: no statistically significant difference; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ ).



**Figure 13.** Statistical representation of ERK/p-38 MAPK pathway-associated proteins and PCSK9 protein expression levels in mouse cardiac tissue. (A). Representative images of Western blot analysis

for protein expression of p38, ERK (1/2), p-ERK (1/2), and c-Jun. (B). Statistical results of grayscale values for protein expression detection of p-p38/p38. (C). Representative images of Western blot analysis for protein expression of p-p38. (D–F). Statistical results of grayscale values for protein expression detection of pERK/ERK, c-Jun, and c-Fos. (G,H). Representative images of Western blot analysis for protein expression of c-Fos, PCSK9, and MMP1. (I,J). Statistical results of grayscale values for protein expression detection of PCSK9 and MMP1 ( $n = 3$  for each group, ns: no significant difference; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ ).

### 3. Discussion

Diabetes can cause widespread structural and functional disorders of the myocardium, and metabolic disorders caused by hyperglycemia, insulin resistance and dyslipidemia can lead to a series of pathophysiological changes. The combined effects of these pathophysiological processes induce calcium ion stress, which simultaneously disrupts mitochondrial and endoplasmic reticulum function. This activates signaling pathways, including protein kinase C (PKC) and MAPK, thereby initiating chronic inflammation. The resulting dysfunction first manifests as diastolic impairment, followed by progressive systolic dysfunction, ultimately leading to myocardial fibrosis [27]. Diabetic cardiomyopathy is defined as clinical ventricular dysfunction in patients without significant coronary artery disease, hypertension or valvular heart disease [8], which significantly impairs quality of life and long-term prognosis. Currently, there is no effective specific treatment.

Beyond its role in familial hypercholesterolemia, PCSK9 is also involved in various biological processes, including apoptosis, autophagy, pyroptosis, ferroptosis, inflammation, energy metabolism, and tumor immunity. It is also associated with diabetes and neurodegenerative diseases. Studies have demonstrated that PCSK9 inhibition not only reduces LDL-C levels but also decreases myocardial infarction area. Furthermore, PCSK9 inhibition has been shown to attenuate LOX-1-induced inflammatory responses and ROS production [28,29]. Given that myocarditis, oxidative stress, fibrosis, mitochondrial damage, apoptosis, and autophagy are all implicated in the pathogenesis of diabetic cardiomyopathy—with inflammation and fibrosis representing key mechanisms underlying disease progression [30]—PCSK9 inhibition may represent a promising therapeutic strategy for this condition.

This study demonstrates that the PCSK9 inhibitor alirocumab exhibits therapeutic effects in enhancing the viability of H9c2 cells under high glucose stress. Alirocumab at concentrations of 500 nM, 1000 nM, and 2000 nM significantly reduced the JC-1 haploid concentration in H9c2 cells (\*\* $p < 0.001$ ), increased the membrane potential, and decreased the number of apoptotic cells. Furthermore, it significantly reduced the fluorescence intensity of DCFH-DA, indicating that alirocumab can also decrease reactive oxygen species production and oxidative stress in myocardial cells of rats with high-sugar injury. Alirocumab treatment similarly reduced the positive rate of Tunnel staining (\*  $p_{\text{low}} < 0.05$ , \*\*\*  $p_{\text{medium}} < 0.001$ , and \*\*\*  $p_{\text{high}} < 0.001$ ), thereby decreasing apoptotic cells. Compared with the high-sugar-injury control group, high concentrations of alirocumab reduced the expression of Drp1, p53, BAX, and IL-1 $\beta$  proteins in high-sugar-treated cells (\*\*\*\*  $p_{\text{Drp1}} < 0.0001$ , \*\*  $p_{\text{p53}} < 0.01$ , \*\*\*\*  $p_{\text{IL-1}\beta} < 0.0001$ , and \*\*\*\*  $p_{\text{Bax}} < 0.0001$ ), while increasing the expression of Mfn2 protein (\*\*\*\*  $p_{\text{Mfn2}} < 0.0001$ ). This further confirms that PCSK9 inhibitors can reduce inflammatory responses and mitochondrial apoptosis in high-sugar-injured cardiomyocytes. Furthermore, this study established a type 2 diabetic cardiomyopathy (T2DCM) mouse model by combining a high-fat diet with intraperitoneal streptozotocin (STZ) injection. The model was divided into four groups: T2DCM+PCSK9 inhibitor treatment group, T2DCM+atorvastatin treatment group, T2DCM control group, and normal control group. In vivo studies further demonstrated that treatment with the PCSK9 inhibitor alirocumab significantly reduced the

LVID (s) and LVID (d) in mice (\*\*  $p < 0.01$ ). The study also showed elevated E/A ratio and left ventricular ejection fraction (LVEF%) compared to the diabetic cardiomyopathy group (\*  $p_{E/A} < 0.05$  and \*\*  $p_{LVEF\%} < 0.01$ ). These findings indicate that alirocumab effectively reduces myocardial hypertrophy, improves ventricular contraction and relaxation functions, and slows the progression of heart failure in diabetic cardiomyopathy. Meanwhile, immunohistochemical analysis revealed that alirocumab not only reduced PCSK9 expression in peripheral circulation but also moderately decreased PCSK9 levels in myocardial tissue. Electron microscopy analysis revealed that the PCSK9 inhibitor alirocumab treatment significantly increased mitochondrial size (\*\*\*\*  $p < 0.0001$ ) and quantity (\*  $p < 0.05$ ) in diabetic cardiomyopathy mice hearts. It also promoted mitochondrial fusion and markedly reversed the reduction in mitochondrial crest density, while decreasing the number of damaged mitochondria.

Although statins are currently the first-line treatment for cardiovascular diseases, they demonstrate significant pharmacological effects, such as lipid-lowering and plaque-stabilizing properties. With well-established cardiovascular protective mechanisms, they can substantially reduce the risk of atherosclerotic cardiovascular diseases. Studies have also confirmed that atorvastatin can inhibit oxidative stress and improve myocardial fibrosis by regulating macrophage polarization in diabetic cardiomyopathy [31]. However, this study demonstrated that the PCSK9 inhibitor alirocumab outperformed atorvastatin in improving myocardial hypertrophy and ventricular remodeling in diabetic cardiomyopathy, as well as reducing the myocardial fibrosis percentage and oxidative stress in diabetic cardiomyopathy mice, based on the heart-to-tibia length ratio and WGA staining and immunohistochemical results. Meanwhile, clinical data indicate that moderate- and high-dose statin use may increase diabetes risk by 11% and 12%, respectively [32], whereas PCSK9 inhibitors show minimal impact on blood glucose levels in real-world studies [15]. Furthermore, the atorvastatin doses used in previous basic research were significantly higher than clinical doses. It remains to be studied whether the cardioprotective effects of statins are dose-dependent and how to balance the risks of liver injury and rhabdomyolysis. Therefore, compared with statins, PCSK9 inhibitors may be more suitable for pharmacological treatment of patients with diabetic cardiomyopathy.

MAPK activation is implicated in the pathogenesis of diabetic cardiomyopathy and heart failure [33]. ERK1/2, p38, and JNK are three key MAPK subfamilies that regulate cardiac hypertrophy and remodeling, and are also involved in the fibrotic, apoptotic, and inflammatory pathological processes associated with diabetic cardiomyopathy [34–36]. Previous studies have confirmed that excessive reactive oxygen species (ROS) production, hyperglycemia, and elevated circulating lipid levels accelerate inflammatory responses, fibrosis, and cardiomyocyte apoptosis in diabetic cardiomyopathy through MAPK phosphorylation and the activation of their downstream targets NF- $\kappa$ B and AP-1 [37]. This study further investigated the mechanism of action of the PCSK9 inhibitor alirocumab in diabetic cardiomyopathy through transcriptome sequencing. The findings revealed that alirocumab's effects may be associated with the mitogen-activated protein kinase (MAPK) signaling pathway, ribosome signaling pathway, interleukin-17 (IL-17) signaling pathway, and tumor necrosis factor (TNF) signaling pathway. Additionally, it was linked to lipid and atherosclerosis, inflammatory responses, vascular development, hormonal responses, and chemotaxis. Notably, a significant number of genes were enriched in the MAPK signaling pathway and inflammatory responses, indicating a close association between these pathways and the therapeutic effects. qPCR results demonstrated that alirocumab treatment significantly reduced gene expression of TNF- $\alpha$ , TGF- $\beta$ , p-38, JNK, and ERK1/2, thereby decreasing c-Jun and c-Fos expression. This inhibition of AP-1 activity further reduced the gene expression level of its downstream signaling molecule MMP1. Western blot analysis

further confirmed that the alirocumab treatment reduced the phosphorylation levels of ERK and p38 compared with those in the diabetic cardiomyopathy group, further decreasing the protein expression levels of c-Fos, c-Jun, and MMP1. Additionally, alirocumab inhibited the upregulation of PCSK9 protein expression in myocardial tissue. These findings suggest that the therapeutic effect of the PCSK9 inhibitor alirocumab on diabetic cardiomyopathy may be mediated, at least in part, by molecules associated with the ERK/p38 MAPK signaling pathway.

A limitation of this study is that, although significant echocardiographic differences were observed, intergroup variability in heart rate may have influenced functional measurements. Additionally, the study lacked LDLR gene knockout experiments to verify whether PCSK9 inhibitors improve diabetic cardiomyopathy through mechanisms independent of their lipid-lowering effects, and did not investigate specific targets of PCSK9 inhibitors in the MAPK signaling pathway or conduct pathway inhibition assays. Although we detected the expression levels of PCSK9 in myocardial tissue, we did not determine the specific distribution of PCSK9 in the heart. These aspects will be further explored in future experiments.

## 4. Materials and Methods

### 4.1. Experimental Cell Culture and Grouping

H9c2 cardiomyocytes were cultured in a medium containing 10% fetal bovine serum and 100 U/mL penicillin–100 mg/mL streptomycin sulfate under the condition of 37 °C and 5% carbon dioxide. They were divided into three groups: (1) CON group: H9c2 cells were cultured in DMEM medium containing low sugar (5 mM glucose); (2) HG group: H9c2 cells were cultured in DMEM medium containing high sugar (40 mM glucose); (3) HG + PCSK9i group: H9c2 cells were cultured in DMEM medium containing high sugar (40 mM glucose) and different concentrations of the PCSK9 inhibitor alirocumab.

### 4.2. Experimental Animals

The animals used in this experiment were 5-week-old male C57BL/6J mice, purchased from Shanghai Bikai Keyi Biology Co. (Shanghai, China), and raised in the animal room of the School of Medicine, Fudan University (SPF-grade animal room under animal room conditions including a standard temperature of  $22 \pm 1$  °C and humidity of  $55 \pm 5\%$ , as well as free drinking water). All experimental protocols were approved by the Animal Ethics Committee of Medical School of Fudan University (ethical approval number: 2024060025). Animal handling is in accordance with the Guidelines for the Care and Use of Laboratory Animals (revised 1996).

### 4.3. Diabetic Cardiomyopathy Mouse Modeling and Experimental Grouping

Male C57BL/6J mice at 5 weeks of age were used to construct a mouse model of type 2 diabetic cardiomyopathy by combining a high-fat diet with intraperitoneal injection of streptozotocin (STZ). The mice were fed a high-fat diet (containing 45% kcal from fat, 35% kcal from carbohydrates, and 20% kcal from protein, with a total caloric value of 4.73 kcal/g) for six weeks. Weekly body weight measurements showed that, when the mice reached over 40 g, STZ (30 mg/kg dissolved in 0.1 M sodium citrate buffer at pH 4.5) was administered via intraperitoneal injection. The treatment continued for three consecutive days. Blood glucose levels were measured using a glucometer on days 3, 5, and 7 post-injection. Mice with a random blood glucose level  $\geq 16.7$  mmol/L were designated as successfully developed type 2 diabetes. After 3 weeks of feeding with a high-fat diet, ventricular diastolic function decreased was confirmed by echocardiography, and a mouse model of type 2 diabetic cardiomyopathy was successfully constructed. The

normal control group was fed with ordinary feed (10% kcal fat, 70% kcal carbohydrates and 23% kcal protein, with a total caloric value of 3.85 kcal/g).

The mice were divided into four groups ( $n = 10$ , based on previous research data): (1) Diabetic cardiomyopathy + PCSK9 inhibitor treatment group: After successful modeling of diabetic cardiomyopathy in mice, the PCSK9 inhibitor alirocumab (10 mg/kg/week diluted with 0.9% NaCl) was subcutaneously injected and the same dose of drug-free 0.9% NaCl solution was administered by gavage, as with the statin treatment group, for 12 consecutive weeks while feeding a high-fat diet. (2) Diabetic cardiomyopathy + atorvastatin treatment group: After the successful modeling of diabetic cardiomyopathy in mice, atorvastatin was dissolved in 0.9% NaCl to prepare a 2 mg/mL solution, and 10 mg/kg/d atorvastatin was administered to the mice by gavage. This group was also subcutaneously injected with the 0.9% NaCl solution at the same dose as the PCSK9 inhibitor treatment group but without the drug for 12 consecutive weeks, while feeding a high-fat diet. (3) Diabetic cardiomyopathy control group: After successful modeling of diabetic cardiomyopathy in mice, the same dose of 0.9% NaCl solution without drug was administered orally and subcutaneously like the other treatment groups, and the high-fat diet was continued for 12 weeks. (4) Normal control group: Synchronized feeding with regular feed, and no medication.

#### 4.4. Drugs and Chemicals

Trypsin and DMEM were purchased from Gibco Chemical Co. (Waltham, MA, USA); CCK-8, mitochondrial membrane potential detection kit, oxygen test kit, TUNEL cell apoptosis detection kit, anti-fluorescence quenching mounting fluid, Tris-HCl, 10% SDS, ammonium peroxydisulfate and TEMED were obtained from Shanghai Biyuntian Biotechnology Company (Shanghai, China). PBS, FBS, BSA, and Triton X-100 were obtained from Beijing Solabao Technology Co., Ltd. (Beijing, China). Alirocumab was purchased from Shanghai Kaimeike Pharmaceutical Technology Co., Ltd. (Shanghai, China). Atorvastatin calcium was obtained from Shanghai Rongweida Industrial Co., Ltd. (Shanghai, China). Primary antibodies included DRP1, MFN2, P53, IL-1 $\beta$ , IL-18, and BAX (Proteintech, Chicago, IL, USA). Secondary antibodies included goat anti-rabbit and goat anti-mouse antibody (Zhongshan Co., Ltd., Beijing, China). The GAPDH primers (133 bp) were: 5'-CCT CGT CCC GTA GAC AAA ATG-3' and 5'-TGAGGTCAATGAAGGGGTCGT-3'; the p-38 primers (143 bp) were: 5'-TGA GCC TGT TGC TGA CCC TTA T-3' and 5'-CAG GTG CTC AGG ACT CCA TTT C-3'; the Mmp1 primers (98 bp) were: 5'-GTT TCT TTA TGG TCC AGG CGA T-3' and 5'-GAC TGG TAA TGG CAT CAA GGG A-3'; the c-fos primers (198 bp) were: 5'-GAG GAG GGA GCT GAC AGA TAC ACT-3' and 5'-AAA TCC AGG GAG GCC ACA GA-3'; the TGF $\beta$  primers (101 bp) were: 5'-GCT GAA CCA AGG AGA CGG AAT A-3' and 5'-GGC TGA TCC CGT TGA TTT CC-3'; the JNK primers (132 bp) were: 5'-GCT GGA ATT ATT CAT CGG GAC T-3' and 5'-AGT CAC CAC ATA AGG CGT CAT C-3'; the ERK1 primers (295 bp) were: 5'-AGG GCT ACA CCA AAT CCA TCG-3' and 5'-CGC TTG TTT GGG TTG AAG GTT A-3'; the ERK2 primers (120 bp) were: 5'-ACC TCA AGC CTT CCA ACC TC-3' and 5'-CTA CGT ACT CTG TCA AGA ACC CTG T-3'; the c-jun primers (258 bp) were: 5'-CCT TCT ACG ACG ATG CCC TC-3' and 5'-GGG TCG GTG TAG TGG TGA TGT-3'; the TNF $\alpha$  primers (223 bp) were: 5'-CCT CAC ACT CAC AAA CCA CCA A-3' and 5'-CTC CTG GTA TGA GAT AGC AAA TCG-3' (Wuhan Sailwell Biotechnology Co., Ltd., Wuhan, China).

##### 4.4.1. CCK-8 Detection of Cell Viability

Cells in the logarithmic growth phase were digested with trypsin and counted under a microscope to make a cell suspension of  $5 \times 10^4$  cells/mL. An amount of 100  $\mu$ L to 96-well

culture plates were taken respectively, and 3 identical holes were inoculated with each cell in each plate as a duplicate hole, with  $5 \times 10^3$  cells per hole; then, a 100  $\mu$ L culture medium was used as a blank control at 37 °C overnight. After treating cells for 24–48 h in CON group, the HG group and the HG combined with 50/100/200/500/1000/2000 nM PCSK9 inhibitor alirocumab, mix the Cell Counting Kit-8 (CCK-8) with serum-free essential basic medium. Add 100  $\mu$ L per well and incubate in a 37 °C and 5% CO<sub>2</sub> incubator for 1 h. The absorbance at 450 nm wavelength was measured with a microplate reader and the values of each plate were recorded.

#### 4.4.2. Flow Cytometry Detection of Membrane Potential (JC-1 Detection)

Take an appropriate amount of JC-1 (200 $\times$ ) and dilute JC-1 according to the proportion of 8 mL ultrapure water per 50  $\mu$ L JC-1 (200 $\times$ ). Vigorously shake to fully dissolve and mix JC-1. Then add 2 mL JC-1 staining buffer (5 $\times$ ) and mix well; this is the JC-1 staining working solution. Collect cells and wash once with 1 $\times$  PBS. Add 0.5 mL cell culture medium to each tube. Add 0.5 mL JC-1 staining working solution and mix thoroughly.

#### 4.4.3. Flow Cytometry Detection of ROS

The processed cells were washed twice with PBS, digested with trypsin, and collected after centrifugation at 1000 rpm for 5 min. Prepare the probe staining working solution by diluting the 10 mM DCFH-DA probe solution with serum-free culture medium at a ratio of 1:1000 to obtain 10  $\mu$ M of staining working solution. Add 1 mL of staining working solution to each sample and resuspend. Invert the mixture every 3–5 min to ensure full contact between the probe and cells. After incubation for 20 min, wash the cells three times with serum-free medium to completely remove unabsorbed DCFH-DA. In the computer test with flow cytometry, the excitation wavelength of 488 nm was used and the emission of 525 nm was measured. ROS-positive cells had strong green fluorescence, corresponding to the FITC detection channel in flow cytometry.

#### 4.4.4. Tunnel Staining

The cell slides were washed with 0.01 M PBS to remove excess medium, and poly-formaldehyde was added to the plate in an appropriate amount for 30 min. After that, the cell slides were washed three times with PBS buffer, each time for 3 min. The cell slides were blocked with 3% BSA for 1 h and washed three times with PBS. The sections were then immersed in PBS containing 0.3% Triton X-100 and incubated at room temperature for 10–20 min. Finally, the slides were rinsed three times with PBS buffer, each time for 3 min. Add the pre-prepared TUNEL detection solution (approximately 50  $\mu$ L) to the slides. Incubate at 37 °C in the dark for 1 h. After incubation, rinse three times with PBS buffer. Aspirate the PBS and add diluted DAPI staining solution to the slides. Incubate at room temperature under light protection for 10–15 min. Finally, rinse three times with PBS buffer. The slide was sealed with anti-fluorescence quenching mounting medium; images were captured under fluorescence microscope.

#### 4.4.5. Western Blot of H9c2 Cardiomyocytes

Rinse the cells with PBS 2–3 times. After the last rinse, pour out the PBS and use a pipette to absorb the residual liquid as much as possible. Add an appropriate volume of RIPA lysis buffer (add the protease inhibitor several minutes before use) into the culture plate for 3–5 min. During this period, the culture plate was shaken repeatedly to ensure full contact between the reagent and cells; the cells were scraped off with a cell scraper and transferred to a 1.5 mL centrifuge tube. Ice-cold lysis was performed for 30 min, during which the cells were completely lysed by repeated pipetting. At 12,000 rpm and 4 °C, centrifuge for 10 min and collect the supernatant to obtain the total protein solution.

Protein concentration was measured with BCA protein concentration assay kit. Different gel concentrations were selected according to the molecular weight of the target protein. The separation gel was prepared according to the formula at different concentrations, and the upper concentration gel was prepared after the lower gel was solidified. According to the protein quantification results, add the required protein with an appropriate amount of loading buffer and centrifuge for 10 min after boiling in water, and then take the upper clear. After that, electrophoresis, membrane transfer, and protein detection on the electrophoretic membrane were carried out according to the conventional procedure. The diluted primary antibody was added as required, and the incubation was performed overnight at 4 °C. After recovery of the primary antibody, the membrane with the primary antibody was washed three times with TBST for 5 min each time. Subsequently, add the secondary antibody according to the specified dosage and incubate the membrane in a room temperature shaker for 1–2 h. After recovery of the secondary antibody, place the membrane in a washing chamber and wash it three times with TBST buffer, each time for 5 min. The ECL chemiluminescence detection is then performed, followed by scanning with the imaging system. Finally, use ImageJ 1.52a software to determine the grayscale values of the bands and perform statistical analysis.

#### 4.4.6. Glucose Tolerance Test in Mice

After fasting overnight, mice were intraperitoneally injected with 40% glucose (2 g/kg). Blood sugar levels were measured with a glucometer 15, 30, 60, 90 and 120 min after injection. In normal mice, blood glucose concentration can be restored to a normal level in about 2 h, while in mice with abnormal glucose metabolism, the time for the blood glucose concentration to return to normal level is significantly delayed; that is, there is impaired glucose tolerance.

#### 4.4.7. Cardiography in Mice

The hair of the heart area of the mouse was removed, and the mouse was fixed on the test table after isoflurane gas anesthesia. The mask ventilation was maintained to keep the anesthesia. The Vevo 3100 imaging system (VisualSonics, Toronto, ON, Canada) and mouse ultrasound probe were used to obtain M-type and B-type images of the cardiac long axis, and LVEF, FS%, LVID (s), LVID (d), IVS (d), IVS (s), and E/A ratio parameters were calculated.

#### 4.4.8. Methylhematin–Hematin Staining

The paraffin section is baked at 70 °C and dewaxed to water according to the conventional procedure. The sections were treated with the HDH pretreatment solution for 1 min. Then the sections were placed in hematoxylin and stained for about 10 to 15 min, and washed with water for 3 to 5 min to remove hematoxylin and floating color. The sections were placed in a differentiation solution containing 1% ethanol hydrochloric acid until the sections turned pale blue after complete discoloration. The sections were washed with tap water, stained with blue-backing solution, and rinsed with running water. The slices were dehydrated in 95% alcohol for 1 min and stained in eosin dye for 15 s, rinsed to remove eosin float, and then immersed in different concentrations of ethanol for dehydration. The dehydrated slices were immersed in xylene to make them transparent, sealed with gum, placed in a warm oven to dry, photographed under the microscope and analyzed.

#### 4.4.9. Maron Trichrome Staining

Slice the tissue wax block, slide under a 42 °C environment, then bake for 30 min (60 °C). Slices were dewaxed according to standard procedures, rinsed under running water for 5 min, and immersed in Masson A solution overnight before rinsing under

running water. The sections were placed in the Masson B solution and Masson C solution mixed in equal proportion, immersed for 1 min, rinsed with tap water, differentiated for a few seconds, and rinsed with tap water. Next, the sections were immersed in Masson D for 6 min and rinsed with tap water. They were then placed in Masson E for 1 min without washing. After slight drying, they were directly transferred to Masson F for 20–30 s of staining. The sections were washed with 1% acetic acid to differentiate, dehydrated with ethanol in turn, and transparented with xylene for three times, each lasting 2 min. The slides were sealed with neutral gum, and then photographed and analyzed under the microscope.

#### 4.4.10. Immunohistochemical

After dewaxing paraffin sections to water, antigen repair was performed. Endogenous peroxidase was blocked and serum was used for blocking. Primary antibody was added in a certain proportion and incubated overnight at 4 °C. The tissue was then placed in PBS and shaken on a decolorization shaker 3 times, each time for 5 min. After slightly drying the slices, add the secondary antibody corresponding to the species of the primary antibody dropwise in the loop to cover the tissue, and incubate at room temperature for 50 min. Then DAB was used for color development, and the color development time was controlled under the microscope. The positive color was brownish yellow, and the color development was terminated by rinsing the slices with tap water. The nuclei were then stained with hematoxylin and dehydrated and mounted. The results were interpreted under a white light microscope, where the nuclei stained with hematoxylin were blue and the positive expression of DAB was brownish.

#### 4.4.11. Wheat Germ Agglutinin Staining (WGA Staining)

The tissue section was placed in a repair box filled with EDTA antigen repair buffer (pH 8.0) and then placed in the microwave oven for antigen repair. After slightly drying the slices, draw a circle around the tissue with a histological brush, add diluted WGA staining solution in the circle, and incubate at 37 °C in a light-shielding constant temperature box for 30 min. The nuclei were stained with DAPI, followed by quenching of tissue autofluorescence and rinsing for 10 min. After slight drying of the sections, the slides were sealed with anti-fluorescence quenching medium. Slices were observed under a fluorescence microscope and images were collected (DAPI excitation wavelength of 330–380 nm and emission wavelength of 420 nm, with blue light; FITC excitation wavelength of 465–495 nm and emission wavelength 515–555 nm, with green light; CY3 excitation wavelength of 510–560 nm and emission wavelength 590 nm, with red light).

#### 4.4.12. Transmission Electron Microscope

The material was fixed, and the tissue was divided into 1 mm<sup>3</sup> sections. After the small tissue block was removed from the body, it was immediately put into a Petri dish containing electron microscope fixing fluid. The small tissue block was cut into 1 mm<sup>3</sup> with a scalpel in the fixing fluid of the Petri dish. The small tissue blocks were then transferred to an EP tube containing a new electron microscope fixative for further fixation. The tissue blocks were first rinsed three times with 0.1 M phosphate buffer PB (pH 7.4), with each rinse lasting 15 min. They were then fixed in a 1% osmium acid solution prepared with the same buffer at room temperature under light protection for 2 h. Finally, the tissue blocks underwent three additional rinses using the 0.1 M phosphate buffer PB (pH 7.4), each rinse lasting 15 min. The tissue blocks were sequentially dehydrated in alcohol solutions at 30%, 50%, 70%, 80%, 95%, 100%, and 100% concentrations for 20 min each, followed by two 15-min immersion steps in 100% acetone. Subsequently, the samples underwent infiltration embedding; after insertion into an embedding plate, they were incubated overnight in a 37 °C oven. The embedded plates were then placed in a 60 °C oven for

48 h to complete polymerization. Finally, the resin blocks were retrieved and prepared for use. The resin block was placed in a semi-thin sectioning machine for 1.5  $\mu\text{m}$  semi-thin sectioning, followed by toluidine blue staining and positioning under light microscope. Then ultra-thin sectioning and staining were performed, and images were observed under transmission electron microscope for image analysis.

#### 4.4.13. Enzyme-Linked Immunosorbent Assay (ELISA)

Whole blood specimens were left at room temperature for 2 h or 4 °C overnight, and then centrifuged at 2–8 °C and 3000 rpm for 15 min; then, the supernatant was taken. Then the coating and sealing were carried out, and a 37 °C incubation was carried out for 1–2 h. Then wash and add samples. Add 100  $\mu\text{L}$  of the diluted test sample to the above coated reaction well, and make blank well and dilution standard well at the same time. After sealing the plate with a sealing film, the specimen was incubated at 37 °C for 1–2 h. Wash again and add antibody. Add 100  $\mu\text{L}$  diluted biotinylated antibody working solution to each well. After sealing the plate with a cover film, the specimen was incubated at 37 °C for 1 h. After washing again, 100  $\mu\text{L}$  of diluted enzyme conjugate working solution was added to each well. Then incubation and washing were carried out again. After that, 100  $\mu\text{L}$  TMB substrate solution was added into each well, and the reaction was carried out at 37 °C in the dark for 10–30 min until a clear color gradient appeared in the standard reference well diluted by the ratio. Then 100  $\mu\text{L}$  2M sulfuric acid was added into each reaction well, and the color changed from blue to yellow. Within 10 min, the OD value of each well was measured at 450 nm on the microplate reader after zeroing at the blank control well. The standard curve was made according to the concentration and OD value of the standard, and then the sample concentration was calculated according to the equation of the standard curve.

#### 4.4.14. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

First, RNA extraction was performed. The tissue block was cut with high-pressure-treated scissors, put into 4 grinding beads, and put into the tissue grinder to grind the tissue into a homogenate. Add 200  $\mu\text{L}$  chloroform (Trizol:chloroform = 5:1), cover the EP tube tightly, shake vigorously up and down, then leave it for 10 min; then, centrifuge for 15 min at 12,000 rpm and 4 °C. Take 150–300  $\mu\text{L}$  of the supernatant into a new EP tube, add the same amount of isopropanol, gently mix 7–8 times, leave at room temperature for about 10–20 min; then, centrifuge for 15 min at 12,000 rpm and 4 °C. White sediment can be seen at the bottom of the tube, which is the RNA precipitation. Discard the supernatant and add 1000  $\mu\text{L}$  of 75% ethanol (diluted with DEPC water) to resuspend the RNA precipitate. At 7500 rpm, centrifuge for 10 min at 4 °C. Discard the supernatant and centrifuge at 100 rpm for 1 min at 4 °C. Aspirate residual ethanol and air-dry for 10–15 min. Add an appropriate volume (15–30  $\mu\text{L}$ ) of DEPC water to dissolve RNA. Measure and record the RNA concentration before proceeding with reverse transcription. Next, remove the genomic DNA. Add the following components to the centrifuge tube in the following order: 4  $\mu\text{L}$  of 4 $\times$  gDNA wiper mix, 1  $\mu\text{g}$  RNA sample, and RNase-free ddH<sub>2</sub>O to make up to 16  $\mu\text{L}$  of reaction solution. Gently aspirate to mix the liquids, then centrifuge at 42 °C for 2 min. Then the reverse transcription reaction was carried out by adding 5  $\times$  HiScript II qRT SuperMix II 4  $\mu\text{L}$  to the aforementioned 16  $\mu\text{L}$  of reaction solution. After beating and centrifugation to mix the liquid, the following procedures were completed separately: 50 °C—15 min and 85 °C—5 s. Finally, quantitative PCR was performed. The cDNA obtained by reverse transcription was diluted 10 times (diluted with ddH<sub>2</sub>O), and blown and centrifuged to mix them. Add the following components sequentially to prepare the mixed solution in the qPCR plate: Primer 1 (0.2  $\mu\text{L}$ ), Primer 2 (0.2  $\mu\text{L}$ ), 2 $\times$  ChamQ Universal

SYBR qPCR Master Mix (5 µL), ddH<sub>2</sub>O (2.6 µL), and cDNA (2 µL). Centrifuge at 800 rpm for 5 min to mix thoroughly. Perform pre-denaturation, annealing, and melting curve analysis according to the programmed sequence.

#### 4.4.15. Transcriptome Sequencing (RNA-Seq)

First, perform RNA sample testing: ① NanoDrop™ One/OneC detects RNA purity (OD<sub>260</sub>/280 and OD<sub>260</sub>/230 ratio); ② Life Invitrogen Qubit® 3.0 fluorescence quantification instrument uses Qubit™ RNA HS Assay Kit for precise quantification (Thermo Fisher Scientific, Waltham, MA, USA); ③ Agilent 4200 TapeStation system accurately measures RNA integrity (RIN value). Next, the library was constructed: most of the mRNA in eukaryotes had the structure of polyA, and magnetic beads with Oligo (DT) were used to capture the mRNA with polyA structure. Using fragmented mRNA as the template and random oligonucleotides as primers, the first strand of cDNA is synthesized in the M-MuLV reverse transcriptase system. The RNA strand is then degraded using RNase H, followed by synthesis of the second strand of cDNA under DNA polymerase I conditions using dNTPs as substrates. The double-stranded cDNA was purified, followed by end repair, A-terminal tailing, and ligation with sequencing adapters. Approximately 200 bp cDNA fragments were screened using AMPure XP beads (Beckman Coulter, Brea, CA, USA), amplified via PCR, and purified again using the same AMPure XP beads to obtain the final sequencing library. Next, the library quality control was carried out: ① Kapa qPCR quantification: precise quantification of library concentration; ② Agilent 4200 TapeStation detection (Agilent Technologies Co., Ltd. (China), Shanghai, China): accurate detection of library fragment size. Finally, the in vitro sequencing and analysis were conducted: After passing quality control, different libraries were pooled according to effective concentration and target output requirements for Illumina PE150 sequencing (Illumina, San Diego, CA, USA). Data quality control, reference genome alignment, gene expression quantification, differential gene analysis, functional enrichment analysis, protein interaction analysis of differentially expressed genes, variant detection, and alternative splicing analysis were performed based on the sequencing results.

#### 4.5. Statistics

For normally distributed data, measurements are expressed as mean ± standard deviation. Independent samples *t*-test is used for intergroup comparisons, while one-way ANOVA is employed for multi-group analyses. Within-group comparisons before and after treatment use the paired samples *t*-test. For non-normal distribution data, measurements are represented by quartiles, with the Kruskal–Wallis nonparametric rank-sum test applied for comparisons.

### 5. Conclusions

This study provides preliminary evidence that the PCSK9 inhibitor alirocumab can improve cardiac function in diabetic cardiomyopathy models by inhibiting the ERK/p38 MAPK signaling pathway. It effectively inhibits myocardial fibrosis, inflammatory responses, and oxidative stress, reduces cardiomyocyte apoptosis, and prevents myocardial hypertrophy and ventricular remodeling. These findings establish a solid experimental foundation for the clinical development of therapeutic agents targeting diabetic cardiomyopathy.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052341/s1>.

**Author Contributions:** S.L. performed the experiments, analyzed the data, and drafted the manuscript. T.S., as the corresponding author, supervised the project, guided all experiments, contributed to the study conception and design, and critically revised the manuscript. B.W. and S.S. provided materials, assisted with data interpretation and critical revisions, and contributed to manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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