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# The complex phenotype of diabetic cardiomyopathy: clinical indicators and novel treatment targets

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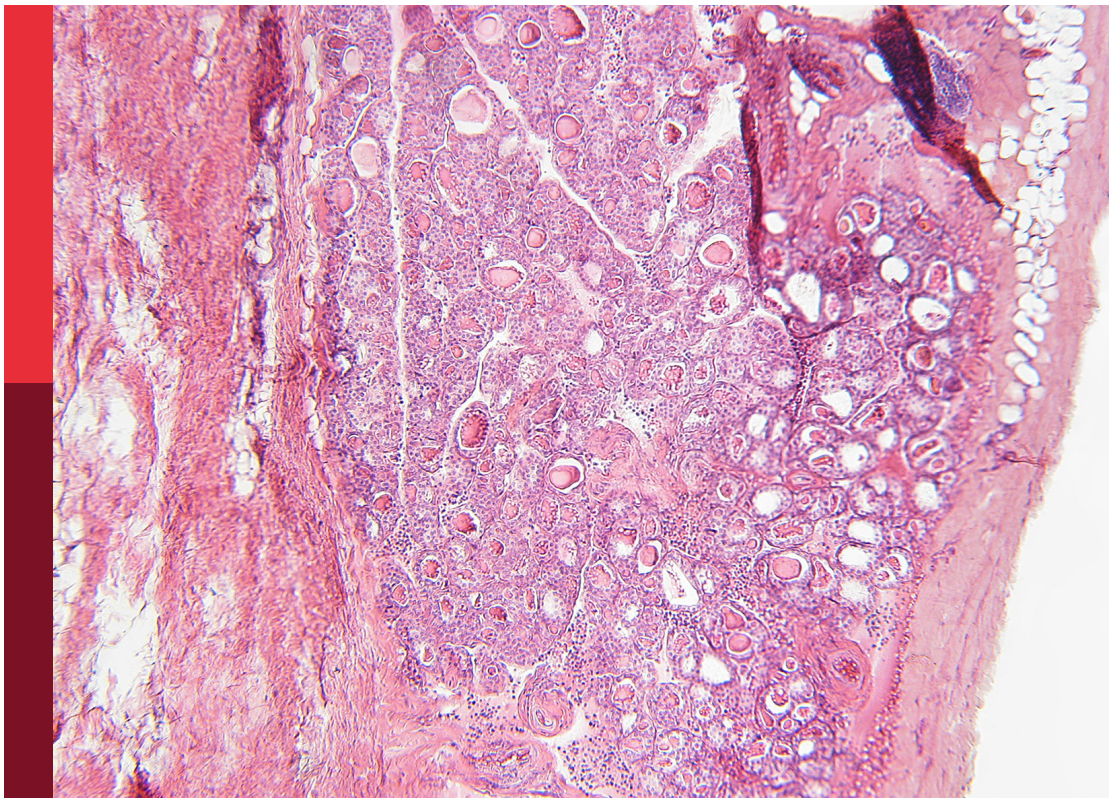

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# The complex phenotype of diabetic cardiomyopathy: clinical indicators and novel treatment targets

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# Editorial: The complex phenotype of diabetic cardiomyopathy: clinical indicators and novel treatment targets

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**diabetic cardiomyopathy, cell death, inflammation, oxidative stress, signaling pathway**

Editorial on the Research Topic
The complex phenotype of diabetic cardiomyopathy: clinical indicators and novel treatment targets

This Research Topic highlights the complex phenotype of diabetic cardiomyopathy (DCM), focusing on its clinical indicators and potential treatment approaches. DCM is a severe complication of both type 1 and type 2 diabetes, characterized by myocardial fibrosis, impaired cardiac function, and increased mortality rates among diabetic patients. The estimated global prevalence of diabetes is expected to reach 12.2% (783.2 million people) by 2045, with a significant percentage of diabetic individuals (11.7% to 67%) developing DCM based on varying diagnostic criteria (1, 2). Moreover, diabetic patients have a 2.45 to 2.99 times higher incidence of myocardial ischemia compared to non-diabetics (3). Therefore, accurate estimates of current and future prevalence of type 2 diabetes are crucial for effective health care planning and targeted interventions to reduce risk factors and reverse increasing trends. However, projections such as those from the International Diabetes Federation's Diabetes Atlas often emphasize urbanization and demographic shifts, overlooking other crucial risk factors like obesity and smoking. To address this, Moreira et al. used the IMPACT TYPE 2 DIABETES model, which incorporates demographic changes along with obesity and smoking trends using a Markov approach in the Brazilian population. Their findings predict a rise in diabetes prevalence, even with aggressive obesity reduction strategies, highlighting the crucial role of tackling obesity to prevent diabetes. The study calls for expanding current initiatives to make a significant impact, suggesting that with stronger efforts, it is possible to lower type 2 diabetes prevalence in line with national and international policies.

Arterial stiffness (AS) is a key factor in the development of cardiovascular disease (CVD), serving as an early indicator of atherosclerosis and a predictor of CVD risk and mortality (4). Lifestyle choices, including sedentary habits and diets rich in processed carbohydrates and saturated fats, contribute to conditions like obesity, lipid disorders, and insulin resistance, which in turn exacerbate AS. Early detection of AS is particularly

important for individuals type 2 diabetes, as they face a higher risk of cardiovascular complications. In response to this, Mao et al. investigated the relationship between lipid accumulation product (LAP) index—based on waist circumference and triglyceride levels—and brachial-ankle pulse wave velocity (baPWV), a key AS indicator, in Chinese type 2 diabetic patients. The study revealed a strong positive correlation between LAP and baPWV, which persisted even after adjusting for various factors and was consistent across different genders and subgroups. This suggests that LAP could be useful tool for assessing AS risk in clinical settings and research.

Additionally, Zeng et al. explored the causal relationship between 1,400 metabolites and dilated cardiomyopathy through a two-sample Mendelian randomization (MR) approach. They identified 52 metabolites with causal association to the disease, some of which were positively linked, while others were negatively correlated. Elevated levels of tryptophan betaine and 5-methyluridine were found to increase the risk of dilated cardiomyopathy, whereas myristoleate and erythronate were linked to a reduced risk. These insights into metabolic factors offer promising avenues for new treatment approaches and biomarker development to improve disease management.

Understanding the underlying causes and pathology of DCM is essential for the development of new drug therapies and clinical indicators. Key contributors to DCM progression include oxidative stress, inflammation, and cell death, with cardiomyocyte death playing a central role. Various forms of cell death – such as apoptosis, autophagy, necrosis, ferroptosis, and pyroptosis – are implicated in the disease. Ferroptosis, a type of programmed cell death driven by iron accumulation and lipid peroxidation, results from deficiencies in oxidoreductases such as glutathione peroxidase 4, which diminishes cellular antioxidant defenses and contributes to myocardial dysfunction (3). Similarly, pyroptosis, a recently identified inflammatory form of programmed cell death, is crucial in DCM progression. This Research Topic includes two key reviews exploring the roles of ferroptosis and pyroptosis in DCM. Zhao et al. discuss the molecular mechanisms linking ferroptosis to DCM and evaluate potential therapeutic approaches using ferroptosis inducers and inhibitors. Wang et al. provide an overview of the mechanisms by which pyroptosis contributes to DCM and explore targeted treatments that focus on NLRP3 inflammasome pathway. Both reviews highlight the importance of understanding these processes, which may lead to new drug developments that can slow or reverse DCM progression, ultimately improving patient outcomes.

Current management of DCM focuses on controlling blood glucose and lipid levels, but no specific or reliable drugs are available for the condition. Other CVD risk factors, such as myocardial infarction (MI), dyslipidemia, and hypertension, also play critical roles in DCM progression. Among these, acute myocardial infarction (AMI) is a leading cause of cardiovascular death worldwide (5). As a result, identifying effective treatments for MI and improving its prognosis are essential for reducing cardiovascular mortality and enhance global health outcomes. In this context, Zhuang et al. conducted a Mendelian randomization analysis to explore the genetic relationship between the metformin use and various MI outcomes. Their findings revealed that metformin does not reduce the risk of acute transmural MI of the anterior wall and might increase the risk of overall MI, old MI, acute MI, and acute transmural MI of the inferior wall. This suggest that metformin may not provide the expected protection against MI and could even increase the risk for various forms of MI.

A meta-analysis by Wang et al. on dipeptidyl peptidase-4 inhibitors (DPP4i) revealed their potential benefits in improving cardiac structure and function, highlighting DPP4i as a promising treatment for preventing MI and other CVDs. Additionally, using a network pharmacology approach, another study by Wang et al. identified the SIRT1, Nrf2, and NQO1 signaling pathways as targets for YuNü-Jian, a traditional Chinese medicinal formula, suggesting its potential for managing DCM. Moreover, Jiang et al. reviewed the cardioprotective properties of salvianolic acid in diabetic patients, particularly its ability to protect against myocardial ischemia-reperfusion injury. The beneficial effects of salvianolic acid are linked to its modulation of oxidative stress, inflammation, mitochondrial dysfunction, ferroptosis, and apoptosis through key pathways such as PI3K/Akt, JAK/STAT, and NF-kB. These findings point to salvianolic acid's promising potential in cardiovascular protection for diabetic patients.

The current research findings offer promising insights, but further detailed clinical studies are critical to effectively address DCM and other CVDs. There is also an urgent need for reliable and sensitive clinical markers to detect early cell death in DCM. Currently, the absence of clear diagnostic criteria for DCM poses challenges in differentiating myocardial injury, hemodynamic changes, and reduced cardiac function caused by cell death from those resulting from other conditions, such as coronary atherosclerosis or ischemic cardiomyopathy. In evaluating the efficacy of treatment for DCM, it is important to consider factors such as drug dosage, timing of administration, and patient characteristics including age, gender, and the presence of comorbidities. Therefore, developing strategies to mitigate risk and intervene in DCM progression is of great clinical and societal importance, especially as it may improve outcomes for those at risk of cardiovascular complications.

## Author contributions

PC: Conceptualization, Project administration, Validation, Writing – original draft, Writing – review & editing. RK: Conceptualization, Supervision, Validation, Writing – review & editing. YC: Formal analysis, Validation, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Ferroptosis: roles and molecular mechanisms in diabetic cardiomyopathy

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Diabetic cardiomyopathy (DCM) is a serious complication of type 1 and type 2 diabetes, which leads to the aggravation of myocardial fibrosis, disorders involving systolic and diastolic functions, and increased mortality of patients with diabetes through mechanisms such as glycolipid toxicity, inflammatory response, and oxidative stress. Ferroptosis is a form of iron-dependent regulatory cell death that is attributed to the accumulation of lipid peroxides and an imbalance in redox regulation. Increased production of lipid reactive oxygen species (ROS) during ferroptosis promotes oxidative stress and damages myocardial cells, leading to myocardial systolic and diastolic dysfunction. Overproduction of ROS is an important bridge between ferroptosis and DCM, and ferroptosis inhibitors may provide new targets for the treatment of patients with DCM.

KEYWORDS

**diabetes mellitus, diabetic cardiomyopathy, ferroptosis, iron metabolism, oxidative stress**

## 1 Introduction

Diabetes mellitus is a chronic metabolic disorder caused by a combination of genetic and environmental factors, and its prevalence is increasing annually. The global prevalence of diabetes in people aged 20-79 years is currently estimated to be 10.5% (536.6 million people) and is expected to rise to 12.2% (783.2 million people) by 2045. There are currently 141 million adults with diabetes in China, and it is expected that by 2045, more than 174 million people will have diabetes (1).

Patients with diabetes are at a significantly higher risk of cardiovascular comorbidities owing to long-term metabolic disorders, persistent high glucose status, and insulin resistance (IR). Diabetic cardiomyopathy (DCM), a specific form of heart disease, has garnered considerable attention. DCM, also known as non-vascular myocardial dysfunction, refers to the development of abnormal myocardial structures and clinical manifestations in patients with diabetes in the absence of other cardiac risk factors, such as coronary artery disease, hypertension, and severe valvular disease (2).

There is still a lack of clear expert consensus on the definition of DCM, and its prevalence and incidence remain unclear. It has been estimated that early features of DCM can be observed in one-quarter to one-third of asymptomatic patients with diabetes. Among them, 15.5% with type 1 diabetes mellitus (T1DM) have abnormal myocardial systolic or diastolic function, whereas patients with type 2 diabetes mellitus (T2DM) are mostly middle-aged and elderly, with a relatively higher risk of cardiovascular system complications (3). A prospective study showed that the prevalence of DCM in subjects diagnosed with clinical or preclinical stages of DCM was approximately 38%, with a prevalence of 48% in female patients, mostly older than 65 years (4). DCM is also a major cause of heart failure (HF) in diabetic patients, and the risk of HF in patients with diabetes increases by 74% (5). The incidence of myocardial ischemia is 2.45 to 2.99 times that of non-diabetic patients (6). DCM is the leading cause of increased mortality among patients with diabetes in both developed and developing countries, and its prevalence increases with the incidence of obesity and T2DM, thereby placing a serious burden on the global economy and health management systems.

Ferroptosis is a new type of programmed cell death that is often accompanied by massive iron accumulation and lipid peroxidation, as well as a deficiency in oxidoreductases, especially glutathione peroxidase 4 ($GPX_4$) (6, 7). In 2012, Dixon et al. named this death pattern of iron-dependent non-apoptotic cell death as “ferroptosis” (8). Ferroptosis can affect $GPX_4$ directly or indirectly through different pathways, causing a decrease in cellular antioxidant capacity and accumulation of lipid reactive oxygen species (ROS), ultimately leading to cell death (7).

In recent years, studies have shown that ferroptosis is strongly associated with various diseases including ischemic heart disease, kidney disease, liver damage, degenerative diseases, and diabetes (6, 9). Ferroptosis inhibitors can improve the progression of DCM-related pathologies and may be used as novel therapeutic modalities for DCM treatment (10). This paper reviews the relationship and molecular mechanisms of ferroptosis and DCM to provide new ideas and targets for the treatment of DCM.

# 2 Roles and regulatory mechanisms of ferroptosis

## 2.1 Ferroptosis and iron metabolism

Iron is an important nutrient in the human body and is involved in a variety of biological processes, such as oxygen transport, lipid metabolism, DNA and protein synthesis, and cellular respiration. Abnormalities in the content or distribution of iron in the body may lead to the occurrence and progression of certain diseases (11). Intracellular iron metabolism mainly involves iron absorption, export, utilization, and storage, and an imbalance between these processes may affect the susceptibility of cells to ferroptosis (12). On the one hand, the redox properties of iron make free $Fe^{2+}$ prone to undergo Fenton reactions with lipid peroxides, which produces a highly toxic hydroxyl radical and induces a strong oxidative stress response, which is a core mechanism for ferroptosis. On the other hand, $Fe^{2+}$ and $Fe^{3+}$ are also cofactors that can enhance the activities of various metabolic enzymes, resulting in the formation of free radicals such as alkoxy (RO) and peroxy ($RO_2$), promoting lipid peroxidation, and inducing ferroptosis (13, 14). In addition, the degradation of ferritin and subsequent autophagy can increase the intracellular unstable iron content and enhance sensitivity to ferroptosis. Iron regulatory protein 1 ($IRP_1$) and iron regulatory protein 2 ($IRP_2$) regulate intracellular iron storage, release, entry, and exit, thereby maintaining cellular iron homeostasis. Changes in their levels may affect the amount of unstable iron in cells, thereby altering the sensitivity of cells to ferroptosis (15, 16). Therefore, an abnormal iron metabolism is necessary for ferroptosis.

## 2.2 Characteristics of ferroptosis

Ferroptosis is mainly characterized by iron overload, which causes substantial lipid peroxide production and, ultimately, cell death (9). Ferroptosis is morphologically, biochemically, and genetically different from cell death by apoptosis, necrosis, and autophagy. Ferroptosis is morphologically characterized by the contraction of mitochondria, reduction in volume, increased membrane density, and the reduction or disappearance of mitochondrial cristae. However, the cell nuclei are normal in size, and there is no chromatin condensation. This is an essential distinction from other cell death modalities such as apoptosis and necrosis. Ferroptosis is biochemically predominantly iron-dependent and is mainly characterized by an increase in $Fe^{2+}$ concentration and lipids. Excessive production of ROS by $Fe^{2+}$ through Fenton reactions causes intracellular lipid peroxidation, whereas iron enhances lipoxygenase (LOX) activity to promote ferroptosis (17). Genetically, ferroptosis is a biological process regulated by multiple genes, and a variety of genes involved in iron metabolism, lipid synthesis, and oxidative stress regulation can modulate ferroptosis (7).

## 2.3 Mechanism of ferroptosis

In addition to iron-dependent cell death, ferroptosis is caused by an imbalance between the production and degradation of intracellular lipid ROS and is regulated by multiple metabolic and redox systems. ROS are mainly produced by the iron-dependent Fenton reaction and mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) family enzymes. Mitochondria are the metabolic centers of most mammalian cells, and are important sources of ROS. Excessive ROS may result in oxidative damage to mitochondrial proteins and DNA, which impairs mitochondrial function, eventually leading to ferroptosis (18).

The system $Xc^-$ -glutathione (GSH)-$GPX_4$ axis plays a central role in limiting lipid peroxidation. Ferroptosis is associated with the inactivation of cellular antioxidant systems, especially system $Xc^-$, which causes the accumulation of lipid peroxides (17). System $Xc^-$ is an amino acid counter-transport protein widely distributed in

phospholipid bilayers and consists of two independent proteins: solute carrier family 7 member 11 ($SLC_7A_{11}$) and solute carrier family 3 member 2 ($SLC_3A_2$) (19). GPX is the main endogenous mechanism that prevents peroxidation, among which $GPX_4$ is a key regulator of ferroptosis and inhibits ferroptosis by inhibiting the production of lipid peroxides. GSH is an essential cofactor for $GPX_4$, which converts reduced GSH to oxidized GSH (GSSG) and reduces toxic lipid peroxides (L-OOH) to non-toxic alcohols (L-OH), thereby decreasing oxidative stress damage and inhibiting the onset of ferroptosis (7, 20).

Cystine and glutamate are exchanged in and out of the cell *via* system $Xc^-$ at a ratio of 1:1. System $Xc^-$ transports extracellular cystine into the cell and converts it to cysteine, which is then used for GSH synthesis. GSH reduces ROS and reactive nitrogen species through the action of GPX (21). Thus, the inhibition of system $Xc^-$ activity can affect GSH synthesis by inhibiting cystine uptake, contributing to a reduction in GPX activity, a decrease in cellular antioxidant capacity, accumulation of lipid ROS, and ultimately the occurrence of oxidative damage and ferroptosis.

Ubiquinone, also known as coenzyme $Q_{10}$ ($CoQ_{10}$), is an electron carrier in the mitochondrial respiratory chain and a potent lipophilic antioxidant (22). Ubiquinol ($CoQ_{10}H_2$) is the reduced form of coenzyme $Q_{10}$ that reduces oxidative stress, inhibits adipocyte differentiation, and suppresses lipid ROS accumulation (20). Mitochondrial $CoQ_{10}$ inhibits apoptosis, whereas non-mitochondrial $CoQ_{10}$ prevents ferroptosis. Ferroptosis suppressor protein 1 ($FSP_1$), a flavoprotein oxidoreductase, may determine the role of $CoQ_{10}$ in apoptosis and ferroptosis, and its pro-apoptotic function may be achieved through the inhibition of redox activity (18). $FSP_1$, originally named apoptosis-inducing factor mitochondrial associated 2 ($AIFM_2$), is also a mitochondrial pro-apoptotic protein (23). It has been found that $FSP_1$ is able to use NADPH to regenerate lipophilic radicals to trap antioxidant; that is, $FSP_1$ is responsible for regenerating $CoQ_{10}$. $FSP_1$ shuttles the reducing equivalents from NADPH to the lipid bilayer, allowing $CoQ_{10}$ to interconvert with $CoQ_{10}H_2$, thereby inhibiting lipid peroxidation and maintaining the effective concentration of $CoQ_{10}H_2$. The $FSP_1$-$CoQ_{10}$-NADPH pathway is an independent parallel system, also known as the non-dependent $GSH/GPX_4$ ferroptosis inhibition pathway, which is the main pathway of endogenous antioxidant enzyme regeneration, and synergistically inhibits lipid peroxidation and ferroptosis with $GPX_4$ and GSH (24). Tetrahydrobiopterin ($BH_4$) is an endogenous antioxidant that protects cells from ferroptosis by reducing lipid peroxidation. Dihydrobiopterin ($BH_2$) can be reduced to $BH_4$ by dihydrofolate reductase (DHFR) (25); therefore, DHFR is also an important regulator of ferroptosis.

Lipid peroxidation is a prerequisite for ferroptosis and is closely associated with lipid metabolism. Fatty acids (FA) are important components of cellular lipid metabolism and have a variety of cellular functions, including energy supply, cell membrane formation, and function as precursors to a variety of signaling molecules (26). The accumulation of polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA), and the reduction in lipid peroxide scavenging capacity lead to ferroptosis (6). It has been shown that phosphatidyl ethanolamine (PE) containing AA or its derivative, adrenic acid (ADA), is a key phospholipid in the induction of cellular ferroptosis. Acyl CoA synthase long-chain family member 4 ($ACSL_4$) and lysophosphatidylcholine acyltransferase 3 ($LPCAT_3$) are two key enzymes involved in the synthesis of PE, activating PUFA and affecting their transmembrane properties. PUFA can be acylated by $ACSL_4$ to form polyunsaturated fatty acid acyl-coenzyme A (PUFA-CoA), which is then esterified by $LPCAT_3$ and finally reacts with PE to form PUFA- phosphatidyl ethanolamines (PUFA-PEs). PUFA-PEs can be oxidized by LOX to L-OOH products such as PE-AA-OOH or PE-ADA-OOH. Therefore, reducing the expression of $ACSL_4$ and $LPCAT_3$ can decrease the accumulation of intracellular lipid peroxides and inhibit ferroptosis (27).

Autophagy is a fundamental cellular homeostatic program, and excessive autophagy may trigger cell death, also known as autophagy-dependent cell death (18). Recent studies have shown that ferritinophagy is a unique form of selective autophagy mediated by nuclear receptor coactivator 4 ($NCOA_4$) (10). Ferritinophagy promotes ferroptosis, which may be attributed to the iron overload caused by increased $NCOA_4$ expression (28). $NCOA_4$ mediates the degradation of ferritin after combining with ferritin heavy chain 1 ($FTH_1$) as a selective autophagic receptor and converts ferritin-bound iron into free iron, thereby inducing ferroptosis (29) (Figure 1).

# 3 Typing and diagnosis of diabetic cardiomyopathy and its pathogenesis

## 3.1 Typing and diagnostic criteria of diabetic cardiomyopathy

The histological features of DCM include myocardial collagen deposition, myocardial hypertrophy, and fibrosis (30). It is conventionally believed that the early stages of DCM are characterized by left ventricular (LV) hypertrophy and diastolic dysfunction, whereas the later stages progress to cardiac fibrosis and systolic dysfunction, eventually leading to HF (31). In 1954, Lundbaek first described DCM as a cardiomyopathy that affects two-thirds of elderly patients with diabetes mellitus (32). Two DCM phenotypes, HF with preserved LV ejection fraction (HFpEF) and HF with reduced LV ejection fraction (HFrEF), were identified based on the predominance of cardiomyocyte hypertrophy or apoptosis, the most common of which is the HFpEF phenotype (30).

The minimum diagnostic criteria for DCM include LV diastolic dysfunction and/or reduced LV ejection fraction (LVEF), LV hypertrophy, and interstitial fibrosis and can be divided into three stages: early, advanced, and end. DCM progresses from an initial subclinical phase, characterized by mild structural and functional abnormalities, then to severe diastolic HF with normal ejection fraction (EF), and eventually to HF with systolic dysfunction accompanied by a reduced EF value (33). One of the earliest features of DCM is abnormal LV wall stiffness, mainly secondary to extracellular matrix and myocardial cell remodeling. Cardiac magnetic resonance imaging (MRI) shows

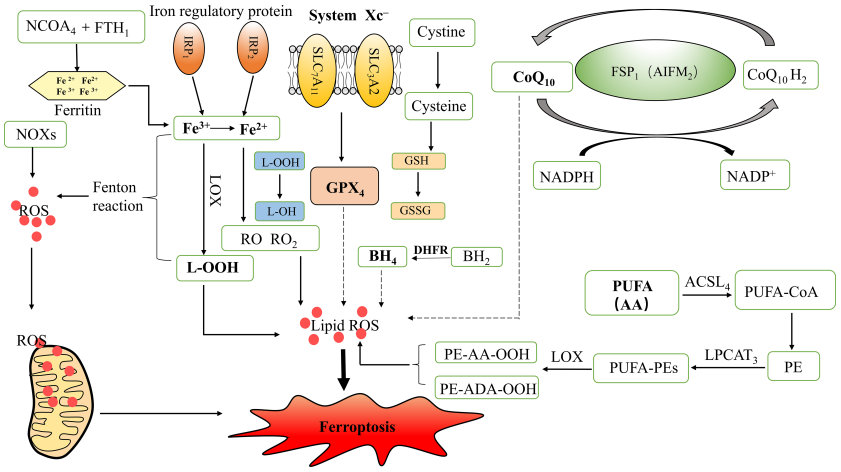


FIGURE 1
Mechanism of ferroptosis and iron metabolism. ROS, Reactive oxygen species; $GPX_4$, Glutathione peroxidase 4; L-OOH, Lipid hydroperoxides; L-OH, Alcohol; RO, Alkoxy; $RO_2$, Peroxy; $IRP_1$, Iron regulatory protein 1; $IRP_2$, Iron regulatory protein 2; LOX, Lipoxygenase; NADPH, Nicotinamide adenine dinucleotide phosphate; NOXs, NADPH Oxidases; GSH, Glutathione; GSSG, Oxidized GSH; $SLC_7A_{11}$, Solute carrier family 7 members 11; $SLC_3A_2$, Solute carrier family 3 member 2; $CoQ_{10}$, Coenzyme $Q_{10}$/Ubiquinone; $CoQ_{10}H_2$, Ubiquinol; $FSP_1$, Ferroptosis suppressor protein 1; $AIFM_2$, Apoptosis inducing factor mitochondrial associated 2; $BH_4$, Tetrahydrobiopterin; $BH_2$, Dihydrobiopterin; DHFR, Dihydrofolate reductase; PUFA, Polyunsaturated fatty acids; AA, Arachidonic acid; ADA, Adrenic acid; PE, Phosphatidyl ethanolamine; $ACSL_4$, Acyl CoA synthase long-chain family member 4; $LPCAT_3$, lysophosphatidylcholine acyltransferase 3; PUFA-CoA, Polyunsaturated fatty acid acyl-coenzyme A; PUFA-PEs, PUFA- phosphatidyl ethanolamines; $NCOA_4$, Nuclear receptor co-activator 4; $FTH_1$, Ferritin heavy chain1.

weakened ventricular wall motion in all components of myocardial deformation (longitudinal, radial, and circumferential strain). Reduced myocardial strain can also be observed in asymptomatic patients and in T2DM patients without other signs of etiologic heart disease. Therefore, reduced myocardial strain can be considered as a marker of preclinical DCM (30).

## 3.2 Pathophysiology of diabetic cardiomyopathy

Glyco-lipotoxicity, IR, inflammation, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress (ERS), and impaired intracellular $Ca^{2+}$ handling may represent the molecular bases for DCM development. In addition, hyperglycemia-related epigenetic modifications may also play an important role in the development of DCM. Among them, histone modifications, especially histone deacetylases, are key regulators of cardiac fibrosis and myocardial hypertrophy (34). The synthesis of microRNA (miRNA) molecules may also be influenced by high glucose levels, and miRNA can affect inflammatory processes and cardiomyocyte survival by regulating oxidative stress response (35).

Oxidative stress can interfere with the balance between antioxidant capacity and ROS production, which is a core mechanism in the development of DCM, and a key regulator of myocardial fibrosis. Myocardial fibrosis is an important feature of cardiac remodeling in DCM (36). Wang et al. reported the induction of the pre- and early stages of DCM using a high-fat diet combined with T2DM rats induced by different doses of streptozotocin (STZ). The results showed increased ROS production in the low- and high-dose STZ treatment groups, with significantly increased cardiac weight index and myocardial collagen deposition in the high-dose group compared to the untreated group. In other words, cardiac structural remodeling, ROS production, and cell death are dysregulated in the pre- and early stages of DCM (37). Excessive ROS in cardiomyocytes leads to mitochondrial DNA damage, lipid peroxidation, post-translational modification of proteins, and eventually cell death and HF (38). ROS production activates redox-sensitive transcription factors and signal transduction pathways, such as protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK), $NH_2$-terminal Jun kinase (JNK), Janus kinase, and signal transducer and activator of transcription (JAK-STAT) pathways, further leading to impaired cardiac structure and function (39). ROS are mainly derived from NOXs, which are oxidative enzymes in the heart, particularly $NOX_2$ and $NOX_4$. $NOX_2$-dependent signaling promotes several deleterious processes in cardiac pathology, including cardiomyocyte hypertrophy, contractile dysfunction, interstitial fibrosis, and cell death. In contrast, $NOX_4$ plays important roles in endogenous detoxification reactions, angiogenesis, ERS, substrate utilization, and stress responses (40).

Increased oxidative stress in the heart is also due to cytoplasmic ROS-induced exacerbation of mitochondrial ROS production, and oxidative damage to the mitochondria further causes mitochondrial dysfunction (36). When mitochondrial ROS production exceeds endogenous clearance capacity, it leads to oxidative stress and inflammation. In addition, increased mitochondrial FA uptake and FA β-oxidation in DCM may induce the accumulation of toxic lipid metabolites, resulting in cardiac lipotoxicity and mitochondrial dysfunction (41). It has been recently shown that upregulation of A-kinase anchoring protein 1 ($Akap_1$) may have the potential to treat myocardial injury in patients with DCM, where $Akap_1$ expression is reduced in a diabetic mouse heart model. Thus, $Akap_1$ deficiency may exacerbate DCM deterioration, increase

mitochondrial ROS levels, and impair mitochondrial function. In contrast, cardiac-specific overexpression of $Akap_1$ restores mitochondrial function and alleviates diabetes-induced cardiac dysfunction and myocardial fibrosis by ectopically regulating NADPH-CoQ in the mitochondria and decreasing mitochondrial ROS (42).

ERS and abnormal $Ca^{2+}$ handling processes are associated with diastolic dysfunction in DCM. In patients with diabetes, persistent hyperglycemia and IR induce ERS and impair $Ca^{2+}$ handling. However, impaired $Ca^{2+}$ handling leads to an increased action potential duration, which consequently causes diastolic dysfunction (41). ERS causes increased myocardial ROS and impaired insulin signaling through activation of the JNK pathway, which is dependent on cellular redox status (43). Cardiomyocyte contractility is affected by impaired insulin signaling and reduced glucose uptake by cardiomyocytes. Additionally, $Ca^{2+}$ is released from cardiomyocytes *via* the ryanodine receptor (RyR), which improves the susceptibility to oxidative stress injury induced by abnormal insulin metabolism and ROS production. Thus, it impairs $Ca^{2+}$ efflux through L-type calcium channels, resulting in a reduction in efflux $Ca^{2+}$ and an increase in intracellular $Ca^{2+}$, which affects the systolic and diastolic functions of cardiac myocytes (44).

Altered cardiac substrate metabolic pathways and impaired energy metabolism are important factors in the pathogenesis of DCM. Under physiological conditions, the heart can employ FA and glucose as energy substrates. In contrast, in diabetic patients, the decrease in glucose uptake due to systemic and cardiac IR promotes a shift of substrates toward increased oxidation of free FA (FFA), resulting in reduced efficiency of cardiac metabolism. The heart loses the ability to utilize glucose, resulting in glucose overload in cardiomyocytes, which promotes the formation of advanced glycation end-products (AGEs) (30, 31). AGEs may play a key role in the development of DCM by stimulating collagen expression and accumulation and promoting collagen cross-linking, which causes increased myocardial fibrosis, reduced myocardial compliance, and impaired myocardial diastolic function (2). In addition, AGEs can also lead to increased intracytoplasmic ROS through activation of the AGE receptor (RAGE), thereby resulting in cardiac diastolic dysfunction (45). Activation of RAGE involves nuclear factor-κB (NF-κB) and its target genes, causing a shift from the α to the β isoform of myosin heavy chain in cardiac myocytes, which reduces myocardial contractility (46).

Alterations in the energy matrix of the heart enable increased FA uptake by cardiomyocytes to exceed the oxidative capacity of mitochondria, leading to excessive lipid storage and production of lipotoxic metabolites that impede cardiomyocyte metabolism and contractility, thereby promoting cardiomyocyte death (47). Excessive accumulation of FA in cardiac tissue and lipotoxicity can also impair insulin signaling, causing reduced cardiac metabolic capacity, increased myocardial oxygen consumption, and abnormal cardiac morphology and structure, ultimately resulting in a significant reduction in cardiac diastolic function (31). In cardiac tissues, the accumulation of lipid metabolites results in the reduced expression of glucose transporter 4 ($GLUT_4$) and reduced translocation of $GLUT_4$ from the cytoplasm to the cell membrane. As a result, glucose uptake by cardiomyocytes is significantly reduced. On the one hand, it may promote cardiac remodeling; on the other hand, it may inhibit cardioprotective pathways (48). Hyperglycemia and IR activate the PKC signaling pathway, allowing for an increase in myocardial endothelial cell permeability, resulting in endothelial dysfunction. In the pathogenesis of DCM, there is an imbalance in the release of vasoconstrictors [for example, prostanoids, endothelin, and angiotensin-II(Ang-II)] and diastolic agents [for example, nitric oxide (NO), prostacyclin ($PGI_2$), bradykinin, and endothelium-derived hyperpolarizing factor (EDHF)] (49). NO, $PGI_2$, and EDHF are released from coronary artery endothelial cells, allowing vasodilation. In the early stages of DCM and IR, NO-induced vasodilation is impaired and EDHF-mediated vasodilation is usually maintained or even enhanced to maintain a normal vascular tone. However, in later stages, both NO- and EDHF-induced vasodilation may eventually be impaired, resulting in microvascular dysfunction (41).

Diabetes mellitus is an inflammatory disease, and increased ROS levels induce an increase in inflammatory factors. Cardiac inflammation in DCM is mostly caused or exacerbated by increased ROS, and this pro-inflammatory state is largely caused by activation of ROS-triggered inflammatory vesicle nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3 ($NLRP_3$) (39). Furthermore, in obesity and IR, M1 macrophages polarize and secrete inflammatory cytokines, causing reduced cardiac insulin signaling and promoting DCM development. Conversely, M2 macrophages secrete IL-10, which inhibits cardiomyocyte hypertrophy and cardiac fibrosis (33). T-lymphocyte inducers contribute to an increase in pro-fibrotic cytokine levels in mouse heart tissue. In addition, T-helper lymphocytes secrete pro-inflammatory cytokines, which can bring about cardiac oxidative stress and coronary artery dysfunction, ultimately leading to cardiac remodeling, fibrosis, and diastolic dysfunction (50). C-reactive protein (CRP) is a cardiovascular pathogenic factor and well-known indicator of inflammation. Mano et al. showed that diabetic mice with CRP overexpression have increased levels of pro-inflammatory cytokines (IL-6 and TNF-α), increased type I collagen, increased expression of brain natriuretic peptide (BNP), and reduced LVEF on echocardiography compared to non-diabetic mice. This means that CRP overexpression may exacerbate LV function, cardiac remodeling, and myocardial fibrosis in DCM patients, possibly through inflammation and oxidative stress (51). Thus, systemic and local adaptive immune responses and inflammation may bring about alterations involving myocardial structure and metabolism, which in turn may cause diastolic dysfunction and eventually HF (Figure 2).

## 4 Role of ferroptosis in DCM

It has been shown that cardiomyocytes in diabetic patients are extremely sensitive to cell death, and their cardiomyocyte mortality is 85 times higher than that of non-diabetic patients. Consequently, cell death significantly affects the development of diabetes and its

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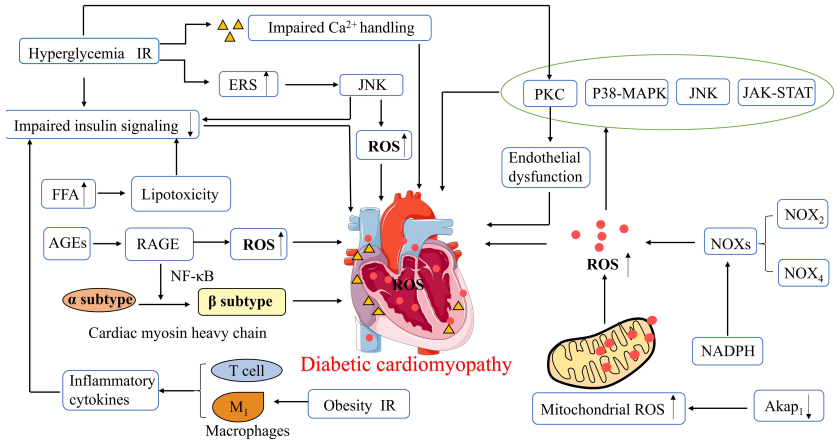


FIGURE 2
Pathophysiological mechanisms of diabetic cardiomyopathy. ROS, Reactive oxygen species; ERS, Endoplasmic reticulum stress; PKC, protein kinase C; p38 MAPK, p38 Mitogen activated protein kinase; JNK, Jun kinase; JAK -STAT, Janus kinase and signal transducer and activator of transcription; NADPH, Nicotinamide adenine dinucleotide phosphate; NOXs, NADPH Oxidases; $Akap_1$, A-kinase anchoring protein 1; FFA, Free fatty acids; AGEs, Advanced glycation end-products; RAGE, AGE receptor; NF-κB, nuclear factor-κB; IR, Insulin resistance.

complications. Apoptosis is the main cause of cell death in DCM and promotes DCM cardiomyocyte injury through multiple upstream signaling pathways (52). However, the role of autophagy in DCM pathogenesis of DCM is unclear. Numerous studies have shown that autophagy protects cardiomyocytes in DCM; however, it has also been shown that autophagy can trigger cardiomyocyte injury. In a fructose-induced T2DM diabetic mouse model, upregulated autophagy was associated with elevated cardiac superoxide production and fibrosis, suggesting that autophagy may contribute to DCM (53). In addition, inhibition of various signaling pathways associated with necrosis may reduce myocardial damage in patients with diabetes (52). Ferroptosis is a specific type of cell death characterized by iron dependence and lipid peroxidation, which allows for increased levels of ROS and may promote the occurrence and development of DCM.

Dysregulation of internal environmental homeostasis and iron metabolism is closely related to the development of cardiac diseases, and iron overload is significantly detrimental to the development of ferroptosis and HF in cardiomyocytes (54). Studies have shown that ferroptosis may cause mitochondrial damage in the heart through iron overload, including abnormal mitochondrial structure, altered mitochondrial membrane potential, and increased mitochondrial ROS, which are also considered important features of ferroptosis (55). Iron overload in diabetic patients not only increases the risk of IR and diabetes progression but may also exacerbate cardiovascular complications through the Fenton response (56). DCM development is primarily associated with excessive ROS production and impaired antioxidant capacity. However, the high correlation between ferroptosis and lipid ROS production suggests that the inhibition of ferroptosis may be an important target for DCM prevention and treatment. Abnormal mitochondrial ferroptosis is observed in the hearts of diabetic mice, mainly manifested by decreased mitochondrial membrane potential, downregulated expression of key enzymes involved in antioxidant defence (superoxide dismutase [$SOD_2$] and glutathione peroxidase 1 [$GPX_1$]) in the mitochondria, and significantly increased mitochondrial ROS levels (57). Another study showed that mice fed a high-iron diet can cause severe myocardial damage and have a typical molecular profile of ferroptosis, including increased lipid peroxidation and reduced GSH levels (58). Thus, iron overload has a detrimental effect on cardiomyocyte function.

In the hyperglycemic state, oxidative stress and impaired antioxidant systems underlie DCM pathogenesis. Ferroptosis, which causes an imbalance between oxidation and antioxidation, typically causes the excessive production of ROS. This is clinically important because cardiomyocytes are highly susceptible to oxidative damage, and lipid peroxidation is involved in ROS-induced cardiac injury. Sampaio et al. reported increased expression of oxidative stress-carbonyl protein markers and myocardial fibrosis (type III collagen) in iron-overloaded T1DM diabetic rats (59). Ghosh et al. showed that GSH was reduced and ROS levels were increased in cardiomyocytes in an STZ diabetic rat model (60).

Glucose-induced persistent cardiomyocyte peroxide accumulation triggers ferroptosis and results in cellular damage at the whole-organism and cellular levels (61). In addition, during DCM onset, the energy metabolism of cardiomyocytes shifts from glycogenolysis to FA oxidation, resulting in increased intracellular lipid accumulation and lipotoxicity (62). However, ferroptosis involves multiple metabolic processes including iron metabolism, lipid metabolism, and in particular lipid peroxide production (63). Therefore, removal of lipid peroxide may attenuate cardiomyocyte injury.

A recent study suggested that the inhibition of ferroptosis by the activation of nuclear factor-erythroid 2-related factor 2 ($NRF_2$) may represent a potential therapeutic target in DCM. $NRF_2$ regulates multiple antioxidants and plays a key role in maintaining cellular redox reactions (64). $NRF_2$ and its target genes have antioxidant, anti-inflammatory, anti-apoptotic, anti-ferroptosis, and anti-fibrotic functions that protect islet β-cells from high-glucose-induced oxidative damage in DCM (65).

Rutin, an $NRF_2$ activator and phytochemical, has multiple pharmacological activities, including hypoglycemic and antioxidant activities. In a diabetic mouse model, rutin improved glucolipid metabolism in diabetic mice, attenuated myocardial damage caused by oxidative stress, including ventricular hypertrophy, ventricular remodeling, and ventricular dysfunction, and prevented the progression of myocardial fibrosis, thereby effectively alleviating DCM (66). Additionally, some key regulators of ferroptosis are also the downstream targets of $NRF_2$, such as $SLC_7A_{11}$ and ferritin. $SLC_7A_{11}$ transports the precursor of GSH, cystine, into the cell matrix, and ferritin plays an important role in iron metabolism by storing excess cellular iron and alleviating the Fenton reaction (67). Almost all genes associated with ferroptosis are transcriptionally regulated by $NRF_2$, including GSH-regulated and NADPH-regenerating genes that are essential for $GPX_4$ activity, lipid peroxidation, and iron metabolism. The $NRF_2$/Kelch-like-epichlorohydrin (ECH)-associated protein 1 ($KEAP_1$)/antioxidant response element (ARE) pathway is the main mechanism of myocardial defence against oxidative damage in diabetes and hyperglycemia. It regulates the expression of several genes, most of which are associated with the reduction of oxidative stress and cell death (68). In addition, it has also been shown that the $NRF_2$/ferroportin 1 ($FPN_1$) signaling pathway is a key mechanism in diabetic myocardial injury, inhibiting ferroptosis by regulating iron metabolic homeostasis, and its activation alleviates diabetic myocardial injury to some extent (64). Furthermore, most patients with diabetes have abnormal lipid metabolism and produce excess saturated FA such as palmitic acid (PA), which plays a role in cardiomyocyte death and the development of DCM. Ferroptosis is associated with PA-induced myocardial injury, and ferroptosis inhibitors significantly reduce cell death in H9c2 and rat cardiomyocytes exposed to PA (10).

$BH_4$ inhibits ferroptosis by inhibiting lipid peroxidation. $BH_4$ is synthesized by GTP (guanosine triphosphate) cyclohydrolase 1 ($GCH_1$) and acts as a cofactor in a variety of biosynthetic pathways, such as the synthesis of aromatic amino acids, neurotransmitters, and NO (69). Overexpression of $GCH_1$ protects the heart from DCM and improves cardiac remodeling and dysfunction in a T1DM mouse heart model; therefore, $GCH_1$ may serve as a new target for DCM therapy (70).

The increase in oxidative stress generated by iron overload through the Fenton reaction promotes the formation of AGEs, leading to lipid peroxidation, which is an important mechanism in DCM pathogenesis. In an ACE-treated diabetic rat model, ferroptosis inhibitors prevented the diastolic dysfunction of DCM, indicating an important role of ferroptosis in DCM. AGEs induce ferroptosis in engineered cardiac tissues (ECT), as evidenced by increased levels of unstable iron and lipid peroxides and decreased levels of GSH and $SLC_7A_{11}$ (67).

Endosomal sorting complexes required for transport (ESCRT) play multiple roles in membrane bending or outgrowth as multi-subunit machinery in the material transport process. Various cellular processes, including cell death, are affected by this membrane-remodeling mechanism. In particular, ESCRT-III initiates membrane repair to prevent various types of cell death, including necrosis, apoptosis, and ferroptosis. Activation of ESCRT-III on cell membranes requires $Ca^{2+}$, confirming the role of calcium homeostasis and ERS in the control of ferroptosis (71). Abnormal calcium homeostasis and ERS are also important mechanisms in DCM development. ERS is a cellular response to endoplasmic reticulum dysfunction that can be induced by ROS, and inhibition of ferroptosis alleviates diabetic myocardial ischemia/reperfusion injury, which may provide a new therapeutic target for DCM. ROS are produced by the interaction between $Fe^{2+}$ and NADPH oxidase during ferroptosis. ERS is characterized by the activation of the transcription factor 4 (ATF4)- CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) pathway. It has been shown that, in a diabetic rat model (high glucose and myocardial injury), the diabetic myocardial injury group showed increased levels of $ACSL_4$, $ATF_4$, and CHOP, severe impairment of cellular arrangement, cell swelling, and most of the myocardial fibers were broken compared to normal control group animals. However, when ferroptosis inhibitors were added, the degree of myocardial injury was significantly reduced in rats, and the levels of ROS, intracellular $Fe^{2+}$ concentration, $ACSL_4$, $ATF_4$ and CHOP decreased, indicating that ferroptosis inhibitors can improve diabetic myocardial injury by reducing ERS (72).

Autophagy is a fundamental intracellular homeostatic process. Appropriate autophagy may be a pro-survival response; however, excessive autophagy, especially selective autophagy, and impaired lysosomal activity may promote ferroptosis. $NCOA_4$-dependent ferritinophagy promotes ferroptosis by releasing free iron from ferritin (73). Many autophagic vesicles have been observed in cardiomyocytes of diabetic patients (74). However, in diabetic mice, 1,25-dihydroxyvitamin-D3 [1-25$(OH)_2VitD_3$] improved myocardial fibrosis and cardiac function in DCM through an autophagy-related vitamin D receptor (VDR)-dependent mechanism and the β-catenin/T-cell factor/lymphoid enhancer factor ($TCF_4$)/glycogen synthase kinase-3β (GSK-3β)/mammalian target of rapamycin (mTOR) pathway (75). Glycogen autophagy plays an important role in DCM pathogenesis. In cultured primary cardiomyocytes, glycogen autophagy is regulated by extracellular glucose and insulin and occurs simultaneously with glycogen accumulation (76). In addition, glycogen autophagy is associated with a higher risk of heart disease (including DCM) in female patients, possibly because glycogen autophagy is associated with selective glycogen accumulation in the female myocardium, in which estrogen may upregulate the expression of signaling intermediates that promote glycogen storage (77) (Figure 3).

In STZ-induced T1DM mice, the heart showed significant features of DCM, such as myocardial systolic and diastolic dysfunction, impaired $Ca^{2+}$ handling, and myocardial fibrosis (78). The inhibition of autophagy may allow an imbalance in $NRF_2$-mediated metabolism as well as redox regulation, which increases iron deposition and lipid peroxidation, promoting cardiomyocyte ferroptosis and the progression of T1DM cardiomyopathy (79). However, in high-fat diet + STZ-induced T2DM mice, the heart is mainly characterized by myocardial steatosis, impaired systolic function, and mitochondrial

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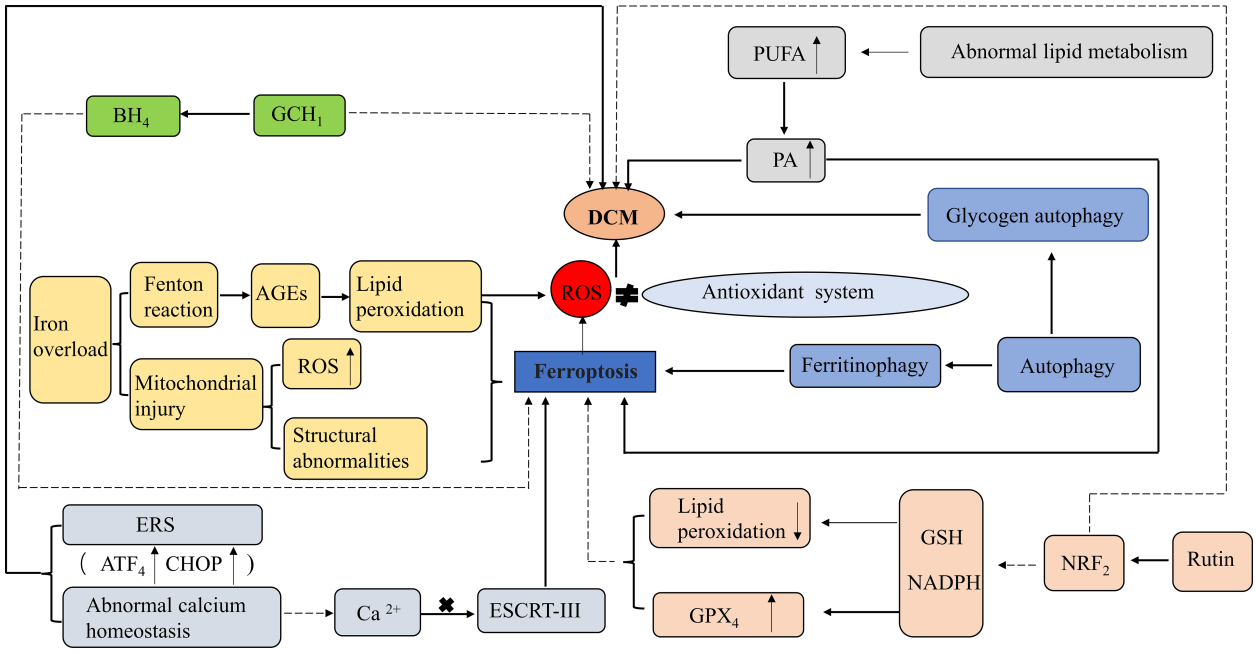


FIGURE 3
Role of ferroptosis in DCM. ROS, Reactive oxygen species;$GPX_4$, Glutathione peroxidase 4; GSH, Glutathione; NADPH, Nicotinamide adenine dinucleotide phosphate; $NRF_2$, nuclear factor-erythroid 2-related factor 2; PUFA, Polyunsaturated fatty acids; PA, palmitic acid; AGEs, Advanced glycation end-products; $BH_4$, Tetrahydrobiopterin; $GCH_1$GTP, (Guanosine triphosphate) cyclohydrolase 1; ERS, Endoplasmic reticulum stress; ESCRT-III, Endosomal sorting complexes required for transport III; $ATF_4$, the activation of the transcription factor 4 (ATF4)- CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP). Activation: Inhibition.

dysfunction (78). Abnormal glucose and lipid metabolism causes the accumulation of AGEs in the extracellular matrix of the heart, leading to iron overload and lipid peroxidation, which ultimately induce ferroptosis in cardiomyocytes (67). The performance of ferroptosis in different animal models of diabetes is summarised in Table 1. Although ferroptosis has been shown to significantly improve cardiac function in animal models and cultured cells, no clinical trials have been performed to date; therefore, population-based studies are needed to determine whether selective blockade of ferroptosis improves the prognosis and/or outcomes of DCM.

## 5 Potential applications of ferroptosis inducers and inhibitors in DCM

Ferroptosis is promoted by class I (e.g., erastin) and class II (e.g., Ras-selective lethal 3 [RSL-3]) inducers, which indirectly and directly inhibit $GPX_4$ activity, respectively (80). Erastin induces ferroptosis by inhibiting system $Xc^-$, indirectly decreasing $GPX_4$ activity, and directly targeting voltage-dependent anion channels 2 and 3 (VDAC2/3). In contrast, class II RSL-3 promotes ferroptosis primarily by inhibiting the endogenous lipid ROS inhibitor $GPX_4$ (81).

TABLE 1 The performance of ferroptosis in different types of animal models of diabetes.

| Animal models | Mechanism | Effects on the heart | Biochemical characteristics | References |
|---|---|---|---|---|
| STZ-induced T1DM mouse model | The inhibition of autophagy may allow imbalance of $NRF_2$-mediated metabolism as well as redox regulation, which increase the iron deposition and lipid peroxidation, promoting cardiomyocyte ferroptosis and the progression of T1DM cardiomyopathy | Cardiac fibrosis, hypertrophy, and cardiomyocyte death | Iron deposition, the increased levels in 4-HNE and $ACSL_4$, and decreased levels in $GPX_4$ and $FSP_1$ | (79) |
| High-fat diet + STZ-induced T2DM mouse model | Abnormal glucose and lipid metabolism cause the accumulation of AGEs in the extracellular matrix of the heart, leading to iron overload and lipid peroxidation, which ultimately induce ferroptosis in cardiomyocytes | Myocardial injury, decreased cardiac function, and cardiac remodeling (hypertrophy and fibrosis) | Significantly increased levels in the labile iron, MDA and $PTGS_2$, and decreased levels in $SLC_7A_{11}$ expression and GSH levels | (67) |

STZ, Streptozotocin; T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus; $NRF_2$, Nuclear factor-erythroid 2-related factor 2; AGEs, Advanced glycation end products; 4-HNE, 4-hydroxy-2-nonenal; $ACSL_4$, Acyl CoA synthase long-chain family member 4; $GPX_4$, Glutathione peroxidase 4; $FSP_1$, Ferroptosis suppressor protein 1; MDA, Malondialdehyde; $PTGS_2$, Prostaglandin-endoperoxide synthase-2; $SLC_7A_{11}$, Solute carrier family 7 members 11; GSH, Glutathione.

Ferroptosis inhibitors act by inhibiting iron accumulation and reducing iron overload (e.g., deferoxamine [DFO]) or by inhibiting lipid peroxidation and reducing lipid ROS production (e.g., liproxstatin-1, vitamin E, and ferrostatin-1 [Fer-1]) (7).

DFO has been shown to ameliorate cardiomyocyte injury in an *in vitro* DCM model of ECT exposed to AGE, reducing the expression of the lipid peroxidation marker malondialdehyde (MDA) and the ferroptosis marker prostaglandin-endoperoxide synthase-2 ($PTGS_2$), improving ECT cardiomyocyte function. Liproxstatin-1 alleviated the decreased diastolic function 3 months after the onset of diabetes, further demonstrating the importance of ferroptosis in the pathogenesis of DCM (67).

Vitamin E is not only a ferroptosis inhibitor but also an endogenous antioxidant defence factor that plays an important role in DCM. Hamblin et al. found that the expression of two myocardial markers of oxidative stress, 8-iso-prostaglandin F2α (8-iso PGF2α) and GSSG, was increased, whereas that of LVEF was decreased in STZ-induced type I diabetic rats. However, after vitamin E supplementation, myocardial oxidative stress decreased and hemodynamic function was enhanced, further demonstrating the role of myocardial oxidative stress in DCM (82).

Fer-1, a potent inhibitor of ferroptosis, acts *via* lipid peroxidation. Treatment with moderate to high doses of Fer-1 reduced $ACSL_4$ levels and enhanced $GPX_4$ levels, thus reducing mitochondrial ROS production, alleviating mitochondrial dysfunction, and improving LV function in rats with cardiac injury (83). Herceptin (trastuzumab), a human epidermal growth factor receptor 2 (Her-2) gene-related targeted therapeutic agent for the treatment of breast cancer, also exerts toxic effects on the heart. H9c2 rat cardiomyocytes treated with herceptin exhibited decreased $GPX_4$ and $SLC_7A_{11}$ expression with increasing doses of herceptin, inducing H9c2 cardiomyocyte injury, oxidative stress, mitochondrial dysfunction, and ferroptosis. However, the addition of Fer-1 restored $GPX_4$ and $SLC_7A_{11}$ expression levels, which were otherwise inhibited by herceptin, and reversed the herceptin-induced increase in $ACSL_4$ expression and increased mitochondrial ROS and iron levels, protecting H9c2 cardiomyocytes from herceptin-induced cardiomyocyte injury and ferroptosis (84).

Heat shock factor 1 ($HSF_1$) is a stress-inducible transcription and defence factor against ferroptosis in cardiomyocytes that acts through the transcriptional activation of various heat shock proteins (HSP). A recent study found that PA induced cell death in cardiomyocytes in a dose- and time-dependent manner. Excess unoxidized PA in cardiomyocytes induces oxidative stress, mitochondrial dysfunction, and ceramide accumulation, whereas $HSF_1$ significantly inhibits the death of H9c2 and rat cardiomyocytes exposed to PA by regulating the expression of $GPX_4$ (10). The potential applications of ferroptosis inducers and inhibitors in DCM are summarised in Table 2.

## 6 Conclusions

An increasing number of studies have confirmed the relationship between ferroptosis and metabolic diseases, one of the more serious complications of diabetes mellitus, mainly through mechanisms such as glucolipotoxicity, inflammatory responses, and oxidative stress, resulting in increased myocardial tissue fibrosis and impaired systolic and diastolic functions. Among these, ROS overproduction may be considered an important bridge between the two. Large amounts of ROS promote oxidative stress and damage cardiac myocytes, which in turn leads to myocardial systolic and diastolic dysfunction. Thus, ferroptosis may be a new therapeutic target for diabetic cardiomyopathy. However, the role of ferroptosis in DCM is still poorly understood, and in-depth clinical studies are lacking. In addition, clinically reliable and sensitive markers for ferroptosis in early DCM are needed. Finally, objective DCM diagnostic criteria are still lacking, making it difficult to determine whether myocardial injury, hemodynamic changes, and decreased cardiac function caused by ferroptosis should be considered DCM or possibly other cardiovascular diseases, such as coronary atherosclerosis or ischemic cardiomyopathy.

TABLE 2 Potential applicationsof ferroptosis inducers and inhibitors in DCM.

| | | Animal/Cell | Mechanisms and effects on the heart | References |
|---|---|---|---|---|
| Inducers | Erastin | Male wild-type mice | Erastin caused cardiomyocyte death by inhibiting system $Xc^-$, indirectly decreasing $GPX_4$ activity and directly targeting VDAC2/3 | (81) |
| | RSL-3 | Male wild-type mice | RSL-3 caused cardiomyocyte death by inhibiting the endogenous lipid ROS inhibitor $GPX_4$ | (81) |
| Inhibitors | DFO | FVB mice and wild-type mice | DFO ameliorated cardiomyocyte injury by reducing iron overload and lipid peroxidation | (67) |
| | Liproxstatin-1 | FVB mice and wild-type mice | Liproxstatin-1 alleviated the decrease in diastolic function at 3 months after the onset of diabetes by inhibiting lipid peroxidation | (67) |
| | Vitamin E | Sprague-Dawley rats | Vitamin E improved cardiac systolic and diastolic function by reducing oxidative stress | (82) |
| | Fer-1 | MaleWistar rats/H9c2 rat cardiomyocytes | Fer-1 reduced $ACSL_4$ levels and enhanced $GPX_4$ levels by inhibiting lipid peroxidation, which further improved mitochondrial oxidative stress and reduces myocardial injury. | (83, 84) |
| | $HSF_1$ | H9c2cardiomyocytes | $HSF_1$ reduced myocardial injury from oxidative stress by regulating $GPX_4$ expression | (10) |

RSL-3, Ras-selective lethal 3; VDAC2/3, Voltage-dependent anion channels 2 and 3; DFO, Deferoxamine; Fer-1, Ferrostatin-1; $ACSL_4$, Acyl CoA synthase long-chain family member 4; $GPX_4$, Glutathione peroxidase 4; ROS, Reactive oxygen species; $HSF_1$, Heat shock factor 1.

## Author contributions

All authors contributed to the study conception and design. The first draft of the manuscript was written by YZ, and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# YuNü-Jian attenuates diabetes-induced cardiomyopathy: integrating network pharmacology and experimental validation

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**Introduction:** Diabetic cardiomyopathy (DCM) is one of the most prevalent complications of diabetes with complex pathogenesis. YuNü-Jian (YNJ) is a traditional Chinese medicinal formula widely used for diabetes with hypoglycemic and cardioprotective effects. This study aims to investigate the actions and mechanisms of YNJ against DCM which has never been reported.

**Methods:** Network pharmacology approach was used to predict the potential pathways and targets of YNJ on DCM. Molecular docking between hub targets and active components of YNJ was performed and visualized by AutoDock Vina and PyMOL. Then type 2 diabetic model was employed and intervened with YNJ for 10 weeks to further validate these critical targets.

**Results:** First, a total of 32 main ingredients of YNJ were identified and 700 potential targets were screened to construct herb-compound-target network. Then 94 differentially expressed genes of DCM were identified from GEO database. After that, PPI network of DCM and YNJ were generated from which hub genes (SIRT1, Nrf2, NQO1, MYC and APP) were assessed by topology analysis. Next, functional and pathway analysis indicated that the candidate targets were enriched in response to oxidative stress and Nrf2 signaling pathway. Furthermore, molecular docking revealed strong affinity between core targets and active components of YNJ. Finally, in rats with type 2 diabetes, YNJ obviously attenuated cardiac collagen accumulation and degree of fibrosis. Meanwhile, YNJ significantly upregulated protein expression of SIRT1, Nrf2 and NQO1 in diabetic myocardium.

**Discussion:** Collectively, our findings suggested that YNJ could effectively ameliorate cardiomyopathy induced by diabetes possibly through SIRT1/Nrf2/NQO1 signaling.

KEYWORDS

**diabetic cardiomyopathy, molecular docking, network pharmacology, Nrf2, NQO1, SIRT1, YuNü-Jian**

# 1 Introduction

Diabetic cardiomyopathy (DCM) refers to diabetes-induced myocardial structural/functional abnormalities in the absence of coronary heart disease, hypertension, and valvular heart disease. The progressive pathophysiological changes of DCM consist of myocardial remodeling, diastolic/systolic dysfunction, heart failure, and even sudden death (1). According to the data from IDF, the global prevalence of diabetes is estimated to be 12.2% (783.2 million) in 2045 (2), among which approximately 11.7% to 67% develop DCM in the most and least restrictive criteria, respectively (3, 4). Therefore, seeking available strategies to reduce the susceptibility and intervene the development of DCM is of enormous clinical and social values.

At present, management of DCM mainly focuses on lowering blood glucose and lipid levels with no reliable specific drugs. However, whether the diabetic patients developed diastolic dysfunction and progressed to heart failure was independent of HbA1c levels, blood pressure, and lipid control (5). Newer glucose-lowering drugs such as GLP-1 agonists, DPP-4 inhibitors, and SGLT2 inhibitors have shown some cardioprotective effects (1). However, meta-analysis found that some drugs did not significantly improve cardiac structural abnormalities (6), and some did not reduce the risk of heart failure in patients with type 2 diabetes (7). This may be due to the complicated mechanisms involved in DCM. Thus, comprehensive medications such as Chinese Medicine may be another potential regimen to counteract DCM.

In traditional Chinese medicine, symptoms associated with DCM have been depicted as early as 1,400 years ago. According to the theory of Chinese medicine, the basic pathogenesis of DCM is dry heat and yin deficiency, which runs through the whole process of DCM (8). Thus, herbs and compound prescriptions with heat-clearing and yin-supporting efficacies can be effective interventions for DCM (8). YuNü-Jian (YNJ) is a traditional Chinese medicinal formula recorded in the medical classic Chinese book Jingyue`s Complete Works (Jingyue Quanshu). The main ingredients of YNJ are Gypsum (Shi Gao), Rehmanniae Radix Praeparata (Di Huang), Anemarrhenae Rhizoma (Zhi Mu), Ophiopogonis Radix (Mai Dong), and Achyranthis Bidentatae Radix (Niu Xi). This compound has been extensively used for the treatment of diabetes by clearing stomach heat and nourishing kidney yin (9). Recent experiments have found that YNJ protected pancreatic islet function and reduced blood glucose level under diabetes by regulating autophagic apoptosis of β-cells (10) and promoting gastric emptying (11). In addition, YNJ could improve ventricular remodeling after myocardial infarction induced by coronary artery ligation (12). These effects offer the possibility of YNJ for prevention and treatment of DCM. However, no relevant reports have been conducted so far.

Chinese medicine formulations are characterized by diverse components and synergistic effects acting through orchestrated biological processes, which makes the study of the mechanisms and actions of herbal formulas difficult. Network pharmacology offers favorable opportunity to assess the possible mechanisms underlying the observed clinical effects of herbal medicines. By constructing a component–target–gene network and performing a series of topological analyses, network pharmacology predicts therapeutic pathways and targets for subsequent validation by *in vivo* and *in vitro* experiments. This makes it possible to investigate the interaction between herbal formulations and disease with multifactorial pathogenesis, which cannot be readily verified by conventional experimental modes based on the "one gene, one drug, one disease" paradigm (13).

In this study, network pharmacology techniques were applied to explore the potential mechanisms of YNJ on DCM, including identification of active components in YNJ, retrieval of DCM differentially expressed genes (DEGs) in the GEO database, drug target prediction, PPI network construction and topology analysis, KEGG pathway analysis, and GO biological analysis. Then, the molecular docking between the active ingredients and the predicted targets was carried out. Finally, a diabetic animal model was established to verify the improvement of cardiomyopathy by YNJ and the potential mechanism through the putative targets. The detailed strategy of this study is summarized in Figure 1.

# 2 Materials and methods

## 2.1 Collection of active compounds of YNJ and their potential targets

The chemical components of each traditional Chinese medicine in the YNJ decoction were searched on the TCMSP (https://tcmsp-e.com/) platform. The oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18 of the absorption, distribution, metabolism, and excretion (ADME) parameters were used as the criteria for screening. In order to ensure the integrity and reliability of the study, active ingredients were supplemented through literature review. Those with high contents and explicit pharmacological effects but not in accord with the above criteria were also considered as active candidate ingredients (14–17).

The screened Pubchem CID was input into the PubChem compound database (https://pubchem.ncbi.nlm.nih.gov/) database, and the SMILES number corresponding to each compound was obtained after eliminating compounds that did not match those in PubChem. Then, the Swiss Target Prediction database (http://www.swisstargetprediction.ch/) was used to predict the targets of active compounds (18). Finally, the UniProt ID of the target was searched through the Universal Protein Resource (UniProt) database (https://www.uniprot.org/) for batch standardization, with species set to *Homo sapiens*.

## 2.2 Construction of the active component–target network of YNJ

The network mapping software Cytoscape (Version 3.8.2) was applied to construct a network of active ingredients of YNJ and their targets. In the network diagram, a node represents an herb, an active ingredient, or a target, and the edge represents the interaction between them. The degree value of a node indicates the number of

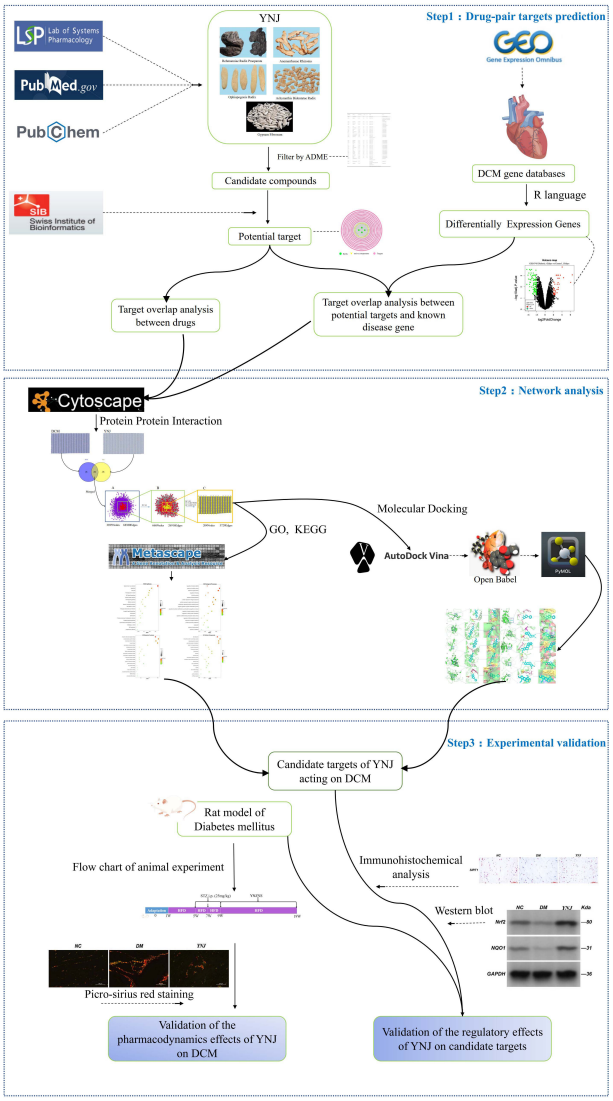


FIGURE 1
Flow diagram of bioinformatics combined with experimental pharmacology for investigating the effects and mechanisms of YNJ in the treatment of DCM.

edges between the nodes in the network; the larger the value, the more likely the target is to be the key target of compounds.

## 2.3 Acquisition of differentially expressed genes of DCM

The gene expression datasets analyzed in this study were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), from which the GSE4745 dataset (GPL85 platform, [RG_U34A] Affymetrix Rat Genome U34 Array) was rigorously selected for further study. Samples from the GSE4745 dataset were grouped by R language (Version 4.1.0) to obtain significantly DEGs with adjusted $p < 0.05$ and $|\log FC| \geq 2.0$ as screening conditions. The DEGs were imported into the HCNC-Tools-HCOP (19) (https://www.genenames.org/tools/hcop/) online tool for species conversion, preserving the homologous genes of rat and human, which are the DCM-related genes of human to be studied.

## 2.4 Construction and analysis of the protein–protein interaction network

Protein–protein interaction (PPI) networks of YNJ targets and DCM-related DEGs were constructed and visually analyzed with the plug-in BisoGenet in Cytoscape software (Version 3.8.2). Core proteins were screened from the central network, which came from the intersection of the above two networks. The CytoNCA plug-in was adopted for multi-center topological parameter analysis and screening according to the degree sorting procedure. The core targets were evaluated according to three parameters: degree centrality (DC), closeness centrality (CC), and betweenness centrality (BC). The screening criteria were set as DC > 2 times of the median to obtain the network of core target from which the key genes for the treatment of DCM by YNJ were further identified with DC, CC, and BC > 1 time of the median.

## 2.5 GO and KEGG pathway enrichment analysis

Metascape (https://metascape.org) was employed to perform GO and KEGG signal pathway analysis with the aforementioned predicted targets. GO biological function analysis mainly describe the function of target genes, including molecular function (MF), cellular components (CC), and biological processes (BP). KEGG enrichment analysis interprets the signaling pathway enriched by the common genes of YNJ active components and DCM. Terms with $p < 0.01$, minimum gene overlap (Min Overlap) of 3, and minimum enrichment factor (Min Enrichment) > 1.5 were collected and grouped according to the similarity of their members. The top 20 crucially significant pathways were selected for further study and are displayed in the advanced bubble chart colored by – log10 ($p$-values).

## 2.6 Verification of the binding of active components of YNJ to target proteins by molecular docking

The 3D protein structure of key targets was downloaded from the RCSB Protein Data Bank (RCSB PDB, http://www.pdb.org/). After adding hydrogen and calculating charges, the crystal structure of core components was downloaded from PubChem. AutoDock Vina software was used to dehydrate and hydrotreat the receptor protein and conduct molecular docking. Afterwards, free energy of binding (in kcal/mol) was obtained as the indicator of the binding likelihood. The more the negative scoring (the greater the absolute value), the higher the binding force is between the compound and the protein. Generally, the binding energy ≤−5.0 kcal/mol can be considered as an excellent binding effect. Finally, PyMOL software was used for visualization.

## 2.7 Pharmacodynamic study of YNJ and experimental validation in diabetic rats

### 2.7.1 Animals and induction of diabetes

Male SD rats aged 8 weeks with a body weight of 180–220 g were obtained from Beijing Charles River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were housed in standard conditions with a 12-h light/dark cycle at 22 ± 2°C and a humidity of 55 ± 5% with free access to food and water. After adaptation for 1 week, animals were divided randomly into a negative control (NC) and a model group. Rats in the model group were fed with a high-fat diet (HFD) for 4 weeks and then intraperitoneally injected with 25 mg/kg freshly prepared streptozotocin (STZ, Sigma, USA) dissolved in citrate buffer (0.1 M, pH 4.5) three times every other week (20). Those in the NC group received continual standard diet and three intraperitoneal injections of citrate buffer alone. Three days after the last injection, rats with a fasting blood glucose of 11.1 mmol/L or higher were considered type 2 diabetes mellitus and subjected to subsequent experiments with persistent HFD feeding. All experimental protocols for animals conformed to the guidelines of National Institutes of Health, and were permitted by the Animal Care and Use Committee of Shandong University of TCM.

### 2.7.2 Experimental protocol

The successfully induced rats were randomly divided into diabetic mellitus (DM) and YNJ-treated group (YNJ). The preparation of YNJ involved mixing of decocting-free granules of Gypsum Fibrosum, Rehmanniae Radix Praeparata, Anemarrhenae Rhizoma, Ophiopogonis Radix, and Achyranthis Bidentatae Radix (Tianjiang Pharmaceutical Co., Ltd, Jiangyin, Jiangsu, China) with distilled water to a final drug concentration of 0.9 g/ml. YNJ was administrated intragastrically at a dose of 4.52 g/kg every day according to the clinical effective dose for diabetic patients. Rats in the NC and DM groups received equal amount of 0.9% saline with the same administration route and duration. After treatment with YNJ or saline for 10 consecutive weeks, all rats were anesthetized and euthanized. Ventricular tissues were rapidly excised and fixed in 4% paraformaldehyde or frozen at −80°C for later assays.

### 2.7.3 Picro-Sirius red staining

After fixation for 24 h, the cardiac tissues were dehydrated in ascending grades of alcohol and embedded in paraffin. Picro-Sirius red (PSR) staining was used to demonstrate cardiac fibrosis. Briefly, sections of 5 μm thickness were conventionally deparaffined with xylene and alcohol. Then, slides were incubated with PSR (S8060, Solarbio, Beijing, China) for 10 min and viewed using a Nikon Eclipse Ci-L microscope (Tokyo, Japan). The collagen volume fraction (CVF, %) within the tissue was identified and assessed with Image Pro Plus 6.0 software as the ratio of stained fibrillar collagen area to the view area. The CVF from six random fields (×200) were averaged and used to show the collagen deposition of this sample.

### 2.7.4 Immunohistochemical staining

Paraffin-embedded sections of 5 μm thickness were kept at 60°C for 3 h in the oven and dewaxed with xylene and hydrated in gradient ethyl alcohol. The slides were subjected to microwave antigen retrieval treatment followed by endogenous peroxidase deactivation using 3% $H_2O_2$ for 10 min. After blocking of nonspecific binding with normal serum for 20 min, the sections were incubated with primary antibody against SIRT1 (8469, Cell Signaling Technology, Boston, USA) at 4°C overnight. The slides were then incubated with secondary antibodies at 37°C for 30 min, stained with DAB, counterstained with Mayer's hematoxylin, and visualized by a highly sensitive inverted microscope (LV200, Olympus). Image analysis was performed by Image Pro Plus 6.0 software.

### 2.7.5 Western blot analysis

Ventricular tissues were homogenized in lysis buffer and quantified for protein concentration with a commercial assay kit (Beyotime Biotechnology, Jiangsu, China). Equal quantities of proteins were separated on 10%–12% SDS-PAGE under denaturing conditions and transferred onto a PVDF membrane. The membrane was blocked using 5% nonfat milk in TBS containing 0.05% Tween 20 (TBST) for 2 h, and then immunoblotted overnight at 4°C with primary antibody, followed by incubation with HRP-conjugated secondary antibody for 1 h at room temperature. The immunoreactive proteins were visualized with an ECL-detection kit (Thermo Fisher Scientific, Pittsburgh, PA, USA) using a Tanon 6600 (Tanon, Shanghai, China) luminescent imaging workstation. Levels of protein were quantified using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) for optical density values. All the target proteins were normalized by GAPDH and depicted as percentage of the NC. Rabbit anti-Nrf2 antibody (ab92946), rabbit anti-NQO1 antibody (ab34173), rabbit anti-GAPDH antibody (ab8245), and goat anti-rabbit IgG H&L (HRP) (ab6721) were all purchased from Abcam (Cambridge, UK).

### 2.7.6 Statistical analysis

All data were expressed as mean ± SD and analyzed and plotted using GraphPad Prism 5 (Version 5.01). One-way analysis of variance (ANOVA) and Tukey's test were used for statistical analyses. *p*-values less than 0.05 were considered to indicate statistically significant differences.

# 3 Results

## 3.1 Bioactive ingredients in YNJ

The candidate bioactive components of YNJ were screened out from the TCMSP database with OB ≥ 30% and DL ≥ 0.18. Together with literature retrieval, we obtained 2, 13, 4, and 17 bioactive ingredients from Rehmannia Radix Praeparata, Anemarrhenae Rhizoma, Ophiopogonis Radix, and Achyranthis Bidentatae Radix, respectively. After removing the duplicate values and those

without targets, 32 bioactive ingredients were screened out and eventually included in the follow-up study (Table 1).

## 3.2 Potential targets and the compound–target network of YNJ

According to the filters described above, 3,579 candidate genes that were targeted by YNJ were harvested from the Swiss Target Prediction database. Then 700 common targets were obtained by overlap analysis, showing synergistical effects among these bioactive components.

By using Cytoscape, we constructed a compound–target network to identify the relationship between YNJ bioactive components and their candidate targets. The network was composed of 736 nodes and 2,504 edges (Figure 2). As revealed in the network, the average degree value of 32 active ingredients was 78.25, certifying the multi-target characteristics of YNJ. The average degree value of all compound targets was 3.53, suggesting systemic actions of diverse active components in YNJ. Moreover, all the four ingredients with highest degree values are from Anemarrhenae Rhizoma. This may be due to the twofold efficacies of clearing heat and nourishing yin, indicating the values of in-depth study aiming at this herb.

## 3.3 Identification of DEGs of DCM

The GSE4745 dataset was standardized and analyzed with the R package. According to the screening criteria of $|\log FC| \geq 2.0$ and adjusted $p < 0.05$, we obtained 113 DEGs, of which 26 were upregulated and 87 were downregulated. The related volcano map was created to show the distribution of these genes (Figure 3). Then, we converted these rat-derived genes into those of human by the HCNC-Tools-HCOP online tool. After getting rid of the ineligible genes, a total of 94 DEGs were obtained, with 24 upregulated and 70 downregulated.

## 3.4 PPI network analysis and hub gene identification

To further illustrate interactions between identified genes, the Cytoscape plugin BisoGenet was used to generate the PPI network of DCM and YNJ, respectively. The PPI network of DCM-related targets was constructed with 2,981 nodes and 70,524 edges, while the network of YNJ-related targets contained 10,601 nodes and 219,193 edges. Then, the two networks were merged into an intersection with 2,695 nodes and 68,380 edges. Afterwards, CytoNCA was used to assess the intersection of the PPI network by topological analysis. A network of YNJ against DCM, with 666 nodes and 26,919 edges, was first screened based on the criteria of DC > 64. A core–target PPI network was further screened with the criteria of DC >101, BC > 0.00074373, and CC > 0.463790583, and consisted of 204 nodes and 5,729 edges (Figure 4). The nodes with top degree values in the core–target PPI network are considered as hub genes, namely, SIRT1 (degree = 939), NQO1 (degree = 736), Nrf2 (degree =569), MYC (degree = 559), and APP (degree = 521).

## 3.5 GO functional enrichment and KEGG pathway analysis

To further elucidate the mechanism of YNJ on DCM, GO function and KEGG pathway enrichment analysis aiming at 204 predicated key therapeutic candidates were performed in the Metascape platform. The top 20 significant GO terms during BP, CC, and MF, and the top 20 significantly enriched KEGG pathways were presented in the form of a bar and bubble diagram (Figure 5). BP that are involved in the treatment of YNJ on DCM were primarily associated with response to regulation to protein stability, response to oxidative stress, response to growth factor, regulation of binding, and so on. In the MF ontology, the targets of YNJ for DCM were mainly related to ubiquitin-like protein ligase binding, protein domain-specific binding, kinase binding, and so on. For the CC ontology, the targets were mainly involved in focal adhesion, intracellular protein-containing complex, ribonucleoprotein complex, perinuclear region of cytoplasm, and so on. KEGG enrichment analysis showed that the pathways significantly influenced by YNJ were cell cycle, proteoglycans in cancer, Nrf2 signaling pathway, ErbB signaling pathway, and so on. Therefore, it could be assumed that YNJ may exert protective effects on myocardium during diabetes mainly through modulation of oxidative stress, especially the Nrf2 pathway, which would be verified in the following docking analysis and *in vivo* experiment.

## 3.6 Molecular docking between hub targets and active components

To further certify the binding activity between the primary active ingredients of YNJ and key therapeutic targets, molecular docking with AutoDockTools was performed. The energy level represents their binding potency, with lower energy level indicating stronger binding capability. Generally, the binding affinity is considered to be potent if the docking calculation score (kcal/mol) is less than −7 in AutoDockTools. As shown in Figure 6, the calculated binding energies between main active components and key therapeutic targets were close to or even lower than −7, indicating specific binding possibility. Among them, diosgenin had the strongest binding affinity with DCM targets, as affinity with SIRT1 = −8.84 kcal/mol, with Nrf2 = −7.33 kcal/mol, with NQO1 = −8.06 kcal/mol. This supported the mechanistic involvement of the corresponding pathways in the effects of YNJ on DCM. In addition, as illustrated in Figures 7B–D, amino acid residue PRO-291 in the crystal structure of SIRT1, ARG-515 in Nrf2, and HIS-162 in NQO1 formed hydrogen bonds with diosgenin, respectively. Figures 7A, E–J shows the visualized images and details of optimal docking of key targets to other active components of YNJ.

## 3.7 Protective effects of YNJ on cardiomyopathy of diabetic rats

To further verify the cardioprotective effects of YNJ, diabetic rats were treated with YNJ for 10 weeks. Cardiac collagen

TABLE 1 Potential effective compounds of YNJ.

| PubChem CID | Compound | Molecular Formula | OB (%) | DL | Filter by | Origin |
|---|---|---|---|---|---|---|
| 12303645 | 3-Epi-beta-Sitosterol | $C_{29}H_{50}O$ | 36.91 | 0.75 | OB ≥ 30% DL ≥ 0.18 | Rehmanniae Radix Praeparata |
| 5280794 | Stigmasterol | $C_{29}H_{48}O$ | 43.83 | 0.76 | OB ≥ 30% DL ≥ 0.18 | Rehmanniae Radix Praeparata/Anemarrhenae Rhizoma/Ophiopogonis Radix/Achyranthis Bidentatae Radix |
| 13855373 | ZINC13374323 | $C_{27}H_{28}N_2O_4$ | 58.02 | 0.52 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 45270099 | Mangiferolic acid | $C_{30}H_{48}O_3$ | 36.16 | 0.84 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 5280863 | Kaempferol | $C_{15}H_{10}O_6$ | 41.88 | 0.24 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma/Achyranthis Bidentatae Radix |
| 5318980 | Icaritin | $C_{21}H_{20}O_6$ | 45.41 | 0.44 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 21160900 | Chrysanthemaxanthin | $C_{40}H_{56}O_3$ | 38.72 | 0.58 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 441594 | Hippeastrine | $C_{17}H_{17}NO_5$ | 51.65 | 0.62 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 5318997 | Icariin | $C_{33}H_{40}O_{15}$ | 41.58 | 0.61 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 6440659 | n-cis-Feruloyltyramine | $C_{18}H_{19}NO_4$ | 118.35 | 0.26 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 99474 | Diosgenin | $C_{27}H_{42}O_3$ | 80.88 | 0.81 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 13939145 | cis-N-p-Coumaroyltyramine | $C_{17}H_{17}NO_3$ | 112.9 | 0.2 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 5372945 | p-Coumaroyltyramine | $C_{17}H_{17}NO_3$ | 85.63 | 0.2 | OB ≥ 30% DL ≥ 0.18 | Anemarrhenae Rhizoma |
| 44575945 | Timosaponin B II | $C_{45}H_{76}O_{19}$ | | | Anti-oxidant effects<br>Inhibits cardiomyocyte apoptosis<br>Cardioprotective effect (1) | Anemarrhenae Rhizoma |
| 5283663 | Chondrillasterol | $C_{29}H_{48}O$ | 42.98 | 0.76 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 5281325 | Spinoside A | $C_{39}H_{56}O_{12}$ | 41.75 | 0.4 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 27545171 | ZINC17147377 | $C_{27}H_{44}O_7$ | 44.23 | 0.82 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 2353 | Berberine | $C_{20}H_{18}NO_4^+$ | 36.86 | 0.78 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 72322 | Coptisine | $C_{19}H_{14}NO_4^+$ | 30.67 | 0.86 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 5281703 | Wogonin | $C_{16}H_{12}O_5$ | 30.68 | 0.23 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 521367 | delta(7)-Stigmastenol | $C_{29}H_{50}O$ | 37.42 | 0.75 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 5281605 | Baicalein | $C_{15}H_{10}O_5$ | 33.52 | 0.21 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 64982 | Baicalin | $C_{21}H_{18}O_{11}$ | 40.12 | 0.75 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 160876 | Epiberberine | $C_{20}H_{18}NO_4^+$ | 43.09 | 0.78 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 222284 | beta-Sitosterol | $C_{29}H_{50}O$ | 36.91 | 0.75 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 455251 | Inophyllum E | $C_{25}H_{22}O_5$ | 38.81 | 0.85 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 5281331 | alpha-Spinasterol | $C_{29}H_{48}O$ | 42.98 | 0.76 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 19009 | Palmatine | $C_{21}H_{22}NO_4^+$ | 64.6 | 0.65 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 5280343 | Quercetin | $C_{15}H_{10}O_7$ | 46.43 | 0.28 | OB ≥ 30% DL ≥ 0.18 | Achyranthis Bidentatae Radix |
| 5319741 | Methylophiopogonanone A | $C_{19}H_{18}O_6$ | | | Anti-oxidant effects<br>Inhibits cardiomyocyte apoptosis (2) | Ophiopogonis Radix |
| 46886723 | Methylophiopogonanone B | $C_{19}H_{20}O_5$ | | | Anti-oxidant effects<br>Protects HUVECs against $H_2O_2$-induced cell death (3) | Ophiopogonis Radix |
| 46173859 | Ophiopogonin D | $C_{44}H_{70}O_{16}$ | | | ROS scavenging<br>Reduced oxidative stress | Ophiopogonis Radix |

*(Continued)*

TABLE 1 Continued

| PubChem CID | Compound | Molecular Formula | OB (%) | DL | Filter by | Origin |
|---|---|---|---|---|---|---|
| | | | | | Maintains $Ca^{2+}$ homeostasis and reduces ER stress in cardiomyocytes (4) | |

accumulation and degree of fibrosis were evaluated by PSR staining. Figure 8A illustrates representative morphological changes showing a larger proportion of Sirius red collagen content in the myocardium of diabetic rats compared with controls. The collagen deposition as indicated by CVF significantly increased in the myocardium of rats with diabetes, which was markedly attenuated by YNJ administration (Figure 8B). The results implied that YNJ could ameliorate cardiac fibrosis induced by DCM, demonstrating cardioprotective effects.

## 3.8 Verification of critical targets of YNJ for DCM with animal experiments

To clarify the effects of YNJ on the screened key targets against DCM, the expression of SIRT1, Nrf2, and NQO1 was detected by immunochemistry and Western blotting analysis. As shown in Figures 9A, B, diabetes induced the declined expression of SIRT1 in the cardiac tissues, which was reversed by YNJ administration. For Nrf2 and NQO1, we found significantly lower expression in the diabetic myocardium than the normal rats. YNJ treatment could effectively upregulate these oxidative-associated markers (Figures 10A–C). These results revealed the possible mechanisms of YNJ on cardiomyopathy initiated by diabetes.

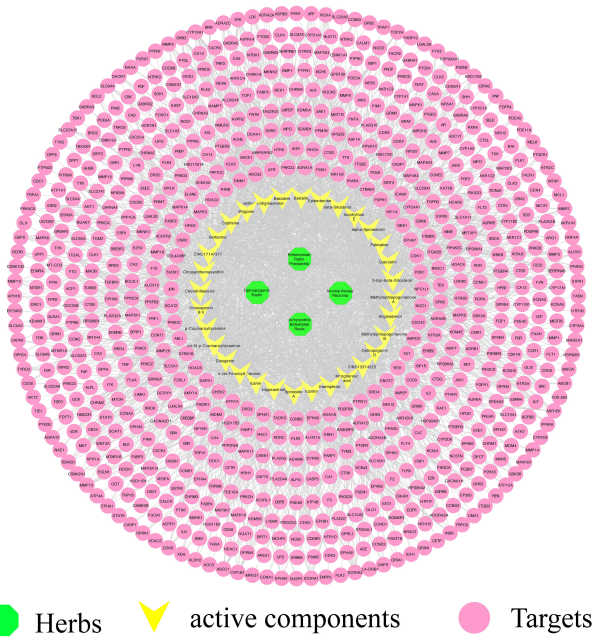


FIGURE 2
Effective component–target network of YNJ. The green nodes represent herbs, the yellow nodes represent active components, and purple nodes represent predicted targets.

# 4 Discussion

Although the treatment of DCM has been developed recently, practically effective interventions are scarce due to the complex etiology and pathogenic mechanisms of DCM. Based on the proprietary characteristics of multicomponents, multitargets, and multipathways, Chinese medicine has shown reliable therapeutic effects for DCM (21). In the present study, for the first time, we discovered the cardioprotective effects of YNJ in diabetic rats as reflected by alleviation of collagen deposition and fibrosis. Additionally, the PPI network of YNJ in the treatment of DCM was constructed, and pathway enrichment analyses were performed. More importantly, the key targets were screened and verified by molecular docking and *in vivo* experiments to illustrate the potential mechanisms of YNJ for DCM.

Cardiac remodeling is the major structural abnormalities of DCM. Here, in the myocardium of diabetic rats, we found obvious collagen deposition and fibrosis, which could be markedly reversed by YNJ administration. Similar protective function of YNJ on pathological remodeling has also been found in coronary artery

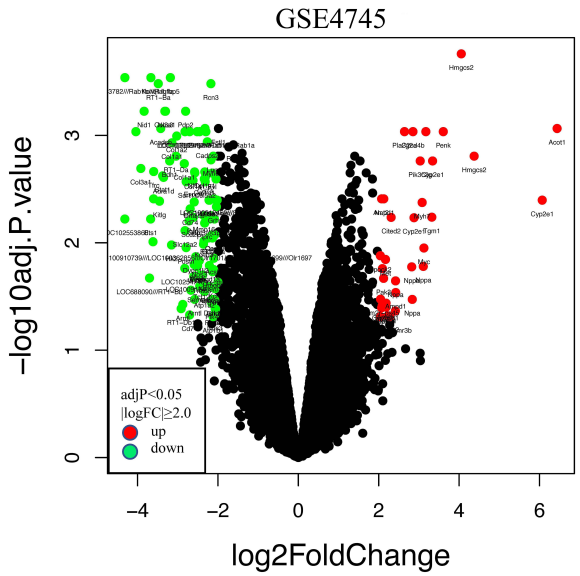


FIGURE 3
Volcano plots of differentially expressed genes (DEGs) in the GSE4745 dataset. Red and green plots represent up- and downregulated genes, respectively (|logFC| ≥ 2.0 and adjusted $p < 0.05$), while black represents no significant difference. FC, fold change.

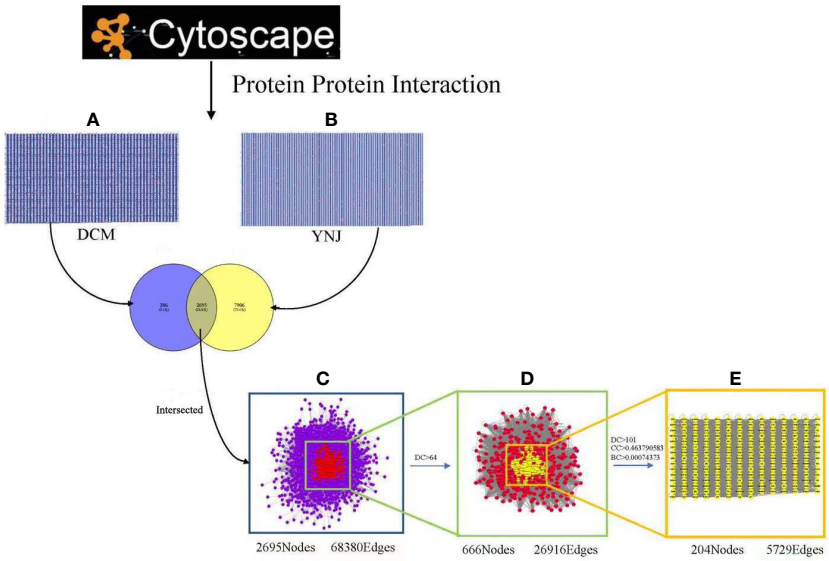


FIGURE 4
Identification of the core targets for YNJ against DCM. **(A)** The PPI network of DCM-related targets (2,981 nodes and 70,524 edges). **(B)** The PPI network of YNJ-related targets (10,601 nodes and 219,193 edges). **(C)** Intersection of PPI networks of YNJ putative targets and DCM-related targets (2,695 nodes and 68,380 edges). **(D)** PPI network of important targets obtained with the screening criteria of DC > 64 (666 nodes and 26,919 edges) from **(C)**. **(E)** Core–target PPI network of YNJ against DCM obtained with the screening criteria of DC >101, BC > 0.00074373, and CC > 0.463790583 (204 nodes and 5,729 edges) from **(D)**. BC, betweenness centrality; CC, closeness centrality; DC, degree centrality.

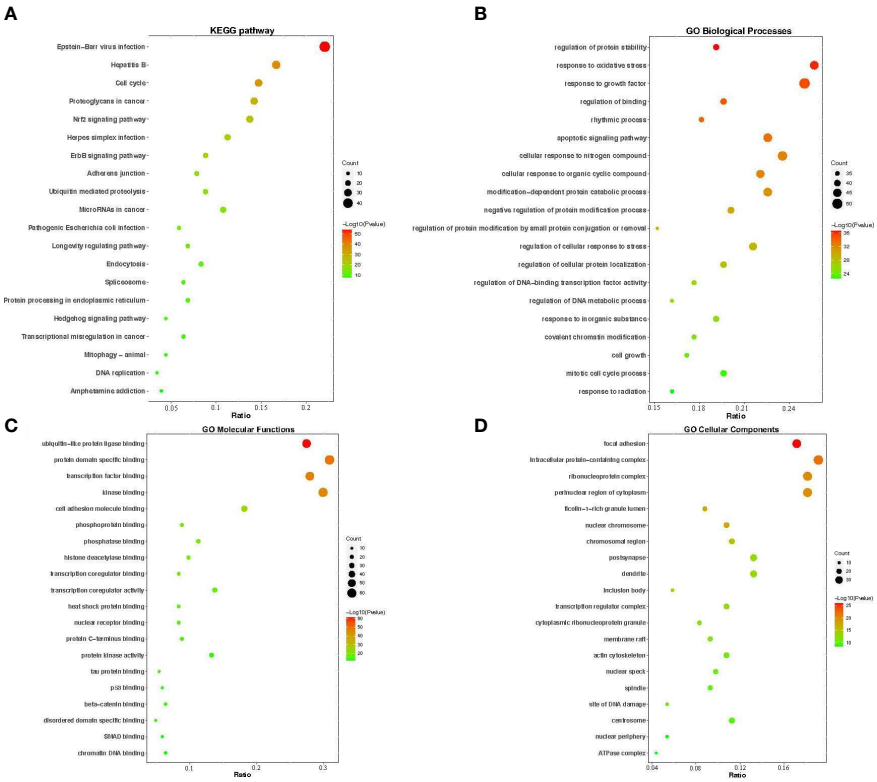


FIGURE 5
GO functional annotation and KEGG pathway enrichments. The bubble plots of the 20 most significant signaling pathways based on KEGG enrichment analysis **(A)**, and the top 20 enriched GO terms of biological process **(B)**, molecular function **(C)**, and cellular components **(D)**. The *X*-axis and *Y*-axis stand for the gene ratios and full names of the processes, respectively. The color and size of each bubble represent the *p*-value and gene count, respectively.

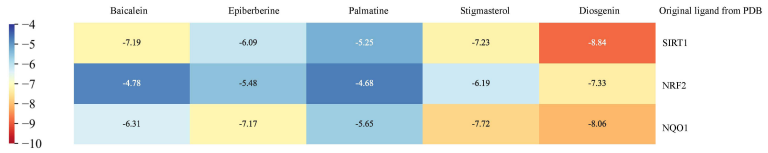


FIGURE 6
Binding affinity of key chemical compounds of YNJ to putative core targets as revealed by molecular docking.

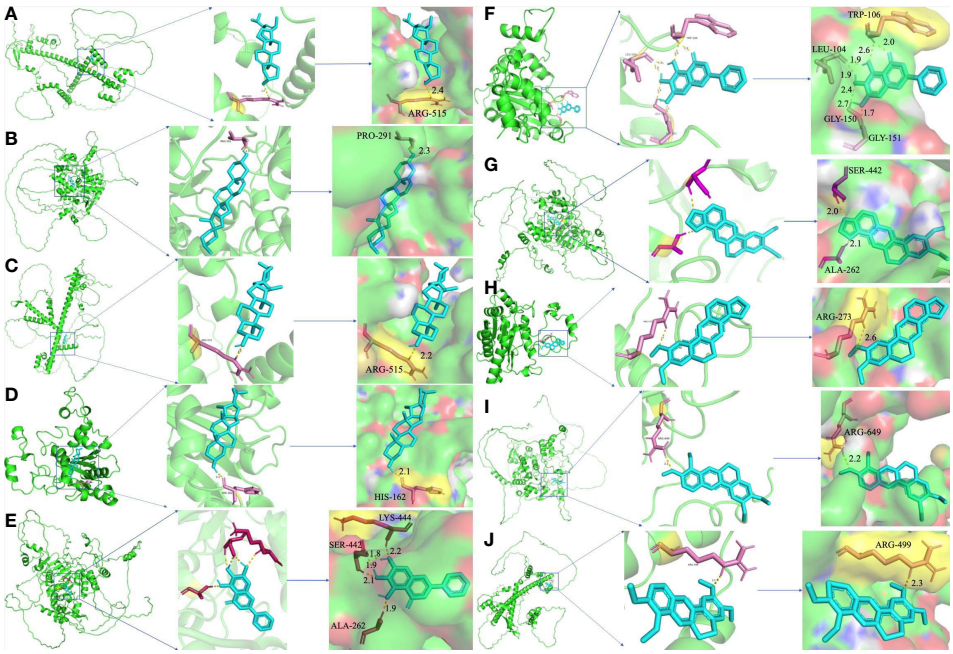


FIGURE 7
Molecular docking of hub targets and active components of YNJ. **(A)** Nrf2-Stigmasterol; **(B)** SIRT1-Diosgenin; **(C)** Nrf2-Diosgenin; **(D)** NQO1-Diosgenin; **(E)** SIRT1-Baicalein; **(F)** NQO1-Baiclein; **(G)** SIRT1-Epiberberine; **(H)** NQO1-Epiberberine; **(I)** SIRT1-Palmatine; **(J)** Nrf2-Palmatine.

ligation-induced myocardium infarction rats (12). Meanwhile, previous studies have found that effective components contained in YNJ possess apparent cardioprotective effects. For example, *Rehmanniae glutinosa*, the minister medicine in YNJ, attenuated adriamycin-induced cytotoxicity and apoptosis in H9C2 cardiac muscle cells (22). Timosaponin B, one of the primary bioactive compounds from *Anemarrhena asphodeloides*, attenuated isoproterenol-induced myocardial injury by inhibiting ER stress-mediated apoptosis pathways (14). In combination with these observations, our results highlight the cytoprotective property of YNJ in diabetic rats. Therefore, the underlined mechanism was next detected.

It is well-documented that oxidative stress characterized by excessive ROS production is one of the leading factors in the development of DCM. The key pathological processes underlying cardiac remodeling in diabetes are highly redox sensitive, including cardiomyocyte hypertrophy, apoptosis, fibrosis, and diastolic/systolic dysfunction (23). Oxidative stress and other risk factors may promote cardiomyocyte death, interstitial fibrosis, and cardiac stiffness, leading to diastolic and systolic dysfunction, and eventually heart failure. Therefore, pharmacological strategies for reducing ROS burden or increasing antioxidant mechanisms may provide a successful strategy for the treatment of DCM (24). Here, the GO analysis revealed that the target genes of YNJ on DCM were numerous and complex, mainly focusing on biological processes such as response to regulation to protein stability and response to oxidative stress. It was revealed that the acetone, ethyl acetate, and water extract of the rhizome of *A. asphodeloides* exhibited strong antioxidant activities (25). Moreover, the protective effect of *R. glutinosa* is associated with the increase of Mn-SOD expression and GSH level as well as the resulting scavenging effect on free radicals in cardiac muscle cells (22). *Achyranthes bidentata* polypeptides showed similar anti-oxidant and protective capacity against myocardial ischemic/reperfusion injury in rats (26). All these indicate that the cardioprotective functions of YNJ are related to alleviation of oxidative stress and the corresponding injuries induced by diabetes.

The cellular antioxidant defense system plays vital roles in the protection against oxidative damage. Nrf2, a basic leucine zipper stress-responsive transcription factor, has been recognized as a

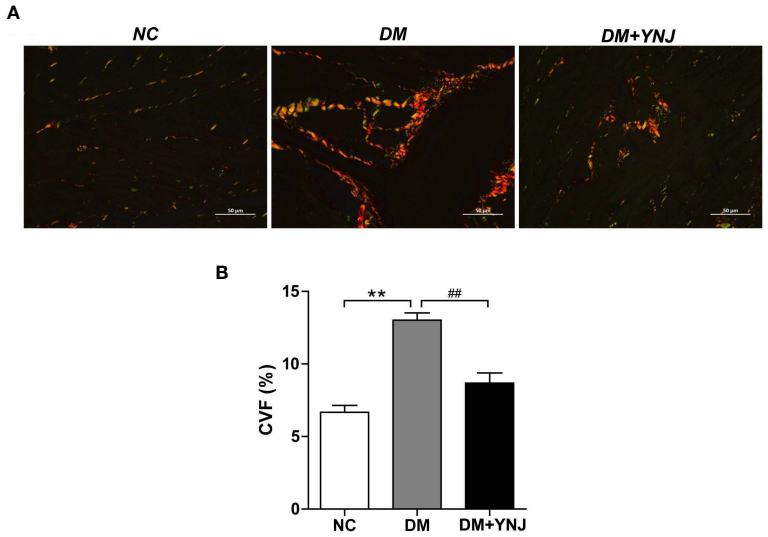


FIGURE 8
Picro-Sirius red (PSR) staining for collagen deposition in the myocardium. **(A)** PSR staining to detected collagen content and fibrosis in the left ventricle (400×). **(B)** Calculation of PSR-positive area as displayed by CVF. Data are presented as mean ± SD. $^{**}p < 0.01$ vs. the normal control group; $^{\#\#}p < 0.01$ vs. the DM group.

crucial mediator in ameliorating oxidative stress and enhancing cell survival by inducing the expression of multiple antioxidants and cytoprotective proteins, particularly NQO1 and HO-1. Both expression level and transcriptional activation of Nrf2, together with its target gene NQO1 level, were decreased in diabetic animals and HG-treated primary neonatal rat cardiomyocytes (27). Moreover, knocking down of Nrf2 with siRNA significantly reduced HG-injured cardiomyocyte viability (27). As a result, targeting Nrf2 signaling by pharmacological entities has been demonstrated to counteract the main pathological processes such as ventricular fibrosis and hypertrophy and then prevent the development of DCM (27, 28). Here, the KEGG pathway enrichment analysis showed that the Nrf2 signaling pathway is one of the key pathways for YNJ in the treatment of DCM. This was further validated in diabetic rats, showing that the lower expression of Nrf2 and its downstream antioxidant enzyme NQO1 was improved by YNJ administration. Moreover, several bioactive ingredients in YNJ, such as stigmasterol, diosgenin, baicalein, epiberberine, and palmatine showed high affinity with Nrf2 and/or NQO1 as indicated by molecular docking. Specifically, Timosaponin B reduced oxidative stress through the stimulation of nuclear translocation of Nrf2 and subsequent gene expression (29). In line with these previous reports, our results suggest the effects of YNJ on DCM are associated with Nrf2 signaling.

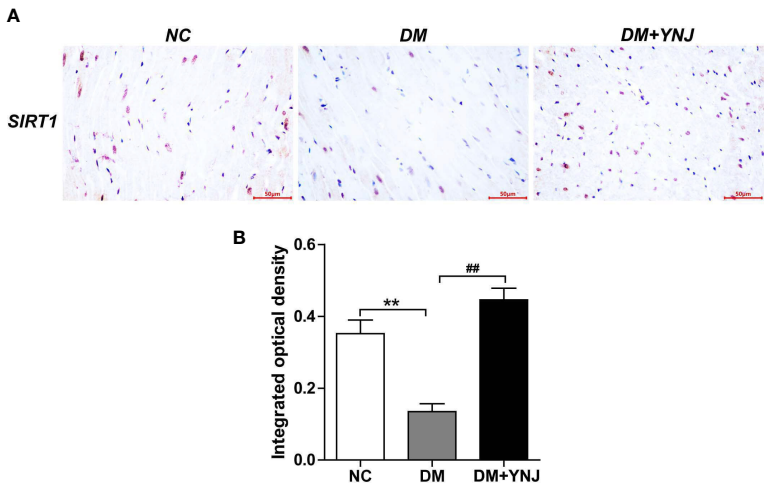


FIGURE 9
Immunohistochemical staining of SIRT1 in cardiac tissues. **(A)** Representative histological images of SIRT1 staining (400×). **(B)** Quantitative analysis of SIRT1 level. Data are expressed as mean ± SD. $^{**}p < 0.01$ vs. the normal control group; $^{\#\#}p < 0.01$ vs. the DM group.

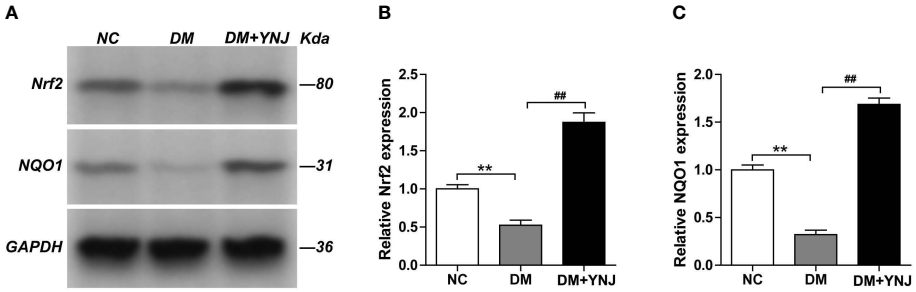


FIGURE 10
Western blotting analysis of Nrf2 and NQO-1 protein expressions in the myocardial tissues. **(A)** Representative blot images of Nrf2 and NQO-1 protein expressions in the myocardium. GAPDH served as the internal control. **(B, C)** Quantitative analysis of Nrf2 and NQO-1 protein levels. Data are presented as mean ± SD. $^{**}p < 0.01$ vs. the normal control group; $^{\#\#}p < 0.01$ vs. the DM group.

SIRT1, a $NAD^+$-dependent histone deacetylase, regulates multiple biological processes, including redox hemostasis, inflammation, apoptosis, and cell metabolism. Decreased SIRT1 expression was detected in myocardial tissue of diabetic rats, along with declined antioxidant defenses and impaired cardiac morphology and function (30). Conversely, normalization of SIRT1 exhibited a protective effect against oxidative stress and hyperglycemia-induced cardiac injury by activating the Nrf2 pathway and Nrf2-dependent antioxidant genes (30, 31), or by promoting mitochondrial fusion/inhibiting mitochondrial fission and subsequent suppression of mitochondria-derived ROS production (32, 33). Moreover, SIRT1 activators may inhibit P300 and MMP-9, deacetylating histone, NF-κB, and P53 or upregulate ERK1/2, NO, and SERCA2a, resulting in increased stress resistance, thus protecting against cardiomyocyte apoptosis, fibrosis, hypertrophy, and inflammation (34). Moreover, active SIRT1 upregulated expression of NQO1, one of the most common targets of Nrf2 (35). Simultaneously, overexpression of NQO1 upregulated SIRT1 expression and activity in db/db mice by regulating the $NAD^+$/NADH ratio, which was responsible for the antioxidant and antiapoptotic effects of NQO1 (36). Also, there is a direct binding between SIRT1 and NQO1 (35). Thus, the crosstalk between SIRT1 and NQO1 may be complex and they may couple in a functional module (35). In this work, we found that SIRT1 was among the key targets of YNJ to DCM. We also provide evidence for the interaction of SIRT1 and several ingredients of YNJ including stigmasterol, diosgenin, baicalein, and epiberberine. Furthermore, the decreased level of SIRT1 in diabetic myocardium was reversed by YNJ administration. These indicated that the cardioprotective effect of YNJ is related to SIRT1.

## 5 Conclusion

In summary, activation of SIRT1 and Nrf2 signaling markedly enhances expression of antioxidant enzymes, attenuates oxidative damage, and is, thus, beneficial by retarding cardiac fibrosis induced by diabetes (37). The chronic supplementation of diabetic rats with YNJ substantially prevents the development of cardiac remodeling, which is at least in part mediated by the SIRT1/Nrf2/NQO1 signaling pathway. In the future, more experiments should be performed to clarify the detailed mechanism of YNJ. Despite this, our findings identify YNJ as a potential strategy for the prevention and treatment of DCM that deserves further investigation.

## Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: NCBI: GSE4745.

## Author contributions

FW conceived and designed the study and provided funding support. WW performed network pharmacology research, molecular docking, and animal experiments and wrote the first draft of manuscript. RL helped analyze the data and prepare the manuscript. YZ revised the manuscript and provided funding support. LW, YT, BD, and ST helped conduct the experiment. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Positive correlation between lipid accumulation product index and arterial stiffness in Chinese patients with type 2 diabetes

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**Background:** Many studies have confirmed that lipid accumulation products (LAP) predict arterial stiffness (AS) in hypertensive patients. But there is little research on the use of LAP in identifying early atherosclerosis in patients with type 2 diabetes mellitus (T2DM). The aim of this study was to determine the relationship between the LAP index and brachial-ankle pulse wave velocity (baPWV) in Chinese patients with T2DM.

**Methods:** A total of 1471 Chinese participants with T2DM, ranging in age from 18 to 80, were included in this cross-sectional study. BaPWV measurements were used to calculate the AS. A baPWV greater than the 75th percentile (1700 cm/s) was defined as indicating increased AS. The LAP index was calculated from the combination of waist circumference (WC) and triglycerides (TG).

**Results:** According to the quartiles of the LAP index, baPWV tended to increase after adjusting for sex and age. Multiple linear regression analysis showed that the beta coefficient (β) of baPWV increased by 31.0 cm/s for each unit of lnLAP that was increased, and the 95% confidence interval (CI) was (6.5, 55.5) cm/s. In multivariate logistic regression analyses, after fully adjusting for confounders, the risk of elevated baPWV increased with each unit increase in lnLAP, with an odds ratio (OR) of 1.3 (95% CI: 1.0, 1.8). According to the generalized additive model (GAM), we found that lnLAP was positively correlated with baPWV and baPWV elevation. The results were the same for males and females. Subgroup analyses showed that the positive correlation between lnLAP and elevated baPWV did not interact across all subgroups.

**Conclusions:** In Chinese patients with T2DM, LAP was strongly and positively correlated with baPWV and elevated baPWV.

KEYWORDS

**lipid accumulation product, brachial-ankle pulse wave velocity, type 2 diabetes mellitus, arterial stiffness, insulin resistance**

# 1 Introduction

Epidemiological surveys reveal that, globally, around 536.6 million persons aged 20 to 79 years had diabetes mellitus in 2021, with a prevalence of approximately 10.5%, and this figure is expected to climb to 783.2 million in 2045, with a prevalence of approximately 12.2% (1). This constantly rising incidence puts a significant strain on the economies of individual nations. Year after year, the challenging public health issue of diabetes prevalence grows. Type 2 diabetes mellitus (T2DM), which accounts for the bulk of the country's diabetic population—more than 90% of all cases—is the most common type of diabetes in China, where the prevalence of the disease among those 18 and older has increased from 0.67% in 1980 to 11.2% in 2017 (2). A nationally representative cross-sectional survey with 173,642 participants in China in 2018 determined the overall prevalence of diabetes to be 12.4% (3).

Patients with T2DM are more likely than healthy people to develop insulin resistance (IR), hyperinsulinemia, lipid metabolism disorders, and elevated blood pressure, which can lead to vascular stiffness and associated cardiovascular disease (CVD) (4, 5). In addition, women with T2DM have faster atherosclerosis than men, especially after menopause (6, 7). Arterial stiffness (AS) is an early predictor of atherosclerosis and is generally considered a predictor of CVD incidence and mortality (8). Most current brachial-ankle pulse wave velocity (baPWV) measurements are now commonly used in research and clinical assessment of AS, and although they have a high degree of consistency, few time requirements, minimal operator dependence, and the ability to capture ankle-arm index waves simultaneously, they are still not universally available in rural areas, community hospitals, and large epidemiologic surveys in China. Because of the Chinese sedentary lifestyle and diet heavy in processed carbs and saturated fats, this can lead to disorders of lipid metabolism, obesity, and increased AS (9). Therefore, given that AS is a slow progression over a long period of time, we require a simple, cost-effective indicator to recognize early AS, especially for Chinese patients with T2DM.

Triglycerides (TG) are the standard lipid that is most strongly linked to AS, according to studies, and they are often linked to the early start of CVD (10). LAP is calculated from the combination of waist circumference (WC) and TG and differentiates between men and women by WC to better reflect central obesity and excessive lipid accumulation (11, 12). There are fewer studies on LAP and baPWV, and they have mostly been studied in the general population and hypertensive population in the past, and the results are controversial (13–15). As far as we know, there is little evidence that LAP can be used to detect early atherosclerosis in Chinese individuals with T2DM, so to fill this gap, we analyzed the correlation between LAP and baPWV in T2DM patients.

# 2 Methods

## 2.1 Characteristics of the population

All information was acquired between May 2020 and January 2022 at the Metabolic Management Center (MMC), Changde Hospital, Xiangya School of Medicine, Central South University, Hunan Province, China. which serves as a platform for standardized diagnosis and treatment of metabolic disorders and long-term follow-up (16). In this cross-sectional survey, 1665 diabetic patients aged 18 to 80 years were included. T2DM was identified using the 1999 World Health Organization diagnostic criteria: fasting blood glucose≥7.0 mmol/L, 2h postprandial plasma glucose≥11.1 mmol/L, or self-reported diabetes diagnosis (17). Exclusion criteria included age < 18 years (n = 3), patients with type 1 diabetes (n = 18), other types of diabetes (n = 4), pregnancy (n = 1), coronary artery disease (n = 52), stroke (n = 17), malignancy (n = 10), and patients with missing data for WC (n = 17), TG (n = 16), and baPWV (n = 56). After the exclusion of the above criteria, 1471 T2DM participants were finally included in this study.

## 2.2 Measurements of variables

All the anthropometric indices and socio-demographic parameters were measured and registered by trained researchers, and socio-demographic data were collected by questionnaire on gender, age, smoking, alcohol consumption, work status, salt intake (≤6 g/day, 6–8 g/day, and ≥8 g/day) (18), regular exercise (physical activity of moderate intensity at least three times per week) (11), duration of diabetes, history of CVD, and use of lipid-lowering, antihypertensive, or glucose-lowering drugs. Smoking (current, previous, and never) and alcohol consumption (current, previous, and never). Current smoking was defined as smoking more than one cigarette per day or more than seven cigarettes per week for more than six months. Former smoking was defined as meeting the above criteria six months ago and not smoking in the past six months. Current alcohol use is defined as drinking more than one "standard drink" (defined as 12 ounces of beer, 5 ounces of wine, or 1.5 ounces of distilled spirits) per week for more than six months. Former alcohol consumption is defined as meeting the above criteria six months ago and not drinking alcohol within the past six months (19). The anthropometric measurements include height, weight, WC, SBP (systolic blood pressure), and DBP (diastolic blood pressure). The participant's height was measured with the shoes removed, and the three points of the head at the occiput, the ridge between the two shoulder blades, and the sacrum should be attached to the height measurement column. Weight (kg)/height$^2$ ($m^2$) was used to compute the body mass index (BMI). In order to calculate the WC, a non-elastic measuring tape was placed at the median point on a line from the superior margin of the iliac crest to the inferior margin of the costal arch (20). After sitting still for a minimum of five minutes, the subjects' blood pressures were measured twice, and the final mean was calculated (21).

## 2.3 Laboratory assays

Laboratory data were obtained by drawing blood from each subject after at least 8 hours of fasting. We measured total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C).

Fasting plasma glucose (FPG), fasting insulin (FINS), glycosylated hemoglobin (HbA1c), homeostasis model assessment for insulin resistance (HOMA-IR), and fasting C-peptide (FCP) were also measured. To evaluate post-load hyperglycemia, postprandial plasma glucose (PPG) was acquired from venous blood samples taken two hours after the steamed bread meal. The triglyceride glucose (TyG) index was calculated as the ln [TG (mg/dl) × FPG (mg/dl)/2]. The visceral adiposity index (VAI) was calculated as [WC(cm)/39.68 + (1.88 × BMI)] × (TG (mmol/L)/1.03) × (1.31/HDL-C (mmol/L)) for men and as [WC(cm)/36.58 + (1.89 × BMI)] × (TG (mmol/L)/0.81) × (1.52/HDL-C (mmol/L)) for women (15). The formula for calculating LAP varies by gender. For men, the formula used to determine the LAP was [WC (cm) - 65] × TG (mmol/L), and for women, the LAP was [WC (cm) - 58] × TG (mmol/L) (22). We corrected 66 cm for males with WC up to 65 cm and 59 cm for females with WC up to 58 cm in order to prevent LAP values that were not positive (14).

## 2.4 Assessment of baPWV

The technician performed baPWV measurements using an automated atherosclerosis detection device (model HBP-8000, Omron HealthCare (China) Co.). All subjects were required to rest for at least 5 minutes prior to the baPWV measurement, and the four cuffs of the automatic recording apparatus were wrapped around the elbow and ankle joints bilaterally. The band is fastened 2 cm above the inner ankle, while the arm band is fastened 3 cm above the elbow socket. The instrument uses the subject's height as a reference and calculates the distance from the humerus to the ankle (La-Lb). The time difference of the waveform between the elbow and the ankle is denoted by Tba. The (La-Lb)/Tba formula was used to determine the baPWV (23, 24). In this study, we evaluated the mean of baPWV (25). Elevated baPWV, which was greater than 1700 cm/s in the study, was identified using the 75th percentile of baPWV measurement results (26, 27).

## 2.5 Statistical analysis

We ln-transformed LAP (lnLAP) because of the skewed distribution of LAP (14). To characterize the distribution of participants' features, we divided them into four equal subgroups based on lnLAP levels. For continuous variables with skewed distributions, the median (1-3 quartiles) was utilized; for continuous variables with normal distributions, the mean ± SD was employed; and for categorical variables, numerical values (percentages) were utilized. ANOVA, or the Kruskal-Wallis H test, was utilized to assess differences in characteristics across lnLAP quartiles for continuous variables, while Fisher's exact test, or the chi-square test, was employed to analyze categorical data.

We characterized the association between lnLAP and baPWV risk by β (beta coefficient) and 95% CI (confidence interval) of multivariate linear regression; meanwhile, multiple logistic regression demonstrated the correlation between lnLAP and elevated baPWV in T2DM patients by OR (odds ratio) and 95% CI. Five models were constructed by adjusting for covariates. Model 1 was not adjusted; Model 2 adjusts for gender and age based on Model 1; similarly, Model 3 added to Model 2 SBP, DBP, BMI, smoking, drinking, and work status. Model 4 added HbA1c, TC, HDL-C, and LDL-C to the previous model, and Model 5 added salt intake, regular exercise, glucose-lowering drugs, anti-hypertensives, lipid-lowering drugs, and duration of diabetes to the previous models. We compared the correlation between lnLAP and baPWV and elevated baPWV across genders by using the generalized additive model (GAM) dose-response relationship. Furthermore, we performed subgroup analyses to examine the relationship between lnLAP and higher baPWV by potential effect modifiers and did an interaction test. Statistical analyses for this study were performed using both R version 4.2.0 and EmpowerStats version 4.0.

# 3 Results

## 3.1 Participants' characteristics

There were 1471 T2DM patients in the study, and the mean age of these participants was 51.81 ± 10.76 years, of whom 851 were men and 620 were women. Table 1 displays the individual's initial characteristics for lnLAP quartiles. Subjects with a higher lnLAP tended to have elevated BMI, WC, SBP, DBP, FPG, PPG, FCP, HbA1c, HOMA-IR, TC, TG, LDL-C, and baPWV. These subjects also had higher rates of smoking, alcohol consumption, participation in the workforce, use of lipid-lowering drugs, antihypertensive drugs, and high salt intake. Of the four groupings, subjects in the greater lnLAP category were younger and had lower HDL-C levels. In contrast, there were no statistically significant variations in the use of glucose-lowering medications or the length of diabetes between the four subgroups. The percentages of high baPWV in lnLAP quartiles were, respectively, 18.21%, 26.16%, 28.26%, and 26.09% in Q1, Q2, Q3, and Q4 ($p<0.05$). The proportion of men also grew, while the number of women declined as lnLAP increased.

## 3.2 Correlation study of lnLAP and baPWV

After correcting for sex and age, as seen in Figure 1, the mean baPWV of subjects showed an increasing trend in the lnLAP index quartile ($F = 11.8622$, $P < 0.001$). The mean baPWV values from the different lnLAP groups (quartiles 1–4) were 1550 (1517, 1582), 1605 (1573, 1638), 1629 (1597, 1661), and 1675 (1643, 1707) cm/s ($P <0.001$), respectively.

A 1-unit increase in lnLAP in the logistic regression model was linked to a 31.1 (95% CI: 6.5, 55.9) cm/s increase in the β coefficient of baPWV after accounting for confounding variables (Table 2). We further grouped the lnLAP quartiles and placed them as categorical variables in a logistic regression model; in model 5, adjusting for all confounders, the β coefficient of the baPWV increased by 75.4 (95% CI: 22.6, 128.3) cm/s in the fourth group of the lnLAP compared with the first group, and in addition, we found that the baPWV's β

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TABLE 1 Baseline characteristics of the participants.

| | lnLAP | | | | |
|---|---|---|---|---|---|
| | Q1 | Q2 | Q3 | Q4 | P-value |
| N | 368 | 367 | 368 | 368 | |
| Age (years) | 52.70 ± 9.90 | 53.17 ± 10.08 | 52.45 ± 11.32 | 48.91 ± 11.19 | <0.001 |
| Sex | | | | | 0.029 |
| Male | 203 (55.16%) | 199 (54.22%) | 213 (57.88%) | 236 (64.13%) | |
| Female | 165 (44.84%) | 168 (45.78%) | 155 (42.12%) | 132 (35.87%) | |
| BMI (kg/m$^2$) | 22.72 ± 2.32 | 25.16 ± 2.71 | 26.60 ± 2.93 | 28.09 ± 3.61 | <0.001 |
| WC (cm) | 82.01 ± 7.55 | 90.36 ± 7.41 | 94.40 ± 8.03 | 98.14 ± 9.01 | <0.001 |
| SBP (mmHg) | 131.05 ± 19.43 | 135.96 ± 18.10 | 136.87 ± 19.44 | 137.48 ± 19.66 | <0.001 |
| DBP (mmHg) | 79.71 ± 10.92 | 83.27 ± 10.68 | 84.05 ± 10.75 | 86.28 ± 11.78 | <0.001 |
| FPG (mmol/l) | 8.11 ± 3.13 | 8.58 ± 3.19 | 8.73 ± 3.31 | 9.84 ± 3.92 | <0.001 |
| PPG (mmol/l) | 13.20 ± 5.69 | 13.41 ± 5.48 | 13.14 ± 4.86 | 14.54 ± 5.22 | <0.001 |
| Fasting C peptide | 0.29 (0.20-0.39) | 0.38 (0.25-0.50) | 0.44 (0.28-0.63) | 0.57 (0.40-0.76) | <0.001 |
| HbA1c (%) | 7.97 ± 2.37 | 8.35 ± 2.20 | 8.49 ± 2.16 | 8.81 ± 2.19 | <0.001 |
| HOMA-IR | 2.27 (1.60-3.62) | 3.44 (2.13-6.08) | 4.29 (2.82-7.30) | 5.67 (3.54-10.10) | <0.001 |
| TC (mmol/l) | 4.48 ± 0.98 | 4.82 ± 0.97 | 5.04 ± 1.14 | 5.47 ± 1.57 | <0.001 |
| TG (mmol/l) | 1.07 ± 0.37 | 1.59 ± 0.42 | 2.32 ± 0.73 | 6.00 ± 6.28 | <0.001 |
| HDL-C (mmol/l) | 1.42 ± 0.41 | 1.24 ± 0.29 | 1.19 ± 0.26 | 1.16 ± 0.26 | <0.001 |
| LDL-C (mmol/l) | 2.46 ± 0.79 | 2.89 ± 0.83 | 3.02 ± 0.97 | 2.85 ± 0.90 | <0.001 |
| Duration of diabetes (month) | 56.00 (15.00-109.00) | 50.00 (14.00-98.00) | 49.00 (17.00-108.00) | 46.00 (14.00-94.75) | 0.448 |
| baPWV | 1549.22 ± 310.62 | 1613.17 ± 332.63 | 1622.86 ± 319.50 | 1624.60 ± 316.14 | <0.001 |
| Smoking (%) | | | | | 0.095 |
| Never | 252 (68.48%) | 230 (62.67%) | 230 (62.50%) | 213 (58.04%) | |
| Former | 24 (6.52%) | 32 (8.72%) | 32 (8.70%) | 27 (7.36%) | |
| Current | 92 (25.00%) | 105 (28.61%) | 106 (28.80%) | 127 (34.60%) | |
| Alcohol consumption (%) | | | | | <0.001 |
| Never | 282 (76.63%) | 260 (70.84%) | 250 (67.93%) | 212 (57.77%) | |
| Former | 22 (5.98%) | 23 (6.27%) | 31 (8.42%) | 28 (7.63%) | |
| Current | 64 (17.39%) | 84 (22.89%) | 87 (23.64%) | 127 (34.60%) | |
| Work (%) | | | | | <0.001 |
| No | 157 (42.66%) | 163 (44.54%) | 169 (46.05%) | 116 (31.69%) | |
| Yes | 211 (57.34%) | 203 (55.46%) | 198 (53.95%) | 250 (68.31%) | |
| Salt intake | | | | | 0.002 |
| ≤6 g/day | 182 (49.73%) | 150 (41.21%) | 146 (40.11%) | 136 (37.47%) | |
| 6–8 g/day | 168 (45.90%) | 188 (51.65%) | 182 (50.00%) | 188 (51.79%) | |
| ≥8 g/day | 16 (4.37%) | 26 (7.14%) | 36 (9.89%) | 39 (10.74%) | |
| Regular exercise | | | | | 0.897 |
| No | 12 (3.26%) | 10 (2.73%) | 9 (2.45%) | 9 (2.46%) | |

*(Continued)*

TABLE 1 Continued

| | lnLAP | | | | |
|---|---|---|---|---|---|
| | Q1 | Q2 | Q3 | Q4 | P-value |
| Yes | 356 (96.74%) | 356 (97.27%) | 358 (97.55%) | 357 (97.54%) | |
| Glucose-lowering drugs | | | | | 0.408 |
| No | 58 (15.76%) | 63 (17.17%) | 47 (12.77%) | 55 (14.95%) | |
| Yes | 310 (84.24%) | 304 (82.83%) | 321 (87.23%) | 313 (85.05%) | |
| Antihypertensive drugs | | | | | 0.006 |
| No | 294 (79.89%) | 282 (76.84%) | 258 (70.11%) | 262 (71.39%) | |
| Yes | 74 (20.11%) | 85 (23.16%) | 110 (29.89%) | 105 (28.61%) | |
| Lipid-lowering drugs | | | | | <0.001 |
| No | 339 (92.12%) | 338 (92.35%) | 317 (86.38%) | 296 (80.87%) | |
| Yes | 29 (7.88%) | 28 (7.65%) | 50 (13.62%) | 70 (19.13%) | |
| High baPWV | | | | | 0.009 |
| No | 301 (81.79%) | 271 (73.84%) | 264 (71.74%) | 272 (73.91%) | |
| Yes | 67 (18.21%) | 96 (26.16%) | 104 (28.26%) | 96 (26.09%) | |
| VAI | 1.19 (0.24-4.14) | 2.04 (0.78-6.18) | 3.09 (1.55-8.29) | 5.81 (1.95-51.18) | <0.001 |
| TyG | 1.34 (0.07-2.92) | 1.82 (0.73-3.28) | 2.18 (0.85-3.84) | 2.96 (1.24-6.05) | <0.001 |

lnLAP, natural logarithm of lipid accumulation product; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; PPG, postprandial plasma glucose; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment for insulin resistance; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; baPWV, brachial-ankle pulse wave velocity; VAI, visceral adiposity index; TyG, triglyceride glucose.

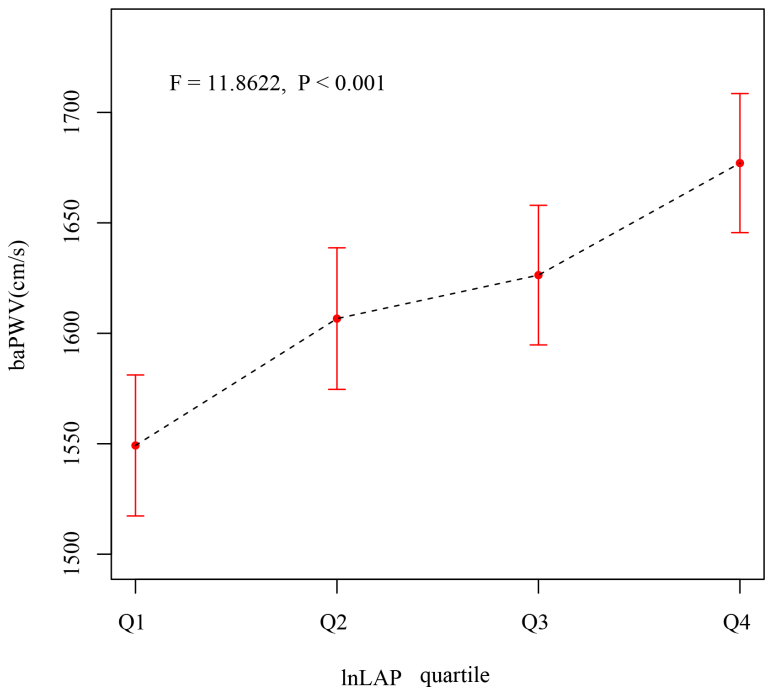


FIGURE 1
Comparison of baPWV after adjusting lnLAP quartiles for age and sex.

coefficient tended to increase between the lnLAP quartiles (P for trend = 0.007). It was also determined in the GAM that lnLAP was positively and linearly correlated with baPWV in both males and females (Figure 2A).

## 3.3 Association between lnLAP and elevated baPWV

Table 3 shows that for every unit rise in lnLAP in the fully adjusted regression model 5, the OR of elevated baPWV was 1.4 (95% CI: 1.0, 1.8). Using the lnLAP quartiles as categorical variables, the OR (95% CI) for elevated baPWV was 1.4 (0.9, 2.3), 1.5 (0.9, 2.5), and 1.9 (1.0, 3.4) for the quartiles 2, 3, and 4 of the lnLAP, respectively, compared with the quartile 1, after full adjustment. The increased risk of elevated baPWV was significant from the first to the fourth quartile (trend P = 0.046). Likewise, the GAM showed a significant positive correlation between lnLAP and elevated baPWV risk in both males and females (Figure 2B).

## 3.4 Subgroup analysis by potential effect modifiers

To learn more about the connection between higher baPWV in each subgroup and lnLAP, we conducted a stratified analysis (Figure 3). The correlation between lnLAP and elevated baPWV was consistent in all subgroups. After excluding stratification variables and adjusting for remaining confounders, we found that lnLAP did not interact with baPWV in all subgroups. (P interaction > 0.05). Sex (P = 0.7505), Age (P = 0.8322), SBP (P = 0.3093), DBP (P = 0.1184), BMI (P = 0.9905), HbA1c (P = 0.0957), Duration of diabetes (P = 0.3798), smoking status (P = 0.9922), drinking status (P = 0.6505), work status (P = 0.9121), use of glucose-lowering drugs or not (P = 0.1111), use of antihypertensive drugs or not (P = 0.8796), use of lipid-lowering drugs or not (P = 0.1975), whether or not females were menopausal (P = 0.1007), low or high VAI (P = 0.5487), and low or high TyG (P = 0.6204).

# 4 Discussion

The present study demonstrated that LAP was positively linked with baPWV and elevated baPWV, and this positive association remained significant when adjusted for multiple regression, with a significant measured response between LAP and AS as assessed by baPWV. This is, as far as we are aware, the first assessment of an independent positive association between LAP and baPWV in Chinese T2DM patients.

There is a limited amount of pertinent research on the connection between LAP and AS, the majority of which has been done on members of the general population or hypertensive patients, and the conclusions are currently controversial (13–15). A study included 954 members of the general Japanese population aged 39 to 64 years and compared four lipid-related indices. It found no significant discriminatory power of LAP for increased AS (13). However, it was discovered that LAP was positively related to greater baPWV and was more significant in women in a different Japanese study of non-industrial workers aged 25 to 55 years (15).

Studies have shown that the mechanism by which LAP is associated with AS may be attributed to insulin resistance (IR). LAP is a stronger predictor of IR than obesity-related indices such as VAI and TyG (28). In addition, a Japanese study including 2818 healthy adults indicated that LAP was positively correlated with elevated baPWV and that LAP was superior to VAI and TyG (15). Our subgroup analysis with two equal groups of VAI and TyG was grouped as low and high. showed that LAP was positively correlated with elevated baPWV in both low and high VAI groups and TyG groups. Our study therefore reinforces the importance of LAP in predicting the risk of AS in patients with T2DM.

Furthermore, a study involving 4926 Chinese hypertensive patients, whose mean age was 64.42 years old, showed that LAP was positively associated with elevated baPWV and did not interact in gender subgroups (14), which is consistent with our findings. This study did not perform a subgroup analysis of menopausal and nonmenopausal women, and the population was mostly comprised of postmenopausal women. The predictive power of LAP in women could be diminished by low estrogen levels. It has been shown that

TABLE 2 Association between lnLAP and baPWV in different models.

| lnLAP | baPWV, cm/s, β (95%CI) | | | | |
|---|---|---|---|---|---|
| | Model I | Model II | Model III | Model IV | Model V |
| Per 1 unit increase | 24.4 (6.0, 42.8) | 49.5 (33.0, 66.0) | 33.9 (15.7, 52.1) | 36.3 (11.5, 61.0) | 31.1 (6.5, 55.9) |
| Quartiles | | | | | |
| Q1 | Ref | Ref | Ref | Ref | Ref |
| Q2 | 64.0 (17.7, 110.2) | 57.4 (16.4, 98.5) | 34.2 (-5.1, 73.6) | 22.1 (-19.0, 63.3) | 23.3 (-17.6, 64.1) |
| Q3 | 73.6 (27.4, 119.9) | 77.1 (36.1, 118.2) | 47.8 (6.2, 89.5) | 34.9 (-10.3, 80.0) | 28.2 (-16.8, 73.2) |
| Q4 | 75.4 (29.2, 121.6) | 127.8 (86.5, 169.2) | 89.4 (44.4, 134.4) | 81.9 (29.0, 134.8) | 75.4 (22.6, 128.3) |
| P for trend | 0.002 | <0.001 | <0.001 | 0.003 | 0.007 |

Model I: adjust for None. Model II: adjust for age and sex. Model III: adjusts for age, sex, SBP, DBP, BMI, smoking, alcohol, and work. Model IV: adjust for age, sex, SBP, DBP, BMI, smoking, alcohol, work, HbA1c, TC, HDL-C, and LDL-C. Model V: adjusts for age, sex, SBP, DBP, BMI, smoking, alcohol, work, salt intake, regular exercise, HbA1c, TC, HDL-C, LDL-C, glucose-lowering drugs, anti-hypertensives, lipid-lowering drugs, and duration of diabetes.
lnLAP, natural logarithm of lipid accumulation product; baPWV, brachial-ankle pulse wave velocity; β, beta coefficient; CI, confidence interval.

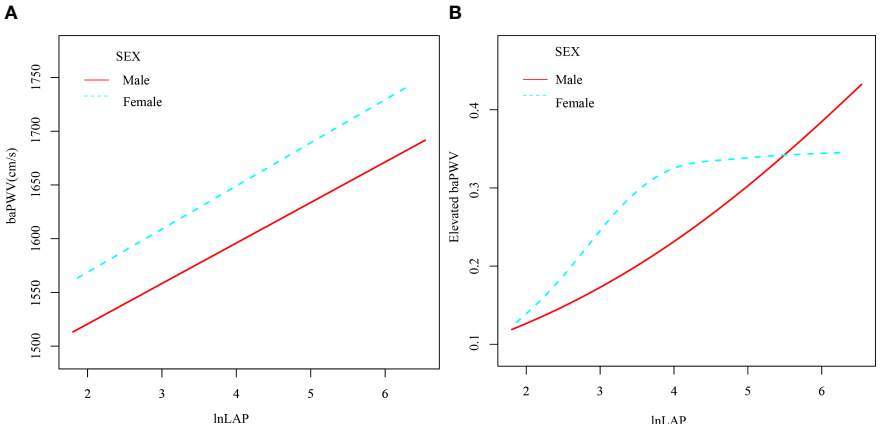


FIGURE 2
Generalized Additive Modeling of the relationship between lnLAP and baPWV **(A)** and elevated baPWV **(B)** across sexes after adjustment for age, SBP, DBP, BMI, smoking, alcohol, work, salt intake, regular exercise, HbA1c, TC, HDL-C, LDL-C, glucose-lowering drugs, anti-hypertensives, lipid-lowering drugs, and duration of diabetes.

men develop carotid atherosclerosis 10 years earlier than women, but the gap between the prevalence of carotid atherosclerosis in men and women narrows gradually after women's menopause. This is associated with postmenopausal hormonal changes, oxidative stress, and changes in abdominal fat (29). Studies suggest that postmenopausal women may have dysregulated lipid metabolism, affecting body fat mass as well as altered abdominal fat distribution due to decreased estrogen levels and increased circulating androgen levels (30, 31).

The reasons for the inconsistent results of several of these studies may be related to variations in participant selection, racial differences, and the definition of AS. Given the controversial results of the above studies, additional studies and analyses of different populations are required to confirm the association between LAP and AS. Many meta-analyses have demonstrated that women with T2DM have faster atherosclerosis than men and are more susceptible to fatal coronary heart disease, myocardial infarction, and stroke, especially after menopause (6, 7, 32). Importantly, to our knowledge, most studies have only explored differences by gender and have rarely assessed differences between whether women are menopausal or not. Therefore, to add to this evidence, we performed an analysis in Chinese T2DM patients aged 18 to 80 years, and the findings revealed that LAP was positively linked with baPWV and did not interact in the gender subgroup after correcting for covariates. In addition, we further demonstrated by subgroup analysis that the positive correlation of LAP with baPWV had no interaction in the subgroup of whether women were menopausal or not. According to the Chinese definition of obesity, BMI <24 kg/m2 is considered non-obese and BMI ≥24 kg/m2 is considered overweight or obese (33), and we divided the subjects into two groups by BMI in the subgroup analysis. After adjusting for all confounders, it was shown that each one-unit increase in LAP increased the risk of AS by 1.3-fold in non-obese, overweight, and obese patients, suggesting that LAP was a better predictor of the risk of AS across all subgroups. Thus, even non-obese patients with T2DM should be closely monitored for LAP to reduce the risk of AS by lowering LAP or maintaining LAP at normal levels.

TABLE 3 Association between lnLAP and elevated baPWV in different models.

| lnLAP | Elevated baPWV, OR (95%CI) | | | | |
|---|---|---|---|---|---|
| | Model I | Model II | Model III | Model IV | Model V |
| Per 1 unit increase | 1.2 (1.0, 1.3) | 1.5 (1.3, 1.7) | 1.4 (1.1, 1.7) | 1.4 (1.1, 1.9) | 1.4 (1.0, 1.8) |
| Quartiles | | | | | |
| Q1 | Ref | Ref | Ref | Ref | Ref |
| Q2 | 1.6 (1.1, 2.3) | 1.6 (1.1, 2.4) | 1.5 (1.0, 2.3) | 1.4 (0.9, 2.2) | 1.4 (0.9, 2.3) |
| Q3 | 1.8 (1.2, 2.5) | 1.9 (1.3, 2.7) | 1.7 (1.1, 2.6) | 1.6 (1.0, 2.6) | 1.5 (0.9, 2.5) |
| Q4 | 1.6 (1.1, 2.3) | 2.5 (1.7, 3.6) | 2.1 (1.3, 3.3) | 2.0 (1.1, 3.5) | 1.9 (1.0, 3.4) |
| P for trend | 0.011 | <0.001 | 0.003 | 0.022 | 0.046 |

Model I: adjust for None. Model II: adjust for age and sex. Model III: adjusts for age, sex, SBP, DBP, BMI, smoking, alcohol, and work. Model IV: adjust for age, sex, SBP, DBP, BMI, smoking, alcohol, work, HbA1c, TC, HDL-C, and LDL-C. Model V: adjusts for age, sex, SBP, DBP, BMI, smoking, alcohol, work, salt intake, regular exercise, HbA1c, TC, HDL-C, LDL-C, glucose-lowering drugs, anti-hypertensives, lipid-lowering drugs, and duration of diabetes.
lnLAP, natural logarithm of lipid accumulation product; baPWV, brachial-ankle pulse wave velocity; OR, odd ratio; CI, confidence interval.

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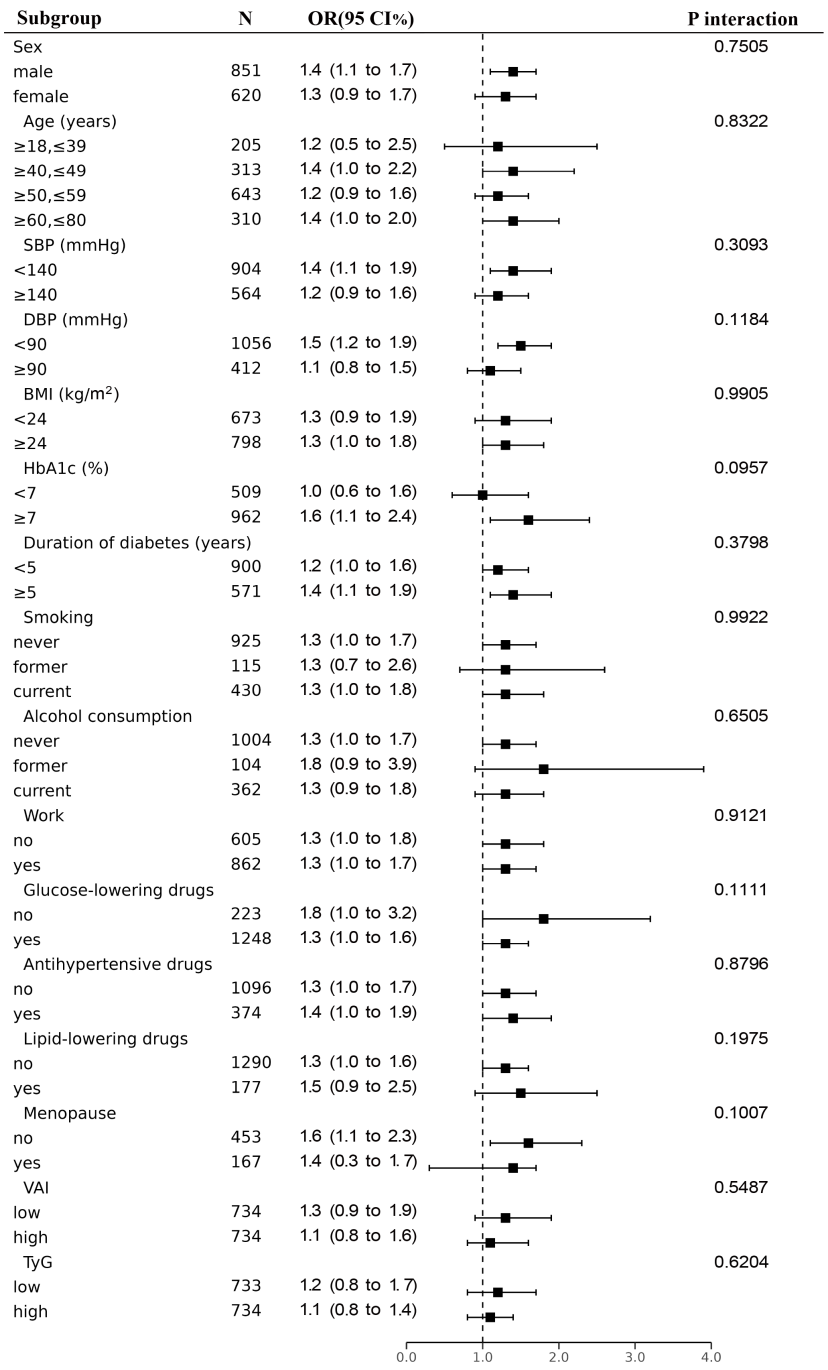


FIGURE 3
Subgroup analyses for the association between lnLAP and elevated baPWV were adjusted for age, sex, SBP, DBP, BMI, smoking, alcohol, work, salt intake, regular exercise, HbA1c, TC, HDL-C, LDL-C, glucose-lowering drugs, anti-hypertensives, lipid-lowering drugs, and duration of diabetes, except for the stratified variable.

Although the mechanism by which LAP is associated with AS is unknown, many studies have suggested that it is likely to be attributable to IR (15), which predisposes the organism to a state of sub-clinical stress and induces a sustained chronic inflammatory response that leads to AS; IR increases the risk of AS after causing hyperinsulinemia (34–36). Physiological doses of insulin increase nitric oxide (NO) release via the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which is blocked by IR (37). Elevated levels of IR and insulin activate vascular endothelial Na+ channels, leading to decreased NO utilization and atherosclerosis. Furthermore, IR activates the renin-angiotensin-aldosterone system. Elevated aldosterone and insulin both increase glucocorticoid kinase-1 (SGK-1) activity, which promotes hypertension, IR, and obesity, increasing the risk of CVD (35).

Studies have also indicated that hyperinsulinemia with IR is also a risk factor for AS. In addition, the interaction of hyperglycemia and hyperinsulinemia exacerbates AS, which, depending on its pathophysiology, allows for the earlier development of hypertension and CVD in individuals with T2DM (38). LAP has been shown to have a high recognition of IR and is strongly associated with the development of T2DM, hypertension, and the metabolic syndrome (MetS) (28, 39, 40). Therefore, it is important to reduce LAP or maintain LAP at normal levels in individuals at risk for T2DM who are prone to a combination of multiple metabolic abnormalities.

However, our study included the following restrictions as well: First, because this study is cross-sectional, we are unable to draw conclusions about the causes of LAP and baPWV or rule out recall bias as a result of cross-sectional studies; future follow-up data from the MMC may provide more precise evidence. Second, this study was conducted only in Chinese T2DM patients, so the applicability of this study to other populations needs to be further verified.

## 5 Conclusions

To summarize, in patients with T2DM, our study found a significant positive correlation between LAP and baPWV. According to the findings, LAP can be utilized in epidemiological studies and actual clinical practice as a quick and accurate instrument for determining the risk of AS.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by the Medical Ethics Committee of the Changde Hospital, Xiangya School of Medicine, Central South University (Program number YX-2023-072-01). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

## Author contributions

JM: Formal Analysis, Methodology, Writing – original draft. SG: Project administration, Writing – original draft. QZ: Methodology, Writing – review & editing. FY: Data curation, Writing – review & editing. HZ: Data curation, Investigation, Writing – review & editing. HL: Investigation, Supervision, Writing – review & editing. JJ: Software, Supervision, Validation, Writing – review & editing. QL: Software, Supervision, Validation, Writing – review & editing. ZD: Conceptualization, Funding acquisition, Resources, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Role of pyroptosis in diabetic cardiomyopathy: an updated review

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Diabetic cardiomyopathy (DCM), one of the common complications of diabetes, presents as a specific cardiomyopathy with anomalies in the structure and function of the heart. With the increasing prevalence of diabetes, DCM has a high morbidity and mortality worldwide. Recent studies have found that pyroptosis, as a programmed cell death accompanied by an inflammatory response, exacerbates the growth and genesis of DCM. These studies provide a theoretical basis for exploring the potential treatment of DCM. Therefore, this review aims to summarise the possible mechanisms by which pyroptosis promotes the development of DCM as well as the relevant studies targeting pyroptosis for the possible treatment of DCM, focusing on the molecular mechanisms of NLRP3 inflammasome-mediated pyroptosis, different cellular pyroptosis pathways associated with DCM, the effects of pyroptosis occurring in different cells on DCM, and the relevant drugs targeting NLRP3 inflammasome/pyroptosis for the treatment of DCM. This review might provide a fresh perspective and foundation for the development of therapeutic agents for DCM.

KEYWORDS

**pyroptosis, diabetic cardiomyopathy, NLRP3 inflammasome, inflammation, mechanism**

## 1 Introduction

Diabetes mellitus (DM) remains a crucial public health concern. Diabetes and its complications have brought a huge medical burden to people all over the world (1, 2). There is an undeniable connection between diabetes and cardiovascular disease. Some studies have confirmed that DM can increase the likelihood of heart failure, irrespective of usual heart failure risk factors including hypertension and coronary heart disease (2). Cardiovascular complications resulting from DM are among the primary factors that contribute to mortality in patients with diabetes (1). There are

currently two main types of diabetes: type 1 diabetes(T1DM), which is mainly characterized by insulin deficiency, and type 2 diabetes (T2DM), which is mainly characterized by insulin resistance (3, 4). These two main types of DM can cause microvascular and macrovascular damage, leading to a variety of diabetes-related complications such as diabetic nephropathy, diabetic cardiomyopathy (DCM), diabetic retinopathy, and so on (5).

DCM is a distinctive cardiomyopathy defined by abnormal cardiac structure and function independent of other cardiac risk factors like coronary heart disease and hypertension (6). The pathophysiological mechanisms of DCM may be partially different in different types of DM, but less is known about the differences in the pathogenesis of DCM between different types of DM. In an analysis of a multiethnic sample, Eguchi et al. found that T2DM was positively associated with increased left ventricular mass, independent of factors such as obesity and race (7). However, few studies have shown that T1DM can promote the increase of left ventricular mass, which may be related to the younger age and insulin therapy in patients with type 1 diabetes (8). Unlike T1DM, T2DM is mainly caused by hyperglycaemia due to insulin resistance and hyperinsulinemia (3, 4). Insulin signaling in cells mainly involves two related pathways: the insulin receptor substrate 1 (IRS-1) pathway and the mitogen-activated protein kinase (MAPK) pathway (9). These two pathways crosstalk to form a complex and balanced insulin signaling system. However, insulin resistance breaks this balance, making the MAPK pathway dominate, and cell growth and metabolism become imbalanced, eventually leading to cardiac fibrosis and diastolic dysfunction (6, 9).

The development of DCM can be roughly divided into two stages: the early stage is marked by left ventricular hypertrophy and diastolic dysfunction, while heart failure with systolic dysfunction characterizes the late stage (10). Various metabolic disturbances, such as hyperglycaemia and hyperinsulinaemia, are present in DM. These disturbances have been found to cause cardiac hypertrophy and diastolic dysfunction, which are the primary manifestations in most DCM patients. As a result, DCM mainly leads to heart failure with preserved ejection fraction (HFpEF) (11–13). The occurrence and development of DCM is the result of a variety of factors. Previous reviews have summarized the possible mechanisms of DCM including insulin resistance, cardiac inflammation, advanced glycation end products (AGEs), and angiotensin II (Ang II) (14, 15). Nevertheless, the precise pathogenesis of DCM remains unclear.

Cardiomyocyte death is one of the key links in the development of DCM. In the last few years, the role of various programmed cell death in DCM has received extensive attention. There are also many ways of cell death in DCM, including cell apoptosis, autophagy, cell necrosis, and ferroptosis (16, 17). Pyroptosis is a recently identified type of programmed cell death that comes with an inflammatory response. In the process of DCM, metabolic disorders such as glucose and lipids are one of the triggers of myocardial injury. Inflammation plays an essential role in it (18, 19). At the same time, many scholars believe that inflammation plays a different role in different types of heart failure. Heart failure with reduced ejection fraction (HFrEF) is mainly related to myocardial ischemia and myocarditis, while HFpEF shows a greater association with inflammation (20). Clinically, the majority of patients with DCM have predominantly HFpEF, and pyroptosis accompanied by inflammation may be an important pathogenic factor of DM and DCM. Therefore, it is imperative to investigate the related mechanisms and therapeutic interventions.

## 2 Conception of pyroptosis

Pyroptosis is one of programmed cell death (21). The term ‘ pyroptosis ‘ was first coined by Cookson and Brennan in 2001. The word consists of ‘ pyro ‘ and ‘ ptosis ‘. Pyro ‘ means fire, which means inflammation accompanied by pyroptosis, while ‘ ptosis ‘ means fall, consistent with other programmed cell death (22). Pyroptosis is mainly triggered by inflammasomes and executed by the caspase and Gasdermin families (23, 24). Furthermore, pyroptosis can manifest in diverse cell types, including the digestive system, urinary system, central nervous system, reproductive system, and cardiovascular system (25). Pyroptosis promotes the development of multiple chronic diseases (26).

## 3 Molecular mechanism of pyroptosis

The Gasdermin family and the Caspase family play an important role in pyroptosis. The Gasdermin family is predominantly found in the gastrointestinal tract and skin (27, 28). The Gasdermin family mainly consists of six proteins: GASDMA, GASDMB, GASDMC, GASDMD, GASDME, and DFNB59 (28). In addition to DFNB59, the family members exhibit two conserved domains: the N-terminal pore-forming domain and the C-terminal repression domain (28). The cleavage and release of the N domains can lead to the creation of oligomeric pores, which have a diameter of approximately 10-20 nm (29). Pyroptosis is also regulated by caspases, an evolutionarily conserved family of cysteine proteases (30). Caspases hydrolyze a variety of cellular protein substrates to coordinate cell apoptosis, and caspases are also the key players in pyroptosis (31). The molecular mechanism of pyroptosis is mainly divided into three pathways (Figure 1): canonical pathway, non-canonical pathway, and Caspase-3/-8-mediated pathway (32). The canonical pathway is mainly mediated by Caspase-1, while the non-canonical pathway is independent of Caspase-1, mainly mediated by Caspase-4/-5/-11 (Caspase-4/-5 in humans, Caspase-11 in mice) (28, 32).

In the canonical pathway, pattern-recognition receptors (PRRs) of the host cell are identified by recognizing a variety of stimuli, including two main categories, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (32, 33). Toll-like receptor 4 (TLR4), which is part of the pattern recognition receptor, is able to interact with PAMPs and DAMPs to initiate the NF-κB pathway (34). NF-κB binds to the inhibitory protein IκB and remains inactive in the cytoplasm (35). However, IKKβ phosphorylation can ubiquitinate IκBα, resulting in its dissociation from NF-κB (36, 37). Activated NF-κB is able to relocate to the nucleus, which ultimately initiates the transcription of genes associated with inflammation (38). In DCM, elevated

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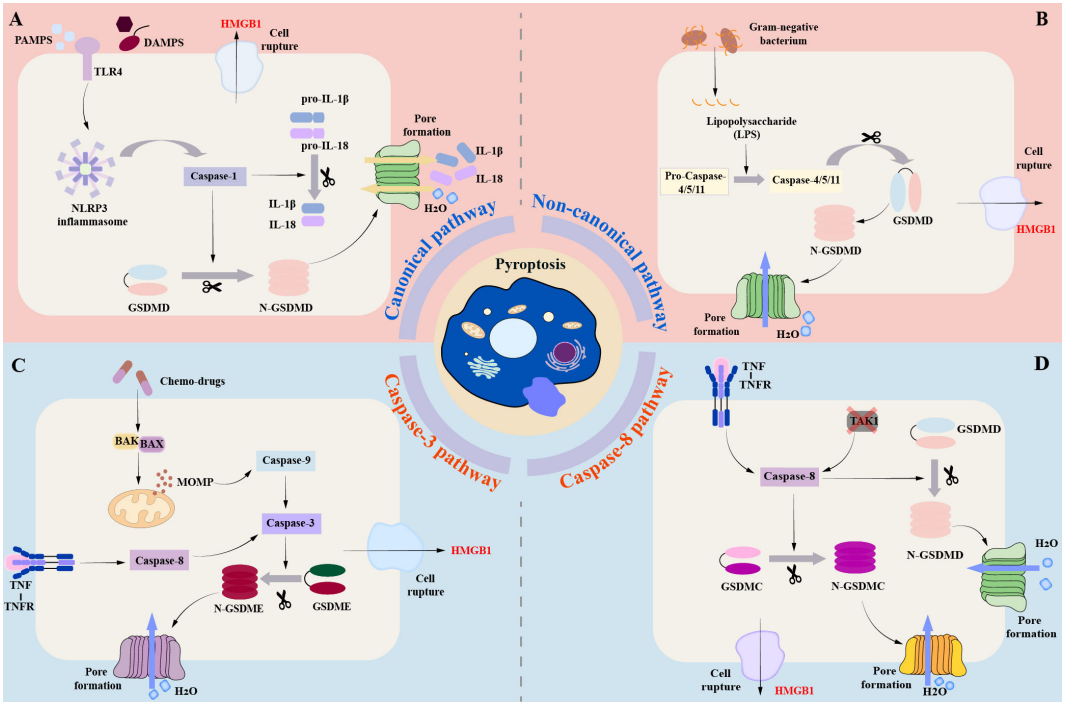


FIGURE 1
Brief molecular mechanism of Pyroptosis. **(A)** (canonical pathway): In the canonical pathway, when the host cell receptor recognizes various stimuli, it mainly includes pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which can promote the activation of downstream pro-Caspase-1 into mature Csapase-1, and promote the assembly of inflammasome. Mature Caspase-1 can cleave GSDMD to form N-GASDMD. Subsequently, the N-terminal poreforming domain of GSDMD can non-selectively penetrate the cell membrane to form membrane pores, which further leads to cell swelling, cracking, and death. **(B)** (Non-canonical pathway): In the non-canonical pathway, LPS can directly bind to the CARD domain of Caspase-4 / 5 / 11 to achieve activation. The activated Caspase-4 / 5 / 11 can also cleave GSDMD to form N-GSDMD, thus promoting the occurrence of pyroptosis. **(C)** (Caspase-3-mediated pathway): Unlike the canonical and non-canonical pathways, activated Caspase-3 mainly mediates the formation of membrane pores by cutting GSDME and promoting the N-GSDME domain to the cell membrane, leading to the occurrence of pyroptosis. **(D)** (Caspase-8-mediated pathway): Under the stimulation of TNF-α, Caspase-8 can also specifically cleave GSDMC to produce N-GSDMC to induce pyroptosis.

glucose levels stimulate advanced glycation end product (AGE) synthesis, which binds with receptors for AGEs (RAGE) causing activation of the NF-κB pathway through phosphorylation of IKKβ (39). Inflammasomes are macromolecular protein complexes that play an important role in the immune system. The NLRP3 inflammasome is the most extensively studied, consisting of pyrin domain-containing NOD-like receptor protein 3 (NLRP3), CARD-containing apoptosis-associated speck-like protein (ASC), and effector protein Caspase-1 (40, 41). Typical activation of the NLRP3 inflammasome consists of two stages: initiation and subsequent activation. Initiation is mainly achieved via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (34). Studies have confirmed that the activation of NLRP3 inflammasome is a key step in pyroptosis, which also plays an essential role in promoting the occurrence and development of diabetic complications such as DCM (42, 43). The activated NLRP3 inflammasome can cleave pro-Caspase to generate active Caspase-1. Activated Caspase-1 can promote the maturation of IL-1β and IL-18, in addition, it can also cleave GSDMD to form C-GSDMD and N-GSDMD. Subsequently, its N-terminal pore-forming domain oligomerizes in the membrane and binds to phosphatidic acid (PA) and phosphatidylserine (PS) to promote the formation of cell membrane pores (44). The GSDMD-mediated membrane pore is a non-selective channel that promotes the release of inflammatory substances such as IL-1β and IL-18 in cells (44–46). At the same time, extracellular fluid can enter the cell to cause cell swelling, so that the cells form bubble-like protrusions (pyroptotic bodies), eventually leading to cell membrane rupture, and finally HMGB1 and ATP flow out of the cell, causing pyroptosis (44–46).

In the non-canonical pathway mediated by Caspase-4/-5/-11, LPS can directly interact with the CARD domain of Caspase-4/-5/-11, promote the cleavage of GSDMD by active Caspase, and trigger pyroptosis (27, 32, 33, 47). Activated Caspase-4/-5/-11 could not cleave pro-IL-1β and pro-IL-18, but activated caspase-11 could cleave pannexin-1 and induce ATP release (27, 32, 33, 47). ATP binds to the P2X7 receptor and mediates potassium ion release. The efflux of ATP and potassium ions can promote the activation of NLRP3 inflammasome (41, 48). The activated NLRP3 inflammasome can promote the maturation of IL-1β and IL-18 and release them into the extracellular space through the membrane pores formed by GSDMD (27, 33, 45, 47).

Caspase-3 and Caspase-8 were previously thought to be apoptosis-related caspases, which did not stimulate GSDM to induce pyroptosis. In recent years, researchers have found that Caspase-3 and Caspase-8 can also mediate pyroptosis (32, 49, 50).

Caspase-3 can induce pyroptosis by cleaving GSDME (51). Caspase-3 is mainly initiated by various chemotherapeutic drugs (52, 53). In the process of chemotherapy, chemotherapeutic drugs can initiate BAK, BAX activation, and oligomerization of the mitochondrial outer membrane, resulting in mitochondrial outer membrane permeability (MOMP) (52, 53). Subsequently, Caspase-9 and downstream Caspase-3 were activated to mediate the cleavage of GSDME, resulting in C-GSDME and N-GSDME.N-GSDME was ectopic to the plasma membrane and promoting the formation of membrane pores (54). Under the stimulation of TNF-α, Caspase-8 can specifically cleave GSDMC to produce N-GSDMC, and then induce pyroptosis (32, 49, 55). In addition, inhibition of TGF-β-activated kinase-1 (TAK1) can cause Caspase-8-dependent GSDMD cleavage during Yersinia infection, which in turn leads to pyroptosis (56). Interestingly, death receptor signaling-induced Caspase-8 can also activate Caspase-3 (57). In addition, recent studies have confirmed that GSDME and Caspase-3 can be cleaved by granzyme B at the same site (58). Granzymes can directly cleave GSDME and induce pyroptosis. At the same time, Liu et al.also found that CAR-T cells can release granzyme to activate Caspase-3 in target cells, causing extensive pyroptosis (59). Therefore, some scholars have also referred to this pathway as the granzyme-mediated pyroptosis pathway (32).

## 4 The differences between pyroptosis and apoptosis

Pyroptosis and apoptosis are both programmed cell death, and there are some similarities between them. For example, both of them rely on Caspases, which damage DNA and cell membrane blebbing (29, 32, 60). However, Regardless of morphology or mechanism, pyroptosis is different from apoptosis (Table 1). Compared with apoptosis, pyroptosis is significantly different in that pyroptosis can promote the occurrence of inflammation, which can lead to cell swelling and incomplete cell membranes (29, 32). In addition, although both of them can cause DNA damage, unlike apoptosis, the nucleus remains intact during pyroptosis, and the nucleus is broken when apoptosis occurs (61, 62). The plasma membrane undergoes diverse morphological alterations during these two processes. During apoptosis, apoptotic bodies can form, while pyroptotic bodies may develop during pyroptosis (29). Apoptotic bodies are subcellular structures that consist of intracellular material, including DNA, organelles, and nuclear debris, and their formation is dependent on membrane blebbing (63, 64). Pyroptotic bodies are a new cellular morphological structure discovered by observing cells undergoing pyroptosis through Time-lapse electron microscopy (29). The nature of pyroptotic bodies remains unclear, but it is interesting to note that pyroptotic bodies share a resemblance to apoptotic bodies, with diameters of about 1-5 μm (45). Furthermore, pyroptosis undermines the integrity of the plasma membrane. Cell death was previously usually classified as apoptosis and necrosis (65, 66). Apoptosis is an active, programmed, non-inflammatory form of cell death. Whereas necrosis is a passive, accidental, non-programmed, with inflammatory response (65, 66). Subsequently, the researchers uncovered another form of cell death that has the characteristics of necrosis but can be highly regulated, called regulated necrosis (67, 68). Both necroptosis, also referred to as programmed necrosis, and pyroptosis are forms of regulated necrosis, characterized by the disruption of cytoplasmic membrane integrity and cellular content leakage (67, 69). There are numerous similarities between necroptosis and pyroptosis, both of which have the potential to initiate inflammation (70). Furthermore, studies conducted on animals have indicated that necroptosis is significant in the occurrence of myocardial infarction, renal ischemia, and ischemia-reperfusion in stroke (71–73). RAW-asc cells have the ability to undergo necroptosis and pyroptosis when exposed to various stimuli. Through electron microscopy observation, Chen et al. noted that necroptotic cells become rounded and eventually rupture explosively, while pyroptosis produces flat pyroptotic bodies before cell rupture occurs. This research also affirms that pyroptosis, in contrast to MLKL channel-mediated necroptosis, requires the creation of non-selective pores by GSDMD-N to enable cell lysis (45).

The caspase family functions critically in pyroptosis as well as apoptosis. Caspases, when activated, can lead to programmed cell death. Additionally, they can also determine the type of cell death that takes place (30, 31, 74, 75). According to their functions, mammalian Caspase can be classified into two types: Apoptotic caspases and Inflammatory caspases (75). Apoptotic caspases encompass Caspase-2, -7, and -10, while inflammatory caspases mainly include Caspase-1, -4, -5, -11and-12 (30, 75, 76). Although the Caspases involved in apoptosis and pyroptosis are not all the same, Caspase-3,-6,-8,-9 have now been shown to play a role in both apoptosis and pyroptosis (32). Inflammatory caspases can accomplish pyroptosis by cleaving different gasdermin. In contrast to inflammatory caspases, apoptotic caspases primarily

TABLE 1 The differences between pyroptosis and apoptosis.

| Characteristics | Pyroptosis | Apoptosis |
|---|---|---|
| membrane rupture | YES | NO |
| Cell swelling; | YES | NO |
| Nucleus intact | YES | NO |
| Inflammation | YES | NO |
| Pore formation | YES | NO |
| Apoptotic bodies | NO | YES |
| pyroptotic bodies | YES | NO |
| Caspase-1 | YES | NO |
| Caspase-2 | NO | YES |
| Caspase-4 | YES | NO |
| Caspase-5 | YES | NO |
| Caspase-7 | NO | YES |
| Caspase-10 | NO | YES |
| Caspase-11 | YES | NO |
| Caspase-12 | YES | NO |

execute a type of programmed cell death termed apoptosis, which is immunologically silent (77). Depending on their role in the process of cell death, caspases can be classified as either initiation proteins, including caspase-2, -8, -9, and -10, or effector proteases, namely caspase-3, -6 and -7 (77). Notably, most of the caspases that induce pyroptosis can also induce apoptosis in the absence of the corresponding GSDM proteins, implying that cleavage of GSDM by inflammatory caspases can convert apoptosis into pyroptosis (78). Meanwhile, specific cleavage of GSDM by inflammatory caspases is necessary for pyroptosis (74).

# 5 Mechanisms of pyroptosis-triggered DCM

The development of DCM is a complex process driven by multiple factors. Chronic inflammation is one of the key drivers of DCM development. Chronic inflammation can lead to cellular death by triggering the activation of inflammatory vesicles such as NLRP3 (79, 80). Pyroptosis can promote the development of DCM through multiple pathways (Figure 2), and both myocardial cells and non-myocardial cells can undergo pyroptosis to accelerate the development of DCM (Figure 3) (25). Understanding the effects of the different pathways of pyroptosis and the cell types that undergo cellular pyroptosis on DCM may provide possible therapeutic targets for DCM.

## 5.1 NLRP3 regulatory pathways associated with DCM

### 5.1.1 NF-κB/NLRP3 inflammasome pathway

The NF-κB pathway promotes the activation of NLRP3 inflammasome, which are involved in multiple chronic inflammatory diseases such as Ulcerative Colitis, lupus nephritis, rheumatoid arthritis, and so on (81–83). In recent years, NF-κB-related signaling pathways have been recognized as crucial in the pathophysiology of DCM. Luo et al. observed severe metabolic disturbances in diabetic rats and increased expression of NLRP3, ASC, caspase-1, and IL-1β in a rat model of type 2 diabetes induced by a high-fat (HF) diet and low-dose streptozotocin (STZ). Furthermore, it was shown that NF-κB is involved in the activation of NLRP3 inflammasome in high glucose-induced H9C2 cells (84). Another study demonstrated that the use of

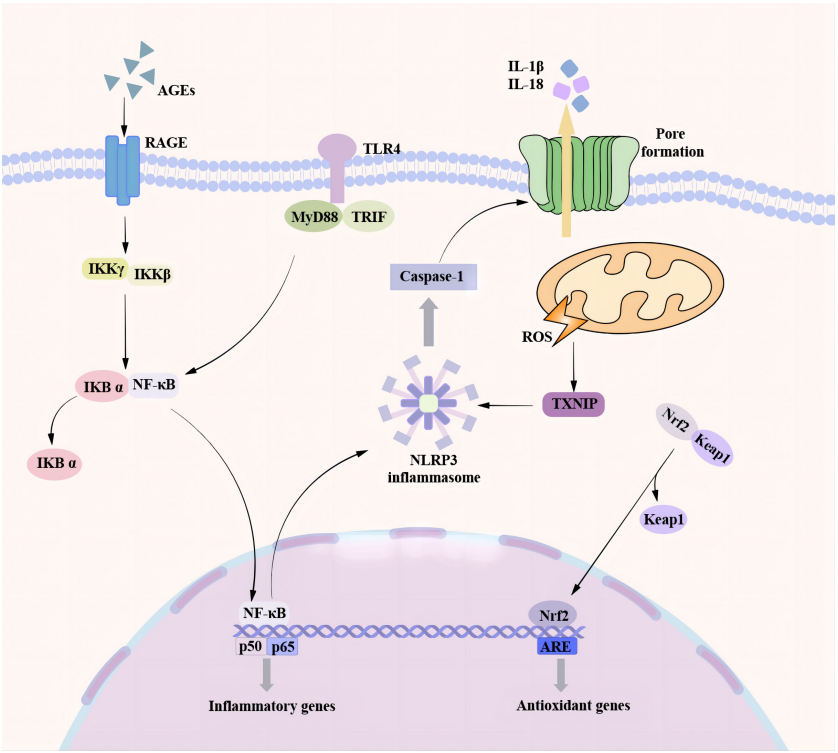


FIGURE 2
Pyroptosis pathway associated with DCM. There are many pathways involved in the process of pyroptosis promoting the occurrence and development of DCM. In the NF-κB / NLRP3 signaling pathway, the TLR4 receptor senses various stimulating factors, separates the NF-κB in the cytoplasm from the inhibitory protein IκB, and transfers to the nucleus, resulting in an increase in the expression of NLRP3 and the activation of NLRP3 inflammasome. A high glucose environment can induce the production of a large number of ROS. TXNIP is a ROS-dependent NLRP3 inflammasome activation regulator. A large amount of ROS can promote the binding of TXNIP to NLRP3 and trigger the activation of NLRP3 inflammasome. As a transcription factor, Nrf2 can regulate cardiac homeostasis by controlling various antioxidant genes to inhibit oxidative stress. Nrf2 is transferred to the nucleus under the action of ROS and oxidative stress and binds to the promoter region of the antioxidant response element (ARE) to promote the production of antioxidant enzymes and protect cardiomyocytes. At the same time, Nrf2 can also inhibit NF-κB to reduce the formation of NLRP3 inflammasome and inhibit pyroptosis.

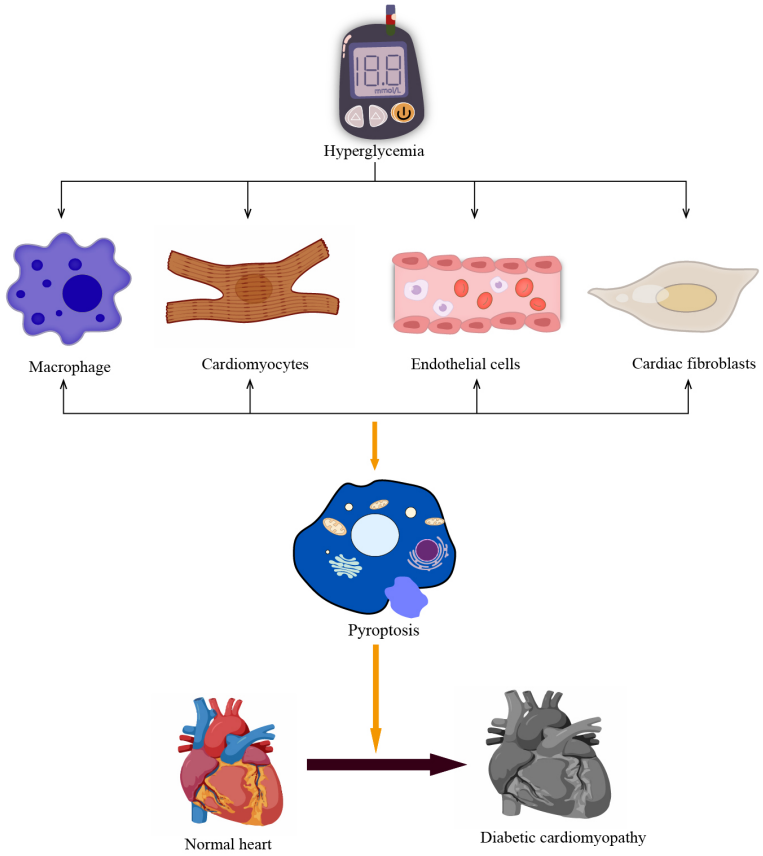


FIGURE 3
Pyroptosis in different cells promotes the development of DCM. The prolonged hyperglycemia causes pyroptosis of non-myocardial cells such as cardiomyocytes and macrophages, vascular endothelial cells, and cardiac fibroblasts, promoting poor cardiac remodeling and accelerating the development of DCM.

tilianin and syringin in this model not only ameliorated metabolic disorders and improved cardiac function in diabetic rats but also significantly increased the expression of TLR4, NF-Kb, NLRP3, IL-1β (85).In the STZ-induced C57BL/6 mouse model, it was observed that DCM was prevented in STZ-induced diabetic mice by inhibiting NF-κB and NLRP3 (86). High glucose (HG) enhances the expression of IL-1β, NLRP3, caspase-1, and ASC along with TLR4 protein levels in HUVECs. These effects were reversed upon TLR4 silencing (87). Besides, the study revealed that in H9C2 cells, high glucose led to an increased expression of NLRP3 inflammatory vesicles, TLR4, and NF-κB. However, the utilization of exogenous H2S effectively inhibited the activation of the TLR4/NF-Kb/NLRP3 pathway (88). All the experiments mentioned indicate the crucial involvement of TLR4/NF-κB/NLRP3 inflammasome pathway in the onset and progression of DCM. Targeting a specific link in this pathway can offer a new therapeutic approach for DCM.

### 5.1.2 Reactive oxygen species/thioredoxin interacting protein/NLRP3 inflammasome pathway

ROS is a by-product produced by specific enzymes during mitochondrial respiration or metabolism (89). ROS has two sides, it has a beneficial effect while also causing damage to the body (89). Long-term adverse environments such as hyperglycaemia, hyperlipidemia, and chronic inflammation can lead to the up-regulation of ROS-producing enzyme activities, resulting in a large amount of ROS and inducing oxidative stress (89, 90). It has been shown that HG causes a significant increase in ROS production in H9C2 cardiomyocytes, leading to the activation of NLRP3 inflammatory complexes via cytochrome C-dependent pathways (91). TXNIP serves as a regulator of NLRP3 inflammasome activation dependent on ROS. Researchers have shown that ROS promotes the binding of TXNIP to NLRP3, leading to the activation of the NLRP3 inflammasome (92–94). Suppression of ROS significantly diminished the expression of TXNIP, NLRP3 inflammatory vesicles, and IL-1β in H9C2 cells induced with high glucose. Meanwhile, suppression of TXNIP expression through the use of TXNIP-siRNA plasmid decreased downstream caspase-1 and IL-1β activation. Moreover, Silencing NLRP3 attenuated the expression of caspase-1 and IL-1β, while alleviating left ventricular dysfunction and reversing myocardial remodeling in diabetic rats on the HF diet and in low-dose STZ-induced diabetic rats (95). These indicate that ROS and the resulting oxidative stress can cause negative changes in the structure of the heart and hasten the progression of DCM (89, 93). Furthermore, in a rat INS-1 pancreatic β-cell-related study, Liu et al. found that HG significantly increased the expression of TXNIP, NLRP3, and caspase-1 in the cells and that TXNIP showed a dose-dependent relationship with glucose concentration and that TXNIP expression increased with

increasing glucose concentration (96). These all suggest that the ROS/TXNIP/NLRP3 inflammasome pathway plays an important role in DM and may accelerate the progression of DCM.

### 5.1.3 Nrf2-related pathways

Nuclear factor-erythroid-2-related factor 2 (Nrf2) is a transcription factor that is expressed in a variety of tissues and organs. Studies have demonstrated that the Nrf2 signaling pathway is closely related to various cardiac diseases, which can regulate cardiac homeostasis by inhibiting oxidative stress by controlling various antioxidant genes (97). Under physiological conditions, Nrf2 binds to and is present in the cytoplasm with its repressor Kelch-like epichlorohydrin-associated protein 1 (Keap1) (97, 98). However, large amounts of ROS and oxidative stress dissociate Nrf2 from Keap1, causing Nrf2 to transfer to the nucleus to bind to the promoter region of the antioxidant response element (ARE), which activates antioxidant genes and promotes antioxidant enzyme production (97, 98). These antioxidant enzymes have anti-inflammatory and antioxidant effects, protecting cardiomyocytes from myocardial damage caused by diabetes and high glucose oxidation (98). A study showed that Nrf2 upregulated the antioxidant protein HO-1 expression in STZ-induced SD rats and HG-cultured H9C2 cells, suppressed cardiomyocyte pyroptosis and impeded the progression of DCM (99). In contrast, blocking the Nrf2 pathway with ML385 did not decrease the expression of proteins associated with cellular pyroptosis (NLRP3, caspase-1, etc.). Furthermore, this study discovered that Nrf2 activation impedes NF-κB, leading to a decrease in the production of NLRP3 inflammasomes and the inhibition of pyroptosis, ultimately leading to cardioprotection (99). Li et al. observed similar results in C57BL/6 mice, where the use of Luteolin increased Nrf2 expression, attenuated cardiac oxidative stress, and provided cardioprotection (100). Another experiment using the Nrf2 knockout vector showed that cardiomyocytes were highly susceptible to high glucose-induced injury. The expression of NLRP3, caspase-1, IL-18, and IL-1β was also significantly increased. Meanwhile, DM rats treated with ML385 to suppress Nrf2 showed more severe myocardial fibrosis and cardiac enlargement (101). All of these studies provide valuable evidence that the Nrf2-related pathway may serve as a future target for treating DCM.

## 5.2 Effect of cardiomyocyte pyroptosis on DCM

Pyroptosis can occur in various cells of the heart, including cardiomyocytes, macrophages, fibroblasts, and endothelial cells. Cardiomyocytes are the main cells that make up the heart. Cardiomyocytes enable the heart to perform systolic and diastolic functions and help the heart pump blood throughout the body (102). The death of cardiomyocytes, whether acute or chronic, can cause irreversible damage to the heart. The activation of NLRP3 inflammasome plays an important role in the process of cardiomyocyte pyroptosis promoting cardiac dysfunction (43, 103, 104). When myocardial ischemia occurs, the potassium efflux in cardiomyocytes increases, thereby up-regulating the expression of NIMA-associated kinase 7 (NEK7) and promoting the activation of NLRP3 inflammasome (105–108). The activation of NLRP3 inflammasome in cardiomyocytes under pressure overload mainly depends on the regulation of NF-κB by calcium/calmodulin-dependent kinase II (CaMKII) (109–111). Hyperglycaemia promotes high ROS production, which binds TXNIP to NLRP3, thereby promoting the activation of NLRP3 inflammasome (43). Moreover, a high glucose environment can promote the pyroptosis of various cells in the heart, thereby accelerating the development of DCM (43). The death of cardiomyocytes plays an important role in the progression of DCM (80). New evidence also confirms that cardiomyocyte pyroptosis induced by NLRP3 inflammasome is a critical step in the progression of DCM (43, 112).

An experiment demonstrated that modulation of the miR-34b-3p/AHR axis in high glucose-induced HL-1 cells inhibited the activation of NLRP3 inflammasome and attenuated NLRP3-mediated pyroptosis in HL-1 cells. Furthermore, the hindrance of pyroptosis resulted in enhanced cardiac function and a reduction in cardiac hypertrophy among DCM mice (113). The expression of the NLRP3 inflammasome has been found to increase when PNRCMs are exposed to HG. This leads to pyroptosis in these cells (114). Likewise, within cardiomyocytes, such as those induced by HGin AC-16 and H9C2 cell lines, as well as primary cardiomyocytes separated from neonatal C57BL/6 mice, there was an increase in the expression of specific focal death-associated proteins, including caspase-1, NLRP3, and other proteins (115, 116). The conducted *in vivo* and *in vitro* experiments exhibit that suppressing cardiomyocyte pyroptosis ameliorates cardiac function and adverse remodeling besides delaying DCM progression. Furthermore, these outcomes highlight pyroptosis as a plausible therapeutic target for DCM.

## 5.3 Impact of non-cardiomyocyte pyroptosis on DCM

### 5.3.1 Macrophages

Macrophage pyroptosis also plays an important role in complications caused by hyperglycaemia (117). Macrophages constitute a vital aspect of the immune system, performing functions such as identification, phagocytosis, secretion, regulation of immunity, and maintenance of body homeostasis (118, 119). A study found that in diabetic mouse models, persistent hyperglycaemia can lead to macrophage dysfunction in which activation of NLRP3 inflammasome plays an important role. Partial macrophage dysfunction induced by hyperglycaemia can be restored by inhibiting the activation of cathepsin B and NLRP3 inflammasome (117). Simultaneously, researchers discovered that the activation of NLRP3 inflammasome in M1 macrophages can exacerbate cardiac dysfunction and encourage myocardial morphological changes in a post-stroke diabetic mouse model. The implementation of CY-09 to inhibit NLRP3 inflammasome has been shown to ameliorate cardiac function in diabetic mice (119). These findings suggest that the activation of NLRP3 inflammasome and pyroptosis in macrophages may contribute to the development of DCM.

### 5.3.2 Fibroblasts

DCM may manifest cardiac interstitial fibrosis in addition to cardiomyocyte hypertrophy. Cardiac fibroblasts are effector cells in

the process of myocardial fibrosis (120). When the heart suffers various injuries, cardiac fibroblasts can repair the heart by promoting the formation of collagen and extracellular matrix. However, when the damage is excessive, this repair matrix causes cardiac fibrosis, resulting in a decrease in cardiac compliance. In the diabetic state, metabolites such as glycosylation end products (AGEs) promote fibroblast differentiation into myofibroblasts (120, 121). Pyroptosis also plays an important role in the process of cardiac fibrosis. Ren et al. discovered that HG caused an increase in caspase-1, IL-1β mRNA, and protein expression in fibroblasts, as well as a similar trend in GSDMD protein expression. However, the administration of ranolazine led to a reduction in cardiac fibrosis in diabetic rats by inhibiting fibroblast pyroptosis and decreasing collagen deposition through the regulation of miR-135b (122). Another study also indicated that the expression of NLRP3, IL-1β, and GSDMD-N increased in fibroblasts following inducement by high glucose. Conversely, the inhibition of inflammation and pyroptosis in fibroblasts improved cardiac function and reduced fibrosis in diabetic C57BL/6 mice (123). These studies all confirm that fibroblasts play an important role in the progression of DCM and that modulation of fibroblast pyroptosis may alleviate cardiac fibrosis and delay the onset of DCM.

### 5.3.3 Vascular endothelial cells

Vascular endothelial cell dysfunction is also the key pathological basis for the occurrence and development of DCM (124). Vascular endothelial cells form a semi-permeable barrier between circulating blood and the extravascular matrix, and this endothelial barrier regulates cellular connectivity to maintain homeostasis in the body (125). During the development of DCM, activated NLRP3 inflammasome induces cellular pyroptosis releasing large quantities of pro-inflammatory factors IL-1β and IL-18. These pro-inflammatory factors subsequently bind to cell-surface receptors and enhance the expression of adhesion and chemokine in the endothelium, while increasing leukocyte adhesion and extravasation. This ultimately leads to the disruption of intercellular junctions, endothelial barrier dysfunction, and increased vascular permeability, which also facilitates the infiltration of pro-inflammatory cells and pro-inflammatory factors and accelerates adverse cardiac remodeling (124, 125). Increased NLRP3, caspase-1, and other cell death-related proteins were observed in HUVEC cells exposed to HG (126). A further study has verified that NLRP3 inflammasome-induced pyroptosis, can harm vascular endothelial cells and reduce the density of cardiac microvessels, resulting in negative cardiac restructuring (127).

## 6 Potential therapies of pyroptosis/ NLRP3 in DCM

The pathogenesis of DCM is very complex. Although its mechanism has been continuously improved for decades, there is still no effective treatment for DCM, which also makes the morbidity and mortality of DCM still high. As many researchers have found that pyroptosis plays an important role in DCM, targeted inhibition of pyroptosis and NLRP3 inflammasome may provide a good direction for the treatment of DCM. At present, these substances mainly include hypoglycemic drugs, phytochemicals, NLRP3 inflammasome, pyroptosis inhibitors, non-coding RNA, and so on (Table 2).

### 6.1 Hypoglycaemic drugs

Sodium-glucose co-transporter 2 inhibitor (SGLT2i) is one of the commonly used hypoglycemic drugs. SGLT2i plays a hypoglycemic role mainly by increasing urinary glucose excretion. In addition to lowering blood glucose, SGLT2i also has an extraordinary cardioprotective effect, which can reduce cardiovascular mortality and heart failure hospitalization rates in patients with type 2 diabetes (128). SGLT2i plays a role in delaying the progression of DCM by regulating metabolism, improving mitochondrial function, inhibiting oxidative stress, and reducing programmed cell death (129).

Empagliflozin is one of the typical SGLT2i, which has been widely used in patients with diabetes (130). A study showed that empagliflozin reduced the activation of NLRP3 inflammasome and attenuated pyroptosis caused by NLRP3 inflammasome in diabetic mice. In addition, this study also found that empagliflozin can inhibit oxidative stress by affecting the activity of the sGC-cGMP-PKG pathway, thus exerting cardioprotective effects (130). Dapagliflozin can inhibit SGLT2 to reduce NLRP3 inflammasome activation and delay the progression of DCM in type 2 diabetic mice. At the same time, it has a better inhibitory effect when combined with DPP-4 inhibitor Saxagliptin (131). It has also been found that ticagrelor, a P2Y12 receptor antagonist, acts synergistically with dagliflozin to slow the progression of DCM by attenuating the activation of NLRP3 inflammatory vesicles via the AMPK/mTOR axis (132).

Metformin is a kind of biguanide drug derived from herbaceous plants. It is the first-line treatment of diabetes. It can protect the heart in a variety of ways (133). Metformin was found to improve DCM by down-regulating the expression of NLRP3, Caspase-1, and IL-1β through AMP-activated protein kinase (AMPK) in cardiomyocytes treated with high glucose and diabetic mouse models (134). Studies have also shown that metformin can reduce the adverse effects of hyperglycaemia on streptozotocin-induced diabetic mice through the PK2/PKR pathway (135). Metformin can also produce incredible results when co-administered with other drugs. Ye et al. found that metformin significantly suppressed oxidative stress and inflammation when co-administered with the lipid-lowering drug atorvastatin, providing better protection against DCM (131). Metformin combined with hydrogen, Cocoa-Carob Blend, and Dendrobium Mixture can also show more effective cardioprotective effects (136–138).

In addition, certain hpoglycaemic drugs have demonstrated the ability to impede cellular pyroptosis in non-diabetic conditions. This implies that glucose-lowering drugs may potentially enhance DCM by inhibiting cellular pyroptosis. Zhao et al. induced HUVEC cells with oxidized LDL and observed that SGLT2i decreased cellular pyroptosis while enhancing endothelial cell dysfunction (139). In the case of ischemia/reperfusion injury, metformin

TABLE 2 Therapies and mechanisms targeting NLRP3 inflammasome/pyroptosis.

| Intervention strategies | Targeted therapies | Models | Mechanisms |
|---|---|---|---|
| **Hypoglycaemic drugs** | Empagliflozin | Spontaneous type 2 diabetic db/db mice | Reducing the activation of NLRP3 inflammasome and inhibiting pyroptosis; Regulating the activity of sGC-cGMP-PKG pathway to inhibit oxidative stress; |
| | Dapagliflozin | diabetic BTBR ob/ob mice | Inhibition of NLRP3 inflammasome activation via AMPK / mTOR axis; Combined with ticagrelor to inhibit the interaction of NLRP3 inflammasome through an AMPK-mTOR interplay; |
| | Metformin | streptozotocin-induced mice; High glucose-treated primary cardiomyocytes | Activate AMPK; Inhibition of mTOR pathway; Activating the PK2/PKR Pathway; |
| **Inhibitor compounds** | MCC950 | High glucose-treated primary cardiomyocytes | Binding to the Waller B site in the NACHT domain of NLRP3; |
| | CY-09 | diabetic db/db mice | Binding to the Waller A site in the NACHT domain of NLRP3; |
| **Non-coding RNA** | miR-30d | streptozotocin (STZ)-induced diabetic rats; High-glucose-treated cardiomyocytes; | Regulating cardiomyocyte pyroptosis by directly targeting foxo3a |
| | LncRNA KCNQ1OT1 | STZ-induced mice; High-glucose-treated AC16 cells and primary cardiomyocytes; | Targeting miR-214-3p and caspase-1 |
| | CircRNA DICAR | DICAR+/− and DICARTg mic | DICAR-VCP-Med12 degradation |
| **Phytochemicals** | Quercetin | STZ -induced diabetic rats; High-glucose-treated H9C2 cells; | Promoting nuclear Nrf2 nuclear translocation. |
| | Pomegranate peel extract | STZ-induced diabetic rats; | Inhibition of NLRP3 / caspase-1 / IL-1β signaling pathway; Down-regulation of lncRNA-MALAT1; |

inhibits NLRP3 inflammasome activation and exerts cardioprotective effects through the AMPK pathway (140).

## 6.2 Inhibiting compounds

NLRP3 inflammasome plays an important role in pyroptosis promoting the occurrence and development of DCM (42, 43). Inhibition of NLRP3 inflammasome can greatly inhibit pyroptosis and delay the progression of DCM. MCC950 is a small molecule that can effectively inhibit NLRP3 inflammasome (141). MCC950 binds to the Waller B site in the NACHT domain of the NLRP3 inflammasome, thereby blocking ATP hydrolysis and inhibiting NLRP3 inflammasome formation (142, 143). In high glucose-induced cardiomyocytes, the use of MCC950 can inhibit the expression of NLRP3, down-regulate the expression of pyroptosis-related proteins, and alleviate high glucose-induced LDH leakage (114). In a study in streptozotocin-induced diabetic mice, MCC950 was also found to delay the development of various complications associated with diabetes, including diabetic retinopathy, diabetes-associated atherosclerosis, and diabetic encephalopathy, by inhibiting the NLRP3 inflammasome and its downstream inflammatory response (144–146). The effect of MCC950 on DCM is still insufficient, but the above experimental results are undoubtedly encouraging. The exact effect of MCC950 on DCM and its related mechanisms still needs to be improved by researchers.

CY-09 is a compound that can effectively inhibit NLRP3 by directly binding to the ATP-binding motif of the NACHT domain of NLRP3 and inhibiting NLRP3 ATPase activity, thereby inhibiting the assembly and activation of the NLRP3 inflammasome (147). Jiang et al.also studied the mechanism of CY-09 inhibiting NLRP3 ATPase activity. The NLRP3 NACHT domain contains two sequences that are important for ATPase activity. The Walker A motif is an important motif for ATP binding, while the Walker B motif is essential for ATPase activity (148). Unlike MCC950, CY-09 binds to the Waller A site in NLRP3, thereby blocking the binding of NLRP3 to ATP, thereby blocking ATPase activity (147). CY-09 significantly ameliorates metabolic disorders in a diabetic mouse model (147), and can also alleviate inflammation, oxidative stress, and fibrosis in diabetic mice by selectively inhibiting NLRP3 inflammasome, thereby improving renal damage in diabetic nephropathy (149). Furthermore, CY-09 also alleviated insulin resistance and hepatocyte steatosis in diabetic mice (150). In terms of cardiac protection, CY-09 can inhibit the activation of NLRP3 inflammasome in M1 polarized macrophages and improve cardiac dysfunction after ischemic stroke (119). The above experiments show that CY-09 may also be used to delay DCM, but there are few studies on the relationship between CY-09 and DCM.

## 6.3 Non-coding RNA

In the process of pyroptosis accelerating the occurrence and development of DCM, non-coding RNA also plays an important regulatory role. Non-coding RNA (ncRNA) includes many types, including microRNA (miRNA), long non-coding RNA (lncRNA),

and circular RNA (circRNA) (151). Among them, miRNA is the most studied type of ncRNA (151). MiRNA is a class of endogenous small non-coding RNA molecules with a length of about 20 nucleotides. It can play a role in biological processes such as cell proliferation, differentiation, and apoptosis by inhibiting or activating gene expression (152). MiRNAs can be stably expressed in different body fluids, and the level of this miRNA in the blood can also change at different stages of the development of DCM (152). Therefore, miRNA may be a potential biomarker for DCM (152, 153). MiR-21, miR-30d, miR-223, and other miRNAs were found to be up-regulated in DCM, whereas miR-1, miR-9, miR-150, and other miRNAs were found to be down-regulated in diabetic conditions (153, 154). Recent studies have found that some miRNAs can slow down the development of DCM by regulating pyroptosis. *In vivo* and *in vitro* experiments confirmed that the expression of miRNA-30d in the diabetic group was significantly increased, and its increased expression up-regulated the expression of pro-inflammatory factors such as Caspase-1 and IL-1βand directly inhibited the expression of foxo3a and its downstream proteins, which promoted the pyroptosis of cardiomyocytes in DCM. However, these effects were reversed after the knockdown of miR-30d (155). Xu et al. also found that miR-223 is highly expressed in H9C2 cardiomyocytes induced by high glucose, and the use of miR-223 inhibitors can attenuate the activation of NLRP3 inflammasome and alleviate myocardial fibrosis, thereby delaying the development of DCM and protecting the heart (154). In the diabetic mouse model induced by high glucose and high fat, Deng et al.also found that miR-223 can regulate the expression of NLRP3 to reduce damage to endothelial cells (156). Meanwhile, it also shows that in the development of DCM, in addition to the myocardial cells themselves, endothelial cell death also plays an important role in promoting it.

In recent years, lncRNAs and circRNAs have also been found to play a regulatory role in DCM. LncRNAs refer to transcription RNA molecules with a length of more than 200 nucleotides, but they do not have protein-coding ability (157). LncRNA KCNQ1OT1 expression is increased in diabetic patients, high glucose-induced cardiomyocytes, and diabetic mouse models. Silencing KCNQ1OT1 inhibits pyroptosis by targeting miR-214-3p and Caspase-1, and also ameliorates abnormal cytoskeleton structure and calcium overload, improving cardiac structure and function (116). CircRNAs are joint regulators in various diseases. CircRNA DICAR has been shown to alleviate DCM, and knockout of DICAR can enhance pyroptosis in DCM (158).

### 6.4 Phytochemicals

In recent years, some substances extracted from herbs have received extensive attention in academia due to their anti-inflammatory effects. Flavonoids are the most abundant phytochemicals in plants, which can alleviate DCM by reducing myocardial oxidative stress and inflammation, so they have also been widely studied (159). Quercetin is a natural flavonoid. Zhang et al.found that quercetin increased the expression of antioxidant proteins such as HO-1 through the Nrf2 pathway and inhibited myocardial pyroptosis (99). Pomegranate peel extract was found to improve cardiac hypertrophy and myocardial fibrosis in diabetic rat models. The study also found that pomegranate peel extract may protect DCM by inhibiting the NLRP3/caspase-1/IL-1β signaling pathway and down-regulating the expression of lncRNA-MALAT1 (160).

Colchicine is a tricyclic alkaloid that is mainly used to treat inflammatory diseases such as gout. In cardiovascular diseases, colchicine is used in acute and recurrent pericarditis, coronary syndrome, atrial fibrillation, and heart failure (161). Colchicine can inhibit the activation of NLRP3 inflammasome by inhibiting the P2X7 receptor and blocking potassium efflux. Moreover, colchicine can also inhibit microtubule synthesis, promote microtubule degradation, and inhibit the assembly of NLRP3 inflammasome (162). Given the important role of colchicine in inhibiting the activation of NLRP3 inflammasome, it may have the potential to treat DCM. However, there are few studies on the role of colchicine in DCM.

## 7 Conclusion

DCM has been recognized for decades, and a large number of studies have been carried out to explore its potential pathophysiological mechanisms. However, the incidence of DCM is still increasing with the increasing prevalence of diabetes. The pathogenesis of DCM has been refined through continuous exploration by researchers, and several new drugs have been shown to have beneficial effects *in vivo* and *in vitro* models. Pyroptosis, as a programmed cell death with inflammation, plays an important role in the occurrence and development of DCM. Understanding the different pyroptosis pathways associated with DCM and the effects of pyroptosis of different cells on DCM will help us to find new therapeutic targets. Some researchers have also found that the intervention of pyroptosis by hypoglycemic drugs and inhibitors targeting NLRP3 inflammasome can delay the progression of DCM. However, the exact mechanism between DCM and pyroptosis has not yet been clarified, and it still needs further exploration by researchers.

## Author contributions

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# Salvianolic acids and its potential for cardio-protection against myocardial ischemic reperfusion injury in diabetes

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The incidence of diabetes and related mortality rate increase yearly in modern cities. Additionally, elevated glucose levels can result in an increase of reactive oxygen species (ROS), ferroptosis, and the disruption of protective pathways in the heart. These factors collectively heighten the vulnerability of diabetic individuals to myocardial ischemia. Reperfusion therapies have been effectively used in clinical practice. There are limitations to the current clinical methods used to treat myocardial ischemia-reperfusion injury. As a result, reducing post-treatment ischemia/reperfusion injury remains a challenge. Therefore, efforts are underway to provide more efficient therapy. Salvia miltiorrhiza Bunge (Danshen) has been used for centuries in ancient China to treat cardiovascular diseases (CVD) with rare side effects. Salvianolic acid is a water-soluble phenolic compound with potent antioxidant properties and has the greatest hydrophilic property in Danshen. It has recently been discovered that salvianolic acids A (SAA) and B (SAB) are capable of inhibiting apoptosis by targeting the JNK/Akt pathway and the NF-κB pathway, respectively. This review delves into the most recent discoveries regarding the therapeutic and cardioprotective benefits of salvianolic acid for individuals with diabetes. Salvianolic acid shows great potential in myocardial protection in diabetes mellitus. A thorough understanding of the protective mechanism of salvianolic acid could expand its potential uses in developing medicines for treating diabetes mellitus related myocardial ischemia-reperfusion.

KEYWORDS

**salvianolic acids A, salvianolic acids B, myocardial ischemia reperfusion injury, diabetics introduction, cardioprotection**

# 1 Introduction

Myocardial ischemia is one of the most common types of cardiovascular disease that increases morbidity and mortality worldwide (1). Effective limitation of infarct size through timely restoration of blood flow to ischemic myocardium is the standard treatment to rescue ischemic myocardium and thus to improve the patient outcomes. Paradoxically, reperfusion itself causes cardiac injury, which is known as myocardial ischemia/reperfusion injury (MI/RI). Moreover, patients with diabetes are more vulnerable to MI/RI than those without diabetes (2), yet the underlying mechanism is incompletely understood. The complicities of the MI/RI which includes oxidative stress, calcium overload, inflammatory response, energy metabolism disorder, mitochondrial dysfunction, and apoptosis were shown in many studies (3, 4). Oxidative stress is known as an essential factor in myocardial ischemia reperfusion (I/R) (5, 6), and oxidative stress levels in the myocardium of diabetic patients were found to be significantly higher than that in non-diabetes. This could be one of the mechanisms attributable to the increased myocardial vulnerability to MI/RI in diabetes. In addition to increases in reactive oxygen species (ROS) and oxidative stress, increases in inflammation, reduction in cardiac Akt and STAT3 all occur in the myocardium of diabetes (7). These elements play important roles in diabetics complicated by MI/RI and may be attributed to the increased myocardial sensitivity to MI/RI (8, 9).

Danshen (Salvia miltiorrhiza Bunge), a traditional Chinese medicine that has been widely prescribed to patients with angina pectoris and hyperlipidemia was found to have a preventive effect in type 2 diabetic patients and in type 2 diabetic rats with nephropathy (10, 11). The chemical constituents of Danshen can be classified into two categories: water-soluble (hydrophilic) phenolic compounds and nonpolar (lipophilic) diterpenoidal compounds. Salvianolic acids are the major hydrophilic constituents amongst all. Among salvianolic acids, salvianolic acid A (SAA), salvianolic acid B (SAB), rosmarinus acid, danshensu, caffeic acid, and lithospermic acid are the main phenolic acids. SAA and SAB, in particular, are polyphenolic compounds known to have powerful antioxidant capacities (12).

Recent studies have demonstrated that SAA can exert anti-diabetic effects, preventing diabetic complications by reducing inflammatory response and improving lipid disorders (13), revealing the possible therapeutic effect of SAA on DM (14). Diabetes with MI/RI are not sensitive to pre-, post-conditioning cardioprotective interventions that are otherwise effective in non-diabetic subjects, while the related mechanisms are unclear. SAA can alleviate diabetes complications like vascular disease (14), but few studies support SAA can reduce MI/RI in DM and the mechanism has not been explored.

This review aims to provide a collective understanding of the potential effect of salvianolic acids in protecting against diabetes and myocardial ischemia-reperfusion in recent years and to explore whether salvianolic acid has the potential protective effects in Diabetes that are complicated by MI/RI. It is hopeful that such a collective understanding will help develop new therapeutic interventions for the clinical treatment of diabetic myocardial ischemia-reperfusion.

# 2 The pathogenesis and mechanism of MIRI

In 1960, Jennings et al. first reported MIRI, which is a condition that occurs when there is a temporary interruption of blood flow to the heart (ischemia) followed by the restoration of blood flow (reperfusion) (15). Restoring blood flow, such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) is the most effective method to improve patient outcome (16). However, reperfusion itself causes damage to the myocardium, leading to exacerbation of the initial ischemic injury, and as such, even effective restoration of blood flow does not attenuate MIRI (4). Reperfusion triggers a series of tissue responses that contribute to the injury. This includes the production of oxygen free radical and mitochondrial damage, release of inflammatory factors, endoplasmic reticulum stress, and amplification of tissue damage (17). MIRI results from complex pathophysiological mechanisms, including oxidative stress, inflammatory response, endothelial cell dysfunction, mitochondrial dysfunction, calcium overload, apoptosis and autophagy (18).

## 2.1 Oxidative stress

Reactive oxygen species (ROS) are small reactive molecules that play a significant role in various cellular functions and biological processes, including cell signaling and homeostasis, in almost all eukaryotic cells. Some examples of ROS include superoxide anion radical, hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^-$), ozone ($O_3$), and singlet oxygen ($O_2$). (19). Under physiological conditions, ROS production is tightly regulated and plays a beneficial role in cell proliferation and metabolism (20). However, when ROS levels becomes excessively high, they can cause oxidative stress, which is a state of imbalance between the production of ROS and the ability of cells to detoxify them or repair the resulting damage (21). Overexpression of ROS and hypoxia in the tissue microenvironment can disrupt normal tissue repair and regeneration. This disruption can contribute to the development of fibrosis, dysfunction, and severity of cardiovascular diseases.

The accumulation of ROS during I/R injury is a major cause of oxidative damage. This phenomenon is also observed in diabetic myocardial injury (22). A study by Liu et al. revealed that I/R-induced apoptosis is mainly a consequence of excessive oxidative stress. Persistent cellular injury including necrosis and apoptosis of cardiomyocytes are the result of intense oxidative stress, which in turn trigger mitochondrial production of ROS in the early stages of ischemia in response to many mildly harmful stimuli to modulate stimulus-induced tolerance to ischemia. During reperfusion, the electron transport chain dysfunction, specifically the dysfunction in complex I (NADH dehydrogenase) and III (coenzyme Q-cytochrome c reductase), leads to excessive release of ROS. This

results in the production of superoxide anions (O2-·), which are converted to hydrogen peroxide (H2O2) by the action of superoxide dismutase. In the presence of $Fe^{2+}$ and $Cu^{+}$, $H_2O_2$ is catalyzed to produce highly reactive hydroxyl radicals (OH), which can cause indiscriminate damage to nucleic acids, proteins, biofilms, and lipid peroxidation. This leads to mitochondrial depolarization, swelling, apoptosis, and cell death (17). Damaged and necrotic cells can activate Toll-like receptor 4 (TLR4) through damage-related molecular pattern (DAMP) activation. This leads to the aggregation of immune cells, which in turn express NADPH oxidase to promote the production of reactive oxygen species and further exacerbate myocardial damage (23). Overall, the overproduction ROS during I/R injury, along with the activation of TLR4 and NADPH oxidase, creates a vicious cycle that intensifies the damage to the myocardium.

## 2.2 Endothelial dysfunction

Endothelium regulates vascular tone, cell adhesion, thromboresistance, smooth muscle cell proliferation, and vascular wall inflammation by producing and releasing vasoactive molecules. This produces and releases vasoactive molecules such as prostaglandins, nitric oxide (NO), endothelium-dependent hyperpolarizing factors, and endothelium-derived contracting factors (24), which impact vascular tone, cell adhesion, thromboresistance, smooth muscle cell proliferation, and vascular wall inflammation. These molecules help to regulate the degree of vasodilation/contraction, tissue oxygen consumption balance, long-term organ perfusions, vascular structure remodeling, and metabolism (25). The integrity of the endothelial barrier depends on the intercellular junction complex located between adjacent endothelial cells (26). Endothelial dysfunction, characterized by impaired endothelial function, is primarily driven by oxidative stress and inflammation (27). Evidence has shown that endothelial injury is a key mediator of myocardial ischemia/reperfusion injury (26, 28). Additionally, I/R injury itself can lead to endothelial dysfunction, manifested by decreased nitric oxide production, vascular dystonia due to endothelial injury, and prolonged vasoconstriction.

During myocardial I/R, there is disruption of endothelial integrity and decreased microvascular permeability of cardiac myocardium after myocardial I/R (29), which increases the permeability of the endothelial barrier by destroying endothelial barrier function and aggravating the inflammation (30, 31). No reflux phenomenon of myocardium is also seen after I/R, leading to vascular leakage and neutrophil infiltration, and eventually apoptosis of cardiomyocytes and damage to myocardial function (30, 31).

## 2.3 Mitochondrial dysfunction

Mitochondria play a crucial role in oxidative stress and cell metabolism, and they are involved in various physiological functions such as endothelial mobilization, aging, proliferation, and growth (32). In cardiomyocytes, mitochondria are responsible for synthesizing about 90% of ATP, which is essential for normal heart functioning or cardiac functional recovery after various injuries (33, 34). Many studies have identified mitochondrial dysfunction as an important prominent mechanism for the progression of myocardial ischemia-reperfusion injury (34, 35). Abnormal mitochondrial fission, decreased mitophagy, and excessive mitochondrial oxidative stress can lead to endothelial dysfunction or death during cardiac reperfusion episodes (36). Mitochondrial dysfunction can lead to cell death through calcium imbalance, overproduction of mitochondrial ROS (mROS), disruption of cellular energy metabolism, impaired ATP production, and the opening of a structure called the mitochondrial permeability transition pore (MPTP), which can ultimately lead to cell death (34, 37). To counteract the effects of ROS and oxidative stress, mitochondria have a complex network of clearance systems (38). Studies have shown that abnormal mitochondrial fission can be an early indicator of mitochondrial dysfunction, and an imbalance between mitochondrial fission and fusion can lead to mitochondrial dysfunction, which in turn can aggravate MIRI damage (28, 34). Another factor contributing to injury is the accumulation of mitochondrial succinate, a metabolite that increases during hypoxia (39). This accumulated succinate is oxidized during reperfusion, resulting in the generation of ROS through a process called reverse electron transport (39). This excessive ROS production further contributes to oxidative stress and tissue damage. During myocardial ischemia, prolonged ischemia induces an increase in mitochondrial fission (40–42). When reperfusion occurs, the uncontrolled production of ROS triggers mitochondrial fission (34, 43, 44). This increased mitochondrial fission reduces mitochondrial membrane potential (MMP), making the MPTP more sensitive and leading to further ROS production. This disruption of the antioxidant balance within the mitochondria can result in the releasing of Cytochrome C (Cyt C) during cardiac microvascular I/R damage, activating caspases and initiating apoptosis through mitochondria-dependent pathways (45–48).

## 2.4 Calcium overload

Calcium plays an important role as the second messenger in various cellular processes, including cell proliferation, division, and energy metabolism. However, excessive calcium levels, known as calcium overload, can lead to detrimental effects in cellular function, as proposed by Zimmerman and Hulsmann in 1966 (49). In a stable internal environment, calcium inflow and outflow are dynamically balanced under the regulation of protein channels (50). Maintaining intracellular calcium homeostasis is crucial for the normal function and growth of cardiomyocytes. Calcium overload in cardiomyocytes can exacerbate ischemic damage, which occurs when blood supply to the heart is compromised (51). When calcium overload occurs, $Ca^{2+}$ dependent protease can promote the conversion of xanthine dehydrogenase to xanthine oxidase, promote the production of reactive oxygen species, and the high

concentration of Ca2+ in the cytoplasm increases mitochondrial uptake of Ca2+, which in turn forms calcium phosphate deposition in the mitochondria, and subsequently adversely affects ATP synthesis. Calcium homeostasis cannot be maintained during myocardial ischemia-reperfusion and intracellular calcium overload is a common pathway for irreversible damage of cells subjected to myocardial ischemia-reperfusion (52). During myocardial ischemia, adenosine triphosphate (ATP) production decreases, leading to intracellular acidosis and the activation of Na+/H+ exchange causing a large influx of sodium ions. This sodium influx, coupled with the high calcium concentration, contributes to calcium overload during reperfusion (53). Reperfusion also disrupts mitochondrial membrane potential and accelerates energy expenditure, resulting in mitochondrial calcium overload and excessive production of ROS. The accumulation of calcium ions in cells inhibits mitochondrial ATP synthesis, leading to instability in mitochondrial membrane potential and subsequent damage, such as contraction disorders and apoptosis (54), exacerbating post-hypoxic or post-ischemic cardiomyocytes injuries (40, 55, 56).

## 2.5 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) regulates the synthesis, folding, and transport of a significant portion of proteins in eukaryotic cells (57). High-quality protein folding can determine cell survival and function as well as normal physiological function. Endoplasmic reticulum homeostasis involves the binding of three ER transmembrane proteins: protein kinase R-like ER kinase (PERK), activated transcription factor 6 (ATF6), and enzyme 1 (IRE1) (58). Such a binding keeps them inactive, and their activation requires specific conditions such as the presence of inositol (58). During environmental injury or disease state, the disruption of endoplasmic reticulum homeostasis can lead to protein misfolding and accumulation of unfolded proteins. This triggers a response called endoplasmic reticulum stress, which activates the unfolded protein response (UPR). The UPR is a cellular mechanism aimed at reducing the burden and damage caused by the ER stress. It helps to restore protein homeostasis within the ER and rebuild the endoplasmic reticulum balance. However, if the endoplasmic reticulum stress becomes chronic or severe, it can promote cell death. Myocardial ischemia is an example that induces ER stress response (59). Activation of the endoplasmic reticulum stress-related pathway induces downstream activation of the apoptotic pathway, thereby promoting the progression of ischemia/reperfusion injury in myocardial tissue (60). Cardiomyocytes express high levels of endoplasmic reticulum stress-related signaling proteins, including transcription factor 6 (ATF6), C/EBP homologous protein (CHOP), glucose regulatory protein 78 (GRP78), etc. Treatments that Inhibit the signaling of endoplasmic reticulum stress can effectively reduce the rate of cell death in conditions like myocardial ischemia-reperfusion injury (61–64). Key cellular events in the pathogenesis of MIRI are summarized in Figure 1.

## 2.6 Current therapeutic interventions against MIRI and the pro-survival cardiac protective signaling pathways

Ischemic heart disease (IHD) is one of the most common diseases that affects the human lifespan. Percutaneous coronary

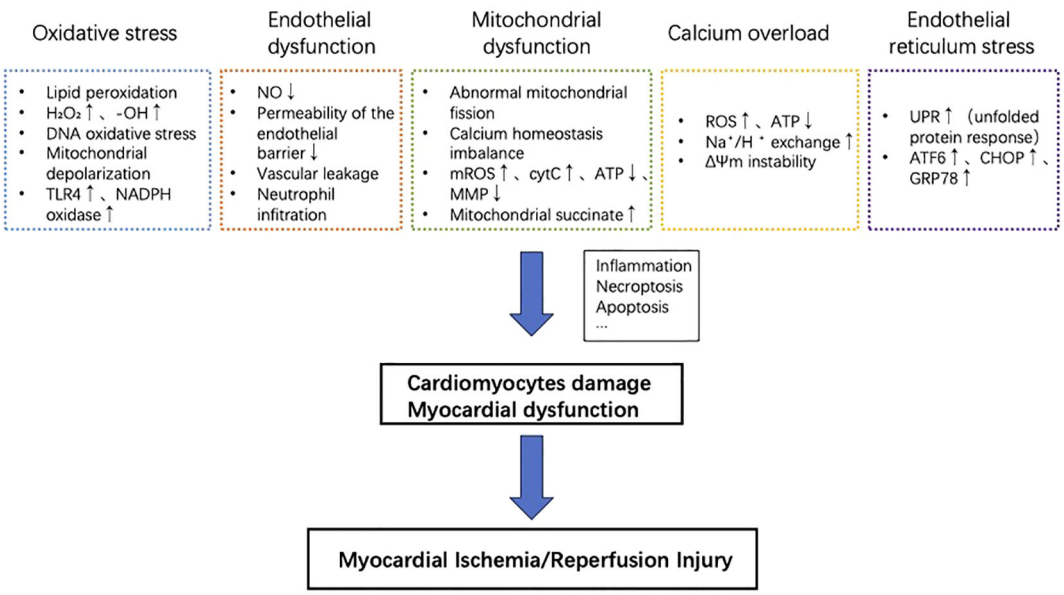


FIGURE 1
Key cellular events in the pathogenesis of MIRI. Oxidative stress, endothelial dysfunction, mitochondrial dysfunction, calcium overload, and reperfusion injury contribution to myocardial cell damage and cardiac dysfunction. These processes promote inflammation, necrotic apoptosis, apoptosis, and other mechanisms, ultimately exacerbating myocardial ischemia/reperfusion injury.

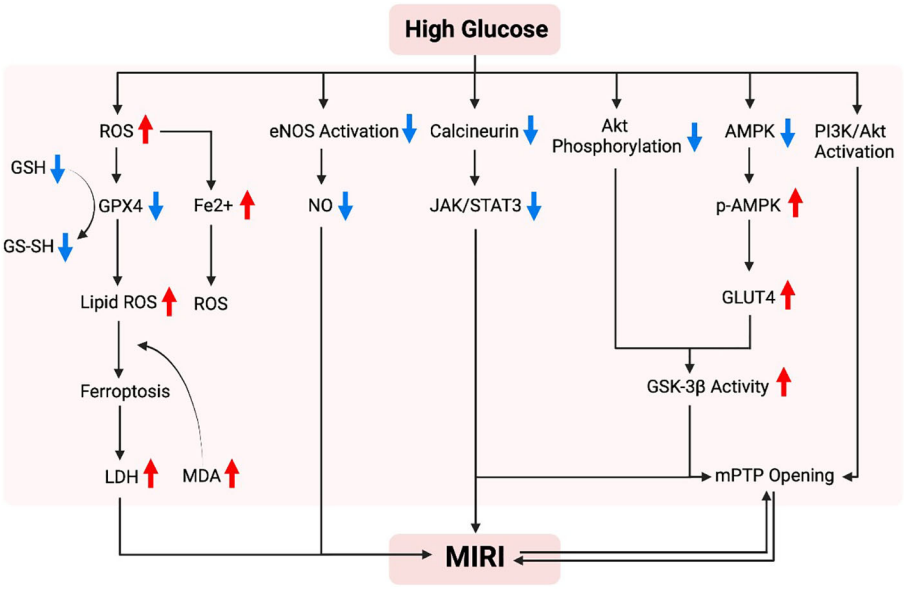


FIGURE 2
Possible mechanism of hyperglycemia in promoting myocardial ischemia-reperfusion injury. In a high-glycemic environment, there is an increased vulnerability to myocardial ischemia/reperfusion injury, which can be attributed to several mechanisms. These mechanisms include the overproduction of reactive oxygen species (ROS), an excessive burden of oxidative stress, abnormal alterations in mitochondria resulting in cell death due to iron overload, and dysfunction of endothelial nitric oxide synthase (eNOS). Furthermore, impaired protection against such injury is often linked to inadequate activation of pro-survival signaling pathways, including Akt, AMPK, JAK/STAT3, and PI3K/Akt.

angioplasty, coronary artery bypass grafting, and other reperfusion methods such as thrombolysis treatment are the most effective treatment methods for myocardial ischemic injury up to date. However, post-ischemic reperfusion itself can cause new heart damage called "ischemia/reperfusion injury". Although a variety of reperfusion therapies have matured, the development of therapies to reduce ischemia/reperfusion injury has been slow. Modern medical treatment has solved the technical problems of myocardial ischemia injury and blood flow recovery, but a series of complex processes of intracellular environmental changes that are caused by reperfusion after blood flow recovery have not been solved. The endogenous adaptive mechanism that occurs in cardiomyocytes in the face of ischemia-reperfusion or other types of metabolic stress challenges is called the pro-survival

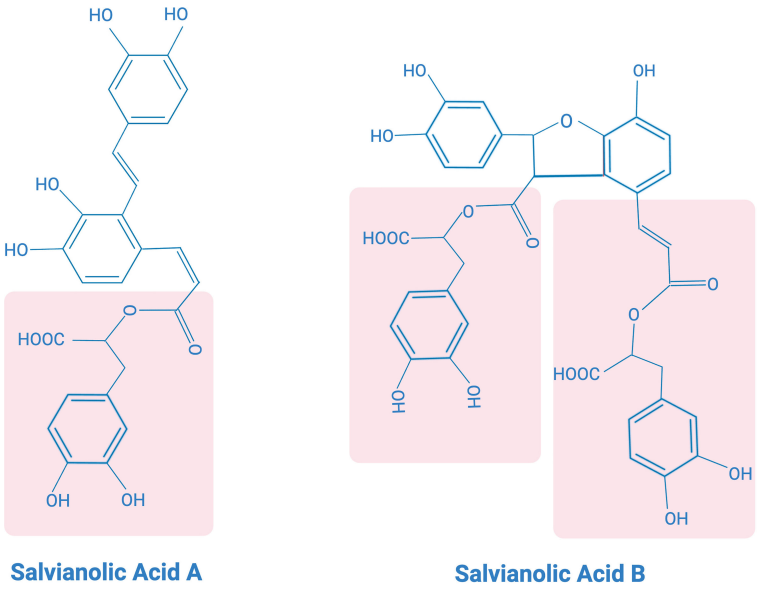


FIGURE 3
Chemical structures of salvianolic acid **(A)** and salvianolic acid **(B)**.

cardioprotective mechanism (65). The SAFE pathway, also known as the Survivor Activating Factor Enhancement pathway, is a signaling pathway that plays a crucial role in I/RI. This pathway is initially identified in studies investigating the cardioprotective effects of erythropoietin (EPO) against I/R injury (66, 67). It has been found that EPO activates the Janus Kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathway, particularly the JAK2/STAT3 pathway, to confer cardioprotection (68). In addition to EPO, other factors and pathways have also been implicated in activating the SAFE pathway. These include cytokines (such as interleukin-6 and interleukin-10), growth factors, and pharmacological agents (such as statins and opioids) (69). These factors can activate JAK/STAT signaling and trigger the downstream protective effects mediated by the SAFE pathway. The Reperfusion Injury Salvage Kinase (RISK) pathway is another signaling pathway that has a cardioprotective effect against I/R injury. It was first identified in studies investigating the cardioprotective effects of Ischemic Preconditioning (IPC), a phenomenon in which brief episodes of ischemia followed by reperfusion protect the heart against subsequent sustained I/R injury. Another cardioprotection mechanism that is relevant to this review is called postconditioning, which involves applying brief episodes of ischemia and reperfusion at the onset of reperfusion after a prolonged period of ischemia. This technique interrupts the initial reperfusion phase to protect the heart against I/R injury. The RISK pathway involves the activation of multiple protein kinases, including phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and extracellular signal-regulated kinase (ERK) (69–71). Activation of these kinases leads to the phosphorylation and activation of various downstream targets that confer cardioprotection. In addition to the aforementioned pro-survival protective signalings, intracellular signaling molecules are also involved in cardioprotective signaling pathways, such as protein kinase C (PKC), protein kinase A (PKA), protein kinase G (PKG), 5' amp activated protein kinase (AMPK), p38 mitogen-activated protein kinase (MAPK), extracellular signaling regulatory kinase 1/2 (ERK1/2) (65, 72, 73). However, the interaction between them has not been fully determined (65, 72, 73).

# 3 Increased myocardial susceptibility to MIRI in diabetes

Diabetes is a major risk factor for IHD. Diabetes not only increases the incidence of acute myocardial infarction and myocardial sensitivity to ischemia-reperfusion injury but also alters or diminishes the myocardial response to cardioprotective interventions such as ischemic conditioning that are otherwise effective in subjects without diabetes. In animal models, ischemia preconditioning has been shown to be cardioprotective and reduce myocardial I/R damage. A recent study conducted in the db/db mouse model of type 2 diabetes shows that diabetes disturbs functional adaptation of the non-ischemic remote myocardium after ischemia/reperfusion (74). However, the effects of pretreatment-mediated cardioprotective in diabetic animal models are still controversial and inconclusive. In the study of Tatsumi et al., diabetic myocardial pretreatment stimulation produces a more substantial protective effect compared to regular myocardial pretreatment stimulation (75), while other studies have shown that diabetes attenuates or inhibits pretreatment-mediated cardioprotective effects (76). Diabetes can trigger various histological, biochemical, and physiological changes that contribute to the aggravation of oxidative stress, apoptosis, inflammation, and other pathways by increasing inflammatory factors, which leads to cardiac dysfunction, and exacerbating myocardial ischemia-reperfusion phenomenon (77).

## 3.1 High glucose induced increase in ROS in the heart

Figure 2 studies conducted in diabetic rodents indicate that high glucose enhance superoxide generation and mitochondrial structural changes that increase the vulnerability of the myocardium to IR injury (78), and treatments that have anti-oxidant property attenuate myocardial IRI through improving mitochondrial homeostasis (28). Thus, excessive oxidative stress and impaired mitochondrial biogenesis in diabetic conditions rendered the diabetic heart more vulnerable to ischemic insults (56, 79). Mechanistically, Nrf2 nuclear translocation triggers Sirt3 upregulation and MnSOD activation, which subsequently reduces mitochondrial levels of ROS (mtROS). However, high glucose levels cause a downregulation of Nrf2 levels in the nucleus, resulting in Sirt3 downregulation and the acetylation of manganese superoxide dismutase (MnSOD), and thus facilitating the production of ROS (80). Energetic stress and mitochondrial ROS formation play critical roles in the pathogenesis of diabetic cardiomyopathy and MIRI (81, 82). AMPK, an important kinase involved in regulating energy homeostasis, plays a role in various metabolic process such as protein metabolism, lipid metabolism, carbohydrate metabolism, autophagy, and mitochondrial homeostasis. It is known that AMPK can sense cellular metabolic conditions and promote mitochondrial biogenesis. In the absence of glucose and ATP, AMPK is activated. Activation of AMPK has been shown to reduce the production of ROS and protect mitochondrial biogenesis. In the presence of high glucose, there is a dual inhibitory effect on AMPK. High glucose reduces the protein level and kinase activity of AMPKα, the catalytic subunit of AMPK. Researchers discovered that high glucose stimulation did not cause an increase in ATP levels, but it did cause an increase in the ratio of AMP/ATP and ADP/ATP (83). This suggests that ATP is not the cause of high glucose inhibition of AMPK signaling. Instead, high glucose promotes the production of ROS in cells (83). Under conditions of persistent hyperglycemia, elevated ROS levels are a causative factor in cell death (84). Under normal physiological conditions, cells have an antioxidant system to remove excess ROS. However, in diabetes, there is a decrease in antioxidant system activity and an increase in ROS production. This impairment between oxidant and antioxidant systems lead to oxidative stress, and results in various forms of damage to cells and tissue (84).

## 3.2 High glucose induced ferroptosis

Iron death, also known as ferroptosis, is a process in which iron-dependent cell death occurs due to increased lipid peroxidation. It is involved in various pathological processes such as cancer drug resistance, neurodegenerative diseases, IR/I and more (85). Several factors and markers are associated with iron death, including lactate dehydrogenase (LDH) activity, lipid peroxidation by reactive oxygen species (ROS), iron (Fe2+) levels, glutathione (GSH) levels, and malondialdehyde (MDA) levels. Studies have shown that high glucose (HG) conditions can increase ROS production with subsequently increased production of the lipid peroxidation product MDA, which in the presences of increased Fe2+ levels but decreased GSH and GPX4 levels, jointly lead to the induction of iron death (86). HG intake also leads to increased production of ROS, exacerbating oxidative stress increasing the production of GSSG, and reducing GSH content (87). This can result in mitochondrial abnormalities, such as decreased size, loss of mitochondrial ridges, and damage to the outer mitochondrial membrane. Glutathione peroxidase 4 (GPX4) is a key regulator of iron death, and its protein levels are significantly reduced under HG condition. The reduction in GPX4 leads to increased lipid ROS formation, lipid peroxidation, and ultimately cell iron death (86). Studies have found that adding ferroptosis cell death inhibitor can reduce cell death in HG environments, indicating the potential therapeutic importance of targeting this pathway (85). Overall, the process of ferroptosis and the proteins associated with it are strongly linked to glucose and lipid metabolism disorders (88). Herb extracts that antioxidant and anti-inflammatory properties such as Astragaloside IV has been shown to attenuate diabetic heart dysfunction in rats via inhibiting ferroptosis (89). However, studies regarding the relative role of ferroptosis in the diabetic myocardial IRI are rare and not definitive (77, 90, 91).

## 3.3 Impaired signaling such as eNOS, STAT3, PI3K/Akt in diabetes

Endothelial nitric oxide synthase (eNOS) is an enzyme that produces nitric oxide (NO) in the endothelial cells of blood vessels. NO is the key regulator of vascular function and homeostasis, and it plays a crucial role in maintaining the health of the cardiovascular system. Dysfunction of eNOS has been found to be associated with the development of diabetes (92). Studies have shown that eNOS dysfunction is closely linked to a high glucose environment, which is characteristic of diabetes. Restoring normal eNOS function is essential for improving vascular health in individuals with diabetes (93). In fact, upregulation of eNOS expression has been found to have a protective effect in diabetic patients (94). It has been discovered that eNOS uncoupling, which is the loss of balance between NO production and ROS generation, is a significant source of increased ROS production in diabetes. Increased oxidative stress further exacerbates eNOS uncoupling and endothelial dysfunction, contributing to cardiovascular damage (95). The PI3K/Akt/eNOS signaling pathway is also affected/impaired by diabetes (96, 97). This pathway involves phosphatidylinositol 3-kinase (PI3K) and AKT/protein kinase B (PKB/AKT), which respond to external signals and regulate various cellular processes, including metabolism, proliferation, cell survival, growth, and angiogenesis (98). Impairment in this pathway can lead to both cardiovascular damages in diabetes (79, 99). The activation of the PI3K-AKT pathway has been shown to play a crucial role in protecting the heart from myocardial IR/I. Cardiac protective interventions such as Ischemic preconditioning, a process that exposes the tissue to brief periods of ischemia before a more prolonged ischemic event, can activate the PI3K-AKT pathway and provide protection to the heart (73, 100). Additionally, the Akt and JAK/STAT3 signaling pathways have been found to be involved in reducing diabetic heart I/R damage (101). However, both the PI3K-AKT pathway and the JAK/STAT3 signaling pathway are impaired in the myocardium of diabetic subjects (102, 103), rendering the diabetic hearts more vulnerable to ischemia reperfusion injury and less or not sensitive to therapeutic interventions that are otherwise effective in non-diabetic subjects (104–106).

# 4 Cardioprotective effects of salvianolic acid A and salvianolic acid B against MIRI

Salvianolic acid is a compound found in the herb salvia, and it has been found to have several beneficial properties, including antioxidant, anti-inflammatory, and antiplatelet properties (107). In the context of myocardial IR/I, salvianolic acid has shown potential cardioprotective effect. Studies have indicated that salvianolic acids, specifically SAA and SAB, can help reduce damage to cardiomyocytes during MIRI in a rat model of ischemia-reperfusion injury (108). However, the exact mechanism by which salvianolic acid exerts its protective effects on MIRI is still not fully understood.

## 4.1 Salvianolic acid A

SAA possesses a polyphenolic acid chemical structure (Figure 3), exhibiting strong antioxidant capacity. It has also been found to have antioxidant, anticancer, antifibrotic, anti-inflammatory and antiplatelet aggregation properties (109). *In vitro* studies have shown that SAA exhibits potent free radical scavenging ability assessed by the methods of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS(+)) radical cation decolorization assay (110). SAA has been shown to attenuate myocardial functional impairment and cell death caused by oxidative stress and attenuate hydrogen peroxide-induced oxidative stress damage to cells both *in vivo* in rodent models and *in vitro* in cultured H9c2 cardiomyocytes (111, 112). Experimental evidence has demonstrated that SAA pretreatment upregulates the anti-apoptotic protein Bcl-2 and inhibits pro-apoptotic proteins Bak and Bax, thereby inhibiting apoptosis (113). Additionally, SAA has been found to significantly ameliorate mitochondrial dysfunction caused by myocardial ischemia in an isoprenaline-induced myocardial ischemia a rat

model (114). The contractile function of cardiomyocytes, as reflected by their shortening, was dose-dependently improved by SAA after myocardial ischemia-reperfusion (115). Furthermore, SAA pretreatment has been shown to reduce lactate dehydrogenase (LDH) leakage in ischemic myocardium, decrease LDH release from the ex vivo heart, and significantly improve cell viability. SAA also downregulates the expression of cleaved caspase-3 protein, thereby inhibiting apoptosis in cardiomyocytes. These findings suggest that SAA pretreatment before ischemia-reperfusion inhibits cardiomyocytes necrosis and apoptosis, thereby reducing ischemia-reperfusion-induced cardiomyocyte damage (115, 116).

## 4.2 Salvianolic acid B

SAB has been shown to effectively attenuate cardiovascular injuries by reducing the expression of related inflammatory factors, inhibiting apoptosis, and reducing oxidative stress in experimental settings (117). Numerous studies have demonstrated the cardiomyocyte protective effects of SAB during myocardial ischemia-reperfusion injury (MIRI) and its ability to reduce oxidative stress-induced damage (118). Similar to SAA, SAB also reduces post-ischemic LDH leakage (119). Experiments investigating the cardioprotective effect of SAB on myocardial ischemia-reperfusion injury, based on cell viability and LDH leakage, have shown that SAB inhibit autophagy, enhances cell viability, reduce LDH leakage, and increases the survival rate of cardiomyocytes after I/R (120). There is evidence showing that the cardioprotective effect of SAB on MIRI is dose-dependent, and both high and low doses of SAB have been found to reduce the size of myocardial infarction after treatment. Moreover, SAB effectively reduces cardiomyocyte apoptosis by significantly increasing the ratio of Bcl-2 expression to Bcl-2/Bax and reducing Bax expression (121). During ischemia-reperfusion, a large amount of ROS is released, accompanied with increased lipid peroxidation product malondialdehyde (MDA) (122) and other specific indicators of ROS-induced lipid peroxidation such as 15-F2t-Isoprostane (123, 124). Studies have demonstrated that SAB treatment can reduce high levels of malondialdehyde measured in rat models of testicular ischemia-reperfusion and myocardial ischemia-reperfusion, with no significant side effects observed throughout the treatment (108, 125). Recent research has also shown that SBB attenuates post-ischemic myocardial apoptosis, inhibits ROS production, decreases MDA levels, and enhances superoxide dismutase (SOD) activity through a mechanism that involves the regulation of the TRIM8/GPX1 axis *in vivo*, making it a potential candidate for the prevention or treatment of MIRI in cultured AC16 cardiomyocytes (118).

## 4.3 Impacts of salvianolic acid A and salvianolic acid B on the signaling pathways affecting MIRI

In rat models of myocardial IRI, Salvianolic Acid A (SAA) pretreatment has been shown to significantly reduce post-ischemic myocardial infarction concomitant with reduced plasma levels of cTnT, CK-MB, TNF-α and IL-1β compared with untreated I/R group (126). Platelets play a critical role in I/R injury, as activated platelets produce various factors that promote blood clot formation. It has been found that SAA treatment can resist ADP and collagen-induced human blood platelet aggregation and thrombosis by inhibiting the abnormal increase of the phosphorylation of Akt and also inhibits PI3K, and these effects of SAA was comparable to that of the PI3K inhibitor LY294002 both *in vitro* and *in vivo* in a mouse model of arterial thrombosis (127). NO is known to play a central role in maintaining cardiovascular homeostasis, and SAA treatment increases rat left ventricle NO content after myocardial I/R (126). Studies have found that SAA can also exerts cardioprotective effects through the ERK1/2 pathway, and this effect is inhibited by the ERK2/098059 inhibitor PD600125 (PD), suggesting that the protective effect of SAA on I/R cardiomyocytes may also depend on the inhibition of the JNK pathway (128). SAA has also been found to inhibit I/R-induced cardiomyocyte apoptosis through the PI3K/Akt, JNK, and ERK1/2 pathways. Among these pathways, ERK1/2 and JNK are regulated by upstream kinases (MAPK kinases), which activate each other in a stepwise manner. Experimental results have shown that inhibition of the p38 MAPK signaling pathway and the JNK signaling pathway can effectively protect and improve MIRI (128). Additionally, Salvianolic Acid B (SAB) has been shown to regulate the PI3K/Akt pathway, and to inhibit apoptosis by downregulating JNK phosphorylation, BCL2-associated X (Bax)/B-cell lymphoma-2 (Bcl-2), and caspase-3 expression (121) In addition, a recent study demonstrated that ubiquitin-proteasome degradation of GPX4 occurs in both MIRI models in rats and in *in vitro* models of cardiomyocyte hypoxia/reoxygenation, while SAB can reduce this degradation and inhibit ferroptosis and apoptosis of cardiomyocytes during MIRI and H/R and protect the cardiovascular system by the GPX4/ROS/JNK-mediated crosstalk mechanism (129). SAA and SAB have respectively been demonstrated to regulate the Jak/STAT3 signaling pathway in the liver (130) and in the intervertebral discs in rats (130). However, the potential impacts of SAA and SAB on the Jak/STAT3 signaling in the heart especially in the context of MIRI have not been explored thus far, which merits in depth future study given the critical role Jak/STAT3 signaling pathway plays during MIRI (45, 131, 132). The current understandings regarding the impacts of SAA and SAB on the signaling pathways that affect MIRI are summarized respectively in Figure 4.

# 5 Cardioprotective potential of salvianolic acid A and salvianolic acid B against MIRI in diabetes

In this study, we provide a systematic summary of the cardioprotective mechanism of salvianolic acid in diabetic myocardial ischemia-reperfusion injury, as well as various signaling molecules and mechanisms associated with myocardial I/R injury. Both salvianolic acid A and B have the potential to exert cardioprotective effects, either through similar or different mechanisms (Table 1). The relevant signaling pathways involved in myocardial ischemia-reperfusion injury include phosphatidylinositol-3 kinase/Akt (PI3K/Akt), mitogen-activated protein kinases (MAPKs), JANUS kinase/signal

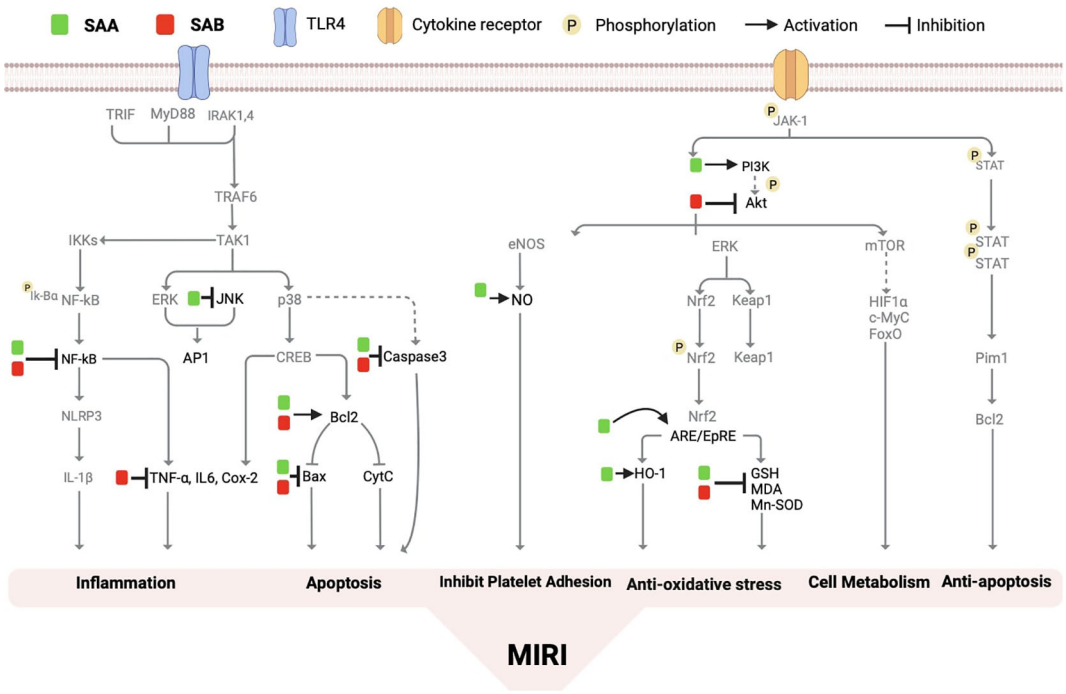


FIGURE 4
The molecular mechanism of salvianolic acid A and salvianolic acid B on myocardial ischemia/reperfusion injury. Salvianolic acid A and salvianolic acid B have the ability to regulate various molecular pathways including nuclear factor kB (NF-kB), stress-activated protein kinase (Bax, Caspase 3, malondialdehyde (MDA), heme oxygenase-1 (HO-1), bcl2, phosphatidylinositol 3-kinase (PI3K), nitric oxide (NO), antioxidant reaction element (ARE), tumor necrosis factor (TNalpha), protein kinase B (PKB/Akt), interleukin-6 (IL-6), and others. By inhibiting the production of reactive oxygen species, these compounds effectively improve inflammation, apoptosis, autophagy, microcirculation disorders, cell growth, and metabolism.

transduction and transcriptional activators (JAK/STAT), nuclear factor-κB (NF-κB), and others (96). Studies have revealed that diabetes can further impair the phosphatidylinositol 3-kinase/Akt/eNOS (PI3K/Akt/eNOS) pathway and activate JAK/STAT3 signaling, thereby exacerbating myocardial ischemia-reperfusion injury in diabetic rats which can be attenuated by treatment with SAA (96).

## 5.1 Salvianolic acid A and/or salvianolic acid B can reduce diabetic myocardial ischemia-reperfusion injury

As mentioned earlier, SAA pretreatment has been shown to have a protective effect on the myocardium during I/R in non-

TABLE 1 Salvianolic Acid potential cardiomyocytes in diabetic myocardial ischemia/reperfusion injury.

| | Cell Line | Animal | Mechanism | Effect Factors | Reference | Year |
|---|---|---|---|---|---|---|
| **Salvianolic acid A** | H9C2 | Mouse | TRL4↓、MyD8↓<br>p-JNK↓、p-ERK1/2↓ | anti-inflammatory mitochondrial dysfunction↑ | (133) | 2021 |
| | – | Rat | Bcl-2↑、Bax↓<br>JNK / PI3K / Akt | anti-apoptosis LDH leakage↓ infarct size↓ | (96) | 2016 |
| | – | – | PI3K↓Rap 1↓<br>late PI3K-dependent<br>Akt phosphorylation↓ | platelet adhesio↓ platelet activati↓ | (127) | 2010 |
| | HK-2 | Rat | MDA↓、HUVECs↓ VCAM-1↓、<br>HO-1↑ Nrf2↑ | oxidative stress↓ inflammation↓ | (134) | 2016 |
| | HepG2 | Mouse<br>Rat | ATP↑ MMP↓<br>CaMKKβ / AMPK↑ | myocardial dysfunction↑ | (135) | 2015 |
| | – | Rat | NF-κB ↓ | anti-inflammatory anti-apoptosis | (109) | 2022 |
| | – | Rat | AST↓、CK↓ LDH↓ | anti-inflammatory | (114) | 2009 |

*(Continued)*

TABLE 1 Continued

| | Cell Line | Animal | Mechanism | Effect Factors | Reference | Year |
|---|---|---|---|---|---|---|
| | – | Rat | Bcl-2↑、Bax↓ Bcl-2/Bax↑ Caspase-3↓ | anti-apoptosis anti-necrosis | (115) | 2011 |
| | H9C2 | Rat | SOD↑、Bcl2↑ H2O2-↓、p-Erk1/2↓ | infarct size↓ anti-apoptosis | (136) | 2011 |
| | – | Rat | TNF-α↓、IL-1β↓ NO↑ | anti-inflammatory myocardial dysfunction↑ platelet aggregation↓ anti-platelet | (126) | 2017 |
| **Salvianolic acid B** | H9C2 | – | NF-κB ↓、IL-6↓ IL-1β↓、TNF-α↓ | anti-inflammatory anti-apoptosis | (137) | 2016 |
| | ESC | – | HIF1α↓ 、BNIP3↓ cleavage caspase 3 ↓ | anti-apoptosis | (138) | 2015 |
| | H9C2 | – | ΔΨm↑、caspase-3↓ LC3-II↓ | Anti-mitochondrial auto-phagy | (139) | 2020 |
| | – | Rat | P-Akt↑、HMGB1↓ TLR4↓ | infarct size↓ anti-apoptosis | (121) | 2019 |
| | INS-1 | – | caspase-9↓ caspase-3↓ MDA↓ | anti-apoptosis | (140) | 2017 |
| | – | – | – | inhibits platelet adhesion | (141) | 2008 |
| | HUVEC | Rat | VEGFR2↑、VEGFA↑ IGFBP3↓、p-Akt↑ | ameliorated left ventricular dysfunction and remodeling cell proliferation↑ | (142) | 2020 |
| | – | Rat | SIRT1↑、Bcl-2↑ Ac-FOXO1↓、Bax↓ | anti-inflammatory anti-apoptosis | (117) | 2015 |
| | AC16 | Rat | TRIM8/GPX1 | oxidative stress↓ anti-apoptosis | (118) | 2022 |
| | primary myocardial cells | – | miR-30a↑、LDH↓ PI3K / Akt | anti-autophagy | (120) | 2016 |
| | – | Rat | P-Akt↑ HMGB1↓ | infarct size↓ anti-inflammatory myocardial dysfunction↑ anti-apoptosis | (121) | 2019 |

"-" means no mention.
"↑" means increased or enhanced.
"↓" means decreased or reduced.

diabetic rats. Further studies have found that Sal A pretreatment significantly improved cardiac hemodynamic and reduced LDH activity after I/R in diabetic rats, with concomitant reduction in post-ischemic myocardial infarction apoptosis (96). Similarly, SAB has been found to significantly reduce intracellular reactive oxygen species and malondialdehyde (MDA) levels, effectively reduce oxidative stress induced by high glucose rat insulinoma cell line INS-1 cells (140). Clinical studies have observed that patients with antiplatelet therapy appears to have similar effects in patients with diabetic coronary artery disease compared to patients with non-diabetic myocardial ischemia-reperfusion injury (143). Sal B has been shown to inhibit platelet aggregation and platelet adhesion by interacting with collagen receptors (141). In subsequent studies, SAA has also been found to significantly inhibit agonist-induced platelet activation by inhibiting PI3K (127). The nuclear factor E2 related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway is involved in the regulation of MIRI damage (144). Pro-inflammatory cytokines also play a significant role in diabetic vascular damage. VCAM-1, a pro-inflammatory cytokine, is known to be inhibited by Nrf2-mediated upregulation of HO-1 in vascular diseases. SAA has been found to reduce VCAM-1 expression by mediating the Nrf2/HO-1 signaling pathway (134).

## 5.2 Signaling pathway for possible cardiac protection of salvianolic acid A and salvianolic acid B in diabetic MIRI

Studies have demonstrated that insulin has cardioprotective effects mediated by the Akt signaling pathway, leading to the activation of eNOS through PI3K/Akt activation (145). The impairment of the PI3K/Akt signaling pathway is involved in myocardial I/R damage in diabetic rats, and high glucose further inhibits the PI3K/Akt pathway. Experimental evidence supports the significant increase in SERCA2 activity through JNK/PI3K/Akt signaling, resulting in anti-apoptotic effects and improvement in cardiac contraction and diastolic function in diabetic rats. Chen et al. found that SAA pretreatment significantly increased the level of the anti-apoptotic protein Bcl-2 in diabetic rats through the JNK/Akt signaling pathway, while reducing the levels of pro-apoptotic proteins Bax and cleaved-caspase-3, ultimately increasing the Bcl-2/Bax ratio and protecting against myocardial I/R damage in diabetic rats (96). Similarly, Sal B has been shown to reduce the expression of insulin-like growth factor binding protein 3 (IGFBP3) induced by high glucose, leading to the phosphorylation of extracellular signal-regulating protein kinase and protein kinase B (AKT) activity in rat models of diabetic cardiomyopathy and in cultured HUVECs under

hypoxia (142). SAB is also considered a potent inhibitor of the Akt/mTOR pathway, reducing the phosphorylation of Akt and its downstream target mTOR (146, 147). Furthermore, Sal A can regulate glucose metabolism by increasing ATP production with concurrent reduction of mitochondrial membrane potential (MMP), and improving mitochondrial function through $Ca^{2+}$/calmodulin-dependent protein kinase kinase-β (CaMKKβ)/AMPK signaling pathway in both type 1 and type 2 diabetic mice (135). NF-κB, which induces inflammatory factors involved in cardiomyocyte apoptosis, is also a downstream target of STAT3. Inhibition of NF-κB activity can prevent H9C2 cardiomyocyte apoptosis (148). Studies have found that SAB can inhibit the activation of the MAPK/NF-κB pathway induced by ox-LDL (149). SAB pretreatment has also been reported to reduce NF-κB levels (150), but whether SAB directly targets NF-κB or acts through its upstream pathway Akt/JAK or STAT3 remains unclear (113).

## 6 Conclusion

This review highlights the evidence and possible mechanisms by which salvianolic acid may reduce diabetic myocardial I/R damage. Possible mechanisms include modulation of oxidative stress, inflammatory response, mitochondrial dysfunction, ferroptosis and apoptosis through pathways such as PI3K/Akt, JAK/STAT, and NF-κB. A recent study has found that aldehyde dehydrogenase 2 (ALDH2) can activate the PI3K/AKT/mTOR pathway to alleviate ischemia and reperfusion injury in diabetic cardiomyopathy (44). However, it should be noted that studies have shown that Sal B can inhibit the Akt/mTOR pathway (147). There is currently no research showing that Sal B can exert cardioprotective effects by mediating the Akt/mTOR pathway. Although salvianolic acid has been studied in various clinical studies as an active ingredient in salvia, its research on diabetic myocardial I/R damage is relatively limited. Further understanding of the mechanisms underlying salvianolic acid-related myocardial protection will contribute to the development of new protective strategies and discovery of more effective therapies against diabetic myocardial I/R damage in the future.

## Author contributions

YJ: Writing – original draft. YC: Writing – original draft. RH: Writing – review & editing. YX: Supervision, Writing – review & editing. ZX: Supervision, Writing – review & editing. WX: Supervision, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Predicting the prevalence of type 2 diabetes in Brazil: a modeling study

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**Aims:** We adopted a modeling approach to predict the likely future prevalence of type 2 diabetes, taking into account demographic changes and trends in obesity and smoking in Brazil. We then used the model to estimate the likely future impact of different policy scenarios, such as policies to reduce obesity.

**Methods:** The IMPACT TYPE 2 DIABETES model uses a Markov approach to integrate population, obesity, and smoking trends to estimate future type 2 diabetes prevalence. We developed a model for the Brazilian population from 2006 to 2036. Data on the Brazilian population in relation to sex and age were collected from the Brazilian Institute of Geography and Statistics, and data on the prevalence of type 2 diabetes, obesity, and smoking were collected from the Surveillance of Risk and Protection Factors for Chronic Diseases by Telephone Survey (VIGITEL).

**Results:** The observed prevalence of type 2 diabetes among Brazilians aged over 25 years was 10.8% (5.2–14.3%) in 2006, increasing to 13.7% (6.9–18.4%) in 2020. Between 2006 and 2020, the observed prevalence in men increased from 11.0 to 19.1% and women from 10.6 to 21.3%. The model forecasts a dramatic rise in prevalence by 2036 (27.0% overall, 17.1% in men and 35.9% in women). However, if obesity prevalence declines by 1% per year from 2020 to 2036 (Scenario 1), the prevalence of diabetes decreases from 26.3 to 23.7, which represents approximately a 10.0% drop in 16 years. If obesity declined by 5% per year in 16 years as an optimistic target (Scenario 2), the prevalence of diabetes decreased from 26.3 to 21.2, representing a 19.4% drop in diabetes prevalence.

**Conclusion:** The model predicts an increase in the prevalence of type 2 diabetes in Brazil. Even with ambitious targets to reduce obesity prevalence, type 2 diabetes in Brazil will continue to have a large impact on Brazilian public health.

KEYWORDS

**type 2 diabetes prevalence, demographic changes, obesity trends, projection, target strategies, modeling**

# 1 Introduction

Diabetes Mellitus (DM) is a chronic metabolic disease that leads, over time, to serious damage to the heart, blood vessels, eyes, kidneys, and nerves (1). Type 2 diabetes (T2D) is the most common, which usually affects adults (1). The prevalence of type 2 diabetes has increased dramatically over the past three decades in countries of all income levels (1). In Brazil, the estimated prevalence of type 2 diabetes is 9.2%, ranging from 6.3% in the North to 12.8% in the Southeast (2).

Risks of type 2 diabetes increase in obese individuals (3), with obesity being an independent risk factor for type 2 diabetes (4). According to the World Health Organization (WHO), the number of adults with obesity has increased more than seven times since 1975 (5). In Brazil, obesity prevalence in adults more than doubled between 2003 and 2019, reaching 26.8%. In the same period, obesity also doubles its prevalence, with men and women prevalence at 30.2 and 22.8%, respectively (6).

In addition, there is a positive association between smoking and the incidence of diabetes (7, 8), with smokers being 30 to 40% more likely to develop type 2 diabetes than those who do not smoke (9, 10). In Brazil, the total percentage of smokers aged 18 years or over is 9.5%, with 11.7% among men and 7.6% among women. Globally, smoking alone is one of the leading causes of preventable disease and death (11).

Obesity and diabetes substantially impact healthcare costs, with 30% of the Unified Health System (*Sistema Único de Saúde – SUS*) cost attributable to diabetes and 11% attributable to obesity (12). Research showed that the total cost to the health system attributable to smoking is 23.3 billion reais per year (13).

Estimates of current and future type 2 diabetes prevalence are essential for managing *SUS* resources and encouraging intensive intervention measures in relation to risk factors to counteract trends of increasing prevalence (14, 15). In the context of Brazil, such projections are characterized by their scarcity and lack of precision since most estimates, such as those produced by the Diabetes Atlas of the International Diabetes Federation, are based on urbanization trends and demographic changes only, without taking into account trends in diabetes risk factors. In this way, forecasts based on trends in key risk factors appear to be more realistic.

The aim of the study was to use the type 2 diabetes-IMPACT model (14) with data from VIGITEL BRASIL and to describe trends in the type 2 diabetes-IMPACT (14) model with data from VIGITEL BRASIL and describe trends in type 2 diabetes and key risk factors, using the model to predict the likely future prevalence of type 2 diabetes, taking into account demographic changes and trends in key risk factors: obesity and smoking. Finally, it uses the model to estimate the likely future impact of different policy scenarios, such as policies to reduce obesity.

# 2 Materials and methods

## 2.1 Model overview

The model is a multistate Markov model that brings together information on population trends, obesity, and smoking at a given time to estimate the prevalence of diabetes in the future. It was previously used to estimate future diabetes prevalence in Tunisia, Turkey, Palestine, and Saudi Arabia (14, 16–19).

The total population is divided into several pools: type 2 diabetes, obese, smokers, and "healthy" (i.e., non-obese, non-smoking, non-diabetic) (Figure 1). Population demographic trends are used to inform the relative size of the "starting states," and transition probabilities are used to estimate the proportion of persons moving from the starting states to the type 2 diabetes and death states. There are two "absorbing states": type 2 diabetes-related death and non-type 2 diabetes-related deaths as competing risks for mortality. Potential overlaps between the healthy, obese, and smoking groups are estimated by calculating the conditional probabilities of membership, which allows for estimating what proportion of diabetic new cases can be attributed to smoking and obesity at each cycle.

## 2.2 Data sources

Data on the Brazilian population in relation to sex and age were collected from the Brazilian Institute of Geography and Statistics (*Instituto Brasileiro de Geografia e Estatística – IBGE*), data from the 2010/2060 population projection, published in 2018 (20).

Data on the prevalence of type 2 diabetes, obesity, and smoking were collected from the Surveillance of Risk and Protection Factors for Chronic Diseases by Telephone Survey (VIGITEL) (21), studies that make up the Surveillance of Risk Factors for Chronic Non-communicable Diseases (NCDs) of the Ministry of Health. Cases of type 2 diabetes are self-reported by individuals surveyed who already have a previous diagnosis of diabetes. The prevalence of obesity is obtained from self-reported data on weight and height to calculate the Body Mass Index (BMI), and individuals with a BMI >30 $kg/m^2$ are considered obese. Individuals who use cigarettes have their smoking status registered.

## 2.3 Estimating the incidence, case fatality, and mortality parameters

We estimated type 2 diabetes incidence for the Brazilian population in 2010 using DISMOD, a freely available software (22). To estimate diabetes incidence, case fatality rates, and mortality rates, we used the following as inputs for DISMOD diabetes prevalence in 2010: diabetes mellitus remission rate and diabetes mellitus relative risk for mortality.

We assumed that the diabetes mellitus remission rate is zero, and the relative risk for mortality can be estimated with the method proposed by Barendregt et al. (23), based on the usual RR for mortality and disease prevalence, using this formula:

$$RRadj = \frac{RR}{p.RR + 1 - p}$$

Where *RRadj* is the relative risk of mortality, *RR* is the usual relative risk for mortality (mortality diseased/mortality healthy) (24), and *p* is disease prevalence.

The potential overlaps between the model health states were handled in three different ways. First, smoking prevalence was multiplied by

Abbreviations: DM, Diabetes mellitus; T2D, Type 2 diabetes.

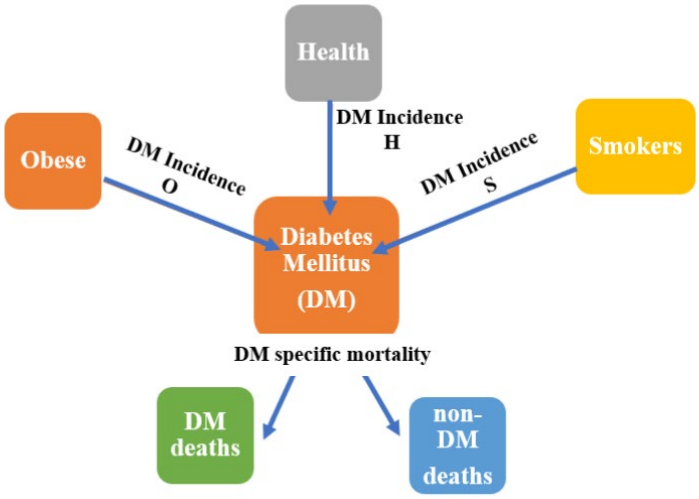


FIGURE 1
The type 2 diabetes model structure.

obesity prevalence to estimate the proportion of the population who were both obese and smokers. Then, such a proportion was subtracted from the 'original' smoking prevalence to leave in the (Smokers) state only those individuals who were smokers but not obese. Second, we estimated the number of individuals with T2DM (in the Diabetes state) in whom the disease was assumed to be 'caused' by obesity as exposure by multiplying the '*population attributable risk*' by the size of (Diabetes) state (25). Then, the number of such individuals was subtracted from the total obese individuals in the population to leave in the (Obese) state only those obese individuals who do not have T2DM. Finally, we applied the same previous approach of the *population attributable risk* to leave in the (Smokers) states only those people who are smokers but do not have T2DM.

## 2.4 Validation

Model validation is very important in any modeling exercise. The model developed for Brazil involves the years 2006 to 2036, with the base prevalence in 2006. As we have data available on the prevalence of diabetes in the country from 2006 to 2020, we compared the model results with the prevalence estimates observed for each year of type 2 diabetes cases at VIGITEL. Type 2 diabetes is diagnosed as self-reported by the population within the study with a previous diagnosis. Thus, we set a correction factor of 1.5, as these cases may underestimate the actual prevalence of the disease since Muzy et al. (2) show that the proportion of underreported cases reaches 50% in Brazil. We then found an equivalence between the model data output and the estimated prevalence demonstrated in the VIGITEL data (validation results are in the Technical Appendix).

## 2.5 Type 2 diabetes forecast and policy scenarios

Effective policy decisions can be modeled by the estimated intervention effect on trends in risk factors, and the trend parameter can be modified to model increasing, decreasing, or stable trends in the prevalence of obesity and/or smoking.

First, we present predictions for type 2 diabetes prevalence and burden through 2036 (30 years from our initial prevalence in 2006), assuming current trends in obesity and smoking continue. Obesity and smoking trends were conducted from the population data divided by age groups, and the data were grouped into six age groups. The projection used a pooled OLS model to control for the fixed-time effect. The pooled data allows you to increase the amount of information and control the effect of time. Next, we explored the potential effect of population-level interventions to reduce obesity.

## 2.6 Policy scenarios

First, we present forecasts for type 2 diabetes prevalence and the burden up to 2036 (26 years from our initial prevalence estimate in 2010), if current trends in obesity and smoking continue as our baseline scenario.

Then, we explored the potential effect of population-level changes in obesity in our first intervention; we are proposing a 1% reduction per year in the prevalence of obesity from the year 2020 to the year 2036, verifying the impact on the diabetes burden. In our second intervention, in a more optimistic scenario, we propose a 5% reduction per year in the prevalence of obesity from the year 2020 to the year 2036, verifying the impact on the diabetes burden.

## 2.7 Sensitivity analysis

The analysis of the extreme method (Briggs) was used (26), which consists of running the model with all parameters adjusted to realistic minimum and maximum values, carried out in Excel; and consists of a very conservative approach, but which allows for a more transparent understanding of the weight of each parameter concerning the model results.

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# 3 Results

## 3.1 Demographic and epidemiological chances

Brazil has approximately 213 million inhabitants. We considered the population aged over 25 years. For this age group, the number of men is estimated to grow from 64 million in 2020 to 76 million in 2036 and from 70 to 84 million for women. The proportion of young people aged 25–34 years will decrease from 25.4% in 2020 to 18.6% in 2036 in relation to the total population of the country. By 2036, 22.8% of the population 25 years and above will be aged over 65 years, compared with 15.3% in 2020 (Table 1).

## 3.2 Observed prevalence of type 2 diabetes, obesity, and smoking

The observed prevalence of type 2 diabetes in Brazil in 2006 among the Brazilian population aged 25 years and above was overall 10.8% (min./max: 5.2–14.3); 11.0% (min./max:2.2–17.4) in men and 10.6% (min./max: 1.7–17.8) in women. The observed prevalence of type 2 diabetes in Brazil in 2020 was overall 13.7% (min./max: 6.9–18.4); 19.1% (min./max: 2.4–23.0) in men and 21.3% (min./max: 2.9–22.2) in women. Obesity is a common risk factor, with 14.5% prevalence among men and 12.5% among women in 2006. This prevalence increased to 22.9% among men and 21.7% among women, respectively, in 2020. Smoking prevalence was decreasing, from 17.8% (2006) to 12.8% (2020) among men and from 11.4% (2006) to 7.9% among women (Table 2).

## 3.3 The effect of obesity and smoking trends on type 2 diabetes prevalence

Changes in the prevalence of obesity and smoking were assumed to be linear, with varying degrees among men and women and in different age groups.

Obesity prevalence rose from 22.9% in 2020 to 31.1% in 2036 in men (annual increase of 0.51%) and from 21.7 to 29.1% in women (annual increase of 0.46%). Obesity prevalence was higher among men than women, with the highest prevalence observed among men aged 35-44, 45-54, and 55-64, and among women aged 55-64 and 65-74. The smoking trends showed a decrease in men and women. The projected prevalence for 2036 is 3.3% in men and 1.8% in women, representing a decrease of almost 82% in men and 84% in women. In some age groups, both for men and women, there is a tendency for the prevalence to be almost non-existent.

Assuming these annual trends in risk factors continue, the forecast prevalence of type 2 diabetes for 2036 is overall 27.0% (min 26.9–max 27.7): 17.1% in men (min 15.8–max 17.7) and 35.9% in women (min 31.1–max 37.7). The total number of Brazilian people with type 2 diabetes is projected to rise from 9 million in 2006 to 43 million in 2036, an increase of almost 400%. Diabetes prevalence is predicted to increase rapidly between 2006 (8.9%) and 2020 (26.3%), and then the increase starts to slow down in men. This trend, however, was not observed for women, who showed an ever-increasing trend in the prevalence of type 2 diabetes.

## 3.4 Scenario projections

If trends in obesity start to decline by 1% per year from 2020 to 2036 (Scenario 1), the prevalence of diabetes decreases from 26.3 to 23.7, representing approximately a 10.0% drop in 16 years. This would prevent approximately 5.2 million Brazilians from developing type 2 diabetes, 4.7 million of whom are women and 0.5 million men. If obesity declined by 5% per year in 16 years as an optimistic target (Scenario 2), the prevalence of diabetes decreased from 26.3 to 21.2, representing a 19.4% drop in diabetes prevalence. It is noteworthy that if only women were observed in the disease projection scenario for 2036, between baseline (35.9%) and Scenario 2 (25.8%), there could be a reduction of more than 30% in the prevalence of diabetes in this group, if obesity were reduced (Table 3). This corresponds to approximately 9.3 million Brazilians who would stop developing type 2 diabetes, 0.9 million men and 8.5 million women. Detailed results with sensitivity analysis are shown in Table 3.

# 4 Discussion

The forecast prevalence of type 2 diabetes for 2036 is 27.0% overall (17.1% in men and 35.9% in women), with almost 400% increase in the number of persons with type 2 diabetes between 2006 and 2036.

TABLE 1 Projection data of the Brazilian population from 2006 to 2036.

| Age groups | 2006 | | | 2020 | | | 2036 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Population N* (million) | | | Population N* (million) | | | Population N* (million) | | |
| | M** | W*** | %**** | M** | W*** | %**** | M** | W*** | %**** |
| 25–34 | 15.6 | 15.5 | 31.0 | 17.6 | 17.1 | 25.4 | 15.0 | 14.6 | 18.6 |
| 35–44 | 12.7 | 13.0 | 25.6 | 15.9 | 16.7 | 24.5 | 16.6 | 16.7 | 20.8 |
| 45–54 | 9.6 | 10.1 | 19.6 | 12.6 | 13.6 | 19.5 | 16.0 | 16.9 | 20.6 |
| 55–64 | 5.8 | 6.5 | 12.2 | 9.6 | 10.9 | 15.3 | 13.0 | 14.5 | 17.2 |
| 65–74 | 3.2 | 3.9 | 7.2 | 5.7 | 7.0 | 9.4 | 9.2 | 11.1 | 12.7 |
| 75+ | 1.7 | 2.6 | 4.3 | 3.2 | 4.8 | 5.9 | 6.4 | 9.7 | 10.1 |
| Total | 48.8 | 51.9 | 100.0 | 64.1 | 70.3 | 100.0 | 76.4 | 83.7 | 100.0 |

N, number; M, men; W, women; %: percentage in relation to the total population.

TABLE 2 Brazilian population aged 25 years and above, obesity, and smoking prevalence by sex and age groups in 2006 and 2020.

| Age groups | 2006 | | | | | | 2020 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T2D % | | Obesity % | | Smoking % | | T2D % | | Obesity % | | Smoking % | |
| | M | W | M | W | M | W | M | W | M | W | M | W |
| 25–34 | 1.3 | 1.0 | 12.2 | 7.1 | 16.7 | 8.4 | 1.7 | 2.2 | 20.1 | 18.0 | 12.8 | 5.5 |
| 35–44 | 2.6 | 2.4 | 15.5 | 10.5 | 17.5 | 12.4 | 3.4 | 3.8 | 25.9 | 22.4 | 11.4 | 7.4 |
| 45–54 | 7.0 | 5.9 | 17.3 | 14.8 | 22.6 | 17.6 | 8.0 | 7.8 | 25.5 | 23.1 | 15.2 | 11.4 |
| 55–64 | 12.6 | 13.5 | 16.1 | 19.0 | 18.5 | 11.3 | 15.8 | 16.0 | 24.4 | 23.5 | 15.4 | 10.6 |
| 65–74 | 16.9 | 18.4 | 13.2 | 19.0 | 13.8 | 7.8 | 22.6 | 22.8 | 19.7 | 23.7 | 11.2 | 6.4 |
| 75+ | 18.3 | 17.7 | 8.9 | 18.3 | 8.3 | 5.4 | 24.6 | 22.7 | 14.2 | 20.8 | 6.5 | 4.4 |
| Total | 11.0 | 10.6 | 14.5 | 12.5 | 17.8 | 11.4 | 19.1 | 21.3 | 22.9 | 21.7 | 12.8 | 7.9 |

N, number; M, men; W, women.

TABLE 3 Scenario projection rate of prevalence of type 2 diabetes by gender with sensitivity analysis (Minimum-Maximum).

| Baseline | Men % | Women % | Total % |
|---|---|---|---|
| 2006 | 8.7 (6.9–10.4) | 9.1 (7.3–10.9) | 8.9 (7.1–10.7) |
| 2020 | 20.4 (17.6–22.4) | 31.7 (30.9–34.0) | 26.3 (26.2–26.8) |
| 2036 | **17.1 (15.8–17.7)** | **35.9 (31.1–37.7)** | **27.0 (26.9–27.7)** |
| Scenario 1 | Men % | Women % | Total % |
| 2006 | 8.7 (6.9–10.4) | 9.1 (7.3–10.9) | 8.9 (7.1–10.7) |
| 2020 | 20.4 (17.6–22.4) | 31.7 (30.9–34.0) | 26.3 (26.2–26.8) |
| 2036 | **16.4 (15.4–16.6)** | **30.2 (29.0–33.4)** | **23.7 (23.2–24.9)** |
| Scenario 2 | Men % | Women % | Total % |
| 2006 | 8.7 (6.9–10.4) | 9.1 (7.3–10.9) | 8.9 (7.1–10.7) |
| 2020 | 20.4 (17.6–22.4) | 31.7 (30.9–34.0) | 26.3 (26.2–26.8) |
| 2036 | **15.9 (15.1–15.9)** | **25.8 (22.4–31.3)** | **21.2 (19.4–23.7)** |

Projected prevalence of T2D by 2036.

If the two proposed scenarios for decreasing the prevalence of obesity were achieved, we would have a 10.0–19.4% (12.2 and 21.48%) reduction in the prevalence of type 2 diabetes by the year 2036. Because the smoking trend among Brazilians for the year 2036 is decreasing, and in some age groups, it will be practically nil, we chose not to include smoking within the proposed scenarios, as the effect would be very small on the prevalence of diabetes.

Obesity is a global epidemic, and rates are particularly high in the USA (42.4% in 2017/2018) (27) and Canada (57.1% in Ontario and 56.2% in Québec) (28) compared with 11.8–31.3% in India (29). In Brazil, the prevalence of obesity has also been growing over the years (30), which has many implications for chronic diseases, including type 2 diabetes. The factors associated with the increase in the prevalence of obesity are multidimensional, related both to the demographic transition and to social inequalities (31, 32) that reflect the high prevalence of food insecurity and the double burden of malnutrition of populations in developing countries, as is the case in Brazil (33, 34).

In developing countries such as Palestine, diabetes mellitus prevalence estimated by the model forecasts was 20.8% for 2020 and 23.4% for 2030 (16), while in Tunisia, the model forecasts a dramatic rise in prevalence by 2027 (26.6% overall, 28.6% in men and 24.7% in women) (14). In Qatar, type 2 diabetes prevalence increased from 16.7% in 2016 to 24.0% in 2050 in the baseline scenario (17). Studies show that if there is a control of the obesity epidemic in these countries, as well as a decrease in the prevalence of smoking, there is a probability of a decrease in the prevalence of DM2 (14, 16–19). In the present model, following the proposed scenarios for the reduction of obesity over the years, a decrease in the prevalence of type 2 diabetes will also be observed in Brazil.

## 4.1 Policy scenarios and their importance

This study proposes two intervention options and assesses their impact on future diabetes prevalence. In Brazil, obesity prevalence is increasing and is predicted to continue to increase. The frequency of overweight adults between 2006 and 2020 ranged from 42.6% in 2006 to 57.5% in 2020 (average increase of 1.04 pp./year). This increase was observed in both sexes, with the highest increase among women, ranging from 38.5% in 2006 to 56.2% in 2020 (1.24 pp./year) (35).

Brazilians have a national strategy for preventing non-communicable diseases. However, this strategy did not set a target for obesity reduction; instead, the strategy is limited to promoting a stabilization of obesity in the country by the year 2030 (36). With this desirable stabilization in mind, our model tends to be more daring, proposing a more plausible scenario of a 1%

reduction per year in the prevalence of obesity and a more challenging scenario of a 5% reduction per year in the prevalence of obesity. Among the goals proposed to stop this growth of obesity and, consequently, of NCDs are the encouragement to practice physical activity in leisure time, increase the consumption of fruits and vegetables by 30%, and stop the consumption of ultra-processed foods (36).

Some initiatives for primary prevention of obesity have been implemented. The National Health Promotion Policy can be considered as a potential inducer of obesity prevention and control actions because it establishes as priority themes: (a) the development of actions for the Promotion of Adequate and Healthy Food (PAHF); (b) the promotion of food and nutrition security, aiming to contribute to the guarantee of the Human Right to Adequate Food; (c) encouragement of bodily practices and physical activity, providing public spaces to enable bodily activities that enhance comprehensive healthcare (37, 38).

Recently, at the national level, "Proteja" was launched, the National Strategy Plan for Prevention and Care of Child Obesity, with several essential strategies that must be implemented by Brazilian municipalities, such as monitoring the nutritional status and markers of children's food consumption, institutional campaigns in the media mass communication on childhood obesity, and ensuring healthy canteens in schools, among other actions (39).

In the National Primary Care Policy (40), two programs with a potential impact on obesity stand out: the School Health Program (*Programa Saúde na Escola—PSE*) and the Health Academy Program (*Programa Academia da Saúde*), which encourages the practice of physical activity outdoors. Furthermore, Brazilian researchers have developed an exemplary proposal regarding healthier eating guidelines for their population, which is a reference for many countries around the world (41), just as there are intersectoral articulation initiatives for the prevention and control of obesity (42), so the scenarios proposed in this study are feasible and can be considered.

Regarding the program that encourages the practice of physical activity, in an evaluation of VIGITEL data in adults and the older adult, it was observed that increased frequency and level of leisure-time physical activity in adults are protective factors in relation to obesity (43). Another factor that can help to reduce the prevalence of obesity is the reduction of sedentary behavior. This has been strongly associated with reducing the occurrence of type 2 diabetes and cardiovascular disease. Last year, a physical activity guide for the Brazilian population was released (44).

Nguyen et al. (45), when building a model (ACE-Obesity Policy Model) simulating BMI, physical activity, consumption of fruits and vegetables, and incorporating the variable sedentary behavior in the Australian population, observed through the most realistic scenario that, if the population spent less than 4 h a day sitting, 3,204 deaths could be avoided per year, and among these 22% of deaths from type 2 diabetes, in addition to preventing the incidence of this disease by 58%. López-González et al. (46), evaluating the consumption of fruits and vegetables in a longitudinal study in adults, revealed that the increase of 100 g in the consumption of these foods in 1 year promoted a significant reduction in glycemic levels in the body weight and waist circumference. These studies provide a basis for nutritional education that should be carried out both within schools and in the practice of population self-care in primary healthcare.

## 4.2 Strengths and limitations

This is the first modeling study involving type 2 diabetes and risk factors for the Brazilian population. In addition, the projections that were carried out used data from an annual study that took place in the country uninterruptedly from 2006 to 2020, which brings greater security in projecting, in view of the trend of real data observed by long series.

A possible limitation of the study was the lack of inclusion of other possible risk factors that are related to diabetes were not considered, such as physical inactivity and poor diet. The use of studies that use self-reporting could also be questioned. However, in Brazil, given the geographic extent of the country, VIGITEL studies have been of high value for annual monitoring of the country's epidemiological situation in relation to chronic non-communicable diseases and related risk factors. In this study, we sought to address this self-report issue in relation to the diagnosis of diabetes, using a correction factor of 1.5, which minimizes the harm of underestimation. Despite these limitations, the model used published prevalence data for obesity and smoking over a 15-year time series, which increases the likelihood of a more realistic trend estimate.

Small and possible variations in population estimates or the groups analyzed do not make the results unfeasible or biased.

## 4.3 Public health implications: a call to actions

Diabetes prevention can be achieved through measures that focus on reducing obesity. Currently, in the country, the discussion of some of these measures is gaining momentum. Some have not yet been implemented, but we can see successful examples in other countries, such as the taxation of junk food, including sweet beverages, clear nutrition labeling, with interest in front-of-pack (FOP) labeling policies based on comprehensive nutrient profiling models, public awareness, regulation of food advertising (especially targeted to children), and school-based health promotion initiatives (47–49). Similarly, the fight against social inequalities needs to be articulated with the obesity reduction action plan since it is proven that socioeconomic factors such as unemployment and poverty are associated with the increase in the prevalence of obesity (50, 51).

Recently, some modeling studies have observed the effect of taxation on foods, especially ultra-processed foods, and the benefit of reducing the prevalence of obesity and other diseases (52, 53). A study developed by Passos et al. (53) showed that a 1% increase in the prices of ultra-processed would lead to a decrease in the prevalence of overweight and obesity (0.33 and 0.59%, respectively), and this effect is even greater in low-income populations (0.34 and 0.63%).

Among the ultra-processed products, a group that deserves special attention is the sugar-sweetened beverages (SSBs), which include soft drinks and industrialized juices, as the relationship between the consumption of SSBs and type 2 diabetes is now supported by substantial epidemiological evidence (54, 55). High levels of free sugars in the diet increase the risk of obesity and diabetes (56, 57). Sugary drinks are often responsible for a large part of the free sugar consumed (58) and have been the main focus of policies (59). Then, a high SSB tax might be an effective fiscal policy to decrease the purchase and consumption of SSB and reduce overweight/obesity

prevalence, especially if the tax were specific for beverage volume and in upper-middle- and middle-income countries, such as Brazil (60).

In a recent meta-analysis, economic tools, product reformulation, and environmental measures were effective in reducing sugar intake or weight outcomes, while labeling, education, and interventions combining educational and environmental measures found mixed effects. The most frequently implemented measures in Europe are public awareness, nutrition education, and labels (61). In addition to these strategies mentioned, Brazil has produced material to support health teams and professionals in the management of obesity in the Unified Health System, with emphasis on the collective approach (62).

## 5 Conclusion

Our study brings promising results for the reduction of type 2 diabetes with actions promoting the reduction of the prevalence of obesity in the Brazilian population in accordance with national policy and highlighting possible and innovative possibilities, such as the consideration of more optimistic scenarios. However, current initiatives will require substantial scaling up and adoption to have a significant impact on current obesity trends.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by the Ethics Committee of Federal University of Paraíba. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because these are secondary data made available on public domain sites of the Brazilian Unified Health System.

## Author contributions

PM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. AdA: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. FF: Writing – original draft, Writing – review & editing. JdA: Formal analysis, Writing – original draft, Writing – review & editing. RR: Data curation, Formal analysis, Methodology, Writing – original draft. RL: Writing – original draft, Writing – review & editing. RV: Writing – original draft, Writing – review & editing. JS: Writing – original draft, Writing – review & editing. MO'F: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1275167/full#supplementary-material

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# Unraveling genetic causality between metformin and myocardial infarction on the basis of Mendelian randomization

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**Background:** In recent years, several studies have explored the effect of metformin on myocardial infarction (MI), but whether metformin has an improvement effect in patients with MI is controversial. This study was aimed to investigate the causal relationship between metformin and MI using Mendelian randomization (MR) analysis.

**Methods:** The genome-wide significant ($P<5\times10^{-8}$) single-nucleotide polymorphisms (SNPs) in patients with metformin and patients with MI were screened from the Open genome-wide association study (GWAS) project as instrumental variables (IVs). The study outcomes mainly included MI, old MI, acute MI, acute transmural MI of inferior wall, and acute transmural MI of anterior wall. The inverse variance weighted (IVW) method was applied to assess the main causal effect, and weighted median, simple mode, weighted mode methods, and MR-Egger regression were auxiliary applied for supplementary proof. The causal relationship between metformin and MI was assessed using odds ratios (OR) and 95% confidence intervals (95% CI). A leave-one-out method was used to explore the effect of individual SNPs on the results of IVW analyses, and a funnel plot was used to analyze the potential bias of the study results, thus ensuring the robustness of the results.

**Results:** In total, 16, 84, 39, 26, and 34 SNPs were selected as IVs to assess the genetic association between metformin and outcomes of MI, old MI, acute MI, acute transmural MI of inferior wall, and acute transmural MI of anterior wall, respectively. Treatment with metformin does not affect the risk of acute transmural MI of anterior wall at the genetic level ($P>0.05$; OR for inverse variance weighted was 1.010). In the cases of MI, old MI, acute MI, and acute transmural MI of inferior wall, metformin may even be a risk factor for patients ($P<0.05$; ORs for inverse variance weighted were 1.078, 1.026, 1.022 and 1.018 respectively). There was no horizontal pleiotropy or heterogeneity among IVs. The results were stable when removing the SNPs one by one.

**Conclusion:** Metformin is not protective against the risk of myocardial infarction in patients and may even be a risk factor for MI, old MI, acute MI, and acute transmural MI of inferior wall.

KEYWORDS

metformin, myocardial infarction, cardiovascular disease, diabetes, Mendelian randomization study

## Background

Mortality and disability rates caused by cardiovascular disease (CVD) are very high worldwide (1), more than twice the mortality rate of cancer, creating a severe burden on global public health (2). In 2019, there were approximately 18.6 million cardiovascular deaths globally, of which 1,080 occurred in Asia, accounting for 35% of the total deaths in Asia (3). According to reports published by the American Heart Association, CVD is the leading cause of death in the United States (4). As an important pathogenesis factor of CVD, type 2 diabetes mellitus (T2DM) causes a variety of large vascular diseases such as coronary heart disease and cerebrovascular diseases and microvascular complications such as diabetic nephropathy and retinopathy because of its insulin resistance (5, 6). CVD has become the main cause of death in T2DM patients. In addition, risk factors for CVD include myocardial infarction (MI), stroke, hypertension, dyslipidemia and so on (7), among which more than half of cardiovascular deaths are caused by acute myocardial infarction (AMI) (8). Therefore, the discovery of effective drugs to treat MI and improve its prognosis is critical to reducing cardiovascular mortality and improving global health. In recent years, researchers continue to explore drugs to improve the prognosis of MI, among which metformin, a drug used in the clinical treatment of T2DM, has attracted great attention.

Metformin is recommended as the basic drug for T2DM treatment by most national guidelines, including the guidelines of the American Diabetes Association, the European Association for the Study of Diabetes and the National Institute for Health and Care Research of the United Kingdom (9). Metformin mainly plays a hypoglycemic role by activating adenosine monophosphate activated protein kinase (AMPK) in cells and reducing glucose output from the liver (10). In addition to hypoglycemic effects, metformin also has many other effects including anti-cancer (11, 12), anti-inflammatory (13, 14) and anti-aging (15, 16). Currently, pharmacogenomic studies of metformin focus on genes such as organic cation transporters (OCTs), plasma membrane monoamine transporter (PMAT) and multidrug and toxic compound extrusions (MATEs) affecting its pharmacokinetics and AMPK, ataxia telangiectasia-mutated (ATM), glucose transporter type (GLUT2) and carboxypeptidase A6 (CPA6) affecting its pharmacodynamics, most of these studies have explored the influence of gene polymorphism on the hypoglycemic effect of metformin. However, the effect of the single-nucleotide polymorphisms (SNPs) on the cardiovascular protective effect of metformin has not been reported (17). Several studies have shown that metformin may have certain cardiovascular benefits for both diabetic and non-diabetic patients (18, 19), but most clinical trials are small in scale, and whether metformin is beneficial for patients with MI remains doubtful. In a 10-year follow-up study of diabetic patients, the risk of MI in diabetic patients taking metformin was significantly reduced (20). Another cohort study found that the use of metformin in T2DM patients increases the risk of cardiovascular disease death during the first occurrence of AMI, while taking metformin after stable MI may have a protective effect (21). The above researches indicate that metformin has a positive effect on improving the outcome in diabetic patients with MI, but this effect does not exclude the benefit of metformin on blood glucose control. While exploring the protective mechanisms of metformin against MI beyond its hypoglycemic effect, Wang M et al. found that metformin reduced MI size in mice by inhibiting Heat shock factor 1 (HSF1) (22). Moreover, some researchers have confirmed that metformin can indeed reduce the fibrosis and inflammation in the hearts of mice after MI (23). In addition, a retrospective study showed that long-term metformin treatment reduced the size of MI (24), which seems to indicate that metformin does have a role to reduce the risk of MI in patients. However, another study showed that no statistically significant cardioprotective correlation was found between metformin and MI size in patients with diabetes and acute ST elevation MI (25). Hartman et al. collected two-year follow-up data after metformin treatment for 4 months in 379 patients with MI without diabetes after PCI. It was found that 4 months of metformin treatment did not reduce the incidence of cardiovascular events compared with placebo (26), which was consistent with the conclusion of a randomized controlled trial conducted by Goldberg (27). One meta-analysis found that combination therapy with metformin may even increase the risk of cardiovascular mortality (28). Therefore, current studies have shown that whether metformin can improve MI is still controversial (29, 30), and the relationship needs to be further explored.

Mendelian randomization (MR) analysis is an emerging epidemiological approach that uses comprehensive statistics from genome-wide association studies (GWAS) to infer causal relationships between certain diseases and exposure factors to identify potential risk factors. The instrumental variable in MR

analysis is the SNP, which uses the known association between SNP and particular trait or disease to randomly group individuals according to their genotype to infer a causal relationship between the SNP and the disease or trait. By using genetic variation as an instrumental variable for exposure factors, MR analysis can overcome common confounding factors in observational studies (31). In this study, the principle of MR was applied to explore the causal relationship between the therapeutic effect of metformin on MI in order to further explore the novel pharmacological effects of metformin and provide alternative therapeutic drugs for patients with MI.

# Methods

## Study design

In this study, SNPs associated with metformin was used as instrumental variables (IVs) to explore the causal relationship between metformin administration and MI using two-sample MR analysis based on the Open GWAS project. IVs need to satisfy three core assumptions (32): (1) hypothesis of correlation: genetic variation is associated with metformin use. (2) hypothesis of independence: genetic variation is not associated with confounding factors affecting exposure and outcome. (3) hypothesis of exclusivity: genetic variation can only affect the outcome variables through exposure. Since the data used in this study were taken from public database, dedicated research ethics approval is unnecessary. The study design is shown in Figure 1.

## Data source

The genetic variation data used in this study were obtained from the Open GWAS project (33). The GWAS ID for metformin is ukb-b-14609, as designated in the National Human Genome Research Institute and European Bioinformatics Institute's (NHGRI-EBI) GWAS catalog (34). Data for MI, Old MI, acute MI, acute transmural MI of anterior wall and acute transmural MI of inferior wall were obtained from the Open GWAS project named ukb-d-19, ukb-b-16662, ukb-b-3469, ukb-b-453 and ukb-b-5126 respectively. The population in the above datasets was the European population, including males and females. The essential information of the dataset is summarized in Table 1.

## IVs selection

We selected IVs at the genome-wide significance level ($P<5.0\times10^{-8}$) (35). To obtain site-independent IVs, we used the "Two Sample MR" package to set the linkage disequilibrium (LD) threshold to $R^2<0.001$ and the clump distance to 10,000 kb from 1000 genomic EUR data.

## Statistical analysis

The statistical analysis workflow of the study is presented in Figure 2. The inverse variance weighted (IVW) method was applied to assess the main causal effects, with the auxiliary application of weighted median, simple mode, weighted mode methods, and MR-Egger regression used for additional supporting evidence. The odds ratio (OR) and 95% confidence interval (CI) value was calculated accordingly. A $P$-value < 0.05 was considered statistically significant. Cochran's Q test was used to analyze the heterogeneity of IVs (36). If $P>0.05$ then it represents no significant heterogeneity. In MR-Egger regression, if the intercept tends to 0, it can be assumed that there is no horizontal pleiotropy. Where MR-PRESSO global test was used to detect pleiotropy ($P < 0.05$) (37). A leave-one-out method was used to explore the effect of individual SNPs on the results of IVW analyses, and a funnel plot was used to analyze the potential bias of the study results, thus ensuring the robustness of the results (38). All tests were two sided and performed using the R package TwoSampleMR version 0.5.6 in R software 4.2.1.

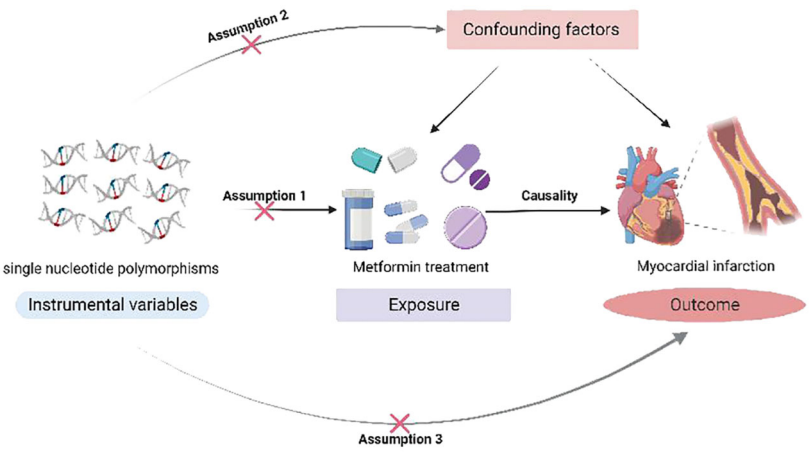


FIGURE 1
A Mendelian randomization study revealing causality between metformin and myocardial infarction.

10.3389/fendo.2024.1376464

TABLE 1 The essential information of the dataset.

| Project name | Dataset | Consortium | Sample size | N case | Population | Year |
|---|---|---|---|---|---|---|
| Metformin | ukb-b-14609 | MRC-IEU | 462933 | 11552 | European | 2018 |
| MI | ukb-d-19 | NA | 361194 | 7018 | European | 2018 |
| Old MI | ukb-b-16662 | MRC-IEU | 463010 | 3340 | European | 2018 |
| Acute MI | ukb-b-3469 | MRC-IEU | 463010 | 2321 | European | 2018 |
| Acute transmural MI of anterior wall | ukb-b-453 | MRC-IEU | 463010 | 1294 | European | 2018 |
| Acute transmural MI of inferior wall | ukb-b-5126 | MRC-IEU | 463010 | 1673 | European | 2018 |

MI, myocardial infarction; MRC-IEU, medical research council integrative epidemiology unit.

# Results

## Acquisition of IVs

Firstly, relevant SNPs were obtained through the screening of IVS, and SNPS associated with confounders of MI were removed through the PhenoScanner database search. Meanwhile, palindromic sequences with intermediate allelic frequency were removed during statistical analysis. In total, the metformin GWAS dataset contains 9,851,867 SNPs. Based on the above screening criteria, 16, 84, 39, 26, and 34 SNPs were identified as IVs to assess the genetic association between metformin and outcomes of MI, old MI, acute MI, acute transmural MI of inferior wall, and acute transmural MI of anterior wall, respectively. Detailed information about SNPs is provided in Supplementary Tables S1–S5. The effect of each SNP on outcomes is displayed in Figure 3.

## Causal relationship between metformin and myocardial infarction

As shown in the forest plots (Figure 4), patients treated with metformin had a higher risk of MI (OR=1.078 (1.013-1.148), *P*=0.018), old MI (OR=1.026 (1.001-1.052), *P*=0.038), acute MI (OR=1.022 (1.003-1.041), *P*=0.023), and acute transmural MI of inferior wall (OR=1.018 (1.001 -1.034), *P*=0.044), but there was no significant change in the risk for acute MI infarction of anterior wall (OR=1.010 (0.995 -1.026), *P*=0.197). The scatter plots (Figure 5) also showed the same variation in the risk of increased risk of MI in patients treated with metformin.

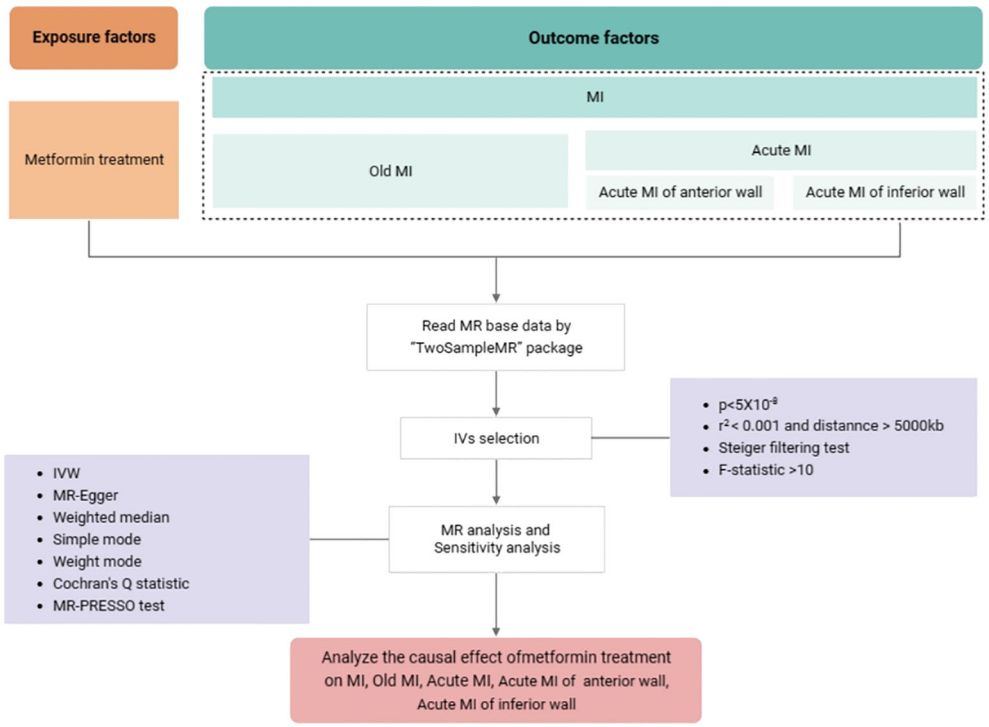


FIGURE 2
The statistical analysis workflow of the study. Abbreviations used: GWAS: genome-wide association study; IVs, instrumental variables; IVW, inverse variance-weighted; MR, Mendelian randomization; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier; MI, myocardial infarction.

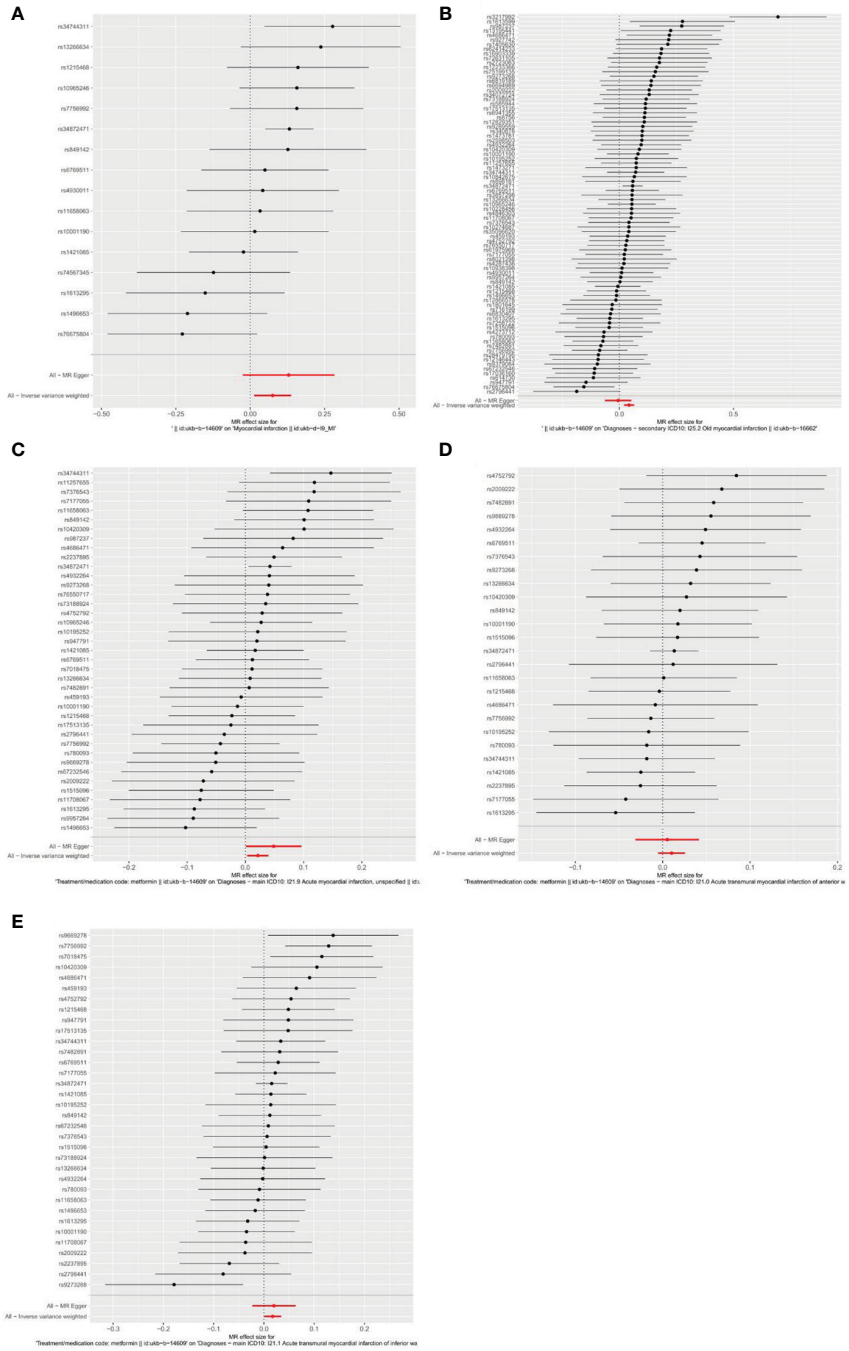


FIGURE 3
Forest plots of the effect of each SNP on outcomes. **(A)** outcome of myocardial infarction; **(B)** outcome of old myocardial infarction; **(C)** outcome of acute myocardial infarction; **(D)** outcome of acute transmural myocardial infarction of anterior wall; **(E)** outcome of acute transmural myocardial infarction of inferior wall. The black line represents the effects produced by a single SNP, and the red line shows the causal estimate using all instrumental variables. If the solid line is completely to the left of 0, the result estimated by this SNP is that metformin can reduce the risk of outcomes. If the solid line is completely to the right of 0, the result estimated by this SNP is that metformin can increase the risk of outcomes. The result is not significant if the solid line crosses 0.

## Heterogeneity and multiplicity analysis

There was no significant heterogeneity among the IVs by Cochran's Q test (*P*>0.05). In terms of pleiotropy, MR-Egger regression showed that the intercept of each group was close to 0, and *P*>0.05. MR-PRESSO global test showed *P* > 0.05, which indicated there were no included SNPs found to have potential pleiotropy or outliers on MI, old MI, acute MI, acute transmural MI of inferior wall, or acute transmural MI of anterior wall (Table 2). Sensitivity analysis using the leave-one-out method showed that the results were stable when removing the SNPs one by one (Figure 6).

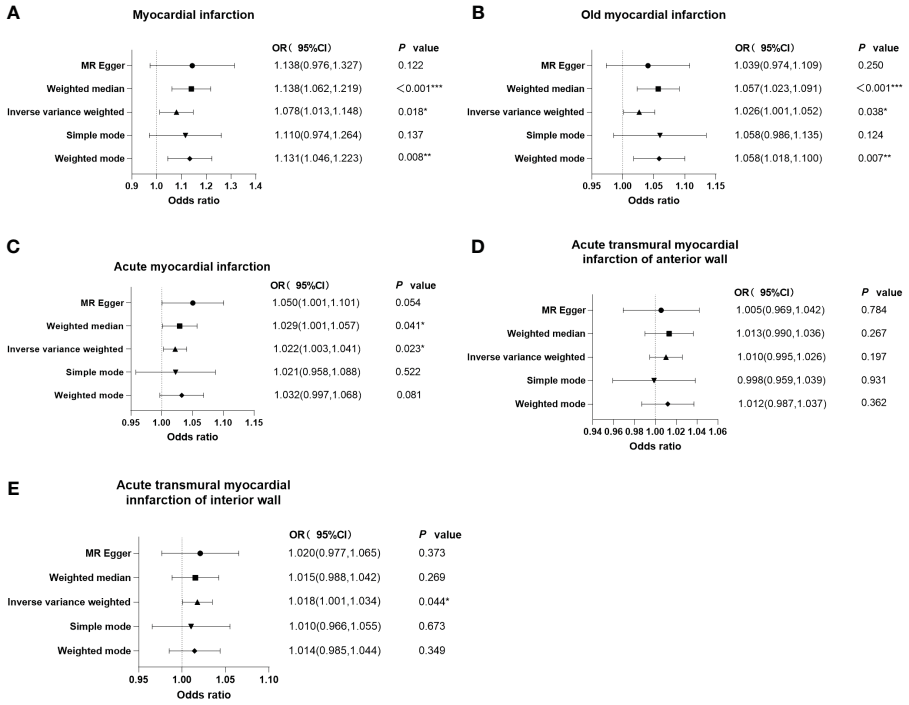


FIGURE 4
Forest plots of the results of MR-Egger regression, weighted median, inverse variance weighted, simple mode, and weighted mode analysis of metformin on outcomes. **(A)** outcome of myocardial infarction; **(B)** outcome of old myocardial infarction; **(C)** outcome of acute myocardial infarction; **(D)** outcome of acute transmural myocardial infarction of anterior wall; **(E)** outcome of acute transmural myocardial infarction of inferior wall. If the solid line is completely to the left of 1, the result estimated by this SNP is that metformin can reduce the risk of outcomes. If the solid line is completely to the right of 1, the result estimated by this SNP is that metformin can increase the risk of outcomes. The result is not significant if the solid line crosses 1.

## Analysis of bias

The results of the funnel plots analysis showed basic symmetry and there was no obvious bias on the impact of the results, so the robustness of the analysis results was excellent and the results were stable (Figure 7).

# Discussion

Diabetes is a risk factor for cardiovascular death (39). As a first-line antidiabetic agent, metformin mainly plays a hypoglycemic role by activating adenosine monophosphate activated protein kinase (AMPK) in cells and reducing glucose output from the liver. Moreover, its activation of AMPK could reduce cardiomyocyte apoptosis and the formation of myocardial AGEs by enhancing the expression of carnitine palmitoyl transferase 1, thus improving the mitochondrial β -oxidation of the fatty acids and benefiting patients with heart failure (40). When further exploring the cardiovascular protective effect of metformin, the researchers found that metformin may have a potential protective effect of atherosclerotic cardiovascular disease due to its effects on lowering blood glucose, improving endothelial dysfunction, regulating blood coagulation, reducing inflammation and regulating intestinal flora. The possible targets of metformin to impact cardiovascular outcomes in patients include liver kinase B1 (LKB1), AMPK, endothelial nitric oxide synthase (eNOS), phosphatidylinositol 3 kinase-protein kinase B (PI3K-Akt), krüppel-like factor 4 (KLF4), nuclear factor-kappa B (NF-κB) and so on (41). However, it remains unclear whether these effects are beneficial. A clinical prospective study conducted by Sardu et al. found that prediabetic patients increase the burden of inflammation in the adipose tissue around coronary arteries (30). Metformin can improve the prognosis of patients with prediabetic AMI by reducing the inflammatory tension in the pericoronary fat and the ratio of leptin to adiponectin. Another cohort study suggested that use of metformin at the first episode of AMI increases the risk of cardiovascular disease and death in patients with T2DM, and that metformin use after AMI may be beneficial. The above studies suggested that metformin may have an effect on improving the outcome of cardiovascular events in diabetic patients with AMI, and is associated with the process of the development of AMI (21). However, for non-diabetic patients, studies have demonstrated that taking metformin does not improve the prognostic outcome of MI (26). In a randomized controlled experiment, metformin was not found to reduce major cardiovascular events (27). The effect of metformin on the treatment of MI in real world studies is still controversial. In addition, most clinical trials examining the relationship between metformin and MI were small, and no studies based on MR exploring the causal effect of metformin therapy on the risk of MI were found during data review. Therefore, this study aimed to use MR theory to select SNPs related to metformin from GWAS database as IVs, so as to indirectly reveal the causal relationship between metformin and

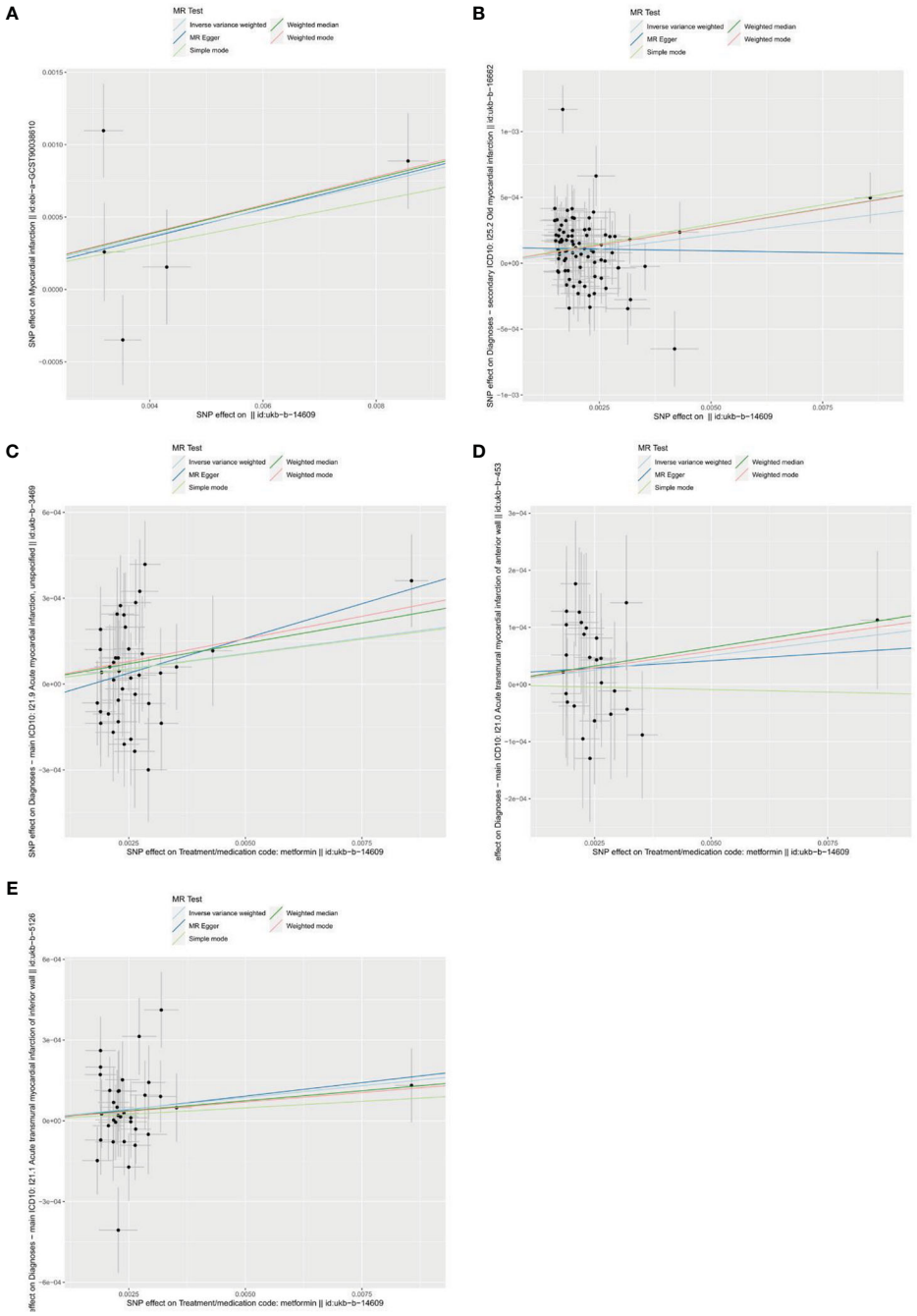


FIGURE 5
Scatters plots of the effect of metformin on outcomes. **(A)** exposure metformin and outcome myocardial infarction; **(B)** exposure metformin and outcome old myocardial infarction; **(C)** exposure metformin and outcome acute myocardial infarction; **(D)** exposure metformin and outcome acute transmural myocardial infarction of anterior wall; **(E)** exposure metformin and outcome acute transmural myocardial infarction of inferior wall. The black points represent instrumental variables. The horizontal axis represents the effect of SNPs on exposure (metformin). The vertical axis represents the effect of SNPs on the outcomes. Colored lines represent the results of MR analysis based on five methods.

MI at different stages and locations from the genetic level. The preliminary results suggested that metformin has no beneficial protective effect on MI, and may even be a risk factor for MI, old MI, acute MI, and acute transmural MI of inferior wall.

MR analysis has been widely used in academic research, although the strength of evidence is not as strong as randomized controlled trials (RCT), it is not limited by ethical and experimental conditions. It is also less susceptible to potential confounders and reverse causality compared to observational studies (32). Therefore, MR analysis is considered to be a natural RCT study, and its results are credible (42, 43). All the IVs included in this study were screened by the PhenoScanner database, and the outcome data used were derived from 6 large GWAS studies. There was no obvious heterogeneity or pleiotropy among the IVs, so the analytical conclusions are robust.

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TABLE 2 Heterogeneity and multiplicity analysis of metformin and outcomes.

| Exposure | Outcome | Method | Q | Q *P* value | egger_intercept | *P* value | MR-PRESSO *P* value |
|---|---|---|---|---|---|---|---|
| Metformin | MI | MR Egger | 23.67 | 0.050 | -2.15550e-04 | 0.464 | 0.075 |
| | | IVW | 24.63 | 0.055 | | | |
| | Old MI | MR Egger | 52.60 | 0.072 | -3.88839e-05 | 0.68 | 0.09 |
| | | IVW | 52.84 | 0.083 | | | |
| | Acute MI | MR Egger | 37.90 | 0.428 | -8.28776e-05 | 0.235 | 0.409 |
| | | IVW | 39.40 | 0.407 | | | |
| | Acute transmural MI of anterior wall | MR Egger | 13.00 | 0.966 | 1.59961e-05 | 0.768 | 0.970 |
| | | IVW | 13.09 | 0.975 | | | |
| | Acute transmural MI of anterior wall | MR Egger | 35.72 | 0.298 | -7.95372e-06 | 0.898 | 0.395 |
| | | IVW | 35.74 | 0.341 | | | |

MI, myocardial infarction; IVW, inverse variance-weighted; MR, Mendelian randomization; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier.

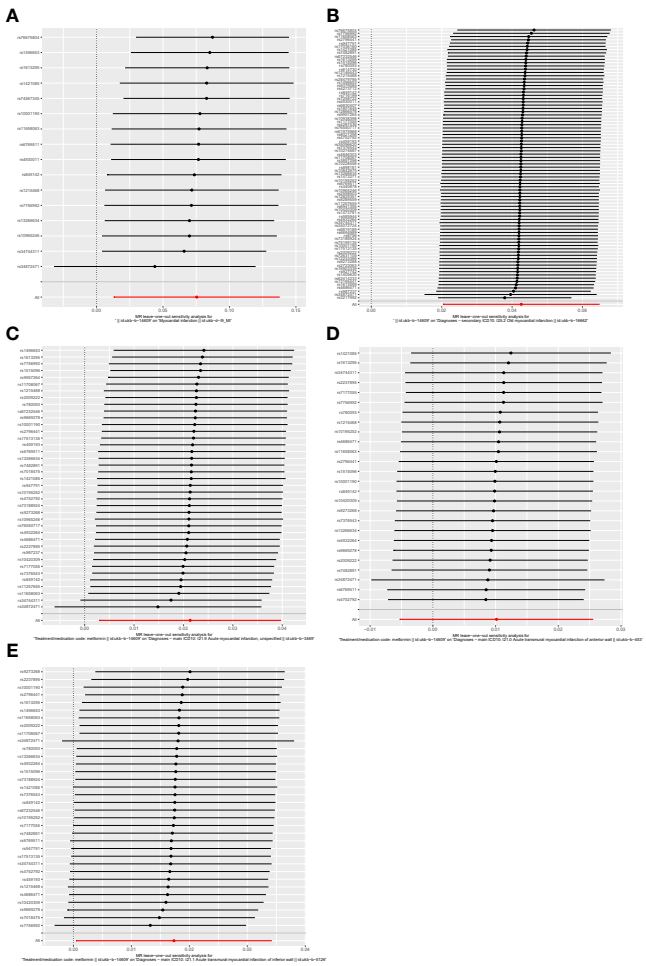


FIGURE 6
Forest plots of leave-one-out analysis. **(A)** outcome of myocardial infarction; **(B)** outcome of old myocardial infarction; **(C)** outcome of acute myocardial infarction; **(D)** outcome of acute transmural myocardial infarction of anterior wall; **(E)** outcome of acute transmural myocardial infarction of inferior wall. The positions of the red points are greater than zero. The black dots are positioned on the right side of the invalid line. This indicates that removing any of the SNPs will not significantly impact the results.

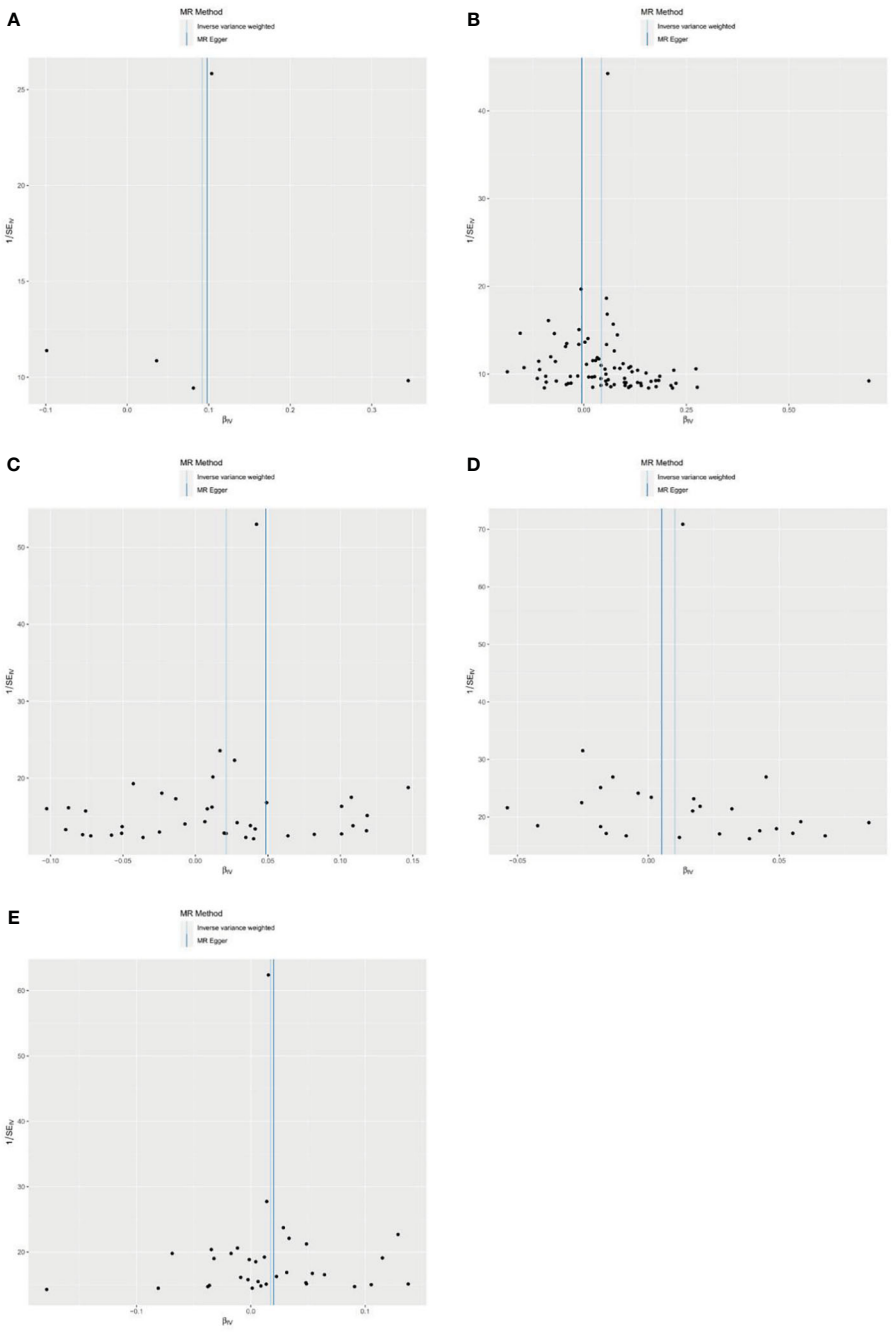


FIGURE 7
Funnel plots of the causal effect of metformin treatment on outcomes. **(A)** metformin and myocardial infarction; **(B)** metformin and old myocardial infarction; **(C)** metformin and acute myocardial infarction; **(D)** metformin and acute transmural myocardial infarction of anterior wall; **(E)** metformin and acute transmural myocardial infarction of inferior wall. Black points represent SNPs, and the distribution of points is symmetric about the inverse variance weighted and MR-Egger line.

This study also has some limitations: using GWAS data, it is impossible to explore any potential non-linear relationships or stratification effects created by age, gender, concomitant diseases and so on, which may bias the results; second, this study cannot verify whether the causal relationship between metformin treatment and MI will change with the dose or timing of metformin; finally, GWAS data only include people of European descent, and the conclusions are not representative of other ethnic groups.

In summary, from the genetic level, there is no obvious causal association between metformin and acute transmural MI of anterior wall, while for MI, old MI, acute MI, and acute transmural MI of inferior wall, it may be a risk factor. Combined with other RCT studies, it may still benefit from metformin in patients with diabetes

and MI, while metformin may not be beneficial or even increase the risk of adverse effects in non-diabetic patients. In order to confirm the conclusion of this study, further standardized and large-sample clinical trials and related MR studies are still needed to further explore the potential effects and clinical significance of metformin in the treatment of MI.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Author contributions

YZ: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Software. XP: Writing – review & editing, Formal analysis. YC: Writing – review & editing, Data curation, Methodology. JS: Writing – original draft, Funding acquisition, Project administration.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1376464/full#supplementary-material.

SUPPLEMENTARY TABLE 1
Detailed information about 16 SNPs identified as IVs to assess the genetic association between metformin and outcomes of MI.

SUPPLEMENTARY TABLE 2
Detailed information about 84 SNPs identified as IVs to assess the genetic association between metformin and outcomes of old MI.

SUPPLEMENTARY TABLE 3
Detailed information about 39 SNPs identified as IVs to assess the genetic association between metformin and outcomes of acute MI.

SUPPLEMENTARY TABLE 4
Detailed information about 26 SNPs identified as IVs to assess the genetic association between metformin and outcomes of acute transmural MI of inferior wall.

SUPPLEMENTARY TABLE 5
Detailed information about 34 SNPs identified as IVs to assess the genetic association between metformin and outcomes of acute transmural MI of anterior wall.

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# The effects of dipeptidyl peptidase-4 inhibitors on cardiac structure and function using cardiac magnetic resonance: a meta-analysis of clinical studies

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**Objective:** The aim of the study was to evaluate the effect of dipeptidyl peptidase-4 inhibitors (DPP4i) on cardiac structure and function by cardiac magnetic resonance (CMR). Research Methods & Procedures: Database including PubMed, Cochrane library, Embase and SinoMed for clinical studies of DPP4i on cardiac structure and function by CMR were searched. Two authors extracted the data and evaluated study quality independently. Mean difference (MD) or standardized MD and 95% confidence intervals (CI) were used for continuous variables. Review Manager 5.3 was used to performed the analysis.

**Results:** Ten references (nine studies) were included in this meta-analysis. Most of the studies were assessed as well quality by the assessment of methodological quality. For clinical control studies, the merged MD values of △LVEF by fixed-effect model and the pooled effect size in favor of DPP4i was 1.55 (95% CI 0.35 to 2.74, P=0.01). Compared with positive control drugs, DPP4i can significantly improve the LVEF (MD=4.69, 95%CI=2.70 to 6.69), but no such change compared to placebo (MD=-0.20, 95%CI=-1.69 to 1.29). For single-arm studies and partial clinical control studies that reported LVEF values before and after DPP4i treatment, random-effect model was used to combine effect size due to a large heterogeneity ($Chi^2$ = 11.26, P=0.02, $I^2$ = 64%), and the pooled effect size in favor of DPP4i was 2.31 (95% CI 0.01 to 4.62, P=0.05). DPP4i significantly

increased the Peak filling rate (PFR) without heterogeneity when the effect sizes of two single-arm studies were combined (MD=31.98, 95% CI 13.69 to 50.27, P=0.0006; heterogeneity test: $Chi^2$ = 0.56, P=0.46, $I^2$ = 0%).

**Conclusions:** In summary, a possible benefit of DPP4i in cardiac function (as measured by CMR) was found, both including ventricular systolic function and diastolic function.

KEYWORDS

dipeptidyl peptidase-4 inhibitors(DPP4i), type 2 diabetes mellitus(T2DM), cardiac magnetic resonance(CMR), cardiac structure and function, meta-analysis

# 1 Introduction

Patients with type 2 diabetes mellitus (T2DM) have a more than doubled risk of developing cardiovascular disease (CVD) than those without (1). More than half of the deaths in DM patients are caused by CVD (2). In addition to CVD, the pathogenesis of cardiac dysfunction caused by diabetes is quite complex, involving cellular, molecular and structural abnormalities. Diabetic cardiomyopathy is also a very important mechanism and is a type of systolic and diastolic dysfunction different from diabetic microangiopathy. Early active and effective hypoglycemic therapy can reduce the risk of complications (including microvascular and macrovascular events) or death in newly diagnosed diabetic patients (3–7). Since 2008, none of the hypoglycemic drugs have shown any cardiovascular safety concerns compared to placebo. Even some hypoglycemic drugs have shown unique cardiovascular benefits (8). Numerous clinical studies have shown evidence of cardiovascular benefits of glucagon-like peptide 1 (GLP-1) receptor agonist compared to placebo (9–15). Dipeptidyl peptidase-4 inhibitors (DPP4i), one of the commonly used hypoglycemic drugs, can improve blood glucose control in T2DM patients by inhibiting the degradation of glucopeptide-1 and glucopeptide-dependent insulin polypeptides, prolongating the action time of endogenous hormones, inhibiting glucagon levels and increasing endogenous insulin secretion (16). However, the cardiovascular effects of DPP4i in patients with diabetes remain unclear. Some studies have shown that DPP4i reduce the risk of adverse cardiovascular events, while others have been neutral about this effect (17–19).

For clinical trials, endocardial biopsy is the main method to evaluate the changes of myocardial pathological in patients. However, due to such shortcomings as the invasiveness, sampling errors, serious complications and poor consistency between observers, evidence of myocardial pathological in heart disease in human is rare (20, 21). For patients with heart diseases or high risk of heart diseases, it is important to evaluate myocardial tissue or cardiocytes by a non-invasive technique. In recent years, cardiac magnetic resonance (CMR) has been widely used in the diagnosis and prognosis assessment of various heart diseases, and is the most commonly used imaging method to evaluate myocardial damage such as myocardial edema and fibrosis (22, 23). Previous studies have found myocarditis (24, 25), dilated cardiomyopathy (26), myocardial infarction (27, 28), heart failure (29, 30) and other heart diseases that have potential myocardial damage by CMR technology, and they were well correlated with myocardial histopathological changes. Early monitoring of global or local myocardial systolic function abnormalities provides important information for early diagnosis and prognosis evaluation of cardiovascular diseases and cardiomyopathy (31). Appling CMR, a sensitive and non-invasive technique, to evaluate the benefit of hypoglycemic drugs, especially the controversial DPP4i, in heart disease may be a good suggestion.

Therefore, this study searched and summarized the previous application of CMR technology to verify the effects of DPP4i on cardiac structure and function, so as to bring better evidence-based medical evidence for the cardiac benefits of DPP4i.

# 2 Materials and methods

This meta-analysis was conducted under the guidance of the Preferred Reporting Items Statement for Systematic Evaluation and Meta-Analysis (PRISMA).

## 2.1 Searching progress

We searched the following databases for clinical studies of DPP4i on cardiac structure and function by CMR: PubMed, Cochrane library, Embase and SinoMed, for clinical studies. A list of references to all eligible articles and related review articles was also manually searched. A literature search of this meta-analysis was limited to published results. Databases were searched from the

earliest data to 29 January 2024 with the following search terms: ("Dipeptidyl-Peptidase IV Inhibitors" OR "DPP-4 Inhibitor*" OR "DPP 4 inhibitor*" OR "DPP4 inhibitor*" OR "Dipeptidyl peptidase-4 inhibitor*" OR "DPP-IV Inhibitor*" OR "DPP IV Inhibitor*" OR "saxagliptin" OR "sitagliptin" OR "vildagliptin " OR "linagliptin" OR "alogliptin" OR "anagliptin" OR "gemigliptin" OR "teneligliptin") AND ("Cardiac Imaging Techniques" OR "CMR" OR "cardiac magnetic resonance" OR "cardiac MRI imaging" OR "cardiac MR imaging"). Eligible studies were screened and selected based on the following criteria (1): published in English or Chinese (2); evaluated the effect of DPP4i on cardiac structure and function by CMR (3); clinical study (either clinical control study or single-arm study) (4); reported at least one outcome of cardiac structure and function by CMR.

## 2.2 Study selection and data extraction

The studies were screened independently by two authors, and any differences were resolved by consensus. If there is still doubt, a third experienced author was invited to join the consultation and reach a consensus finally. The following data were extracted from the eligible studies (1): characteristic of populations, interventions, number of participants (2); follow-up time (3); MR system (4); outcome index.

## 2.3 Methodological quality assessment

Risk of bias is used for randomized/clinical control studies to assess the methodological quality. A designed tool for assessing risk of bias in single-arm studies considered the following items: selection bias, lead time bias/immortal time bias, confounding by indication, misclassification bias/information bias, bias from natural recovery/regression to the mean, bias due to adjunctive therapies, attrition bias, selective reporting of outcomes. For risk of bias table, a point of 4 or less is considered "poor methodological quality", while a point above 4 is defined as "good methodological quality". Two authors scored these items independently.

## 2.4 Statistical analysis

The main outcome was the change of LVEF over the treatment duration. We also analyzed the change of other index that reflect the cardiac structure and function (including left ventricular function parameters, right ventricular function parameters, heart fat content and myocardial strain parameter) by CMR technique. For continuous variables, mean difference (MD), standardized MD and 95% confidence intervals (CI) were used. Fixed-effect model was used for data analysis. The $I^2$ was calculated as an indicator of the inter-study heterogeneity. If the heterogeneity test showed a large heterogeneity, the random-effects model was replaced. All data analysis was performed by Review Manager 5.3 (Cochrane Collaboration, United Kingdom, http://www.cochrane.org).

# 3 Results

## 3.1 Search results and characteristics of included studies

After retrieval form the database showed above and re-checking, 127 articles of potentially relevant studies need further classified. After screening the abstract, 26 articles were required to be read in full. Of these, 10 articles (9 studies) were eligible (32–41). Figure 1 shows the searching progress. Seven studies are published in English (34–41), the rest two were Chinese (32, 33). Two countries, China and Japan, each had two studies included. Canada, the UK, Germany and Australia each had one study included. And a clinical study conducted simultaneously in several countries around the world was also included in the article. Two of the studies included patients with myocardial infarction (36, 41), while the remaining studies included patients with type 2 diabetes. These studies included diabetic patients with heart failure (37, 40) or without history of heart failure (32–34, 39), or with known or suspected cardiovascular disease (38), or obese T2DM patients (35). In the nine included studies, different DPP4i were applied, including saxagliptin, sitagliptin, alogliptin and dutogliptin, and the doses vary. Totally, for clinical control studies, there are 347 patients received DPP4i treatment and 346 patients assigned to the control group. And the sample size ranges from 10 to 112 in DPP4i treatment group while 10 to 120 in control group (32, 35, 36, 38–41). For single-arm studies, 32 patients were included totally (33, 34, 37). The follow-up ranged from 28 days to 26 weeks. Table 1 summarizes the detailed characteristics of these nine included studies.

## 3.2 Quality assessment of included studies

The quality assessment of these nine studies is shown in Figure 2. Of the seven controlled clinical studies, four studies got a point of 4 or less and were considered to be of poor methodological quality. The rest three studies were considered as good (Figure 2A). For two single-arm studies, 7 points were obtained, which we can consider to be of high methodological quality (Figure 2B).

## 3.3 Effect on global cardiac function

Of the nine included studies, eight studies reported the LVEF as an outcome, and six were clinical control studies (32, 35, 36, 38, 40, 41), five studies reported LVEF both before and after DPP4i treatment, respectively (32, 33, 37, 38, 41). Totally, for clinical control studies, the merged MD values of ΔLVEF by fixed-effect

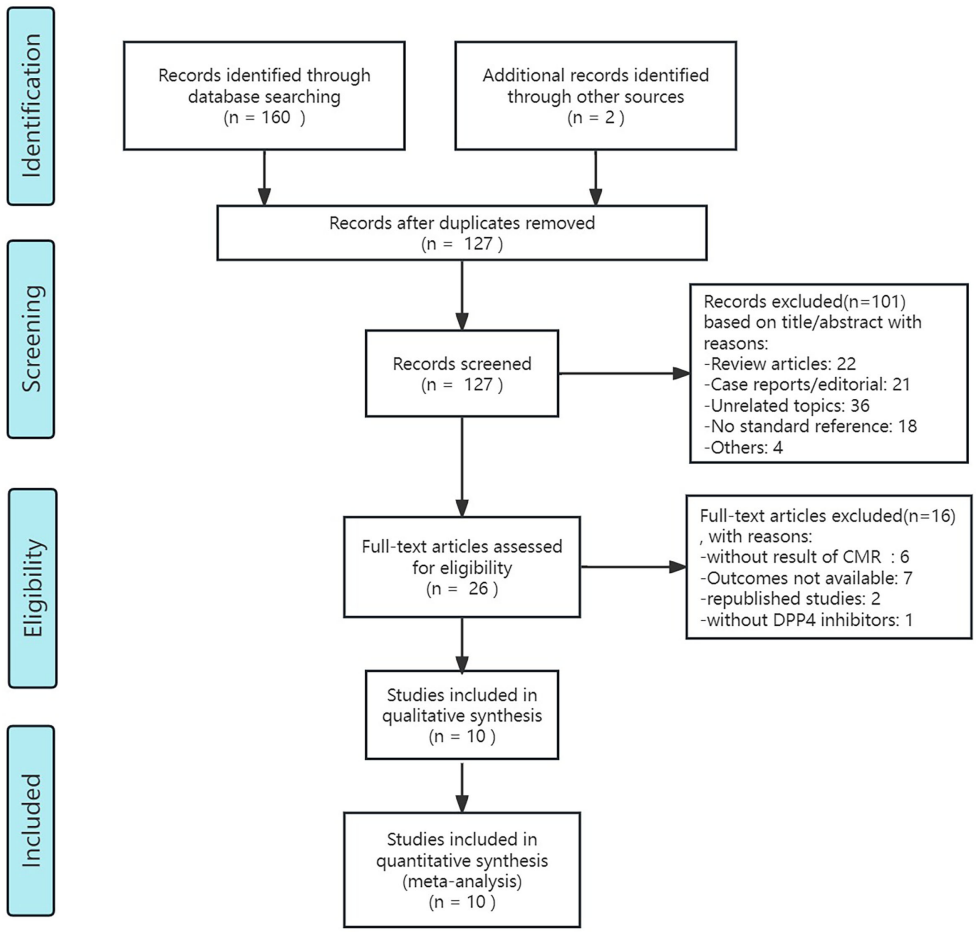


FIGURE 1
Flow chart of the systematic search process.

model and the pooled effect size in favor of DPP4i was 1.55 (95% CI 0.35 to 2.74, P=0.01). Heterogeneity analysis showed a huge heterogeneity ($Chi^2$ = 23.44, P=0.0003, $I^2$ = 79%) (Figure 3). Subgroup analysis was performed according to whether the control group was a placebo or not. Compared with positive control drugs, DPP4i can significantly improve the LVEF (MD=4.69, 95%CI=2.70 to 6.69), whereas, a big heterogeneity existed ($Chi^2$ = 8.11, P<0.00001, I2 = 75%). However, there was no such change compared to placebo (MD=-0.20, 95%CI=-1.69 to 1.29). For single-arm studies and partial clinical control studies that reported LVEF values before and after DPP4i treatment, random-effect model was used to combine effect size due to a large heterogeneity ($Chi^2$ = 11.26, P=0.02, $I^2$ = 64%), and the pooled effect size in favor of DPP4i was 2.31 (95% CI 0.01 to 4.62, P=0.05), which indicated that DPP4i could improve LVEF (Figure 4). Overall, from the above results, we can still see the trend of DPP4i's effect on improving LVEF.

Four studies reported the RVEF value (33, 36, 37, 41), two were clinical control studies (36, 41) and three analyzed the outcome both before and after DPP4i treatment (33, 37, 41). For clinical control studies, the merged MD values of △RVEF by fixed-effect model and the pooled effect size was 0.99 (95% CI -1.64 to 1.83, P=0.92). No heterogeneity was found in the heterogeneity test ($Chi^2$ = 0.76, P=0.38, $I^2$ = 0%). For three single-arm studies, no changes in RVEF were found before and after treatment (MD=-0.06, 95% CI -1.67 to 1.55, P=0.94), and no heterogeneity existed ($Chi^2$ = 0.03, P=0.98, $I^2$ = 0%). In total, DPP4i has no effect on RVEF.

## 3.4 Impact on further left ventricular structure and function parameters

Other outcome representing left ventricular structure and function parameters, such as left ventricular end-diastolic volume (LVEDV), left ventricular end-diastolic volume indexed (LVEDVI), left ventricular end-systolic volume (LVESV), left ventricular end-systolic volume indexed (LVESVI), left ventricular mass (LVM), left ventricular mass indexed (LVMI), peak filling rate (PFR) and time to peak filling rate (TPFR) have also been reported and been further analyzed here. Both in two types of studies, we have not observed the effect of DPP4i on the

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TABLE 1 Characteristic of nine included studies.

| First author, year | Country | Populations | Comparations | | Sample size | | Follow-up |
|---|---|---|---|---|---|---|---|
| | | | Experimental group | Control group | Experimental group | Control group | |
| NCT02917031 | Globally | T2DM patients with heart failure | saxagliptin | placebo | 112 | 120 | 24 weeks |
| Christoph Brenner, 2016 | Germany | NSTEMI patients | sitagliptin 100mg/day | placebo | 87 | 86 | 28 days |
| Shingo Kato, 2016 | Japan | T2DM patients with known or suspected CAD | alogliptin | glimepiride | 10 | 10 | 3 months |
| Xiaofang Wang, 2018 | China | Newly diagnosed T2DM patients with normal systolic function | sitagliptin | non-DPP4i | 58 | 59 | 12 months |
| David R. Webb MBChB, 2020 | UK | Obese T2DM patients | sitagliptin 100mg qd | liraglutide | 33 | 28 | 26 weeks |
| Shigenori Hiruma, 2021 | Japan | T2DM patients | sitagliptin 50-100mg/day | empagliflozin 50mg/day | 21 | 21 | 12 weeks |
| Paul Sandhu, 2021 | Canada | T2DM patients without a preexisting history of heart failure | saxagliptin 5mg qd | / | 16 | / | 6 months |
| Kathy CK. Wong, 2024 | | | | | | | |
| Likong Fu, 2022 | China | T2DM patients without a preexisting history of heart failure | saxagliptin 5mg qd | / | 16 | / | 6 months |
| Dirk von Lewinski, 2022 | Austria | Successfully treated STEMI but reduced LVEF | dutogliptin 60mg bid | placebo | 26 | 22 | 90 days |

T2DM, type 2 diabetes mellitus; NSTEMI, non-ST-elevation myocardial infarction; CAD, coronary artery disease; STEMI, ST-elevation myocardial infarction; LVEF, left ventricular ejection fraction; MR, magnetic resonance.

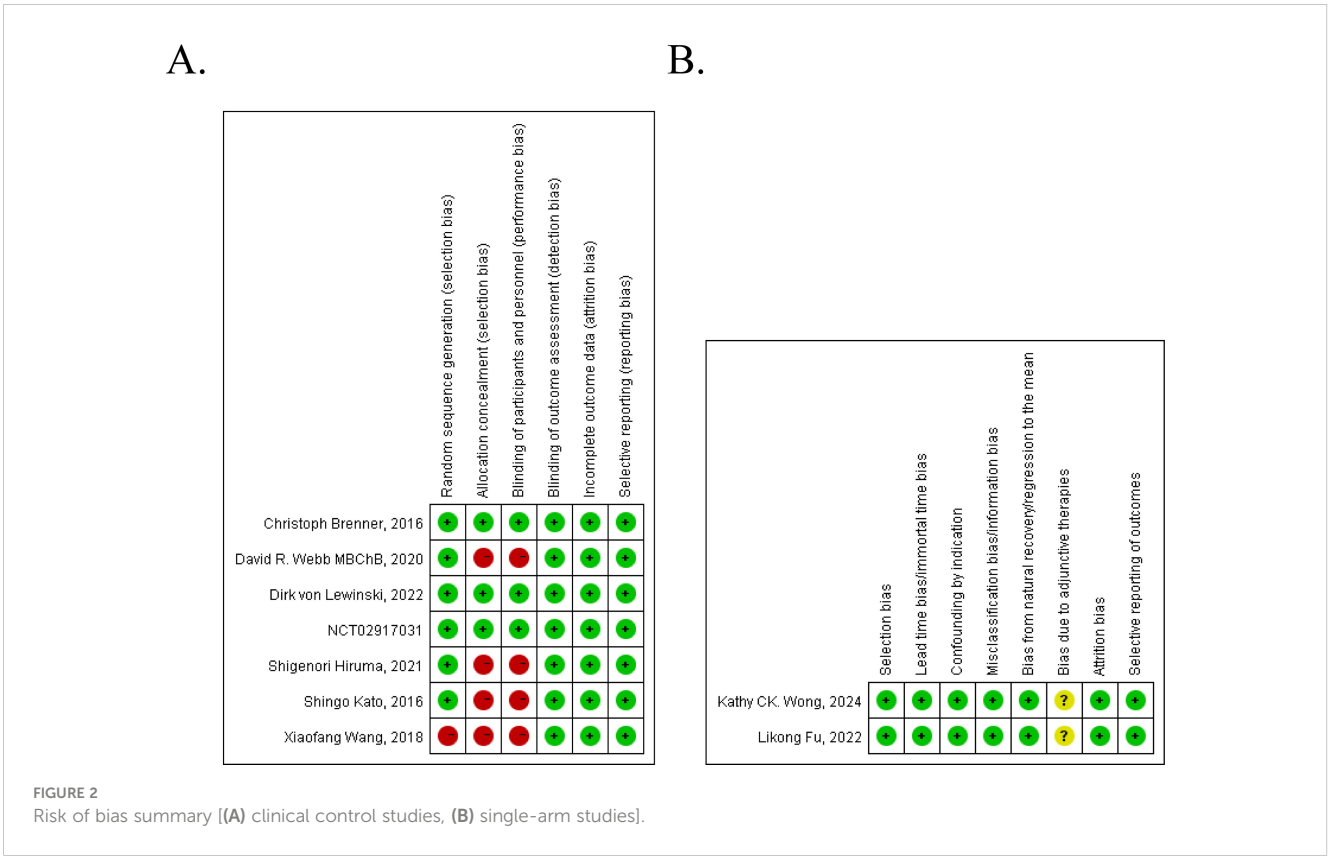


FIGURE 2
Risk of bias summary [**(A)** clinical control studies, **(B)** single-arm studies].

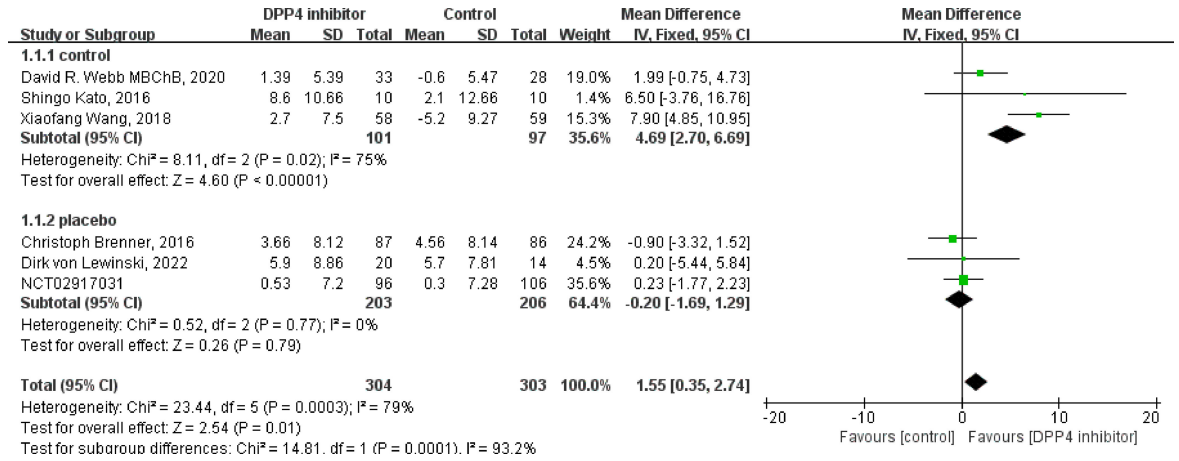


FIGURE 3
Forest plot of the LVEF through clinical control studies.

LVEDV, LVEDVI, LVESVI, LVESV, LVM and LVMI, namely DPP4i don't change these left ventricular structure and function parameters (Table 2). It was found that DPP4i significantly increased the PFR without heterogeneity when the effect sizes of two single-arm studies were combined (MD=31.98, 95% CI 13.69 to 50.27, P=0.0006; heterogeneity test: $Chi^2$ = 0.56, P=0.46, $I^2$ = 0%) (32, 34). But this result has not been confirmed when combined the results from two clinical control studies (P=0.76) (32, 35).

## 3.5 Impact on further right ventricular structure and function parameters

Only one clinical control study reported the change of right ventricular end-diastolic volume (RVEDV), right ventricular end-diastolic volume indexed (RVEDVI), right ventricular end-systolic volume (RVESV), right ventricular end-systolic volume indexed (RVESVI) compare to control group (36). At the same time, two single-arm studies reported the above indicators both before and after treatment (33, 37). Both two study types, DPP4i did not significantly change the above right ventricular structure and functional parameters and DPP4i also did not alter the RVM and RVMI in self-controlled single-arm trials. The details were shown in Table 3.

## 3.6 Other parameters

Shigenori Hiruma et al. reported the changes in accumulation of pericardial fat and myocardial triglyceride content between sitagliptin and empagliflozin groups (39). DPP4i did not significantly change the heart fat content when the statistics were pooled (accumulation of pericardial fat: MD=-79.8, 95%CI -190.13 to 30.53, P=0.16; myocardial triglyceride content: MD=0.80, 95%CI -2.49 to 4.09, P=0.63). Results of myocardial strain parameters (including global radial strain (GRS), global circumferential strain (GCS), and global longitudinal strain (GLS)) were reported in two single-arm studies (33, 37), but no changes were found.

## 3.7 Publication bias

Funnel plot was done to show the publication bias and results were shown in Figure 5. Due to the limited numbers of included studies, selection bias is significant but inevitable.

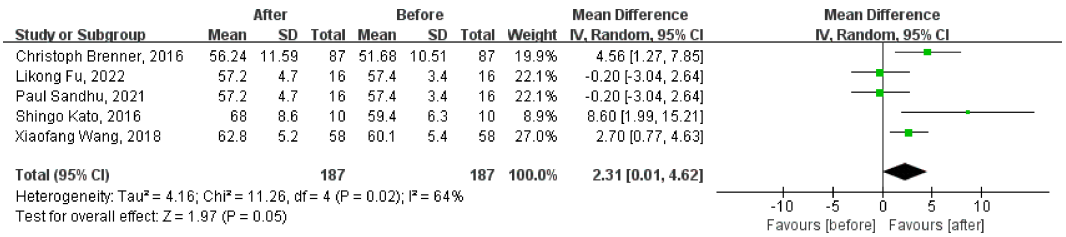


FIGURE 4
Forest plot of the LVEF through single-arm studies (self before-after comparison).

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TABLE 2 Summary of left ventricular structure and function parameters by CMR.

| Index | Reference | Model | Mean difference | 95%CI | *P* valve | $I^2$(%) |
|---|---|---|---|---|---|---|
| Clinical control studies | | | | | | |
| LVEDV | 1, 5 | Fixed-effect | 1.00 | (-9.92,11.92) | 0.86 | 0 |
| LVEDVI | 4, 5, 7, 9,10 | Fixed-effect | -1.88 | (-4.19,0.44) | 0.11 | 0 |
| LVESV | 1, 5 | Fixed-effect | 0.51 | (-6.52,7.54) | 0.89 | 0 |
| LVESVI | 5, 7, 9,10 | Fixed-effect | -1.15 | (-3.46,1.17) | 0.33 | 0 |
| LVM | 4, 5, 9 | Fixed-effect | -2.38 | (-5.87,1.11) | 0.18 | 0 |
| LVMI | 4, 5, 7 | Fixed-effect | -1.28 | (-3.86,1.30) | 0.33 | 0 |
| PFR | 1,4 | Random-effect | 12.70 | (-68.08,93.48) | 0.76 | 92 |
| Single-arm studies | | | | | | |
| LVEDV | 1,2,6 | Fixed-effect | 3.65 | (-3.90,11.20) | 0.34 | 0 |
| LVEDVI | 2,6,7,10 | Fixed-effect | 0.06 | (-3.12,3.24) | 0.97 | 0 |
| LVESV | 1,2,6 | Fixed-effect | -0.16 | (-3.82,3.50) | 0.93 | 0 |
| LVESVI | 2,6,7,10 | Fixed-effect | -0.83 | (-2.71,1.06) | 0.39 | 0 |
| LVM | 2, 6 | Fixed-effect | 3.00 | (-7.03,13.03) | 0.56 | 0 |
| LVMI | 2,6,7 | Fixed-effect | 0.78 | (-2.80,4.36) | 0.67 | 0 |
| PFR | 1,3 | Fixed-effect | 31.98 | (13.69,50.27) | 0.0006 | 0 |
| TPFR | 1,3 | Random-effect | Std. -0.45 | (-1.17,0.27) | 0.22 | 71 |

LVEDV, left ventricular end-diastolic volume; LVEDVI, left ventricular end-diastolic volume indexed; LVESV, left ventricular end-systolic volume; LVESVI, left ventricular end-systolic volume indexed; LVM, left ventricular mass; LVMI, left ventricular mass indexed; PFR, peak filling rate; TPFR, time to peak filling rate.

# 4 Discussion

Previous clinical studies have suggested the cardiac safety and even cardiac benefits of a variety of hypoglycemic drugs, including DPP4i. Through this study, we found that DPP4i can indeed improve the level of LVEF, and the results from single-arm studies suggest the potential role of DPP4i in improving PFR.

LVEF is an indicator of left ventricular systolic dysfunction, which is of great significance for the prognosis of heart disease. Studies conducted by Read et al. have shown that DPP4i can increase the

TABLE 3 Summary of right ventricular structure and function parameters by CMR.

| Index | Reference | Model | Mean difference | 95%CI | *P* valve | $I^2$(%) |
|---|---|---|---|---|---|---|
| Clinical control studies | | | | | | |
| RVEDV | 5 | – | 6.50 | (-12.20,25.20) | 0.50 | – |
| RVEDVI | 5 | – | 2.10 | (-7.40,11.60) | 0.66 | – |
| RVESV | 5 | – | -0.30 | (-13.88,13.28) | 0.97 | – |
| RVESVI | 5 | – | 3.30 | (-1.49,8.09) | 0.18 | – |
| Single-arm studies | | | | | | |
| RVEDV | 2,6 | Fixed-effect | 2.00 | (-11.16,15.16) | 0.77 | 0 |
| RVEDVI | 2,6 | Fixed-effect | 1.00 | (-4.22,6.22) | 0.71 | 0 |
| RVESV | 2,6 | Fixed-effect | 1.00 | (-5.58,7.58) | 0.77 | 0 |
| RVESVI | 2,6 | Fixed-effect | 0.00 | (-2.71,2.71) | 1.00 | 0 |
| RVM | 2, 6 | Fixed-effect | 0.00 | (-2.26,2.26) | 1.00 | 0 |
| RVMI | 2, 6 | Fixed-effect | 0.00 | (-0.88,0.88) | 1.00 | 0 |

RVEDV, right ventricular end-diastolic volume; RVEDVI, right ventricular end-diastolic volume indexed; RVESV, right ventricular end-systolic volume; RVESVI, right ventricular end-systolic volume indexed; RVM, right ventricular mass; RVMI, right ventricular mass indexed.

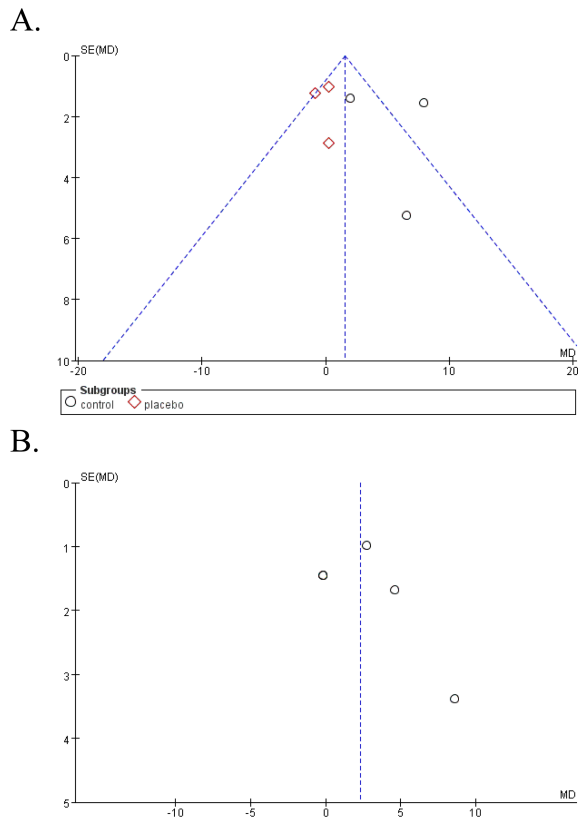


FIGURE 5
Funnel plot of publication bias [**(A)** clinical control studies, **(B)** single-arm studies].

LVEF assessed by dobutamine stress in patients with ischemic heart disease (42). In addition, echocardiography is a clinical first-line imaging examination of cardiovascular diseases, mainly used to evaluate the heart structure, function and hemodynamics, is currently the highest time resolution of non-invasive imaging technology, little affected by heart rate and rhythm. Compared with other imaging methods, echocardiography has the advantages of real-time, dynamic, convenient and economical, but low spatial resolution, low signal-to-noise ratio, small scanning field of view and large operator dependence are its main shortcomings. Obviously, the use of echocardiography to evaluate the effect of DPP4i on LVEF has also been adopted by some scholars, and the effect of DPP4i on improving LVEF has also been reported (32, 43). However, some studies have come to the opposite conclusion (44). Could this be due to the limitations of echocardiography? Compared with echocardiography, CMR has higher spatial resolution, can accurately delineate the endocardium and the epicardium, and then obtain more accurate cardiac function parameters, and it has become the recognized gold standard for non-invasive evaluation of cardiac structure and function (45, 46). Therefore, CMR was used to evaluate left ventricular systolic function in this study, which has a high sensitivity and makes the study results more accurate. However, the mechanism by which DPP4i increase the level of LVEF is not fully understand. According to Frank-Starling's law, an increase in LVEDV leads to an increase in ejection fraction. However, our study found that despite an increase in LVEF, there was no increase in LVEDV. It has also been suggested that anti-inflammatory effect may be one of the mechanisms, but it has not been widely verified and recognized (38, 47). Another more accepted theory may be that because DPP4i can increase endogenous GLP-1 and glucose-dependent insulin stimulating polypeptide concentrations by inhibiting DPP4 enzyme activity, and enhance their effects. GLP-1 agonists have been shown to increase the level of LVEF (44). This may due to its role in regulating PI3K/Akt1 and AMPKα signaling that to inhibit angiotensin II and pressure overloading inducing cardiac remodeling (48). Wang et al. (49) also found that the cardioprotective effect of GLP-1 agonists may depend on the inhibition of oxidative stress through the mammalian target rapamycin complex 1/p70 ribosomal protein S6 kinase pathway. In experimental animal models of heart failure, GLP-1 agonists protect the heart during acute ischemia and improve mitochondrial function, microvascular function and myocardial glucose uptake (50, 51). GLP-1 agonists were found in left ventricular cardiomyocytes, thus it may have a direct effect on the ventricle (52). In addition, GLP-1 agonists can reduce inflammation, reduce ischemic injury, increase heart rate, promote plaque stabilization, and reduce smooth muscle proliferation (53). Studies have also shown that the positive effects of GLP-1 agonists on cardiovascular diseases may be the result of direct action on the arteriosclerosis process (54). Therefore, the principal mechanisms underlying this cardioprotection likely involve the suppression of cardiac oxidative stress, apoptosis, ferroptosis, necroptosis, and pyroptosis, which may also directly contribute to enhanced cardiac resistance to ischemia/reperfusion (I/R) injury (55). Furthermore, DPP4i mitigate the progression of diabetic microvascular and cardiovascular complications by reversing alterations associated with hyperglycemic memory. This process is linked to epigenetic modifications, which represent a significant area of current research within the context of metabolic memory phenomena related to diabetic complications (56).

PFR is an important indicator of diastolic function, which decreases when this function is impaired. Diabetic cardiomyopathy is strongly linked to diastolic dysfunction (57). Our research indicates that DPP4i can enhance PFR, highlighting its potential as a treatment for diastolic dysfunction. Earlier studies found that sitagliptin delayed left ventricular diastolic dysfunction in diabetic mice (58). In essence, DPP4i effectively enhances cardiac function.

However, this meta-analysis has some limitations. Only nine studies were included, potentially introducing biases and methodological errors due to their varying designs and poor methodological quality. The small number of studies in subgroup analyses could impact the findings' reliability. Additionally, DPP4i directly affects ventricular function, but changes in body weight, blood pressure, and waist size could indirectly influence cardiometabolic parameters. While DPP4i's cardiac benefits seem consistent in patients with or without CVD or HF history, but more research is needed to assess their advantages based on different HF and CVD risk levels due to limited clinical data.

## 5 Conclusions

In conclusion, the results of our meta-analysis show that DPP4i ameliorates PFR and LVEF levels in patients, as measured by CMR technology.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Author contributions

HW: Data curation, Formal analysis, Funding acquisition, Writing – original draft. SYG: Data curation, Formal analysis, Writing – original draft. SG: Data curation, Writing – original draft. CL: Data curation, Writing – original draft. FW: Data curation, Writing – original draft. JZ: Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Causal association between 1400 metabolites and dilated cardiomyopathy: a bidirectional two-sample Mendelian randomization analysis

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**Background:** Dilated cardiomyopathy (DCM) is a cardiac disease with a poor prognosis of unclear etiology. Previous studies have shown that metabolism is associated with DCM. This study investigates the causal relationship between 1400 metabolites and DCM using a two-sample Mendelian randomization (MR) approach.

**Methods:** The study utilized data from the OpenGWAS database, comprising 355,381 Europeans, including 1,444 DCM cases. A total of 1,400 metabolites were evaluated for their causal association with DCM. Instrumental variables (IVs) were selected based on genetic variation and used in the MR analysis. The primary analysis method was inverse variance weighting (IVW), supplemented by weighted median-based estimation and sensitivity analyses.

**Results:** Of the 1,400 metabolites analyzed, 52 were identified as causally associated with DCM. The analysis revealed both positively and negatively correlated metabolites with DCM risk. Notable findings include the positive correlation of Tryptophan betaine and 5-methyluridine (ribothymidine) levels, and an inverse association of Myristoleate and Erythronate levels with DCM.

**Conclusions:** The study provides significant insights into the metabolites potentially involved in the pathogenesis of DCM. These findings could pave the way for new therapeutic strategies and biomarker identification in DCM management.

KEYWORDS

**dilated cardiomyopathy, metabolites, Mendelian randomization, causal analysis, genetic variants, epidemiology**

# 1 Introduction

Dilated cardiomyopathy (DCM) is a serious cardiac disease, the pathogenesis of which is still unclear (1). DCM is mainly characterized by the dilation and impaired contraction of one or both ventricles. It is a common cause of heart failure and has a high mortality rate (2). The incidence of DCM is about 36 cases per 100,000 people. It is a common cause of heart failure and has a high mortality rate, with an incidence of about 36 cases per 100,000 people (3). The risk of DCM is 1.5-2 times higher in men than in women. DCM may occur in families, but most cases are sporadic. DCM can be caused by a variety of factors, including genetic factors, viral infections, autoimmune reactions, malnutrition, and alcoholism (4, 5). Some genetic variants are known to increase susceptibility to DCM, such as genes encoding proteins such as myosin and nuclear membrane proteins. Titin (TTN) is recognized as the major candidate gene for DCM, with mutations in TTN accounting for a significant proportion of familial and idiopathic cases (6), and LMNA, known for its role in laminopathies affecting cardiac function. Missense mutations in SCN5A have also been identified in DCM and carry a higher risk for arrythmias (7, 8). Mutations in RBM20 are responsible for 1–5% of genetic DCM (62) (9). These genetic abnormalities disrupt normal cardiac structure and function, exacerbating the effects of diabetes and leading to the development of DCM. Understanding these genetic contributions is essential for identifying at-risk individuals and developing targeted therapeutic strategies.

The development of metabolomics technology has provided new insights into the pathogenesis of DCM. Metabolomics can detect changes in metabolites in body fluids and tissues that reflect underlying biological processes (10). Recent metabolomics studies have identified altered serum and urinary metabolic profiles in patients with DCM. For example, serum levels of amino acids, carnitine, and creatine are elevated in patients with DCM, while levels of guanosine and inositol are reduced. This suggests that metabolic abnormalities may be involved in the pathogenesis of DCM. However, large-scale metabolomics studies have not systematically assessed the role of a wide range of metabolites in the pathogenesis of DCM (11). Clarifying the causal relationship between metabolites and the risk of DCM is crucial, as it could inform mechanistic studies and drug target discovery in DCM.

Current statistical methods for inferring causal relationships between metabolites and disease risk have limitations. Traditional observational studies are susceptible to confounding factors (12). In addition, it is difficult to distinguish whether metabolite changes are a consequence or a cause of DCM from observational data alone. To overcome these limitations, the present study adopted the Mendel randomization (MR) method, which uses genetic variation as an instrumental variable to assess the causal relationship between metabolites and the risk of DCM (13). The MR method utilizes the principle of random assignment of alleles inherited from parents to enhance the reliability of causal inference. It is less susceptible to confounding than observational studies. Therefore, the MR method provides more reliable causal hypothesis testing (14).

In this study, the causal associations between 1400 metabolites and DCM risk were assessed by a two-sample MR design using genetic and DCM data from the OpenGWAS database. The identified candidate causal metabolites may provide insights into the pathogenesis and therapeutic targets of DCM. More broadly, this study demonstrates the potential of MR methods in elucidating the metabolic mechanisms of complex diseases.

# 2 Materials and methods

## 2.1 Study design

Based on two-sample MR Analysis, we evaluated the causal relationship between 1400 metabolites and dilated cardiomyopathy. MR Uses genetic variation to represent risk factors, so valid instrumental variables (IVs) in causal reasoning must satisfy three key assumptions: (1) genetic variation is directly associated with exposure; (2) genetic variation is not associated with possible confounders between exposure and outcome; and (3) genetic variation does not affect outcome through pathways other than exposure (15, 16). This study used dilated cardiomyopathy data from the OpenGWAS, which included 355381 Europeans, including 1444 cases and 353937 as the control group.

## 2.2 Metabolite GWAS data sources

Aggregate GWAS statistics for each metabolite are publicly available from the GWAS catalog (registration numbers from GCST90199621 to GCST90201020). This is a large-scale GWAS study that includes 1091 metabolites and 309 metabolite ratios from 8299 individuals in the Canadian Longitudinal Aging Study (CLSA) cohort (17, 18).

## 2.3 Selection of instrumental variables

Since genetic variation is directly related to exposure, the significance level of IVs for each metabolite was set at $1\times10^{-5}$, the significance level of IVs for each metabolite was set at $1\times10^{-5}$. To obtain IVs for independent sites, we used the "Two Sample MR" packet data with a linkage unbalance (LD) threshold set to $R^2<0.001$ and an aggregation distance of 10,000 kb (19, 20). For dilated cardiomyopathy, we adjusted the significance level to $5\times10^{-6}$, which is commonly used to represent genome-wide significance in GWAS, with a LD threshold of $R^2<0.001$ and an aggregation distance of 10,000 kb (21).

## 2.4 Statistical analysis

For the statistical analysis portion of our study examining the causal influence of metabolite on dilated cardiomyopathy risk, all procedures were conducted using R software, version 4.2.1, which is a widely used environment for statistical computing and graphics, available at (http://www.Rproject.org). To ascertain the causal relationships between the 1400 metabolite and dilated cardiomyopathy, we primarily employed methods including

inverse variance weighting (IVW), weighted median-based estimation. These analyses were facilitated by the 'TwoSampleMR' package, version 0.5.7, within the R software environment. This package is specifically designed for conducting MR analyses, providing tools for estimation, testing, and sensitivity analysis of causal effects (22). The IVW method is a standard approach in MR that combines the Wald estimates (ratio of the SNP-outcome association to the SNP-exposure association) from multiple genetic variants, weighting by the inverse variance of each SNP-outcome association. The weighted median and mode-based methods serve as supplementary approaches that provide robust causal estimates even when some of the instrumental variables are invalid, as long as certain assumptions are met. These analyses were backed up by rigorous sensitivity analyses, including Cochran's Q test to examine heterogeneity amongst the instrumental variables (23). Such thorough statistical evaluation ensures that the findings regarding the relationship between metabolite and dilated cardiomyopathy are as reliable and accurate as possible given the data. The whole process was shown in Figure 1.

# 3 Results

## 3.1 Exploration of the causal effect of metabolite on dilated cardiomyopathy risk

At the significance level of 0.05, a total of 52 metabolite were identified as causally associated with the development of dilated cardiomyopathy (as shown in Figure 2).

The Tryptophan betaine levels(P=0.029,OR=1.174, 95% CI =1.016~1.356),5-methyluridine (ribothymidine) levels (P=0.042, OR=1.130, 95%CI =1.004~1.271), 2-acetamidophenol sulfate levels (P=0.039, OR=1.219, 95%CI =1.009~1.473), Linolenoylcarnitine (C18:3) levels(P=0.041, OR=1.238, 95%CI =1.008~1.520), Dihomo-linolenoylcarnitine (C20:3n3 or 6) levels (P=0.009,OR=1.146, 95% CI =1.034~1.271), Glucuronide of C10H18O2 (7) levels (P=0.014, OR=1.223, 95%CI =1.040~1.438), Eicosenoylcarnitine (C20:1) levels (P=0.001, OR=1.305, 95%CI =1.110~1.534), 3-hydroxy hexanoylcarnitine (1) levels(P=0.047,OR=1.225, 95% CI =1.002~1.496), Cis-3,4-methyleneheptanoylcarnitine levels (P=0.016, OR=1.203, 95%CI =1.034~1.399), Stearate (18:0) levels (P=0.022,OR=1.297, 95%CI =1.037~1.623), Palmitate (16:0) levels (P=0.038,OR=1.246, 95%CI =1.011~1.535), Malate levels (P=0.017, OR=1.253, 95%CI =1.040~1.511), Trans-urocanate levels (P=0.001,OR=1.282, 95%CI =1.101~1.492), Cytosine levels (P=0.047, OR=1.248, 95%CI =1.002~1.554), Arachidate (20:0) levels (P=0.023, OR=1.262, 95%CI =1.031~1.545), Maltose levels in coronary artery disease (P=0.045,OR=1.208, 95%CI =1.003~1.455), X-18913 levels (P=0.007, OR=1.284, 95%CI =1.067~1.545), X-21736 levels (P=0.005,OR=1.255, 95%CI =1.067~1.476), X-23997 levels (P=0.038, OR=1.224, 95%CI =1.011~1.483),X-19141 levels (P=0.009, OR=1.249, 95%CI =1.055~1.478), Adenosine 5'-monophosphate (AMP) to inosine 5'-monophosphate (IMP) ratio (P=0.017,OR=1.173, 95%CI =1.027~1.339), Cortisone to cortisol ratio (P=0.025,OR=1.269, 95%CI =1.030~1.564), Spermidine to choline ratio(P=0.042, OR=1.254, 95% CI =1.007~1.562), Adenosine 5'-diphosphate (ADP) to glycine ratio (P=0.020, OR=1.149, 95%CI =1.021~1.293),1-oleoyl-GPE (18:1) levels (P=0.008,OR=1.258, 95%CI =1.061~1.493)are positively correlated with dilated cardiomyopathy.

While Myristoleate (14:1n5) levels (P=0.012,OR=0.746, 95%CI =0.592~0.940), Erythronate levels (P=0.021,OR=0.783, 95%CI =0.635~0.965), N6-carbamoylthreonyladenosine levels (P=0.018,

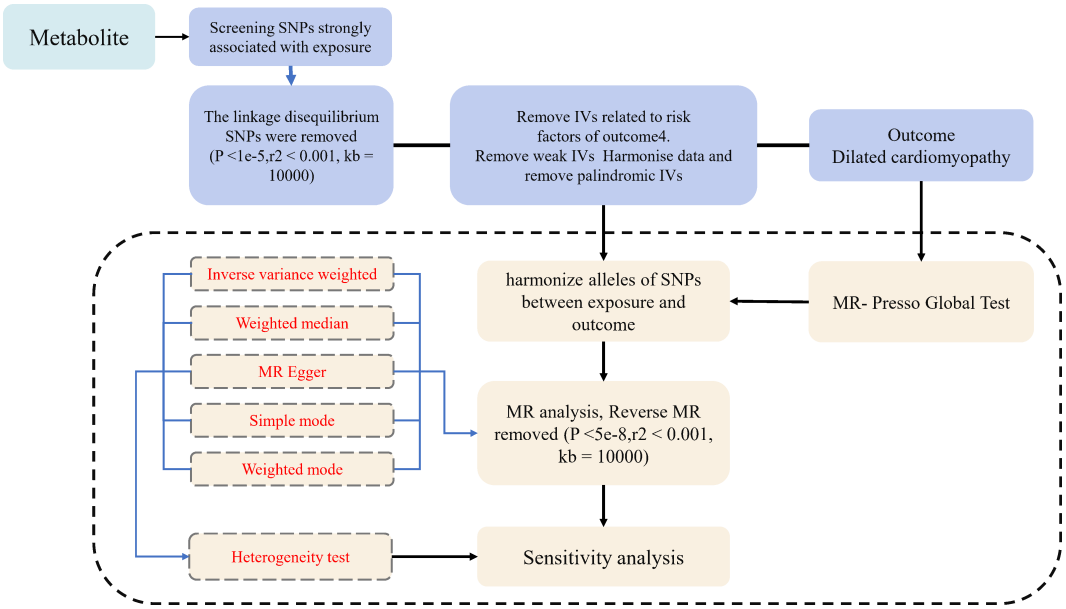


FIGURE 1
Flow diagram for quality control of the instrumental variables (IVs) and the entire Mendelian Randomization (MR) analysis process. SNPs, single-nucleotide polymorphisms; IVW, inverse variance weighted; MR, Mendelian Randomization; MR Presso, Mendelian Randomization Pleiotropy RESidual Sum and Outlier.

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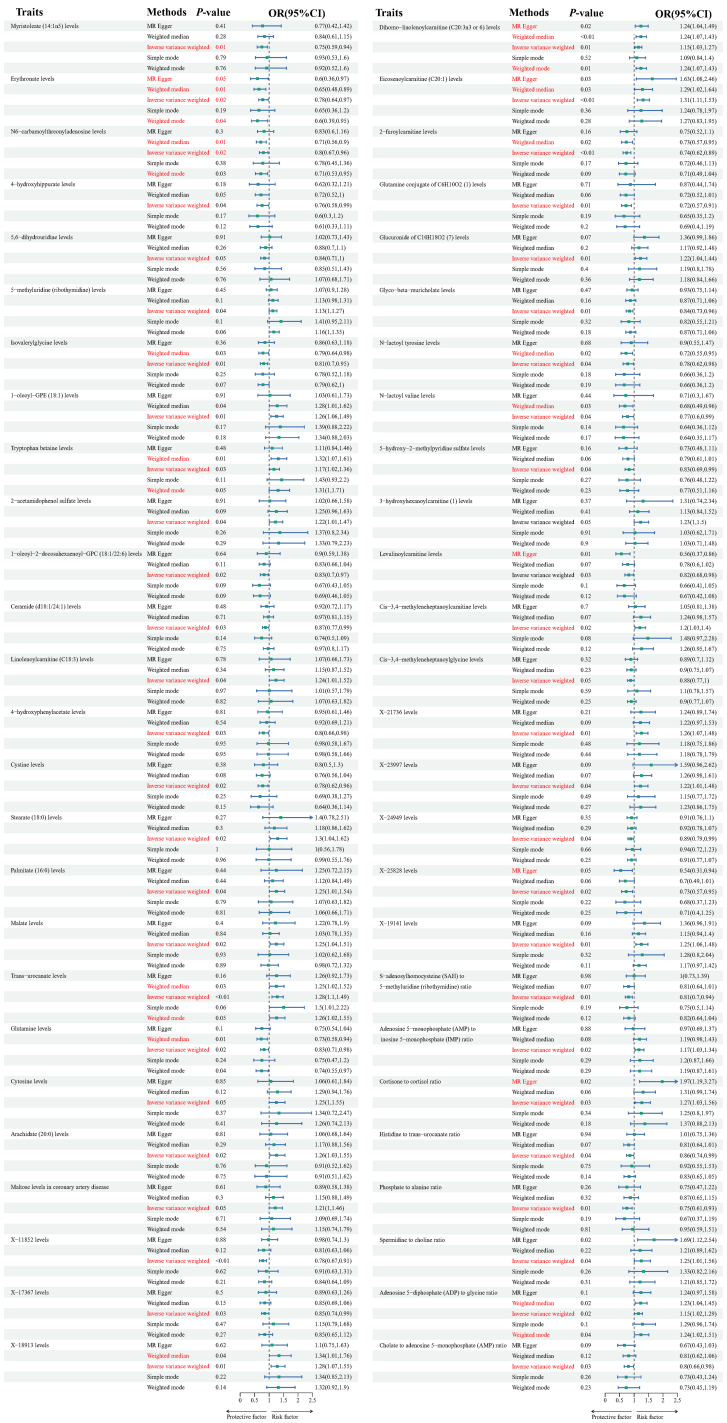


FIGURE 2
Forest plots depicting the causal associations between dilated cardiomyopathy and specific metabolites. IVW, inverse variance weighting; CI, confidence interval.

OR=0.804, 95%CI =0.671~0.964), 4-hydroxyhippurate levels (P=0.039, OR=0.755, 95%CI =0.577~0.987), 5,6-dihydrouridine levels (P=0.047,OR=0.840, 95%CI =0.707~0.998), Isovalerylglycine levels (P=0.009, OR=0.812, 95%CI =0.695~0.950), 1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6) levels (P=0.021, OR=0.827, 95% CI =0.704~0.972), Ceramide (d18:1/24:1) levels (P=0.029,OR=0.871, 95%CI =0.769~0.986), Glutamine conjugate of C6H10O2 (1) levels (P=0.005,OR=0.717, 95%CI =0.567~0.906), 2-furoylcarnitine levels (P=0.001,OR=0.742, 95%CI =0.618~0.890), Glyco-beta-muricholate levels (P=0.010,OR=0.835, 95%CI =0.728~0.957), N-lactoyl tyrosine levels (P=0.035,OR=0.783, 95%CI =0.623~0.984), N-lactoyl valine levels (P=0.040,OR=0.768, 95%CI =0.596~0.988), 5-hydroxy-2-methylpyridine sulfate levels (P=0.038,OR=0.825, 95%CI =0.688~0.990), Levulinoylcarnitine levels (P=0.027, OR=0.818,

95%CI =0.684~0.978), Cis-3,4-methyleneheptanoylglycine levels (P=0.046, OR=0.879, 95%CI =0.774~0.998), 4-hydroxyphenylacetate levels (P=0.033, OR=0.802, 95%CI =0.655~0.982), Cystine levels (P=0.021,OR=0.775, 95%CI =0.624~0.962), Glutamine levels (P=0.023, OR=0.832, 95% CI =0.710~0.976), X-11852 levels (P=0.001,OR=0.780, 95%CI =0.667~0.912),X-17367 levels (P=0.033,OR=0.854, 95%CI =0.739~0.987),X-24949 levels(P=0.040,OR=0.887, 95% CI =0.790~0.994),X-25828 levels (P=0.019,OR=0.734, 95%CI =0.567~0.952),S-adenosylhomocysteine (SAH) to 5-methyluridine (ribothymidine) ratio (P=0.006,OR=0.813, 95%CI =0.701~0.944), Histidine to trans-urocanate ratio (P=0.039,OR=0.856, 95%CI =0.739~0.992), Phosphate to alanine ratio(P=0.008,OR=0.750, 95% CI =0.606~0.928), Cholate to adenosine 5'-monophosphate (AMP) ratio(P=0.029,OR=10,802, 95%CI =0.658~0.977) are inversely associated with dilated cardiomyopathy. Results from sensitivity analyses demonstrate the robustness of the observed causal association (Supplementary Figure 1). Scatter plot and funnel plot also show the stability of the results (Supplementary Figures 2, 3).

### 3.2 Exploration of the causal effect of dilated cardiomyopathy risk on metabolite

To investigate the causal relationship between dilated cardiomyopathy and metabolites, a two-sample Mendelian randomization (MR) analysis was employed, with the Inverse Variance Weighting (IVW) method as the primary analytical approach and other methods serving as supplementary. Subsequently, reverse MR was used to explore the impact of dilated cardiomyopathy onset on the aforementioned 50 metabolites. The results showed that there was no causal relationship between dilated cardiomyopathy and the 52 aforementioned diseases.

## 4 Discussion

In this study, we performed a comprehensive Mendelian randomization analysis to assess causal associations between circulating metabolites and dilated cardiomyopathy (DCM) risk. Our analysis identified 52 metabolites across multiple classes exhibiting significant causal relationships with DCM. These results provide unique insights into the metabolic pathways that may be involved in DCM development and progression. More broadly, this work highlights the utility of MR methods in elucidating complex disease mechanisms.

We found metabolites from diverse biochemical families, including amino acids, lipids, microbial metabolites, nucleotides, and carbohydrates, that demonstrated causal impacts on DCM risk. Both positive and inverse associations were observed, indicating potential pathogenic and protective effects of various metabolites. Several of the identified compounds have known links to cardiac physiology or dysfunction, further supporting their causal implications in DCM revealed by this analysis.

For instance, we found that higher levels of tryptophan betaine were causally associated with increased DCM risk. Tryptophan betaine, also known as ergothioneine, is a dietary phytochemical and antioxidant. While its cardioprotective properties have been previously described, our findings suggest it may exert adverse effects in DCM (24). Consistent with our conclusions, one recent reports supporting the association of typtophan betaine with heart failure (25). This compound is transported into tissues via organic cation transporters, whose expression and activity are altered in cardiovascular disease (26). The resultant accumulation of tryptophan betaine could potentially disturb redox homeostasis or cation homeostasis in ways that promote cardiomyopathy. Additional work is warranted to clarify the mechanisms underlying its causal relationship with DCM observed here.

Among the lipids, myristoleate was found to be negatively associated with DCM risk, indicating a potential protective effect. Myristoleate is an omega-5 unsaturated fatty acid that can act as a bioactive lipid mediator. In mice, it was shown to attenuate cardiac dysfunction and remodeling following experimental myocardial infarction (27). Myristoleate supplementation also improved left ventricular performance in a rat model of ischemic cardiomyopathy (28). Our results provide orthogonal population-level evidence that higher myristoleate levels may preserve cardiac structure and function, thereby lowering susceptibility to DCM. The cytoprotective effects are thought to derive from its capacity to resolve macrophage inflammation.

Another notable finding was the positive causal association between ribothymidine levels and increased DCM risk. Ribothymidine is a modified nucleoside found in transfer RNA. Though its functional roles are incompletely defined, it may be involved in mediating cellular responses to stress. Our results further indicate this nucleoside associates with and may promote cardiomyopathic changes. Altered ribothymidine metabolism could perturb RNA biology in ways that undermine myocardial viability.

We also found multiple microbial metabolites exhibiting causal links with DCM, including p-cresol sulfate, phenyllactate, and imidazole propionate. This aligns with the recognized role of the gut microbiome and its products in shaping cardiovascular health (29). For example, p-cresol sulfate, generated by gut bacterial fermentation, positively associated with major adverse cardiovascular events in chronic kidney disease patients (30). The absorptive transport of microbial metabolites and their effects on factors like vasoreactivity, inflammation, and redox balance may thus be an important conduit through which the microbiota influences DCM development.

Overall, our findings nominate a number of metabolites across diverse classes that may be involved in DCM pathogenesis through both novel and established mechanisms. By leveraging genetic anchors for improved causal inference, this study moves beyond simply observing metabolite associations to provide evidence these molecules may directly modulate DCM susceptibility. Additional research is warranted to elucidate their underlying molecular mechanisms in cardiac pathogenesis.

Notably, our MR results revealed several metabolites exhibiting inverse causal relationships with DCM, including glutamine, erythronate, glycocholate, and cystine. Lower levels of these compounds associated with higher DCM risk, suggesting potential cardioprotective effects. Glutamine supports myocardial

metabolism and function, particularly under stressed conditions (31). Its depletion could starve cardiac myocytes of a vital substrate. Erythronate is involved in carnitine synthesis, which is essential for fatty acid oxidation. Diminished erythronate could thereby reduce energy production. Finally, bile acids like glycocholate possess signaling activities affecting diverse physiological processes (32, 33). Their reduction may remove beneficial signaling that helps maintain cardiac performance. These protective metabolites or their related pathways warrant further evaluation as therapeutic targets or biomarkers in DCM.

A major strength of our study was the use of two-sample MR leveraging data from large GWAS datasets. This enabled interrogation of a broad panel of metabolites for causal effects on DCM in a sample size sufficiently powered to detect modest effects for this rare disease. Furthermore, the extensive panel of genetic instruments from metabolomics GWAS enhanced specificity for assessing metabolites. Application of multiple MR methods and sensitivity analyses ensured robust causal estimates. Overall, MR overcomes limitations of conventional observational studies for assessing metabolite-disease relationships.

However, some limitations should be considered when interpreting the findings. The MR results require confirmation through experimental models given the constraints of population-level analyses. The study population was predominantly of European ancestry, warranting caution in extrapolating conclusions to other ethnic groups (34). Only metabolites with available GWAS data could be evaluated, providing an incomplete metabolomic portrait. Additionally, we were unable to account for potential effects of DCM medications on circulating metabolites. Nevertheless, this work provides a significant advance in evaluating metabolite causal effects in DCM free of confounds using genetic anchors. Furthermore, MR offers substantial insights into causal relationships, yet it has inherent limitations. One key issue is the potential for pleiotropy, where genetic variants influence multiple traits, which can confound causal inferences. To address this, we have employ methods such as multivariable MR or MR-Egger regression, which help to detect and adjust for pleiotropic effects. the reliance on large sample sizes may limit applicability in smaller or underrepresented populations. To mitigate this, future studies could integrate data from biobanks or consortiums, thereby enhancing power and ensuring diversity. Measurement error in exposures or outcomes can further complicate interpretations. Utilizing more accurate phenotyping methods and conducting sensitivity analyses can help strengthen findings. Lastly, while MR suggests causation, it often lacks insight into underlying mechanisms. Incorporating complementary approaches, such as bioinformatics analyses or functional studies, can provide a more comprehensive understanding of the relationships under investigation. By acknowledging these limitations and implementing rigorous methodologies, the robustness of MR findings can be significantly improved, enhancing its utility in epidemiological research.

## 5 Conclusion

The findings have several important clinical implications. First, delineation of causal metabolites enhances our mechanistic understanding of DCM etiopathogenesis. The identified metabolic pathways could be leveraged for development of prognostic biomarkers, which are lacking for DCM. For example, panels incorporating multiple MR-supported metabolites may enable earlier DCM diagnosis or risk stratification. Second, the results reveal novel targets for potential pharmacologic intervention. Therapeutic normalization of harmful metabolites like tryptophan betaine or elevation of beneficial metabolites like glutamine could ameliorate DCM severity. Nutritional or microbiome-based approaches to modulate implicated metabolites may also hold promise. Overall, the findings pave the way for metabolite-centered precision prevention and treatment strategies. Finally, this work highlights the broader utility of MR for dissecting complex disease mechanisms. Two-sample MR with large GWAS datasets is a powerful tool for probing cause-effect relationships, free of confounds affecting conventional observational studies. Our study demonstrates the promise of applying this technique to map causal metabolic pathways involved in cardiovascular diseases. Integrating MR with multi-omics data represents an emerging frontier to unravel disease pathogenesis. Elucidating causal molecular networks through genetic instrumental variable approaches will be critical for guiding development of targeted diagnostics and therapies. As GWAS data continues expanding, MR applications leveraging massive sample sizes for precise etiologic insights will become increasingly feasible and impactful.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Author contributions

XZ: Data curation, Formal analysis, Methodology, Supervision, Writing – original draft. QZ: Data curation, Formal analysis, Writing – original draft, Methodology, Software. XW: Methodology, Project administration, Writing – original draft. KL: Data curation, Methodology, Writing – original draft. JW: Data curation, Writing – original draft. JL: Data curation, Investigation, Methodology, Project administration, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1423142/full#supplementary-material

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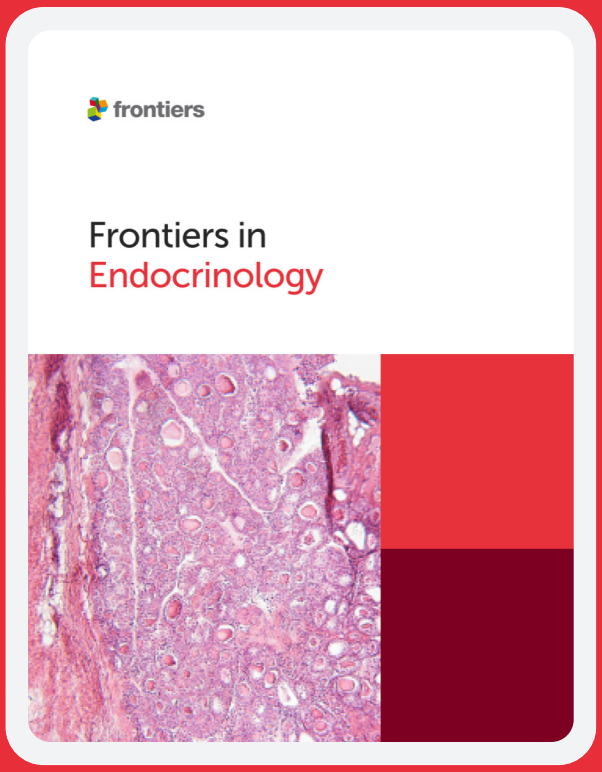


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