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Cytokines, novel cell death models and pathways in cardiovascular diseases

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Cytokines, novel cell death models and pathways in cardiovascular diseases

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Editorial: Cytokines, novel cell death models and pathways in cardiovascular diseases

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Editorial on the Research Topic
Cytokines, novel cell death models and pathways in cardiovascular diseases

Cardiovascular disease (CVD) persists as a major global health issue and remains one of the leading causes of mortality worldwide. Cell deaths, especially programmed cell deaths, are critical processes in the development of various CVDs (1). Recently, accumulating studies have shed light on emerging cell death modalities, such as ferroptosis, necroptosis, pyroptosis, PANoptosis, and their relevance to the onset and progression of CVDs (2–4). A comprehensive understanding and targeted exploration of different types of programmed cell death could provide novel insights for the therapeutic targets of CVDs.

Cytokines also play an essential role in CVD development. They are considered to have crucial regulatory roles in CVDs through autocrine, paracrine, and endocrine actions (5, 6). For instance, we have previously reported the important roles of IL-10, sST2, and IL-33 in vascular and myocardial diseases (7–9). Furthermore, many cytokines, such as TNF-α, IL-1β, IL-6, and IL-11 are critically involved in CVD development (6, 10). Importantly, pro-inflammatory cytokines, particularly IL-1β, IFN-γ and TNF-α can directly initiate the cell death program, such as apoptosis and PANoptosis (11, 12). Concurrently, cell death mortalities like pyroptosis and PANoptosis can also promote the release of intracellular components and cytokines, triggering an inflammatory cascade response, thereby contributing to CVDs (13). Research focusing on the crosstalk between cytokines and the cell death pathway may offer novel therapeutic perspectives for heart-related diseases.

Building on this, the research topic "Cytokines, Novel Cell Death Models, and Pathways in Cardiovascular Diseases" published in Frontiers in Cardiovascular Medicine aimed to discuss recent advances and offer insights in this field.

Among the contributions to this special issue, Li et al. presented a comprehensive review on the pivotal role of ferroptosis in CVDs. Ferroptosis, an iron-dependent form of cell death characterized by phospholipid peroxidation, was first identified in 2012 (14). The review by Li et al. delves into the molecular and metabolic mechanisms underlying ferroptosis, including its regulation through lipid oxidation metabolism, glutamate metabolism, and iron metabolism. They summarized the research progress regarding the significance of

ferroptosis in various CVD conditions, including arrhythmia, myocardial ischemia-reperfusion injury, atherosclerosis, chemotherapeutic drug-induced cardiotoxicity, heart failure, hypertension, diabetic cardiomyopathy, and septic cardiomyopathy. In addition, the review highlights promising therapeutic strategies targeting ferroptosis in CVDs. Various ferroptosis inhibitors, including ROS inhibitors, iron chelators, and traditional Chinese medicine, have shown potential in mitigating myocardial injury and preserving cardiac function in different CVD scenarios, particularly in myocardial infarction, ischemia-reperfusion injury, and cardiomyopathy. This comprehensive review significantly enhances our understanding of the crucial pathogenic role of ferroptosis in multiple CVD conditions and underscores its promising potential as a therapeutic target for CVDs. Further studies focusing on the regulatory mechanisms and therapeutic applications of ferroptosis in CVDs are urgently warranted.

Diabetic cardiomyopathy is characterized by myocardial dysfunction in diabetic patients, independent of hypertension and structural or coronary heart disease (15). Cardiomyocyte death in metabolic disorders caused by diabetes is a major contributor to the development of diabetic cardiomyopathy. Ke et al. provided a comprehensive review, highlighting the significant roles of ferroptosis, necroptosis, and cuproptosis in the pathogenesis and progression of diabetic cardiomyopathy. They highlighted that targeting these novel regulated cell death pathways could offer potential therapeutic benefits for the treatment of diabetic cardiomyopathy. The review emphasized the need for further researches to explore the similarities and potential overlaps among different regulated cell death pathways to identify optimal drug targets for therapeutic purposes.

Another area of focus in the research topic was coronary heart disease (CHD), a prevalent cardiovascular disorder primarily caused by atherosclerosis and narrowing of the coronary arteries. CHD can lead to severe outcomes such as myocardial infarction, ischemic cardiomyopathy, and heart failure, resulting in significant morbidity and mortality rates. Several studies in this research topic examined different aspects of CHD. Wang et al. evaluated the prognostic value of insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 2 (IGFBP-2) in patients with acute coronary syndrome (ACS) and found that IGFBP-2 levels were associated with a poor prognosis after ACS. Yu et al. demonstrated that combining Lp(a) levels with carotid intima-media thickness could provide a favorable predictive value for CHD. Wali et al. identified that early atrial remodeling could predict hospitalization for cardiovascular events in patients with new-onset metabolic syndrome. Tong et al. conducted a bioinformatics study and revealed that circRNAs (circRNA0001785, circRNA0000973, circRNA0001741, and circRNA0003922) possess promising predictive capabilities for CHD. Liu et al. conducted a mendelian randomization analysis to investigate the genetic causal relationship between whole-body iron status and CHD development.

In conclusion, this special issue of Frontiers in Cardiovascular Medicine sheds light on the intricate interplay between cell death modalities, cytokines, and their involvement in CVDs. It underscores the importance of further researches on the crosstalk between cell death pathways and cytokine regulation, as it holds significant promise for developing more effective preventive and treatment strategies to address the increasing burden of CVDs worldwide.

Author contributions

YC: Writing – original draft. MW: Funding acquisition, Writing – review & editing. QG: Writing – review & editing. JW: Funding acquisition, Writing – review & editing. YH: Writing – review & editing. YH: Writing – review & editing. RQ: Writing – review & editing. WM: Writing – review & editing. HZ: Conceptualization, Writing – original draft, Writing – review & editing. All authors contributed to the article and approved the submitted version.

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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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Research progress on the role of ferroptosis in cardiovascular disease

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The cardiovascular disease pathogenesis is extremely complex and seriously threatens human health. Cardiomyocyte death plays a significant role in cardiovascular disease occurrence and development. In addition to the previously revealed modes of cell death (apoptosis, autophagy, and pyroptosis), ferroptosis is highly related to the development of cardiovascular diseases, including arrhythmia, atherosclerosis, and myocardial ischemia/reperfusion. Ferroptosis is a novel cell death pathway driven by lipid peroxidation and iron overload. Lipid, amino acid, and iron metabolism regulate the ferroptosis pathway. Small molecule compounds (iron chelators, antioxidants, and ferroptosis inhibitors) and genetic programming can alleviate or prevent cardiovascular disease by inhibiting the ferroptosis pathway. Ferroptosis plays a key role in various cardiovascular disease occurrence and development, and inhibiting ferroptosis in cardiomyocytes is expected to become a feasible treatment method. In this mini-review, we systematically summarize the molecular mechanisms of ferroptosis in different cardiovascular diseases, delineate the regulatory network between ferroptosis and cardiovascular diseases, and highlight its potential therapeutic targets.

KEYWORDS

ferroptosis, cardiovascular disease, iron overload, lipid peroxidation, arrhythmia

1 Introduction

Obesity, hypertension, a high-cholesterol diet, and other factors contribute to cardiovascular disease, endangering human physical and mental health (1). Recently, cardiovascular disease has been associated with multiple cell death pathways, such as ferroptosis, pyroptosis, and autophagy (2). Ferroptosis was officially identified as a novel mode of regulating cell death in 2012, attracting attention to the study of cardiovascular disease (3). Ferroptosis is mainly caused by iron-dependent cell death, accumulating lipid peroxidation to lethal levels, resulting in cell membrane damage (4). The ferroptosis mechanism might result from glutathione depletion, excess iron, and

reactive oxygen species (ROS) overgeneration (5). This mini-review mainly describes ferroptosis metabolic pathways and their strong correlation with cardiovascular disease.

2 Molecular and metabolic mechanisms of ferroptosis

2.1 Lipid oxidation metabolism

In cell membranes, phospholipid-related polyunsaturated fatty acids (PUFAs), such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), are responsible for inducing lipid peroxidation to induce ferroptosis (6, 7). Acyl-CoA synthetase long-chain 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) are two important enzymes in lipid metabolism (5). These two enzymes can activate PUFAs, such as arachidonic acid (AA) and adrenic acid (ADA), to generate corresponding PE-AA and PE-ADA, respectively (8). Subsequently, lipoxygenase (LOX) can oxidize them to PE-AA-OOH and PE-AdA-OOH to promote ferroptosis product synthesis (8). The glutathione peroxidase 4 (GPX4) transforms toxic lipid hydroperoxides into non-toxic alcohols. Nevertheless, inhibiting GPX4 enzymatic activity causes significant lipid hydroperoxide accumulation, leading to ferroptosis (8).

2.2 Glutamate metabolism

A heterodimer containing a light chain SLC3A2 (4F2hc) and a heavy chain SLC7A11 (xCT) is known as cystine/glutamate antiporter (System Xc^-) on the cell membrane that promotes the glutathione (GSH) synthesis by exchanging extracellular cystine with intracellular glutamate (9). Erastin, an authoritative System Xc^- inhibitor, inhibits cysteine absorption to the cellular membrane, leading to glutathione depletion (10). GSH can reduce ROS and reactive nitrogen under the activity of GPX4. Furthermore, GPX4 converts GSH into glutathione disulfide (GSSH) in the oxidation reaction, expunges excessive peroxides and hydroxyl radicals during cell metabolism, and alleviates the PUFAs peroxidation (11). The GPX4 inhibition, like Ras-selective Lethal small molecule 3 (RSL3), causes ferroptosis by activating lipid peroxidation (10). Herein, the System Xc^--GSH-GPX4 axis system plays a significant position during ferroptosis.

2.3 Iron metabolism

Iron absorption, transport, storage, and excretion affect iron homeostasis, which is crucial for human health (5). Transferrin receptor 1 (TFR1) recognizes ferric (Fe^{3+}) by binding to transferrin through the cellular membrane, and iron reductase reduces it to ferrous (Fe^{2+}) (12). Then, Fe^{2+} is transferred into the cytosolic labile iron pool (LIP) through divalent metal transporter 1 (DMT1) (5). Moreover, a part of iron is stored in the ferritin as Fe^{3+}, while another part is released into the extracellular by membrane transporters ferroportin (FPN) (5).

Iron homeostasis is important for cellular metabolism, especially for cardiomyocytes with high energy requirements (13). Iron overload is a consequence of iron intake exceeds the capacity of transferrin iron-binding, leading to the accumulation of iron in the parenchymal cells of various tissues and organs (14, 15). Ferroptosis is dependent on iron-dependent lipid peroxidation, which can be inhibted by iron chelators (14, 16). Cellular labile iron contents may affect the ferroptosis sensitivity (17). Overflowing Fe^{2+} catalyzes the hydroxyl and high ROS generation through the Fenton reaction, inducing lipid peroxidation and cell damage (5, 11). Heme oxygenase-1 (HO-1) also affects ferroptosis sensitivity by degrading heme to release iron (17).

2.4 Other related signaling metabolism

The redox-sensitive transcription factor, nuclear factor erythroid-2 related factor 2 (NRF2) regulates its downstream target genes to modulate lipid peroxidation and ferroptosis (18, 19). NRF2 targets, such as FPN and ferritin, are involved in iron/heme metabolism, which is crucial for cellular antioxidant defense (20). Besides, NRF2 plays a critical role in mediating glutamate metabolism targets, SLC7A11 and GPX4 (19). Of note, NRF2 target protein NAD(P)H:quinone oxidoreductase 1 (NQO1) also plays a key role in regulating ferroptosis (21).

3 Ferroptosis in cardiovascular disease

3.1 Arrhythmia

Atrial fibrillation (AF) is the most clinically diagnosed arrhythmia (22). Most patients with AF are prone to recurrent attacks, presenting clinicians with a dilemma (23). The mechanical function and electrical activity are progressive deterioration in iron-overloaded hearts. Iron overload can affect calcium, sodium, and potassium channels, interfering with cardiac electrophysiology and confirming the connection between iron ions and arrhythmia (24).

Recently, ferroptosis has gained increased attention in the field of arrhythmia. Antioxidant factor NRF2 overexpression can reduce the arrhythmia, inflammation, and cardiac fibrosis induced by AF (23). Down-regulation the expression of FPN, a downstream factor of NRF2, causes intracellular iron accumulation in the new-onset AF model, leading to ferroptosis

(25). Moreover, excessive alcohol consumption can lead to iron dysregulation, increased serum non-heme iron concentration, and atrial tissue iron accumulation. This progress promotes ferroptosis and increases the susceptibility to AF. Ferrostatin 1 (Fer-1) can partially or completely reverse the atrial damage caused by excessive alcohol intake (26).

Arrhythmia is a common clinical feature of coronavirus disease 2019 (COVID-19) (27). According to a recent study, severe acute respiratory syndrome coronavirus 2 infections derange human sinoatrial node-like pacemaker cells, facilitating ferroptosis. Early deferoxamine and imatinib administration reduces viral infection in human embryonic stem cell cardiomyocytes. This result suggests that ferroptosis is involved in the arrhythmia progression in COVID-19 patients (28).

3.2 Myocardial ischemia-reperfusion injury

Myocardial ischemia-reperfusion injury (IRI) is tissue injury due to the recovery of blood supply in myocardial tissue, leading to life-threatening clinical complications after a period of ischemia (12). Iron and lipid metabolism are strongly linked to the pathological process of IRI. In the early stage of ischemia and reperfusion, ferritin degradation releases iron, promotes free iron-mediated Fenton reaction and induces oxidative damage (29). Cardiomyocytes are more vulnerable to injury than endothelial cells during the ischemic phase (30). The early ischemic stage, a disorder of PUFAs-phospholipids, may initiate peroxidative conditions, providing a priming signal for oxidative injury in the reperfusion stage. Studies have found that oxidative phosphorylation of the core enzyme ALOX15 can initiate PUFA-phospholipid peroxidation and enhance the susceptibility to ferroptosis in ischemia-induced myocardial injury (31). Consequently, inhibiting ferroptosis during the early stage of ischemia can reduce myocardial injury caused by reperfusion as soon as possible.

Ferroptosis can also regulate IRI through other metabolic pathways. A novel long non-coding RNA LNCAABR07025387.1 is up-regulated in myocardial tissue of IRI rat models, efficiently activates ACSL4 expression by down-regulating miR-205, accelerates the lipid peroxidation and exacerbates IRI (32). Moreover, ROS is strongly related to endoplasmic reticulum (ER) stress during ferroptosis. In diabetic rats during IRI, ER stress factor expressions, such as activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and ACSL4, are elevated. Meanwhile, the GPX4 level is decreased, exacerbating myocardial injury (33).

Furthermore, IRI can be mitigated by interfering with ferroptosis-related targets. The deubiquitinating enzyme USP22 can stabilize the sirtuin-1 (SIRT1) level to inhibit ferroptosis. USP22 overexpression can increase the SIRT1 protein level and decrease the p53 acetylation level, promoting SLC7A11 expression. Overall, this mechanism suppresses lipid peroxidation and attenuates ferroptosis-induced myocardial damage in IRI through SIRT1/P53/SLC7A11 axis (34). In the future, we can explore the clinical practice of USP22 on myocardial IRI to offer a novel diagnosis and therapeutic target for IRI patients.

3.3 Atherosclerosis

Atherosclerosis is a metabolic disease characterized by lipid metabolism and endothelial dysfunction. Atheromatous plaque formation is associated with iron deposition and peroxidation of lipids in vascular endothelial cells (35, 36). ACSL4 is up-regulated, while GPX4 is down-regulated in the coronary arteries of atherosclerosis patients (37). GPX4 controls the balance of reductive and oxidative states. GPX4 knockout can promote lipid peroxidation, leading to highly cytotoxic oxidation products for the cell and aggravating the atherosclerosis effect. Oppositely, GPX4 overexpression can alleviate atherosclerotic lesions of the aortic by inhibiting ferroptosis in $ApoE^{-/-}$ mice (38). In addition, high level of uric acid has been shown to promote atherosclerotic plaque formation and inhibit the protein level of the NRF2/SLC7A11/GPX4 signaling pathway in $ApoE^{-/-}$ mice (39). Fer-1 can inhibit iron deposition and lipid peroxidation in high-fat diet-fed $ApoE^{-/-}$ mice by limiting SLC7A11 and GPX4 levels (36). Consequently, we can further explore the novel molecular targets that continue to involve in the atherosclerosis pathogenesis mechanism.

3.4 Chemotherapeutic drugs induced cardiotoxicity

Chemotherapeutic drugs induced cardiotoxicity remains an intractable issue for cancer patients, which is mostly associated with anthracycline drug (40). Doxorubicin (DOX), an anthracycline drug isolated from streptomyces, is frequently used to treat cancer patients (41). In DOX-induced cardiac injury mice, NRF2 induces HO-1 expression with an antioxidant effect, catalyzing hemoglobin degradation and promoting the free iron release, leading to ferroptosis and heart failure (42). Interestingly, protein arginine methyltransferase 4 (PRMT4) can modulate oxidative stress and autophagy, interacts with NRF2 to limit NRF2 nuclear translocation, and subsequently inhibits GPX4. A subsequent study confirms that PRMT4 overexpression raises ROS levels and intensifies DOX-induced myocardial dysfunction (43).

Additionally, mitochondria-dependent ferroptosis is involved in the pathology of DOX-induced cardiotoxicity. In the DOX-induced heart failure model, the GPX4 expression is

downregulated, triggering lipid peroxidation of the DOX-Fe^{2+} complex and inducing mitochondria-dependent ferroptosis (44). Fer-1 and iron chelators can reduce DOX-induced cardiac damage by maintaining mitochondrial function (42). Therefore, these drugs can reduce DOX-induced cardiotoxicity by inhibiting the ferroptosis pathway, bringing good news to patients (42).

Tyrosine kinase inhibitors (TKIs) are also a class of anticancer agents for various cancers (45). Regorafenib, a molecule structurally related to sorafenib, is an effective xCT inhibitor, which can induce ferroptosis by decreasing cellular GSH (46, 47). In addition, lapatinib is usually used with the combined treatment of DOX to improve the anti-tumor efficacy. In H9C2 cells, lapatinib aggravates DOX-induced cell injury by decreasing GPX4 activity but increasing ACSL4 level (48). Together, these findings suggest that further in-depth research is required to study ferroptosis regulator genes as promising therapeutic targets in protecting TKIs-induced cardiotoxicity.

3.5 Heart failure

Heart failure is the terminal stage of various cardiovascular diseases (49). As mentioned above, iron homeostasis is crucial for maintaining cardiac function. Iron overload is closely related to heart failure and cardiomyopathy (50). For example, left ventricular diastolic function may be more sensitive to early markers of iron overload than systolic function (51). Furthermore, heart failure with preserved ejection fraction (HFpEF) patients has complex pathological processes, such as chronic inflammatory and oxidative stress stages. Elevated ROS level often promotes cardiomyocyte injury by increasing lipid peroxidation products, destroying the antioxidant mechanisms, and decreasing GSH levels. This progression implies an underlying connection between inflammation, ferroptosis, and HFpEF (51).

Of note, ferritin heavy chain (FTH), a significant component of ferritin, is down-regulated in transverse aortic constriction mice (49). SlC7A11 expression is decreased in FTH-deficient cardiomyocytes, while selectively, SlC7A11 overexpression increases the GSH level in cardiomyocytes (52). Puerarin, an antioxidant reagent, can alleviate heart failure by increasing FTH1 and GPX4 expression in H9C2 cells and aortic banding rats (53). Therefore, it can be shown that genes related to ferroptosis deserves for further exploration in heart failure.

3.6 Hypertension

Hypertension is a common comorbidity in HFpEF patients with high angiotensin II (Ang II) levels and myocardial fibrosis (51). Pathological cardiac remodeling mediated by hypertension leads to heart failure (54). The peptide hormone Elabela (ELA) is an endogenous ligand of the apelin receptor that can inhibit Ang II signal transduction, thus preventing pressure overloading. Intraperitoneal injection of ELA in Ang II-induced hypertensive mice model can inhibit interleukin-6/signal transducer and activator of transcription 3/GPX4 (IL-6/STAT3/GPX4) signaling, reducing the lipid peroxidation accumulation. Thus, ELA treatment in mice reduces myocardial fibrosis and cardiac injury with hypertensive heart failure (55). Similarly, it has been studied that the expression of GPX4 and GSH is decreased in hypertensive brain damage rat models (56). Moreover, SLC7A11 overexpression in mice alleviated Ang II-mediated cardiac fibrosis, hypertrophy, and dysfunction (57). At present, there is poorly existing basic research on the association between ferroptosis and hypertension, which is needed to deeper explore the underlying mechanisms, and provides new targets for the treatment of hypertension.

3.7 Other cardiovascular disease

Iron overload in diabetic patients increases the insulin resistance risk and aggravates cardiovascular complications through the Fenton reaction (50). Oxidative stress has become the main mechanism of diabetic cardiomyopathy (58). Heat shock factor 1 (HSF1) can resist oxidative stress response caused by ferroptosis-related lipid metabolism disorder. HSF1 overexpression alleviated palmitic acid-induced cell death and regulated the transcription of iron metabolism-related genes (FTH, TFRC, and FPN) to improve disturbed iron homeostasis (59). NRF2 is also a master regulator factor of antioxidant proteins in ferroptosis. Ferroptosis exacerbates diabetic cardiomyopathy by down-regulating the SLC7A11 expression through the AMPK/NRF2 pathway in the later stages of diabetes. Sulforaphane, an NRF2 inhibitor, prevents diabetes-induced oxidative stress and cardiac dysfunction by activating NRF2 (60). Likewise, AMPK/P38/NRF2 pathway is activated as an anti-oxidative stress mechanism during IRI in diabetic rats and is involved in the cardioprotective effect of resveratrol (61). Therefore, more investigation is needed to explore antioxidant drugs for treating diabetic cardiomyopathy.

Patients with severe sepsis often present with cardiac injury and dysfunction. Ferroptosis metabolic pathways such as mitochondrial autophagy and iron metabolism are involved in septic cardiomyopathy progression. Lipopolysaccharide (LPS) can increase ferritin and nuclear receptor coactivator 4 (NCOA4) expressions in H9C2 cells. NCOA4 increased the cytoplasmic Fe^{2+} and activated sideroflexin on the mitochondrial membrane to transport Fe^{2+} to mitochondria. This progress can lead to iron overload and elevate ROS in mitochondria, triggering lipid peroxidation and cardiomyocyte ferroptosis (62). However, FPN, as the only iron exporter

in ferroptosis progression, is crucial for maintaining iron homeostasis. LPS can down-regulate FPN and up-regulate ferritin light chain (FTL) and FTH expressions, promoting iron deposition in myocardium-septic rats. Fer-1 and dexrazoridine can reduce cardiac inflammation and dysfunction and improve septic cardiomyopathy (62, 63).

4 Targeted therapy of ferroptosis in cardiovascular disease

Inhibiting ferroptosis-related targets has excellent therapeutic potential for treating and preventing heart disease, based on research into the pathogenesis of these conditions. In this section, we summarize various drugs that inhibit the ferroptosis pathway and their application in various models of heart diseases.

4.1 ROS inhibitors

Fer-1 can reduce ROS-induced cell damage and thus inhibit ferroptosis. Fer-1 is widely applied in various cardiovascular diseases (7). As described previously, Fer-1 has a protective effect on myocardial damage in DOX-induced and septic cardiomyopathy (42, 63). Fer-1 treatment reduced total creatine kinase release and neutrophil recruitment during heart transplantation (64).

Additionally, ferroptosis inhibitor liproxstatin-1 (LIP-1) has potentially cardioprotective properties. LIP-1 can reduce the myocardial infarction size by reducing voltage-dependent anion channel 1 (VDAC1) to maintain mitochondrial structure and function (65). MitoTEMPO, as a mitochondria-targeted superoxide scavenger, reduces lipid peroxides and thus significantly reduces cardiac dysfunction and mitochondrial damage (42). Hence, ferroptosis inhibitors are essential to treat cardiovascular disease. These drugs' actual clinical development and utilization still need further exploration.

4.2 Iron chelators

Iron chelators can protect the myocardium from injury by regulating intracellular free iron levels. Dexrazoxane, a common iron chelator, easily passes through the cell membrane and chelates intracellular free iron. Dexrazoxane can act on high mobility group box 1 (HMGB1) protein to inhibit ferroptosis and reduce DOX-induced cardiotoxicity in rats (7, 66). Moreover, histochrome has better iron-chelating and antioxidant effects on alleviating myocardial IRI. Intravenous

TABLE 1 Summary of traditional Chinese medicine in cardiovascular disease.

Drug	Mechanisms	Test in	Disease	References
Resveratrol	Increase the level of GPX4 and FTH, decrease the level of TFR1	Rat and H9C2 cells	Myocardial ischemia-reperfusion	(75)
Baicalin	Increase the level of GPX4, decrease the level of ACSL4, decrease the generation of ROS and Fe^{2+} deposition	Rat and H9C2 cells	Myocardial ischemia-reperfusion	(72)
Betulinic	Enhance the induction of nuclear NRF2 and HO-1 expression	Rat and H9C2 cells	Myocardial ischemia-reperfusion	(74)
Ginsenoside Rd	Increase the expression of nuclear NRF2 and HO-1	Rat	Myocardial ischemia-reperfusion	(73)
Cyanidin-3-glucoside	Increase the level of GPX4 and FTH1, decrease the level of TFR1	Rat and H9C2 cells	Myocardial ischemia-reperfusion	(76)
Hesperidin	Reduce non-heme iron deposited and lipid peroxidation	Mice	Iron-overload	(77)
Coumarin	Reduce lipid peroxidation	Mice	Iron-overload	(77)
Epigallocatechin-3-gallate	Increase the level of GPX4, decrease iron accumulation, inhibit excess ROS generation and oxidative stress	Mice and H9C2 cells	Dox-induced cardiomyopathy	(78)
Curcumin	Promote nucleus translocation of NRF2, increase the level of HO-1 and GPX4	Rabbit and H9C2 cells	Diabetic cardiomyopathy	(70)
Puerarin	Increase the expression of P-AMPK/T-AMPK; Increase the level of GPX4 and FTH1	Rat and H9C2 cells	Sepsis-induced myocardial injury; heart failure	(53, 79)
Shensong Yangxin	Increase the expression of TFR1 and FPN, decrease intracellular iron overload and ROS production	Rat and HL-1 cells	Syndrome-induced atrial fibrillation	(80)
Tanshinone	A coenzyme for NQO1, accept electrons from FAD to generate reduced tanshinone to reduce lipid ROS and ferroptosis	Mice	Myocardial ischemia-reperfusion	(81)

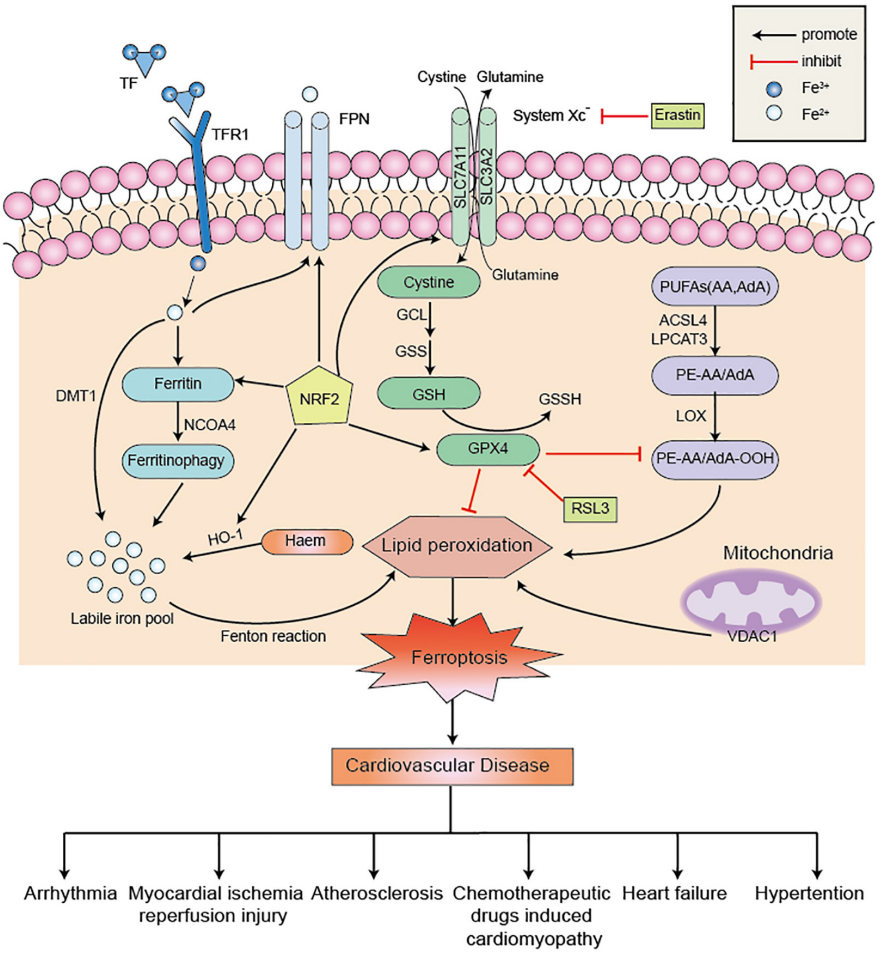

FIGURE 1
The regulatory metabolic pathways in ferroptosis. Ferroptosis is a form of cell death characterized by iron overload and lipid peroxidation. Excessive iron uptake or decreased iron excretion leads to intracellular iron overload, which promotes Fenton reaction. GPX4 is an essential regulator factor in the System Xc^{-}-GSH-GPX4 axis system to blocking ferroptosis. Besides, PUFAs can be catalyzed by ACSL4 and LPCAT3 to form PE-AA and PE-AdA, which is further oxidized by LOX to promote the occurrence of lipid peroxidation. These three metabolic pathways promote ferroptosis and further aggravate cardiovascular disease. NRF2 also acts as an antioxidant factor to inhibit ferroptosis by operating its downstream factors. Erastin and RSL3 are common ferroptosis inducers to promote ferroptosis. PUFAs, polyunsaturated fatty acids; AA, arachidonic acid; AdA, adrenic acid; ACSL4, acyl-coa synthetase long chain 4; LPCAT3, lysophosphatidyl acyltransferase 3; LOX, lipoxygenase; System Xc^{-}, cystine/glutamate antiporter; GSSH, glutathione disulfide; GSH, glutathione; TF, transferrin; TFR1, transferrin receptor 1; DMT1, divalent metal transporter 1; FPN, ferroportin; HO-1, heme oxygenase-1; NCOA4, nuclear receptor coactivator 4; NRF2, nuclear factor erythroid-2 related factor 2; RSL3, Ras-selective lethal small molecule 3; VDAC1, voltage-dependent anion channel 1; GPX4, glutathione peroxidase 4; PE, phosphatidylethanolamine.

injection of histochrome in rats can inhibit ferroptosis by maintaining GSH level and GPX4 activity, thereby reducing infarct size and arrhythmia potential. The iron chelators deferiprone and deferoxamine can also inhibit IRI (67). However, further study is necessary to determine the effects of iron chelators on the body's iron homeostasis.

4.3 Traditional Chinese medicine

Traditional Chinese medicine is a treasure trove of precious natural compounds with multiple targets and minor side effects (30). Some active ingredients of traditional Chinese medicine contain natural antioxidants and have regulatory effects on ferroptosis, such as artemisinin (68), curculigoside (69), curcumin (70), and glycyrrhiza (71). Several studies on alleviating cardiovascular disease with traditional Chinese medicine have progressed with in-depth research on ferroptosis (**Table 1**). For instance, baicalin can inhibit erastin-mediated GPX4 degradation and ACSL4 expression to enhance cell resistance to ferroptosis (72). Studies also showed that betulinic acid and ginsenoside Rd could inhibit oxidative stress markers and protect the heart from ischemia-reperfusion *via* NRF2/HO-1 signaling (73, 74). Besides, resveratrol can increase the

GPX4 and FTH to reduce cardiac damage (75). Therefore, there is an urgent requirement to explore more traditional Chinese medicine with the ability to decrease lipid peroxidation and ROS to protect against cardiovascular disease with fewer side effects.

5 Discussion

As a non-apoptotic form of cell death, the underlying mechanism of ferroptosis is complex and intimately connected with other regulatory cell death signaling pathways (17). In-depth research on ferroptosis in cardiovascular disease has revealed that many signaling factors have been found to directly or indirectly regulate ferroptosis, thereby affecting iron metabolism and lipid peroxidation (**Figure 1**; 82). Recently, scientists are increasingly focusing on ferroptosis inhibitors to alleviate myocardial injury and cardiac dysfunction, which will provide insights into the molecular mechanisms of cardiomyocyte death after cardiac injury.

However, there are still many issues worth discussing: (1) The precise mechanisms involved in ferroptosis remain to be elucidated on cardiovascular disease. As mentioned above, studies exist on the experimental basis of ferroptosis and hypertension have been rare; (2) Further research is needed on the clinical application of ferroptosis and cardiovascular diseases, such as magnetic resonance imaging and serum-based biomarkers. By extension, we can explore predictive specific biomarkers of ferroptosis in cardiovascular disease, thereby providing a novel idea for early diagnosis and treatment of heart disease. (3) Traditional Chinese medicine has the advantage of its unique therapeutic effects, such as reduced toxicity and few side effects, in preventing and treating cardiovascular disease. There are more traditional Chinese medicine with anti-ferroptosis effect needs to be further investigated.

As a therapeutic target, ferroptosis has a good application prospect on cardiovascular disease (4). With the rapid development of molecular detection in the field of precision medicine, it need to be further explore more specific ferroptosis-related targets. Therefore, exploring the regulatory mechanism related to ferroptosis and actively promoting clinical verification is necessary to provide new treatment ideas and directions for clinical diagnosis and treatment of cardiovascular disease.

Author contributions

HL and LL contributed to the literature review and manuscript drafting. Y-LX contributed to provide the funding. YX and XY were responsible for all 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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Predictive effect of different blood lipid parameters combined with carotid intima-media thickness on coronary artery disease

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Background: Blood lipids disorder and atherosclerosis are closely related to coronary artery disease (CAD). This study aims to compare different blood lipid parameters combined with carotid intima-media thickness (cIMT) in predicting CAD.

Methods: This was a retrospective study including patients who underwent coronary angiography for highly suspected CAD. Blood samples were taken for lipid profile analysis and cIMT was evaluated by carotid ultrasound. Logistic analysis was used to establish different models of different lipid parameters in predicting CAD. The area under the receiver operating characteristic curve (AUC) was used to examine the predictive value. The optimal lipid parameter was also used to explore the relationship with multi-vessel CAD.

Results: Patients were classified into two groups based on whether CAD existed. Compared with non-CAD patients, the CAD group had higher lipoprotein (a) [Lp (a)], apolipoprotein B/apolipoprotein A, total cholesterol/high-density lipoprotein cholesterol (HDL-C), triglyceride/HDL-C and LDL-C/HDL-C. According to the AUCs, Lp (a) combined with cIMT (AUC: 0.713, $P < 0.001$) had the best performance in predicting CAD compared to other lipid parameters. High level of Lp (a) was also associated with multi-vessel CAD (odds ratio: 1.41, 95% confidence interval: 1.02–1.95, $P = 0.036$).

Conclusion: For patients with highly suspected CAD, Lp (a) better improved the predictive value of CAD rather than most of blood lipid indices, especially in the absence of high levels of LDL-C. Lp (a) also can be used to predict the multi-vessel CAD.

KEYWORDS

lipoprotein (a), blood lipid indices, carotid intima-media thickness, carotid ultrasound, coronary artery disease

Introduction

Although the treatment of coronary artery disease (CAD) has greatly improved in recent years, the incidence and mortality of CAD remains high (1). Atherosclerosis, as the main pathophysiological mechanism of CAD, closely related to lipid disorders that is poor-controlled (2). Early screening of CAD based on atherosclerosis is of great importance, and there is an urgent need to predict CAD using clinically available information.

The role of lipid parameters in CAD is controversial. Previous studies have shown that blood lipid indices, such as triglyceride/high-density lipoprotein cholesterol (TG/HDL-C) ratio, apolipoprotein B/apolipoprotein A-I (Apo B/Apo A-I) ratio, total cholesterol (TC)/HDL-C ratio or low-density lipoprotein cholesterol (LDL-C)/HDL-C have a better predictive value of CAD than a single parameter (3–8). Recent findings showed that blood lipid indices were significantly correlated with the risk of intracranial atherosclerotic stenosis and metabolic syndrome (9, 10). The combination of lipid parameters represents relative status of blood lipids, which can comprehensively reflect the balance between atherosclerosis and anti-atherosclerosis and accurately assess lipid deposition (11, 12). Lipoprotein (a) [Lp (a)], which has LDL-like moiety comprising Apo B and apolipoprotein (a) [Apo (a)], is independently associated with the diagnosis and prognosis of CAD (13). In an Asian population study, Lp (a) concentration was a risk factor for CAD, and higher levels of Lp (a) increased the risk of CAD (14). As a marker of the residual risk of cardiovascular disease, Lp (a) has a stronger correlation with CAD than LDL-C when LDL-C is in low levels (15, 16). However, it is still unknown whether Lp (a) is superior than the new lipid indices in predicting CAD.

Carotid intima-media thickness (cIMT), a widely used non-invasive marker of subclinical atherosclerosis, can also be used as a preliminary screening tool for CAD (17–19). However, a meta-analysis suggested that the sensitivity and specificity of cIMT for the diagnosis of CAD were quite low, indicating that using cIMT alone as CAD screening tool was still insufficient (20). Since the total load of atherosclerosis is closely related to the concentration of blood lipids, morphological assessment of atherosclerosis based on cIMT has certain limitations in predicting CAD (21–24). Considering subclinical atherosclerosis status and blood lipid profiles together would effectively improve the accuracy of the diagnosis of CAD.

In clinical screening for CAD, blood lipid parameters and cIMT are considered to be available tools. However, it is still unknown which blood lipid parameters combined with cIMT has the optimal accuracy in predicting CAD. Therefore, this study was aimed to compare the predictive value of different blood lipid parameters combined with cIMT on CAD.

Materials and methods

Study design and participants

This retrospective study included 1,598 consecutive patients who underwent coronary angiography for highly suspected CAD, including chest pain with typical change in ECG or severe lesion in coronary CT angiography, from September 2014 to July 2015 in Guangdong Provincial People's Hospital. A total of 105 patients with a history of stroke, 24 patients without cIMT measurement, and 74 patients with missing information on blood lipid parameters were excluded.

This study was approved by the Ethics Committee of Guangdong Provincial People's Hospital, and informed verbal consent was obtained from all patients. This study was conducted in accordance with the Declaration of Helsinki.

Data collection and measurements

Demographic data, laboratory test results, and carotid ultrasonography and coronary angiography results from the electronic medical records were collected.

The measurement of cIMT by carotid ultrasonography was detailed in our previous study (25). Carotid artery ultrasound was performed by experienced sonographers using the GE Vivid E95 (GE Healthcare, Milwaukee, WI, USA) interfaced with a 7.5–12 MHz phased array probe. The ultrasound probe scanned the entire length of the carotid artery longitudinally (from the bottom of the neck to the angle of the mandible). The region of interest for cIMT measurement is located at the far wall of the bilateral carotid arteries proximal to the bifurcation, along with $\geq$ 10 mm of plaque-free lesions on each side. Mean cIMT was calculated as the mean of two sides of cIMT. Two sonographers performed the cIMT measurements and determined the final results together. If there was any discrepancy, the results were determined together with a third sonographer. All patients underwent coronary angiography, and the degree of coronary stenosis was judged by two experienced cardiologists. Hypertension was diagnosed according to the European Society of Cardiology guidelines (26). Diabetes mellitus (DM) was defined based on the presence of diabetes or was diagnosed during hospitalization following the criteria of the European Society of Cardiology guidelines (27). Chronic kidney disease (CKD) was defined as previous medical history. Smoking was defined as previous or current smoking. Alcohol consumption was defined as previous drinking habit. Lp (a) was measured by AU5800 spectrophotometer (Beckman Coulter, USA) *via* immunoturbidimetry, with trihydroxy aminomethane buffer and anti Lp (a) antibody sensitized granules. HDL-C, LDL-C, TC, TG, Apo A-I, and Apo B were also detected using AU5800 spectrophotometer (Beckman Coulter, USA) *via* colorimetry or immunoturbidimetry. Based on previous studies (3–10), we selected six lipid ratios, Apo B/Apo A-I, LDL-C/Apo B, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, and HDL-C/Apo A-I, that may be associated with CAD.

Definitions

As suggested by the American College of Cardiology in 2016, a $\geq$ 70% luminal diameter narrowing of an epicardial stenosis or $\geq$ 50% luminal diameter narrowing of the left main artery observed by visual assessment was considered as severe lesion that used as the diagnostic criteria for CAD (28). Two or more coronary arteries with severe stenosis were defined as multi-vessel CAD.

Statistical analysis

The total procedure of statistical analysis was divided into four steps. First, Student's *t*-test was used for normally distributed data, the

Mann–Whitney U test was used for non-normally distributed data, and the Chi-square test or Fisher's exact test was used for categorical variables to identify significant differences between two groups. Second, except for blood lipid parameters, the basic prediction model considered potential confounding factors that were both clinically and statistically significant in a backward stepwise logistic regression model (with 0.1 significance level for removal), and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Third, a receiver operating characteristic (ROC) curve was constructed to evaluate the sensitivity, specificity, and area under the ROC curve (AUC) of different lipid parameters in predicting CAD based on the basic prediction model. Furthermore, net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were calculated to evaluate the improvement of the new model when compared with the basic prediction model at low (< 50%)/intermediate (50–80%)/high risk (> 80%) of CAD. The 95% CI of NRI was obtained after bootstrapping 10,000 times. Fourth, a fully adjusted logistic model was used to explore the relationship between the optimal blood lipid parameter and multi-vessel CAD in the subgroup of diagnosed CAD. Comparisons with $P < 0.05$ (two-sided) were considered to be statistically significant. All of the analyses were performed with Stata 15.0 (StataCorp LLC, College Station, TX, USA), R version 3.4.3 (The R Project for Statistical Computing, Vienna, Austria), and EmpowerStats (X&Y Solutions, Inc., Boston, MA, USA).

Results

Study population

A total of 1,395 patients were eventually enrolled in this study (**Figure 1**). Baseline information of patients with and without CAD is presented in **Table 1**. There was no significant difference in LDL-C between the two groups. Hypertension, DM, and CKD were more prevalent in the CAD group. As for blood lipid indices, TC/HDL-C, LDL-C/HDL-C, Apo B/Apo A-I, and TG/HDL-C ratios were higher, whereas the LDL-C/Apo B ratio was lower in the CAD group. Lp (a) and mean-cIMT were also significantly higher in patients with CAD than in those without CAD. In addition, for medication history, statins was more commonly used in the CAD group.

Univariate and multivariate analysis

Univariate and stepwise multivariate logistic models were performed to select both clinically and statistically significant risk factors (**Table 2**). Age, sex, hypertension, smoking, alcohol consumption, DM, and mean-cIMT were associated with CAD and included in the basic prediction model. Among these, mean-cIMT was the strongly associated with the presence of CAD (OR 2.73, 95% CI: 1.64–4.52, $P < 0.001$).

Predictive value of different blood lipid parameters on CAD

The ROC curves of the different blood lipid parameters on the basic prediction model for predicting CAD are shown in **Figure 2**. The addition of Lp (a) as well as Apo B/Apo A-I ratio improved the prediction effect of the basic prediction model [AUC: 0.7129 for Lp (a), $P < 0.001$; AUC: 0.6848 for Apo B/Apo A-I ratio, $P < 0.05$]. However, when LDL-C/Apo B, TC/HDL-C, TG/HDL-C, and LDL-C/HDL-C ratios were added into the basic prediction model, the AUC of the new model was not significantly improved.

The new model that included Lp (a) achieved an NRI of 12.8%, as compared with the basic prediction model for predicting CAD (**Table 3**), which means that 12.8% of patients were correctly reclassified. The IDI is also listed in **Table 3** and also showed a significant improvement in accuracy generated with the new model. These results indicated that the new model including Lp (a) had better predictive capability for CAD than the other blood lipid indices.

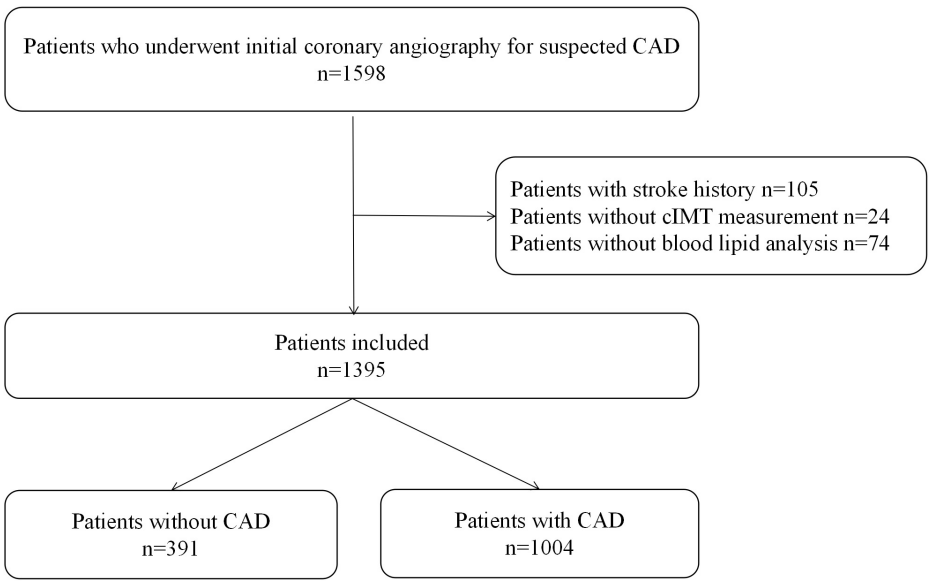

FIGURE 1
Study flowchart.

TABLE 1 Baseline information of CAD and non-CAD patients.

	Non-CAD n = 391	CAD n = 1004	P-value
Age, year	62 ± 11	63 ± 11	0.009
Male sex	222 (56.78%)	757 (75.40%)	<0.001
Hypertension	204 (52.17%)	615 (61.25%)	0.002
Diabetes	97 (24.81%)	382 (38.05%)	<0.001
Smoking	107 (27.37%)	378 (37.65%)	<0.001
Drinking	26 (6.65%)	56 (5.58%)	0.445
CKD	45 (11.51%)	179 (17.83%)	0.004
TC, mmol/L	4.61 ± 1.18	4.43 ± 1.25	0.019
TG, mmol/L	1.55 ± 1.13	1.65 ± 1.23	0.161
LDL-C, mmol/L	2.68 ± 1.05	2.60 ± 1.07	0.245
HDL-C, mmol/L	1.15 ± 0.28	1.05 ± 0.27	<0.001
Apo A-I, mmol/L	1.28 ± 0.31	1.17 ± 0.29	<0.001
Apo B, mmol/L	0.78 ± 0.21	0.79 ± 0.24	0.448
Apo B/Apo A-I	0.61 (0.48–0.77)	0.68 (0.53–0.86)	<0.001
LDL-C/Apo B	3.35 (2.98–3.75)	3.22 (2.90–3.59)	<0.001
TC/HDL-C	4.00 (3.34–4.77)	4.15 (3.48–5.04)	0.002
TG/HDL-C	1.15 (0.80–1.76)	1.41 (0.92–2.06)	<0.001
LDL-C/HDL-C	2.21 (1.74–2.97)	2.42 (1.82–3.16)	0.007
HDL-C/Apo A-I	0.91 (0.82–0.99)	0.90 (0.82–0.98)	0.361
Mean-cIMT, mm	0.90 (0.75–1.00)	1.00 (0.85–1.10)	<0.001
Lipoprotein (a), mg/L	113.72 (70.48–215.14)	180.80 (93.34–395.25)	<0.001
Medication history			
ACEI/ARB	57 (14.58%)	171 (17.03%)	0.266
β-blocker	56 (14.32%)	185 (18.43%)	0.069
CCB	41 (10.49%)	149 (14.84%)	0.033
Diuretic	17 (4.35%)	36 (3.59%)	0.504
Statin	66 (16.88%)	432 (43.03%)	<0.001

Data are expressed as mean ± standard deviation or median (Q1–Q3) for continuous variables and n (%) for categorical variables.

CAD, coronary artery disease; DM, diabetes mellitus; CKD, chronic kidney disease; TC: total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; cIMT, carotid intima-media thickness; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium calcium entry blocker.

Association between Lp (a) and multi-vessel CAD

As shown in **Figure 3**, after adjusting for other risk factors in the CAD group (n = 1004), high levels of Lp (a) was associated with multi-vessel CAD than those with low levels of Lp (a) (OR 1.41, 95% CI 1.02–1.95, P = 0.036).

Discussion

By comparing the predictive value of different blood lipid parameters combined with cIMT on CAD, the study had three major findings: (1) Lp (a) better improved the prediction accuracy of CAD in comparison with other blood lipid parameters; (2) in the absence of high levels of LDL-C, the predictive value of Lp (a) on CAD was highlighted; (3) Lp (a) can also be used to predict multi-vessel CAD.

Atherosclerosis is a systemic vascular disease caused by a lipid metabolism disorder and most likely involves the carotid and coronary arteries (29). Although coronary angiography is the gold standard for the diagnosis of CAD, it is an invasive examination that is unsuitable for general screening of CAD. In addition to identifying traditional risk factors such as demographic information and healthy behavior to predict CAD, screening for subclinical atherosclerosis is also important. Carotid ultrasound is a non-invasive examination (18). cIMT is widely recognized as a marker of early stage atherosclerosis and the severity of CAD, which can be used to indirectly assess coronary artery conditions (19, 30). Many studies have shown that cIMT is associated with cardiovascular risk factors and cardiovascular disease (CVD) (31, 32). Furthermore, several cohort studies have found that increased cIMT is associated with future cardiovascular events (33, 34). However, the results of a meta-analysis indicated that the sensitivity and specificity of cIMT for the diagnosis of CAD were 0.68 (95% CI: 0.57–0.77) and 0.70 (95% CI: 0.64–0.75), respectively, which indicated that only cIMT has limited effectiveness as a diagnostic tool for CAD screening (19). In addition, since lipid metabolism disorders cannot be reflected directly, it is of limited value in the use of single cIMT in actual clinical application (21, 35). Combining cIMT and lipid parameters could further improve the diagnostic efficiency of CAD. In recent years, research progress regarding lipid abnormalities has focused on the blood lipid indices, which can reflect lipid metabolism disorders better than a single one (36–38). Wu et al found that multivariate logistic regression analysis showed that Apo B/Apo A-I ratio, a composite index, had a larger OR value than a single index (LDL-C or Apo B) and was better than a single lipid index in the prediction of CAD (36). Similarly, in a cohort study by Rabizadeh et al. the analysis showed that LDL-C/Apo B ratio ≤ 1.2 can independently predict CAD (OR = 1.841, P = 0.002) (37). In a cohort study conducted by Kappelle et al. Apo B/Apo A-I ratio and TC/HDL-C ratio were able to predict CAD and the first major adverse event during follow-up (38). Consistent with previous studies, this study found that Lp (a), TC/HDL-C, LDL-C/HDL-C, Apo B/Apo AI, TG/HDL-C, and LDL-C/Apo B ratios were significantly associated with CAD. In terms of effectively predicting CAD, the addition of lipid parameters on traditional risk factors model improved the accuracy of the estimates, and Lp (a) was the best.

Many studies have reported that elevated plasma Lp (a) is an independent risk factor for CAD, which mainly participates in the pathophysiological process of CAD *via* prothrombotic/anti-fibrinolytic effects and promoting the deposition of cholesterol in the vascular intima (39–41). On an equimolar basis, Lp (a) is more likely to cause atherosclerosis than LDL because it not only contains all proatherogenic components of LDL-C but also those of Apo (a) (13). Because the structure of Lp (a) has one more Apo (a) than that of LDL-C, its ability to enhance atherosclerotic thrombosis through other mechanisms including inflammation is stronger than that of LDL-C (42). In a chart-controlled study of 143,087 subjects, Lp (a) concentration was associated with the risk of CAD in a dose-dependent manner, with the risk of CAD increasing as the percentile level of Lp (a) increased (43). With the development of genetic technology, the relationship between Lp (a) and CVD has been well elucidated at the genetic level (44). Many large observational and genetic epidemiological studies have shown that high Lp (a) levels

TABLE 2 Backward stepwise logistic analysis.

Variable	Univariate		Multivariate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age	1.01 (1.00–1.03)	0.009	1.01 (1.00–1.03)	0.024
Male sex	2.33 (1.82–2.98)	<0.001	2.41 (1.82–3.19)	<0.001
Hypertension	1.45 (1.15–1.83)	0.002	1.36 (1.05–1.75)	0.018
Smoking	1.60 (1.24–2.07)	0.000	1.35 (1.00–1.82)	0.052
Alcohol consumption	0.83 (0.51–1.34)	0.445	0.57 (0.34–0.96)	0.035
CKD	1.67 (1.18–2.37)	0.004	–	–
DM	1.86 (1.43–2.42)	<0.001	1.75 (1.33–2.30)	<0.001
Mean-cIMT	3.63 (2.25–5.88)	<0.001	2.73 (1.64–4.52)	<0.001

CKD, chronic kidney disease; DM, diabetes mellitus; cIMT, carotid intima-media thickness; OR, odds ratio; CI, confidence interval.

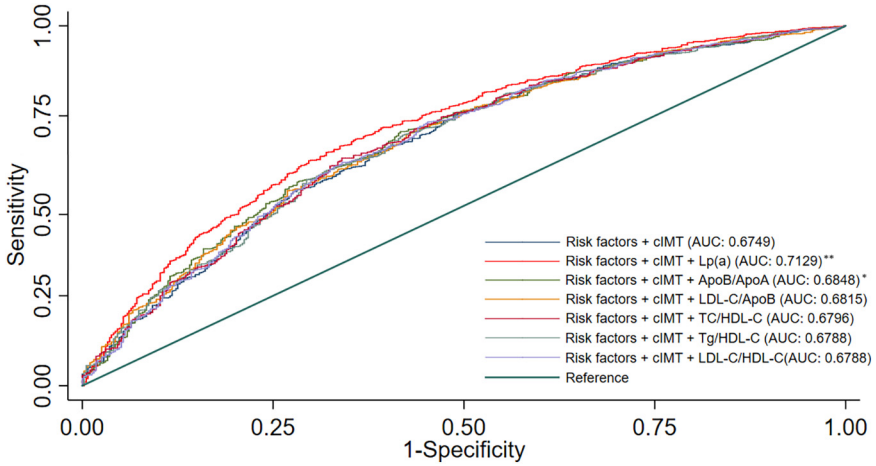

FIGURE 2
Receiver operating characteristic curves for models with different lipid parameters. Illustration: Basic prediction model adjusted for age, sex, hypertension, smoking, alcohol consumption, DM, and mean-cIMT; *$P < 0.05$ compared with basic prediction model; **$P < 0.001$ compared with basic prediction model.

TABLE 3 Comparison of different lipid parameters for predicting CAD based on basic prediction model using the NRI and IDI with cut-off point at low (< 50%)/intermediate (50–80%)/high risk (> 80%) of CAD.

	Lp (a)	Apo B/Apo A-I	LDL-C/Apo B	TG/HDL-C	TC/HDL-C	LDL-C/HDL-C
NRI	0.126** (0.051 to 0.224)	0.028 (−0.014 to 0.113)	0.040* (−0.014 to 0.105)	0.010 (−0.019 to 0.094)	0.005 (−0.024 to 0.086)	0.012 (−0.023 to 0.071)
IDI	0.032** (0.024 to 0.040)	0.008* (0.003 to 0.012)	0.005* (0.000 to 0.009)	0.004* (0.000 to 0.008)	0.005* (0.00 to 0.008)	0.003* (0.000 to 0.006)

NRI > 0 or IDI > 0 means the parameter improve the predictive value of disease.
*$P < 0.05$.
**$P < 0.001$.
NRI, net reclassification improvement; IDI, integrated discrimination improvement; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; Lp (a), lipoprotein(a); CAD, coronary artery disease.

and corresponding LPA genotypes increase the risk of CVD (45, 46). In addition, elevated level of LDL-C is strongly associated with the development of CAD (47). The 2019 ESC guidelines also recommend using LDL-C as the primary indicator of lipid-lowering therapy (48). In the baseline information of the study patients, the LDL-C of the CAD group was similar to that of the non-CAD group, possibly because the CAD group took more statins. In such condition, the levels of LDL-C were comparable in both groups, and the additional effect of Lp (a) could be investigated. Previous studies have shown that patients still have a certain risk of CAD after the treatment for reducing LDL-C, and this residual risk was related to elevated Lp (a) (49–53). In our study, the mean LDL-C level in CAD group was 2.6 mmol/L, meeting the LDL-C management target at a low level. Among patients whose target blood lipids are normal or below the target, Lp (a) will play an important role in the prediction of CAD. This may explain the results that the AUC value of Lp (a) was greater than that of LDL-C-associated blood lipid indices in the ROC curve.

In addition, elevated Lp (a) levels were correlated with multi-vessel CAD. This result is consistent with a recent finding demonstrating that high Lp (a) was significantly associated with

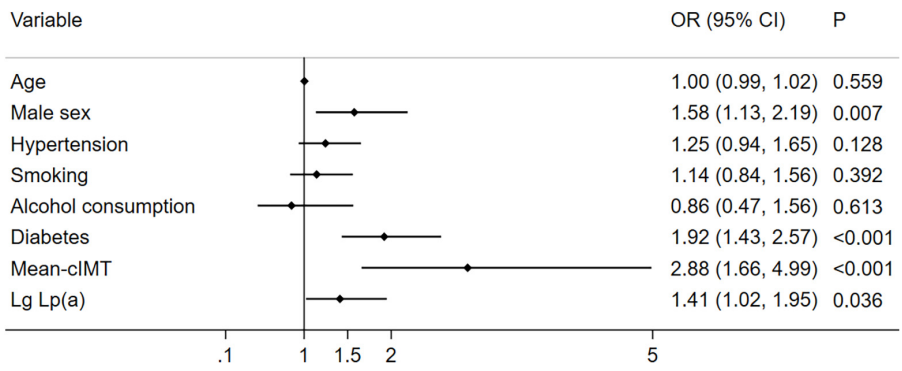

FIGURE 3
Relationship between Lp (a) combined with cIMT and multi-vessel CAD (n = 1004). Illustration: Lp (a) was transferred to Lg Lp (a).

increased CAD severity, evaluated using the SYNTAX score (44). The findings of the present study highlight the importance of Lp (a) combined with cIMT in assessing the risk of CAD, especially for those with normal level of LDL-C, which may be useful for guiding primary prevention decisions.

This study has some strengths. This study compared the effectiveness of different lipid parameters combined with cIMT in predicting CAD in the absence of high LDL-C for the first time, and recognized that Lp (a) was superior to other lipid parameters in CAD discrimination, through NRI and IDI. This study has certain limitations. First, this was a retrospective study and no causal conclusion can be drawn. Second, Some people with CAD have a history of statin use, which may have an effect on Lp (a) levels. Third, this study lacked height and weight data to calculate individual body mass index (BMI). As the aim of our study was to compare the predictive value of different lipid parameters rather than to build a predictive model for CAD, the effect of BMI on the primary outcome of this study was not significant. Finally, the severity of CAD was based on the number of coronary artery lesions, and the corresponding degree of stenosis in each branch was not well quantified.

Conclusion

For patients with highly suspected CAD, Lp (a) combined with cIMT better improved the predictive value of CAD compared with other blood lipid parameters, especially in the absence of high levels of LDL-C. High concentration of Lp (a) was also associated with the multi-vessel CAD. In the future, Lp (a) may need more attention and management in the prevention of CAD.

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 human participants were reviewed and approved by the Ethics Committee of Guangdong Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BY and YW: manuscript preparation and writing—original draft. WL, LZ, and YL: data collection and collation. BY, YW, and WW: data analysis. GL and YZ: writing—critical revisions. XH and XL: conceptualization and approval of the final version of the manuscript for submission. All authors read and approved the final manuscript.

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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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Development of risk prediction model for cognitive impairment in patients with coronary heart disease: A study protocol for a prospective, cross-sectional analysis

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Background: Ischemic heart disease and degenerative encephalopathy are two main sources of disease burden for the global elderly population. Coronary heart disease (CHD) and cognitive impairment, as representative diseases, are prevalent and serious illnesses in the elderly. According to recent research, patients with CHD are more likely to experience cognitive impairment and their cognitive ability declines more quickly. Vascular risk factors have been associated with differences in cognitive performance in epidemiological studies, but evidence in patients with CHD is more limited. Inextricably linked between the heart and the brain. Considering the unique characteristics of recurrent cognitive impairment in patients with CHD, we will further study the related risk factors. We tried to investigate the potential predictors of cognitive impairment in patients with CHD through a prospective, cross-sectional study.

Methods: The cross-sectional study design will recruit 378 patients with CHD (≥65 years) from Xiyuan Hospital of China Academy of Chinese Medical Sciences. The subjects' cognitive function is evaluated with MoCA scale, and they are divided into cognitive impairment group and normal cognitive function group according to the score results. Demographic data, disease characteristics (results of coronary CT/angiography, number of stents implanted, status of diseased vessels), laboratory tests (biochemistry, coagulation, serum iron levels, pulse wave velocity), metabolites (blood samples and intestinal metabolites), and lifestyle (smoking, alcohol consumption, sleep, physical activity) will be assessed as outcome indicators. Compare the two groups and the correlation analysis will be performed on the development of mild cognitive impairment. Mann-Whitney U or X^2 test was selected to describe and evaluate the variation, and logistics regression analysis was employed to fit the prediction model. After that, do the calibration curve and decision curve to evaluate the model. The prediction model will be validated by a validation set.

Discussion: To explore the risk factors related to mild cognitive impairment (MCI) in patients with CHD, a new predictive model is established, which can achieve advanced intervention in the occurrence of MCI after CHD. Owing to its cross-sectional study

design, the study has some limitations, but it will be further studied by increasing the observation period, adding follow-up data collection or prospective cohort study. The study has been registered with the China Clinical Trials Registry (ChiCTR2200063255) to conduct clinical trials.

KEYWORDS

coronary heart disease, cognitive impairment, risk factors, prediction model, cross-sectional analysis

1. Introduction

With the deepening of population aging, the incidence of cardiovascular and cerebrovascular diseases is increasing annually, which brings heavy social and economic burden. Coronary heart disease (CHD) and cognitive impairment are major diseases that endanger human health, especially for the elderly. Cognitive impairment includes varying degrees from mild cognitive impairment (MCI) to dementia. When memory or other cognitive functions are impaired but not to the point of dementia, this is referred to as MCI (1). Epidemiological investigation have shown that 10–15% of MCI patients develop dementia each year among people over 65 years old (2), while about 70 % of Alzheimer's disease (AD) patients in China are evolved from MCI (3). Hence, it has significant clinical value for the early recognition, early diagnosis and transformation prediction of the disease.

Studies have shown that those with CHD are more likely to experience cognitive impairment. Compared to the general population, the prevalence rate is much greater at roughly 35–46% (4–6). A clinical study published in The New England Journal found that the incidence of cognitive decline in patients with CHD was 42% five years after coronary-artery bypass grafting (CABG) (6). A significant longitudinal cohort research with 7,888 participants (mean age 62.1 ± 10.2 years) indicated that CHD incidents were linked to accelerated cognitive decline. The decline in cognitive ability (including overall cognitive, verbal memory, and time orientation scores) occurred in the years after the diagnosis CHD (7). Similar findings were made by other researchers who discovered that after a follow-up of 3.2 ± 0.37 years, patients with stable CHD had a 42.4% incidence of cognitive impairment (4). In view of this, our study intends to focus on elderly patients with a history of coronary heart disease over 3 years, because of the high incidence of MCI at this stage. Additionally, cardiovascular disease risk factors such as hyperlipidemia, hypertension, diabetes, obesity, smoking and so on are also risk factors of cognitive impairment (8, 9). Clinical observation revealed that cognitive impairment was more pronounced in patients with significant three-vessel disease or coronary artery stenosis (10). Researchers discovered that people with a history of myocardial infarction had a 2–5 times higher chance of developing dementia (7).

The impact of cardiovascular event chain (from cardiovascular risk factors to end-stage heart diseases) characterized by coronary atherosclerosis has expanded to organs outside the heart, particularly the brain, and affects cognitive performance (11, 12). It is of great significance to further study the relationship between CHD and cognitive impairment. Therefore, our study soughts to assess the correlation between CHD and MCI in the elderly by examining the differences of coronary heart disease and its risk factors between patients with cognitive impairment and patients with normal cognition. At the same time, a risk prediction model will be established to provide reference for early clinical identification and intervention of cognitive impairment in patients with CHD. In order to achieve the objective of preventing risk factors from occurring and enhancing cognitive performance.

2. Methods and analysis

2.1. Study design and participants

This is a prospective, cross-sectional study with 378 participants. After screening according to the inclusion and exclusion criteria, the informed consent of the subjects was obtained. According to whether CHD patients are accompanied by mild cognitive impairment, the researchers will divide them into a cognitive impairment group (observation group) and a normal cognitive function group (control group). It is based on the program "Research on the Transformation and Application of TCM in the Prevention and Treatment of Secondary Cognitive Impairment of Coronary Heart Disease" sponsored by the Chinese Academy of Chinese Medical Sciences (CI2021A01406) to identify the risk factors for cognitive impairment in CHD patients. The fieldwork for this study will be conducted at Xiyuan Hospital of China Academy of Chinese Medical Sciences in China. Both outpatients and inpatients are allowed to be included. Depending on the feasibility and specific implementation, additional hospital sites may be added. In addition, this study has been registered as a clinical trial by the Chinese Clinical Trials Registry (ChiCTR2200063255), which further standardizes our research. The specific overview of study procedure is shown in Figure 1.

2.2. Recruitment and screening

Eligible patients were identified according to the diagnostic description and the International Classification of Diseases (10th and 11th editions). The recruitment of subjects was carried out through offline and online announcements, focusing on offline treatment process. Eligible patients are expected to be enrolled between September 2022 and December 2023. The diagnostic criteria of CHD refer to "guidelines for diagnosis and treatment of stable coronary heart disease" (13) and "guidelines for emergency rapid diagnosis and treatment of acute coronary syndrome (2019)" (14).

For the diagnostic criteria of MCI, refer to the Chinese guidelines on mild cognitive impairment (15). Specifically: (i) cognitive impairment was reported by patients or insiders, or experienced clinicians found cognitive impairment; (ii) objective evidence of

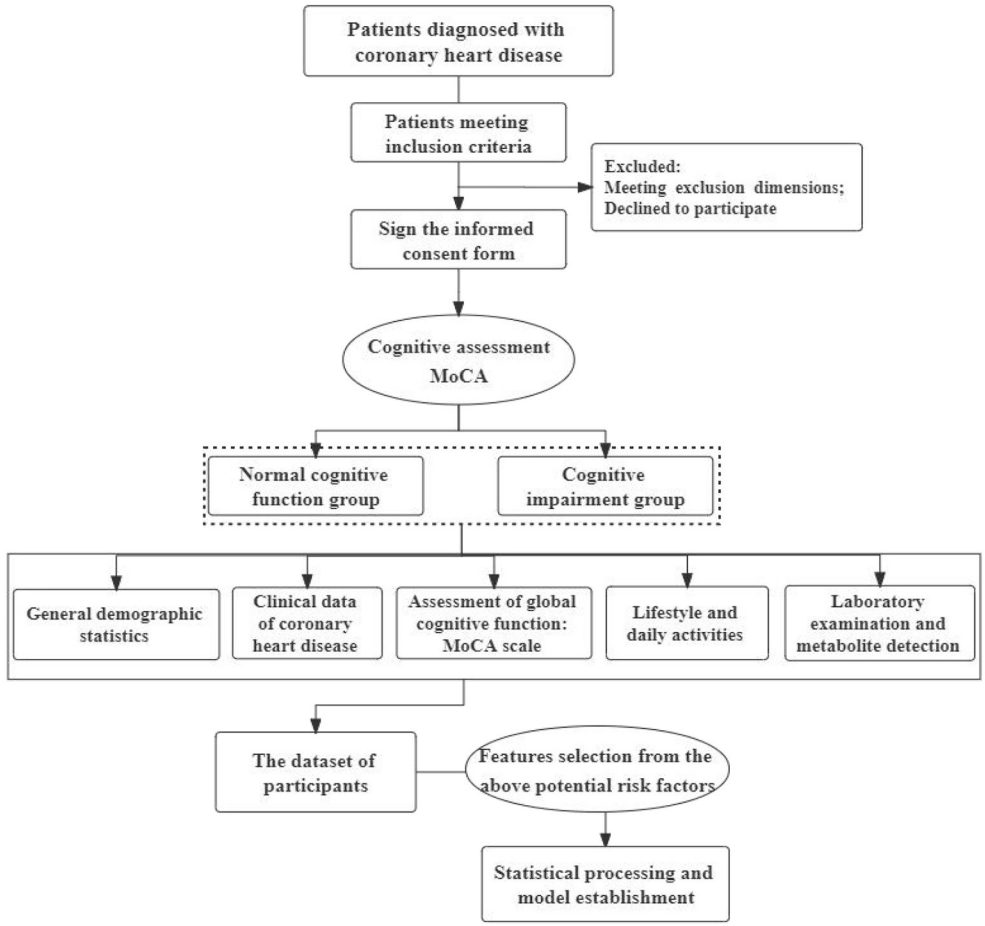

FIGURE 1
Flow chart of specific steps.

impairment of one or more cognitive domains (from cognitive tests); (iii) sophisticated instrumental daily abilities might be slightly hampered, but still preserve independent daily living abilities; iv unable to reach the diagnosis of dementia. Auxiliary diagnostic criteria: the score of Montreal Cognitive Assessment (MoCA) scale <26 points; the score of Clinical Dementia Rating (CDR) scale was 0 or 0.5. MoCA: the full score of the scale is 30, and the score ≥26 is normal. If the subjects' years of education are <12 years (senior high school level), the results can be increased by one point.

All the above are diagnosed by the attending physician. Under the premise of full informed consent, it will be screened by the researcher or the attending physicians according to the requirements. The inclusion and exclusion criteria for this study are shown in Table 1.

2.3. Sampling and sample size

For the exploratory analysis of multiple risk factors, the sample size of this study is set to be >5–10 times the number of observed factors (16, 17). According to previous research reports, after 3.2 ± 0.37 years of follow-up, the incidence of cognitive impairment in patients with stable coronary heart disease is 42.4% (4). Clinical observations showed that patients with CHD had a relatively higher incidence of MCI. Based on the actual conditions of patients in the hospital, and the 18 independent variables pre-estimated in the study (detailed in the Outcomes Measures part), the sample range should be at least 90–180. Considering the absence and effectiveness of 5% of the samples, the total sample size finally determined by the two groups should ≥378. The final sample size of each group shall be determined according to the actual situation and can be adjusted appropriately.

2.4. Outcomes measures

Clinical, biological, social and environmental determinants of interest in this study were selected based on the potential or established association with cognitive impairment in patients with CHD and/or the fact that they are modifiable through preventive strategies.

2.4.1. Demographic and clinical data

Standardized questionnaires will be used to collect demographic, symptom, and medical history data. Social demographic information includes age, gender, race, height, weight, BMI, marital status, education level, nature of work, and type of occupation. Clinical data include past and present diseases (acute and chronic), drugs, and

TABLE 1 Inclusion criteria and exclusion criteria of the study.

Inclusion criteria
Age ≥ 65 years
Meet the diagnostic criteria of CHD, and the medical history ≥3 years
Visual and auditory discrimination is relatively intact, enough to cooperate with relevant tests
Voluntarily participate in the study and sign informed consent form
Exclusion criteria
Alzheimer's disease or other types of dementia
Patients with neurological and mental disorders, such as severe depression, schizophrenia, bipolar disorder, etc.
Have suffered from other diseases that affect cognitive function, such as cerebrovascular history, hypothyroidism, obvious folic acid and vitamin B12 deficiency, or previous intracranial surgery
Patients with severe heart disease and perioperative hemodynamic instability other than CHD, primary diseases with important organs, blood system or endocrine system
History of alcohol dependence and special drug abuse, including drugs* that can cause cognitive changes
Patients with severe hepatic and renal insufficiency
Had been diagnosed with cognitive impairment or dementia before the first diagnosis of CHD
Participating in other clinical trials

*Drugs affecting cognitive function mainly include the following categories: anticholinergics, antiepileptics, antiparkinson's, antihistamines, tricyclic antidepressants, skeletal muscle relaxants.

family history of cardiovascular disease. Disease-related information includes the course of CHD, the results of coronary CT/ angiography, the number of coronary stents implanted, cardiac function class, the number of diseased vessels, types of statins, and drug treatment. Previous disease history and drug use also need to be recorded. Vital signs such as temperature, respiration, pulse and blood pressure are also covered.

2.4.2. Cognitive assessment

At baseline, all participants underwent cognitive assessment. Global cognitive function will be assessed using the MoCA scale. Neuropsychological scale screening tests are crucial in the identification of cognitive impairment. Currently, the Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) are the most commonly used cognitive assessment tools. Of the two, MoCA covers a wider variety of cognitive domains than MMSE. In addition, meta-analysis findings (18) revealed that the sensitivity, specificity and AUC value of MoCA are higher than those of MMSE in elderly patients (over 60 years old) with MCI. Therefore, we choose MoCA as the screening method to determine whether there is MCI.

As a screening tool specially developed to detect MCI, it takes around 10 min to administer MoCA when executed (19). The MoCA covers 14 topics with a total of 30 points, including visual spatial structure skills, executive function, attention and concentration, short-term memory and delayed recall, language, abstract thinking, calculation and orientation. Due to varied geographies, cultures and populations, there are some differences in the cut-off values of MoCA screening for MCI among elderly individuals in various regions. Studies have shown that when using a cutpoint of 25/26, the MoCA has a sensitivity of 80–100% and specificity of 50 to 76% for detecting MCI (20). According to a survey to evaluate MCI in Beijing, China's urban and rural old communities, MoCA has excellent sensitivity and fairness specificity under the recommended critical score of 26 (21). The systematic review of MCI screening for the elderly in China also concurs that using a diagnostic threshold of 25/26 is the most practical option (22). Thus, the score of MoCA <26 is chosen as the benchmark to ascertain whether MCI exists in this study.

2.4.3. Health and lifestyle

According to the investigation, researchers found that an insomnia history was associated with an increased risk of MCI, and a sleep disorder history may usefully predict MCI (23). Therefore, we specially selected the commonly used Pittsburgh Sleep Quality Index (PSQI) to analyze the sleep characteristics of the subjects. Sleep quality, falling asleep time, sleep time, sleep efficiency, sleep disorders, hypnotic drugs and daytime dysfunction are included in this scale. The World Health Organization (WHO) guidelines recommend that elderly people exercise at least 150 min medium intensity or 75 min high intensity aerobic exercise per week. So the physical activity of the participants will also be monitored to explore if it is related to MCI. Physical activity levels will be measured by using a Chinese version of the International Physical Activity Questionnaire-short form (IPAQ-S) (24).

2.4.4. Laboratory indicators

Laboratory examination can accept the test results within 7 days before and after screening. It mainly includes the following contents: fasting glucose, glycosylated hemoglobin (HbA1c), total cholesterol, triglyceride, low-density lipoprotein, high-density lipoprotein, total bilirubin, serum creatinine, serum iron level (Fe), coagulation function (PT, APTT, TT, FIB), vascular ultrasound pulse wave velocity (PWV).

In addition to the above-mentioned indexes, in the early morning of the 2nd day after the selection, two tubes of peripheral venous blood of 5 ml will be drawn from a qualified phlebotomist and placed in the EDTA anticoagulant tubes. Centrifuge the blood samples (3,000 rpm at 4°C for 15 min), store them at −80°C, and then conduct untargeted metabolomics detection. 2 g of fresh fecal samples will be collected and put into aseptic collection tube and stored at −80°C immediately for metabolic detection and analysis of intestinal flora.

2.5. Quality control

All researchers can enter and conduct this trial only after they have completed the training and obtained the authorization of the principal researcher. The involved researchers were trained in Good Clinical Practice (GCP) prior to the start of this study. Every researcher has received training in research procedure and plan. Including the collection of the MoCA scale, the researchers mastered the relevant questioning skills and evaluation criteria after strict and standardized training in advance. Ensure the rigor and

consistency of the implementation process. All investigators have a thorough understanding of the information in this clinical research protocol, master the GCP principles, unify the recording methods and judgment standards, and strictly follow the protocol.

2.6. Statistical analysis

Among the outcomes of this study, the measurement data in accordance with normal distribution are expressed by mean ± standard deviation, and independent sample *t*-test is used for comparison between the two groups. The measurement data that don't conform to the normal distribution are expressed in the median (quartile), and the Mann-Whitney U test is used for comparison between the two groups. Counting data were expressed as frequency or percentage, and X^2 test was used to compare the two groups. Single factor analysis shall be conducted for all factors. In order to avoid the omission of important variables, the covariates with $P < 0.1$ in the univariate analysis will be included in the multivariate logistic regression analysis. Stepwise forward regression analysis is used to analyze the independent risk factors of cognitive impairment in patients with CHD, and the OR value and 95% CI of each related factor will be estimated. The risk prediction model of cognitive impairment in patients with CHD would be established by adding independent risk factors. ROC curve will be drawn and the area under the curve (AUC) will be calculated to evaluate the effectiveness of the prediction model. Use Bootstrapping method to verify the accuracy of the final model internally. All tests with $P<0.05$ on both sides indicate that the difference is statistically significant, and other statistical analysis can be carried out by SPSS25.0.

2.7. Data management

In this study, standardized case report form (CRF) tables will be used to collect data. Each participant will be assigned a unique identifier with the hospitalization number or patient ID code. Medical and other records of the participants will be kept by the researchers. EpiData3.1 software will be used for data entry and establishing the database. Once the data input is completed, the integrity and reasonableness of all data will be evaluated and checked by the researchers item by item. Check the problem with the original survey data and correct it after confirmation.

3. Discussion

Age-related cognitive decline is a series of natural cognitive changes that may develop into MCI or dementia. MCI is a state of cognitive impairment between healthy aging and dementia, which is considered as the prophase of dementia (25). But at this stage, the function of daily life remains basically intact. Delaying the onset of cognitive decline is the optimal strategy but where there are some earlier measures to slow the decline is important. Through comparative analysis of the population characteristics of the two groups, our study will screen out the specific markers of cognitive impairment in patients with CHD. So as to provide reference for early clinical identification and intervention of the disease.

The objective of this study is to ascertain the relationship between CHD and its risk factors and cognitive impairment in the elderly. At the same time, establish the risk prediction model of cognitive impairment in patients with CHD. The study will make available data regards CHD and MCI risk prediction. We propose leveraging the progress of machine learning to systematically compare different modeling approaches (26) to develop predictive tools for cognitive impairment in patients with CHD and verify their effectiveness. Comprehensive application of clinical observation, multi-source data processing, machine learning and other methods is also the innovation of the study.

Still, this research is subject to certain limitations. As an observational study, cross-sectional design has limitations e.g., causality cannot be inferred, we can only address the association. However, we try to include more observation factors, which are widely related to basic demographic data, disease characteristics, blood chemistry, metabolites and lifestyle. It is expected that some risk factors with clinical value can be found on the basis of this exploratory analysis. In order to broaden the current knowledge on CHD and its relationship with cognitive impairment in the elderly. Additionally, it will serve as a valuable foundation for hypothesis generation for future longitudinal studies and / or randomized controlled trials. In the future, it is necessary to conduct a larger sample of prospective cohort studies and add follow-up time to determine the incidence of secondary MCI in patients with CHD.

Ethics statement

The study was approved by the Ethics Committee of Xiyuan Hospital of Chinese Academy of Chinese Medical Sciences (2022XLA060-3). This study involves elderly patients with cognitive impairment and the legitimate rights and interests of the subjects should be fully protected. Trained researchers will provide the subjects with informed consent forms, which are easy to understand and clear. Researchers will patiently and completely explain the purpose, procedure, potential benefits, and risks of this study before each subject is recruited for it. The principles of complete information disclosure and voluntary choice must be adhered to during the informed consent procedure. Subjects have the right to refuse to participate without giving reasons. After participants have voluntarily signed the informed consent form, attention will be paid to the questioning skills during the study, so as to avoid the unfamiliar emotions of the elderly subjects due to their participation in clinical research. Any modification of the research protocol will be submitted to the Research Ethics Committee for approval. Currently, the researchers are recruiting participants. The research results of this study will be published in medical journals in Chinese or English, but the patients' information will be kept private as required by law. When necessary, government administrative departments, hospital Ethics Committees, and their relevant personnel may consult the patient's data according to the regulations.

Author contributions

FX and YuL conceived and designed the study protocol, helped to critically revise the draft, and conduct the final manuscript. QW, SX, and FL drafted the manuscript. YaL and QW made critical

revision. All authors contributed to this 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.

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circRNA, a novel diagnostic biomarker for coronary heart disease

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Objective: This study aimed to identify the potential diagnostic biomarkers of coronary heart disease (CHD) from exosome-derived circRNA.

Methods: The microarray data of circRNA derived from the exosomes of patients with CHD and mRNA in acute myocardial infarction was retrieved from exoRBase website and GEO database (GSE61144), respectively, to identify the differentially expressed genes (DEGs). Our findings detected the differentially expressed circRNAs and mRNAs and predicted their correlation with microRNAs using the microRNA target prediction website, thus ascertaining the corresponding circ-microRNA and micro-mRNAs. Then, we performed systematic Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis on the differentially expressed mRNA. Protein-Protein Interactions (PPI) of these DEGs were examined using STRING. The receiver operator characteristic (ROC) curve was used to validate the diagnostic efficacy of circRNA in patients with CHD. Finally, the RNAs identified in this study were verified by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: A total of 85 differentially expressed circRNAs (4 up-regulated and 81 down-regulated) were identified by screening the circRNAs in exosome of CHD patients. Based on the prediction data of circRNA, mRNA, and the corresponding microRNA, a ceRNA network was constructed, including 7 circRNA nodes, 5 microRNA nodes, and 2 mRNA nodes. Finally, validated by qRT-PCR testing, we found circRNA0001785, circRNA0000973, circRNA0001741, and circRNA0003922 to be the promising candidate for the effective prediction of CHD. These potential diagnostic markers can provide insight for further research on the occurrence of CHD or even acute coronary syndrome (ACS).

KEYWORDS

diagnostic marker, plasma exosome, coronary heart disease, ceRNA network, differentially expressed genes

1. Introduction

Ischemic heart disease (including acute myocardial infarction) is the leading cause of death globally and accounted for 17.3% of all deaths in 2016 (1, 2). It is well known that myocardial injury leads to narrowing of coronary arteries and exacerbates oxygen deficiency in myocardial cells (3). Although PCI and coronary artery bypass grafting are widely used to treat patients with acute myocardial infarction, post-operative complications and reduced cardiac function remain inevitable (4). Elucidating the mechanism underlying atherosclerotic plaque formation and triggering of plaque rupture may facilitate the development of treatments for patients with CHD.

Exosomes are intracellular membrane-bound vesicles with diameters of 30–150 nm that originate from a variety of intracellular cell types and transfer their bioactive molecules between cells (5). Exosomal contents change in response to environmental stimuli on their parental cells (5–7), thus modulating downstream biological effects of the exosomes. Moreover, exosomes can deliver their cargo, such as functional nucleic acids (microRNAs, mRNAs, and other RNA types), to recipient cells, thereby regulating these cells at the post-transcriptional level (8–10). Therefore, exosomes are intercellular signaling factors that can deliver bioactive proteins, lipids, and RNA species in both paracrine and autocrine fashions (11, 12). For example, exosomes isolated from human atherosclerotic plaques were shown to directly transfer functional ICAM1 to target cells, and plaque exosomes from symptomatic patients could induce monocyte adhesion and migration more strongly than those from asymptomatic patients, indicating functional differences among these exosomes (13). Therefore, the detection of differentially expressed genes of exosomal origin enables a more accurate determination of diagnostic biological markers of disease.

Circular RNA (CircRNA) is a novel endogenous non-coding RNA, characterized by a covalent closed-loop structure called post-clipping through a special type of alternative splicing (14). In recent years, the development and application of microarray technology, RNA sequencing analysis (RNA-seq), and new bioinformatic methods have led to the discovery of many circRNAs (15–19). For example, has-circ-0124644 can be used as a diagnostic biomarker of CHD (20, 21), and has-circ-0005870 can be used as a diagnostic biomarker of hypertension (22). In addition, some circRNAs have also been identified as new prognostic biomarkers for patients with heart failure after cardiac infarction (23–25). Similarly, the enrichment and stability of circRNAs in exosomes were also identified in body fluids, such as human blood, saliva, and cerebrospinal fluid, indicating that these Exo-circRNAs have potential applications as disease biomarkers and novel therapeutic targets (20). Some databases also indicate that the number of circRNAs in exosomes is even higher than the number of mRNAs. However, the role of exosomal circRNAs in cardiovascular disease is not fully understood.

In this study, we downloaded microarray data of exosome-derived circRNAs from exoRBase[1] and microarray data of mRNAs involved in acute myocardial infarction (GSE61144) and identified the differentially expressed genes (DEGs) (26). The differentially expressed circRNAs and mRNAs were predicted using the microRNA target prediction websites (Targetscan, StarBase, miRanda) and the relationship between the two and microRNA. Furthermore, starBase and Circular RNA Interactive were used to identify the corresponding circ-microRNA and micro-mRNA (27, 28). Then, systematic Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed with the DEGs identified following the analysis of the mRNAs (29, 30). STRING was used to study the protein interactions between these DEGs (31), ROC curves were used to verify the diagnostic effect of circRNAs on patients with CHD (32). Finally, we extracted peripheral blood from healthy and CHD individuals, and verified the above-derived RNAs by PCR. In conclusion, our study provides new insights into the potential of circRNAs as diagnostic biomarkers for patients with CHD based on exosomes as delivery vehicles for genes.

1 http://www.exorbase.org/

2. Experimental methods

2.1. Microarray data

The expression profiles of exosomal RNA, including circRNA, in blood samples of patients suffering from CHD (6 cases) and healthy individuals (32 cases) were downloaded from the exoRBase (see text footnote 1). mRNA expression profiles in blood samples of patients with acute myocardial infarction were downloaded from the GEO (GSE61144) database, including 7 datasets of patients with acute myocardial infarction and 10 datasets of blood samples from healthy individuals.

2.2. Filtering DEGs

Differentially expressed genes (DEGs) screening: "Limma" and "sva" R packages were used to identify the DEGs. RNA conforming to $|\log2FC| > 0$ and $P < 0.05$ were designated as DEGs, including DEmRNAs and DEcircRNAs, and the corresponding volcano map was generated.

2.3. Constructing the ceRNA network

This study was conducted using Target Scan Human 7.2[2] and miRanda[3] to predict the microRNA corresponding to the mRNA. ENCORI[4] was used to predict the corresponding microRNAs of the circRNA. Circular RNA interactive identified corresponding circ-microRNA and micro-mRNA targets. Finally, the prediction data of circRNA with differential expression in the exosomes of patients diagnosed with CHD, mRNA derived from the blood of patients with acute myocardial infarction and their corresponding microRNA were obtained to construct a circRNA-microRNA-mRNA-related ceRNA regulatory network.

2.4. Functional enrichment analysis

To evaluate the potential biological functions of the differentially expressed mRNAs corresponding to the circRNA in exosomes, we performed Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using clusterProfiler, EnrichPlot, and GGploT2 softwares. GO enrichment analysis primarily included biological process (BP), cell component (CC), and molecular function (MF) groups, and the significance analysis was conducted based on this database. KEGG enrichment analysis was used to analyse the pathway significance of differentially expressed genes based on the KEGG database.

2.5. The general information of patients

Thirty-one patients with CHD, who were being treated in the Department of Cardiology of the Second Affiliated Hospital of

2 https://www.targetscan.org/vert_72/

3 cbio.mskcc.org/miRNA2003/miranda.html

4 http://starbase.sysu.edu.cn/

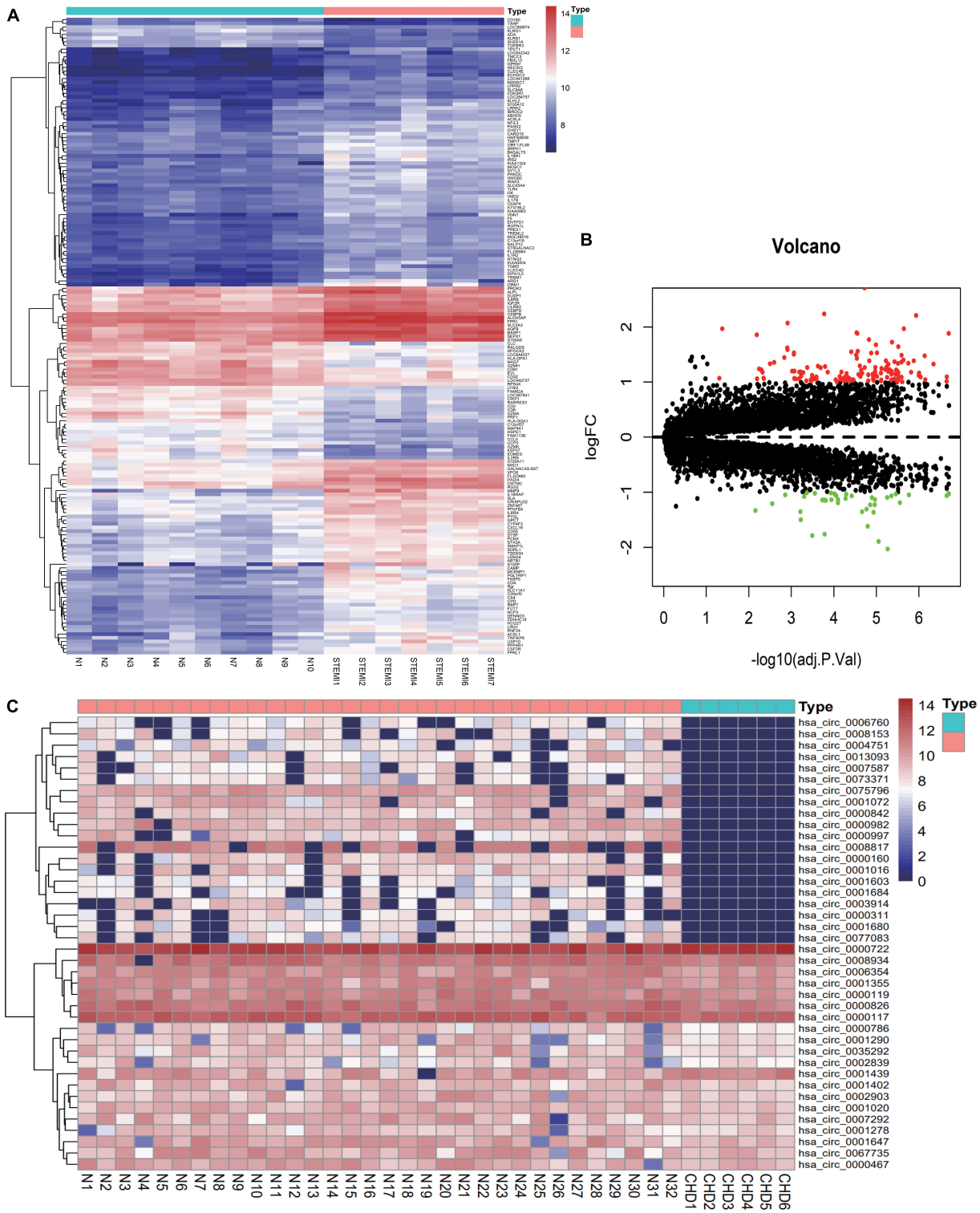

FIGURE 1
Distribution of differentially expressed genes. **(A)** Heat maps of differentially expressed genes in plasma derived from patients with acute myocardial infarction were divided into acute myocardial infarction (ACS) group (7 cases) and healthy control group (10 cases). Blue to red successively represent gene expression levels from low to high. **(B)** Volcano map of ACS differentially expressed genes. **(C)** Heat maps of differentially expressed circRNA genes in patients with CHD derived from exosomes were divided into CHD group (6 cases) and healthy control group (32 cases). Blue to red represent gene expression from low to high.

Harbin Medical University, were selected for the study. Twenty-four arrhythmia or other non-CHD patients of the same gender and age were selected at the same period. The clinical data are shown in **Supplementary Table 1**. The diagnostic criteria for CHD were set according to the report "Nomenclature and Diagnostic Criteria for Ischemic Heart Disease" formulated by the Joint Task Group on "Clinical Nomenclature Standardization of the International Cardiology Societies and Associations" and the World Health

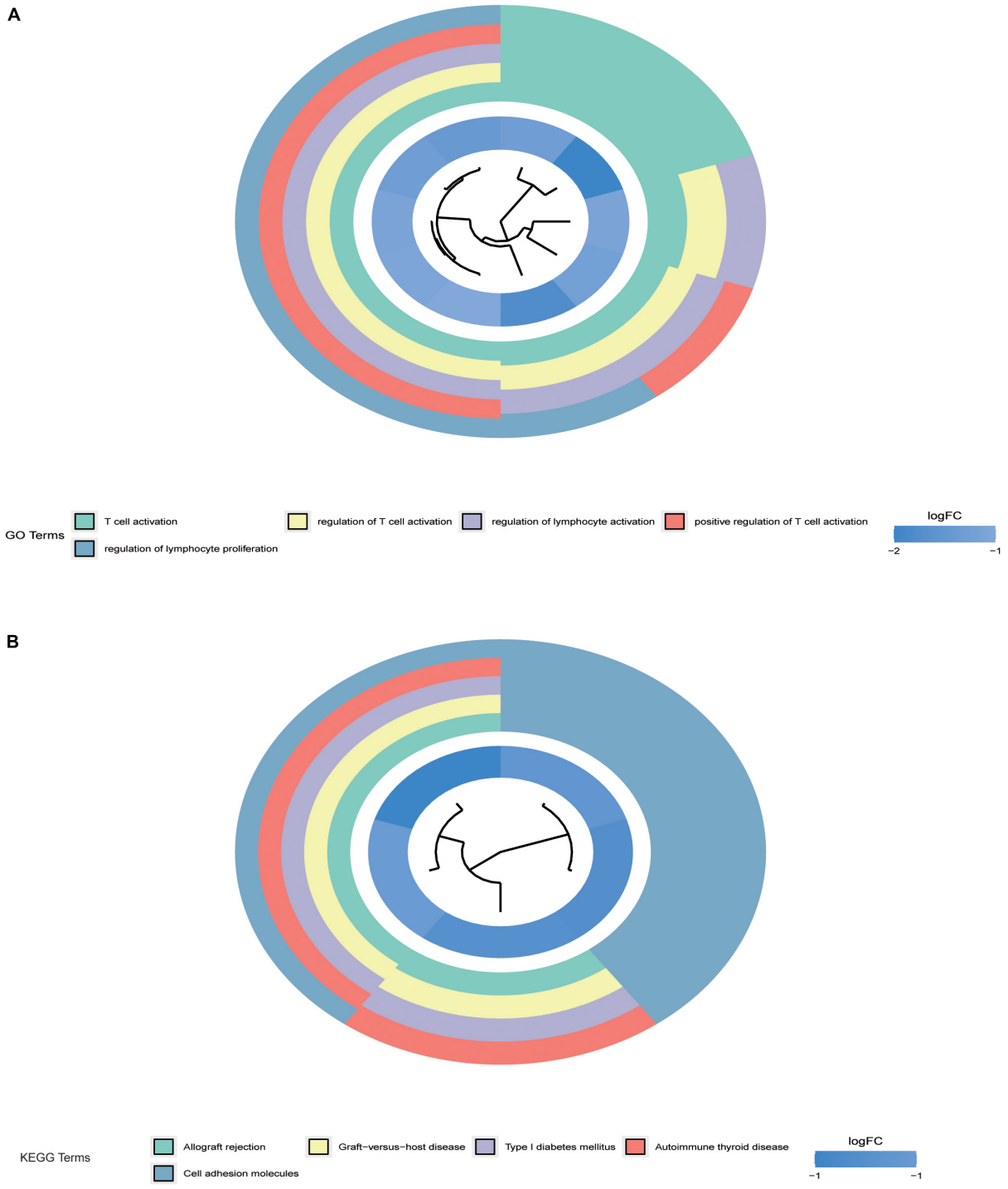

FIGURE 2
Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis. **(A)** Shows the enrichment results of GO pathway analysis of differential genes. **(B)** Shows the enrichment results of KEGG pathway analysis of differential genes. $P < 0.05$ indicated that the enrichment pathway was statistically significant.

Organization (WHO). The criteria for excluding patients with severe infection and complications and coronary angiographic features were determined by a combination of history, physical examination, serological examination, and angiography with stenosis greater than or equal to 50%. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (KY2022-072). All patients participating in the study provided informed consent.

2.6. Diagnostic merit of characteristic biomarkers in CHD

To test the efficiency of the prediction of CHD by differential expression of circRNA, we generated receiver operating characteristic (ROC) curves using circRNA expression data from the dataset of 31 CHD patients and 24 healthy individuals. The area under the

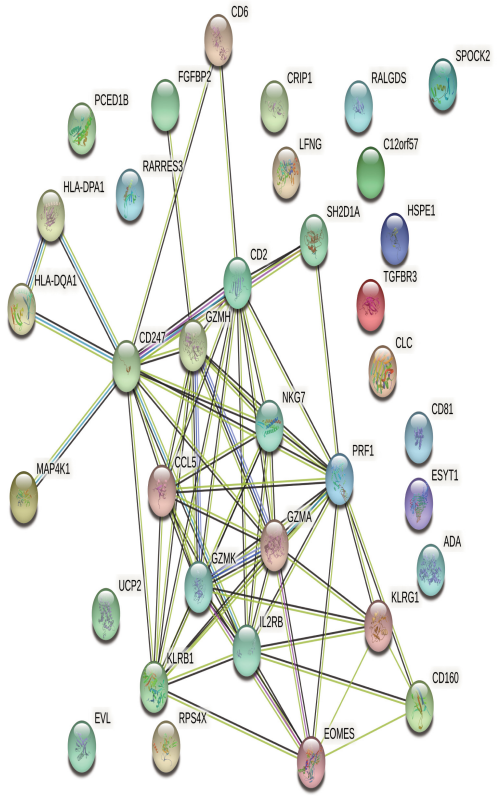

FIGURE 3
STRING network of protein-protein interactions (PPI) of differentially expressed genes (DEGs) related to AMI. In PPI networks, there are seven different colored lines: 1. Light blue: supported by the evidence from database; 2. Purple: experimentally proved; 3. Red: gene-gene fusion; 4. Yellow-green: supported by the evidence from previous studies; 5. Green: genetic proximity; 6. Blue: co-existing genes; 7. Black: co-expression between genes. Filled nodes indicate the proteins which have their three-dimensional structure known or predicted, while empty nodes designate those whose three-dimensional structures are yet to be resolved.

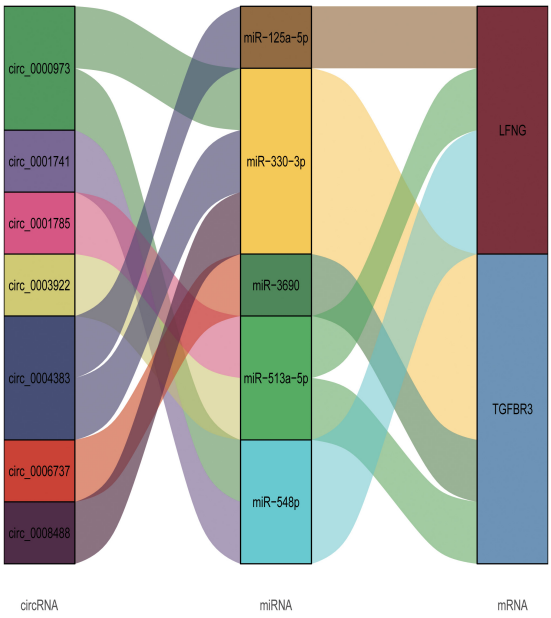

FIGURE 4
circRNA-microRNA-mRNA related ceRNA regulatory network. In this figure, the first column represents the differentially expressed exosomal circRNAs from patients with CHD, the third column depicts the mRNAs from patients with acute myocardial infarction with differentially expressed mRNAs, and the second column denotes the microRNAs with intersection with circRNA and mRNA as predicted by bioinformatic analysis.

ROC curve (AUC) was used to evaluate the diagnostic value of the identified hub genes.

2.7. qRT-PCR verification

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the variations of gene expression levels in peripheral blood with specific primers. The experiment was performed with biological triplicates. The reaction system was 10 μl. β-actin was used as the internal control. Quantitative analysis of differential expression was assessed by qRT-PCR using SYBR green reaction system on qRT-PCR machine (Bio-Rad, Hercules, CA, USA). The relative expression levels of circRNA were calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for qRT-PCR are enlisted in **Supplementary Table 2**.

2.8. Statistical analysis

All statistical analyses related to the bioinformatics study were assessed using the R 3.6.3 statistical software. Graphpad prism 7.00 statistics Mann-Whitney test was used to analyse differences in PCR of circRNA between the CHD group and the healthy control group. Multiple alterations in circRNA expression were assessed by comparing the gene expression levels of patients with CHD and the control group. Screening criteria to examine the differential expression of circRNA was set to $P < 0.05$.

3. Results

3.1. DEG screening results

Based on the analysis of circRNAs in exosomes from patients with CHD and healthy subjects, 85 differential expressed circRNAs were screened, including 4 up-regulated genes and 81 down-regulated genes. From the analysis of peripheral blood mRNAs in patients with acute myocardial infarction, 173 differentially expressed mRNAs, including 133 up-regulated genes and 40 down-regulated genes, were screened. The DEG heat map and volcano map are shown in **Figure 1**.

3.2. Enrichment analysis of GO terms and KEGG pathways

To further detect the potential function of differentially expressed mRNAs in acute myocardial infarction in CHD, we performed the GO term and KEGG pathway functional enrichment analyses. The GO term enrichment analysis showed that exosomal mRNAs were mainly enriched in T cell activation, regulation of T cell activation, regulation of lymphocyte activation, positive regulation of T cell

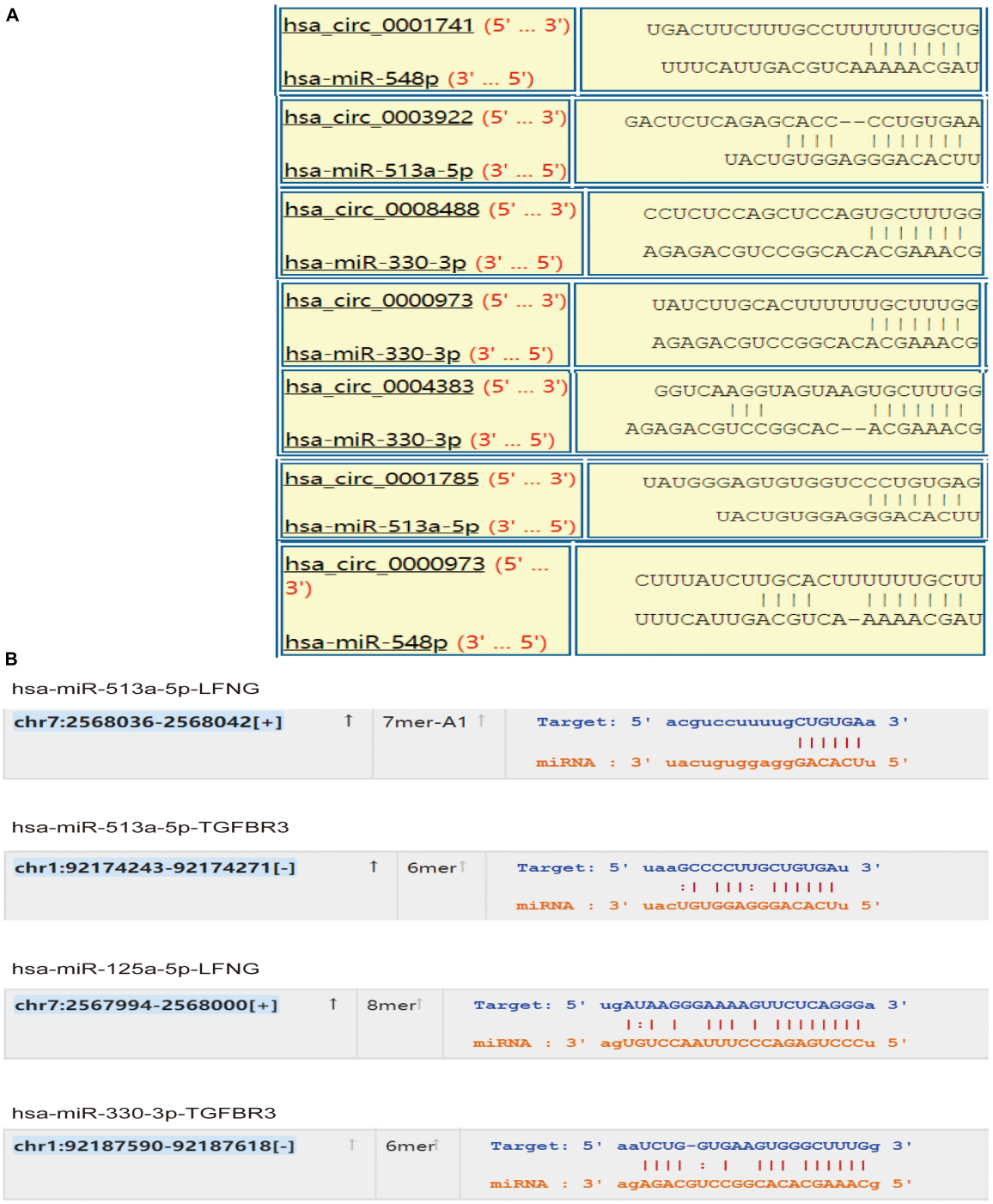

FIGURE 5
Target prediction of circ-microRNA and micro-mRNA. To further corroborate the accuracy of the target of the ceRNA network, we predict the target location using bioinformatic analysis. **(A)** Binding target of circRNA and microRNA, and **(B)** predicted target binding site of microRNA and mRNA.

activation, and regulation of lymphocyte proliferation (Figure 2). The KEGG pathway enrichment analysis showed that the differentially expressed mRNAs in the regulatory network are mainly enriched in Allograft rejection, Graft-versus-host disease, Type I diabetes mellitus, Epicardial thyroid disease, and Cell adhesion molecules. Studies have shown that CHD is correlated with cell adhesion molecules (32, 33). These results suggest that the differentially expressed circRNAs may play an important role in the occurrence and development of CHD.

3.3. STRING analysis of protein interactions

STRING is an online biological database that offers gene analysis and constructs networks of gene interactions at the protein level (31). In this study, we used STRING (version 11.0) to construct the PPI network of DEGs. To further explore central genes related to CHD and their mechanism of action, 40 genes with down-regulated expression among the 173 differentially expressed genes in the CHD

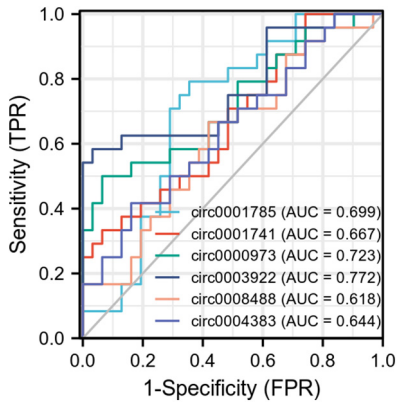

FIGURE 6
Receiver operator characteristic (ROC) curve of circRNA for the diagnosis of coronary heart disease (CHD). As shown in the figure, each point on the curve corresponds to the FPR and TPR at different thresholds. TPRate refers to the proportion of all samples of true category 1 that are predicted to be in category 1. FPRate refers to the percentage of all samples with true category 0 that are predicted to be in category 1. AUC refers to the random selection of a positive sample and a negative sample from a given category. The classifier predicted that the probability of a positive sample being positive was P1 and that of a negative sample being positive was P2. AUC is the probability that P1 > P2.

group were identified and uploaded to STRING online database to build a PPI network. A PPI network with 40 nodes and 80 edges was obtained (**Figure 3**). The nodes represent differentially expressed genes enriched in the STRING database, whereas the edges reflect the interactions between differentially expressed genes. As genes with a high binding degree and high clustering coefficient are important in maintaining the stability of the entire network, we searched for genes with a high binding degree and a clustering coefficient greater than 0.4 using the PPI network. The average node degree was 4.57, average local clustering coefficient was 0.453, and *P*-value of PPI enrichment was <1.0e−16.

3.4. circRNA-related ceRNA regulatory network construction

Based on the prediction data of circRNAs, mRNAs, and corresponding microRNAs with differential expression in CHD, we constructed a ceRNA network with 7 circRNA nodes, 5 microRNA nodes, and 2 mRNA nodes (**Figure 4**). To prove the reliability of the ceRNA network, we obtained the corresponding target information of circRNAs, microRNAs, and mRNAs from StarBase, Circular RNA Interactive (**Figure 5**).

3.5. Diagnostic effect of characteristic biomarkers on CHD

Six biomarkers were used to distinguish CHD from the prediction of high diagnostic value in healthy samples (**Figure 6**). The AUC value of circ0004383 was 0.644 (95% CI = 0.495–0.792), AUC of circ0001741 was 0.667 (95% CI = 0.521–0.812), AUC of circ0000973 was 0.723 (95% CI = 0.583–0.863), ACU of circ0008488 was 0.618 (95% CI = 0.467–0.769), AUC of circ0003922 was 0.772 (95% CI = 0.638–0.905), and AUC of circ0001785 was 0.699 (95% CI = 0.557–0.840). Finally, we produced another joint ROC curve for each circRNA, which showed an area under the curve of 0.784 (**Supplementary Figure 1**).

3.6. PCR validation and analysis results

After screening the differentially expressed circRNAs, we performed PCR verification of circRNA0000973, circRNA0001741, circRNA0001785, circRNA0003922, circRNA0004383, and circRNA0008488. RNA sequence information is shown in **Supplementary Table 2**. We designed primers for circRNA0006737 for three times, but the results failed to reach the peak value in the qRT-PCR experiment, so we did not conduct further experiments on it. The results showed that the expression of circRNA0001785, circRNA0000973, circRNA0004383, and circRNA0001741 were down-regulated in patients with CHD on peripheral blood. While the expression of circRNA0001785 in CHD was significantly reduced, which was statistically significant. These results validate the results of our bioinformatics analysis (**Figure 7**). Finally, we built a flow chart for the whole experiment design (**Figure 8**).

4. Discussion

CAD is associated with high morbidity and mortality rates worldwide. In recent years, the number of patients with CHD has been increasing. Thus, there is a need to control the number of patients with CHD and explore its molecular mechanism. Studies have shown that in acute myocardial infarction, myocardial cells secrete exosomes rich in tumor necrosis factor, which leads to myocardial cell damage. On the contrary, cardiac stem-cell-derived exosomes can reduce scar tissue formation (34). Exosome microRNAs play a major role in limiting the development of atherosclerosis (35). However, the role of exosome-derived circRNAs in coronary artery disease is still poorly studied.

With the development of gene chip technology, microarray analysis has been applied in the study of exosomes from patients with CHD. Here, the GEO gene expression dataset was used to detect the differential gene expression between exosomes from patients with CHD and those from healthy subjects, and the ceRNA network of circRNA, mRNA, and the corresponding microRNA was constructed using the bioinformatic prediction website. The GO term and KEGG pathway enrichment analysis were performed on mRNAs differentially expressed in acute myocardial infarction to determine the underlying mechanism of acute myocardial infarction in CHD. Potential biomarker genes were preliminarily verified using the ROC curve. To further confirm the diagnostic function of exosome-derived circRNAs in CHD, basic experiments were performed to verify them. PCR analysis was performed on circRNAs from the healthy and disease groups. Our study contributes to a better diagnosis of CHD and offers potential biomarker for predicting the risk of CHD in acute myocardial infarction.

Finally, through differential expression analysis and functional basis verification, we found that the down-regulation of circRNA0001785, circRNA0000973, circRNA0001741, and circRNA0003922 was statistically significant. Also, we constructed a ceRNA network corresponding to circRNAs related to acute myocardial infarction. The increase in circ0001785 promoted

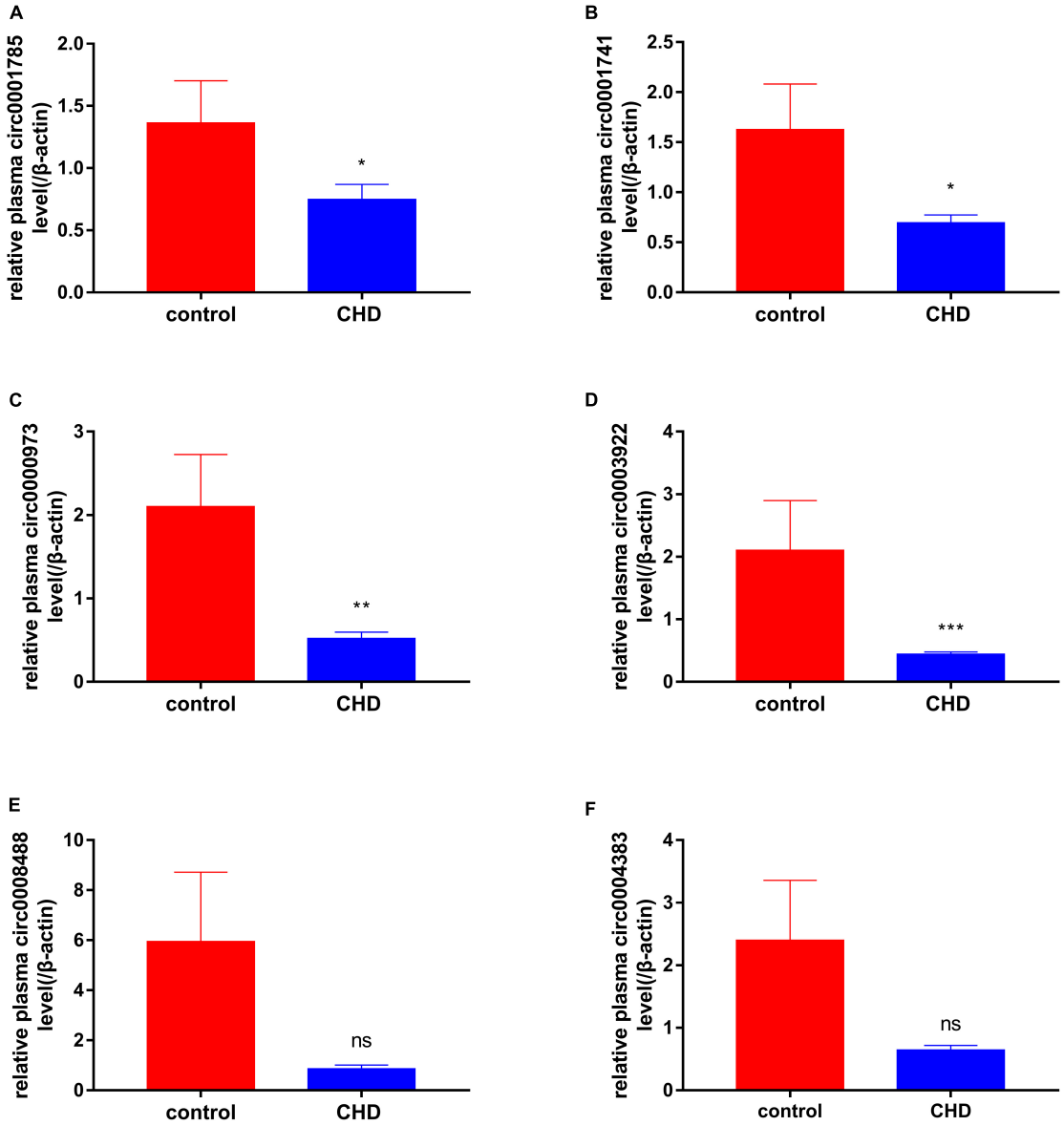

FIGURE 7
qRT-PCR results of peripheral blood-derived circRNA. The expressions of peripheral blood-derived circRNAs were further confirmed using qRT-PCR. **(A)** Represents the expression of circ0001785 in white blood cells of healthy and coronary patients, **(B)** represents the expression of circ0001741 in white blood cells of healthy and coronary patients, **(C)** represents the expression of circ0000973 in white blood cells of healthy and coronary patients, **(D)** represents the expression of circ0003922 in white blood cells of healthy and coronary patients, **(E)** represents the expression of circ0008488 in white blood cells of healthy and coronary patients, and **(F)** represents the expression of circ0004383 in white blood cells of healthy and coronary patients. Red represents healthy control group, blue represents coronary heart disease (CHD) group, $P < 0.05$ was statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

the combination of circ0001785 and miR513a-5p and reduced the combination of miR513a5p and TGFBR3, thus leading to the increased expression of TGFBR3. Similarly, the increased circ0000973 promoted the expression of LFNG by competitively combining with miR-330-3p and miR-548p. The increased circ0001741 that is prone to combine with miR-548p caused the increased expression of LFNG. Also, the increased circ0003922 promoted the expression of TGFBR3 or LFNG by competitively combining with miR-513a-5p. Studies have shown that up-regulated circ0001785 expression in osteosarcoma cells can enhance its carcinogenic effect by up-regulating HoxB2 by sponge miR-1200 (36). Circular RNA hsa_circ_0001785 inhibits the proliferation, migration, and invasion of breast cancer cells *in vitro* and *in vivo* by sponging miR-942 to up-regulate SOCS3 (37). miR-125a-5p can induce the release of gastrin from vascular endothelial cells and thus affect gastrointestinal function (38). miR-513a-5p can mediate TNF-α and LPS-induced apoptosis by down-regulating X inhibitors of apoptosis proteins in endothelial cells (39). TGFBR3 signal can conduct and regulate apoptosis of myocardial cells after infarction. In addition, TGFBR3 signaling is a potential negative regulator that can protect myocardial cell-induced apoptosis (40). Because of the role of these genes in CHD, we confirm that exosome-derived circRNAs can serve as a stable biomarker for diagnosing plaque stability in patients with CHD in future clinical applications.

There are some limitation in this study. First, we did not compare microRNA and mRNA expression in the plasma of

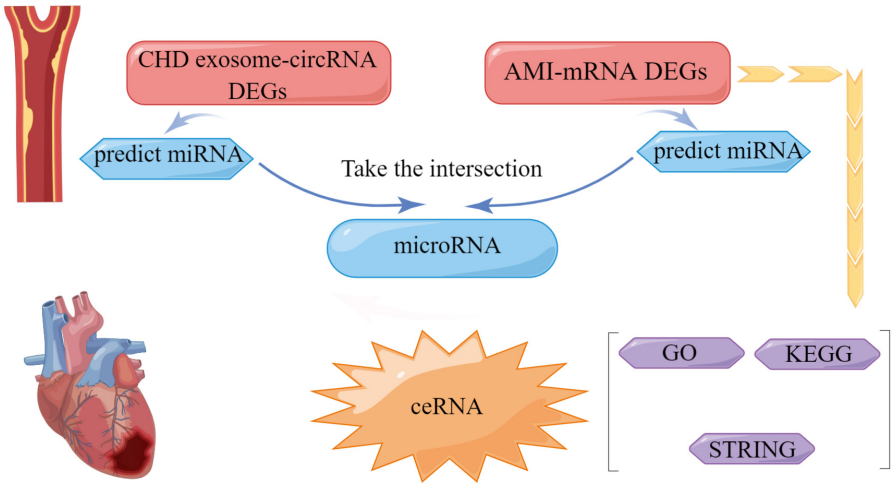

FIGURE 8
Flow chart of coronary heart disease (CHD).

patients. This will be further verified in our subsequent experiments. Second, we used bioinformatic methods to infer that exosome-derived circRNA0001785, circRNA0000973, circRNA0001741, and circRNA0003922 can predict the possibility of myocardial infarction in patients with CHD, and these results need to be verified in large-scale studies. Third, according to the current results, the expression of these circRNAs are all at a lower diagnostic level in the ROC curve, but their specificity is higher and their expression is more stable in humans, also its combined ROC curve has a high diagnostic efficiency, so we believe that they are still of great clinical importance.

In conclusion, we found that circRNA0001785, circRNA0000973, circRNA0001741, and circRNA0003922 are potential biomarkers for the diagnosis of CHD, and can affect the stability of plaque by down-regulating these genes. This study provides new insight for studying pathogenesis and prevention strategies of acute myocardial infarction in patients with CHD.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61144.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements.

Author contributions

XT: conceived and designed the study, conducted the experiments, and analyzed the data. XZ and XD: interpreted the results and prepared the charts. YK and JK: drafted the manuscript and edited it. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1070616/full#supplementary-material

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Case report: An elderly woman with recurrent syncope after pacemaker implantation

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Syncope caused by atrioventricular block may occur as a result of a cardiac vasodepressor reflex. This article reports on a case of recurrent syncope in an 80-year-old woman with high-grade atrioventricular block, documented by electrocardiographic monitoring after pacemaker implantation. Pacemaker testing revealed stable impedance and sensing but a clear increase in the ventricular capture threshold at outputs. This case is unusual because the patient's primary diagnosis was non-cardiac. However, a combination of high D-dimer, hypoxemia, and computerized tomography scan of the pulmonary artery confirmed the diagnosis of pulmonary embolism (PE). With 1 month of anticoagulant therapy, the ventricular capture threshold gradually dropped to the normal range and syncope resolved. This is the first report of an electrophysiological phenomenon detected by pacemaker testing in a patient with syncope arising from PE.

KEYWORDS

permanent pacemaker implantation, loss of capture, pulmonary embolism, syncope, electrophysiology phenomenon

Introduction

The clinical picture of pulmonary embolism (PE) is variable, and it remains challenging to diagnose and a frequent cause of death. Close attention should be paid to the symptom of syncope, although data show that PE is identified in <1% of patients with syncope and in <3% of hospitalized patients with syncope (1, 2). Bradycardia-induced syncope during PE is rarely reported, and the underlying mechanism is not well-understood. Some studies suggest that PE can trigger the vasovagal reflex (3, 4), which leads to bradycardia. However, no studies have yet confirmed the changes in electrophysiology induced by the vagal response in PE, and investigation of the relationship between electrocardiographic (ECG) abnormalities and electrophysiology under the purview of pacemaker research has been limited. The purpose of the present case study was to further assess the relationship between acute PE and cardiac electrical signals.

Case presentation

A patient in her 80's with a history of pacemaker implantation presented with recurrent syncope for the past 2 months. She denied having chest pain, palpitations, or shortness of breath before the onset of syncope. She exhibited Mobitz II atrioventricular (AV) block and underwent a dual-chamber pacemaker implantation (St. Jude Medical Zephyr XL DR 5826) 3 years ago. She had a history of hypertension and diabetes for the past 5 years and

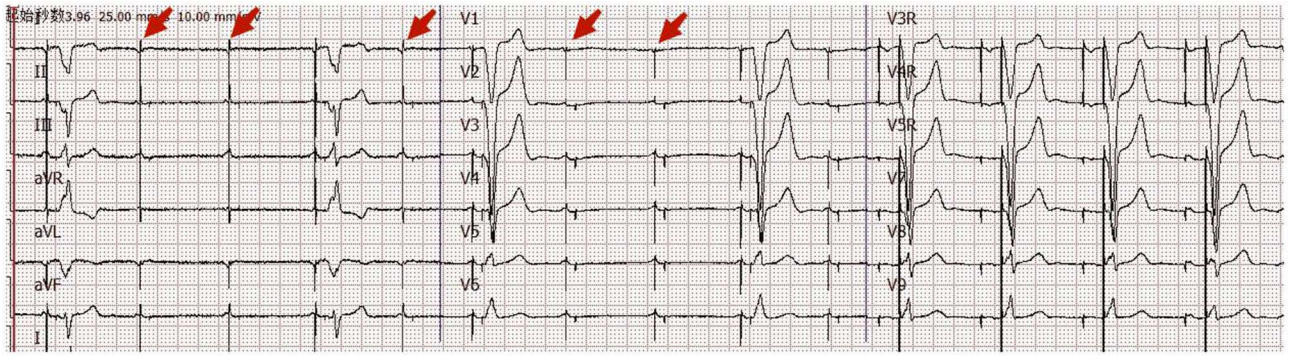

FIGURE 1
Eighteen-channel surface ECG. ECG on admission showed transient loss of ventricular pacing capture.

was treated in the outpatient clinic. On initial evaluation, her blood pressure was 170/68 mmHg, heart rate 70 beats per minute, and oxygen saturation 92% on room air. Physical examination, including examination of the heart and lungs and a neurological examination, was normal. There were no recent changes in medication. She experienced recurrent syncope during the loss of ventricular pacing capture. ECG (Figure 1) showed a transient loss of ventricular pacing capture. At the last follow-up visit, pacing outputs were set at A: 2.0V, V: 2.2 V, with a programmed atrioventricular (AV) delay of 200 ms (DDD). Pacemaker testing revealed both stable impedance and stable sensing in the atria and ventricles (Figure 2A) and a normal atrial output threshold (0.5 to 0.75 volts under a 0.4-ms pulse width) but a clear increase in ventricular capture threshold at outputs 2 months previously (Figure 2B). The period of threshold elevation was consistent with the reported syncopal episodes. However, an anteroposterior chest x-ray showed correct atrial and ventricular pacing lead positions (Figures 3A, B). The patient had also undergone craniocerebral CT examination before admission, which revealed no brain-related diseases.

What are the possible causes of the change in the ventricular capture threshold? We adjusted the pulse width and improved the output power of the pacemaker to ensure normal pacing. The results of initial testing showed normal cardiac enzymes and electrolytes but markedly high D-dimer levels (4.41 mg/L) and hypoxemia (PaO_2 62 mmHg). Therefore, a computerized tomography (CT) scan of the pulmonary artery was performed in view of the presenting symptoms; this showed PE in the right lower pulmonary arterial branch (Figure 4A). Further testing for hypercoagulable disorders, autoimmune diseases, tumors, and deep vein thrombosis revealed no abnormalities. An echocardiogram revealed ventricular septal hypertrophy (12 mm) and a mild elevation in pulmonary arterial systolic pressure (35 mmHg). Furthermore, a single-photon emission computed tomography (99m-Tc -pyrophosphate nuclear scintigraphy) was performed to rule out cardiomyopathy, and the results were normal. Subsequently, following treatment with anticoagulation and adjustments to the pacing output, the patient was discharged without symptoms after 10 days. After 1 month, the ventricular capture threshold gradually fell back to the normal range; a reexamination CT scan of the pulmonary artery indicated no evidence of PE (Figure 4B), and arterial oxygen pressure (PaO_2 82 mmHg) and D-dimer levels (0.2 mg/L) were normal. Figures 4C, D depict the positions of the right ventricular and atrial leads, respectively, from a posteroanterior view, at the time of admission and 1 month later. The patient was well for 10 months, with no evidence of any recurrence of syncope.

Discussion

Bradycardia-induced syncope in cases of PE is rarely reported, and the mechanism underlying this phenomenon remains poorly understood. This report presents an unusual case of PE characterized by symptomatic bradycardia and transient loss of ventricular pacing capture. Despite the absence of chest pain or shortness of breath, the patient suffered from recurrent syncope due to bradycardia. In one series of angiographically confirmed cases of PE, it was discovered that 13% of the patients experienced an episode of syncope (5, 6). Of these, more than 80% had a massive embolism, defined as an obstruction of 50% of the pulmonary circulation. Acute right ventricular failure in these patients impairs left ventricular filling, and the ensuing hypotension causes syncope. However, in our case, the patient did not have a massive embolism, her blood pressure was normal, and there were no malignant arrhythmias. Furthermore, the patient had not started any new medications since late December 2021, making it unlikely that syncope was related to any of these factors.

The common causes of an increase in pacing threshold include acute myocardial ischemia, hypoxia, hyperkalemia, and electrode-related problems, such as electrode dislocation, perforation, and fracture. However, these causes were ruled out in this case. Large cohort clinical studies have shown that sinus bradycardia and first-degree AV block may occur in over 2 and 3.5% of patients with PE, respectively (7). Some studies suggest that PE induces bradycardia, as a result of triggering a vasovagal reflex (3, 4). Patients with PE are in a state of high adrenergic energy, which stimulates the mechanoreceptors in the inferior ventricle to reduce sympathetic tone and increase parasympathetic excitability. In

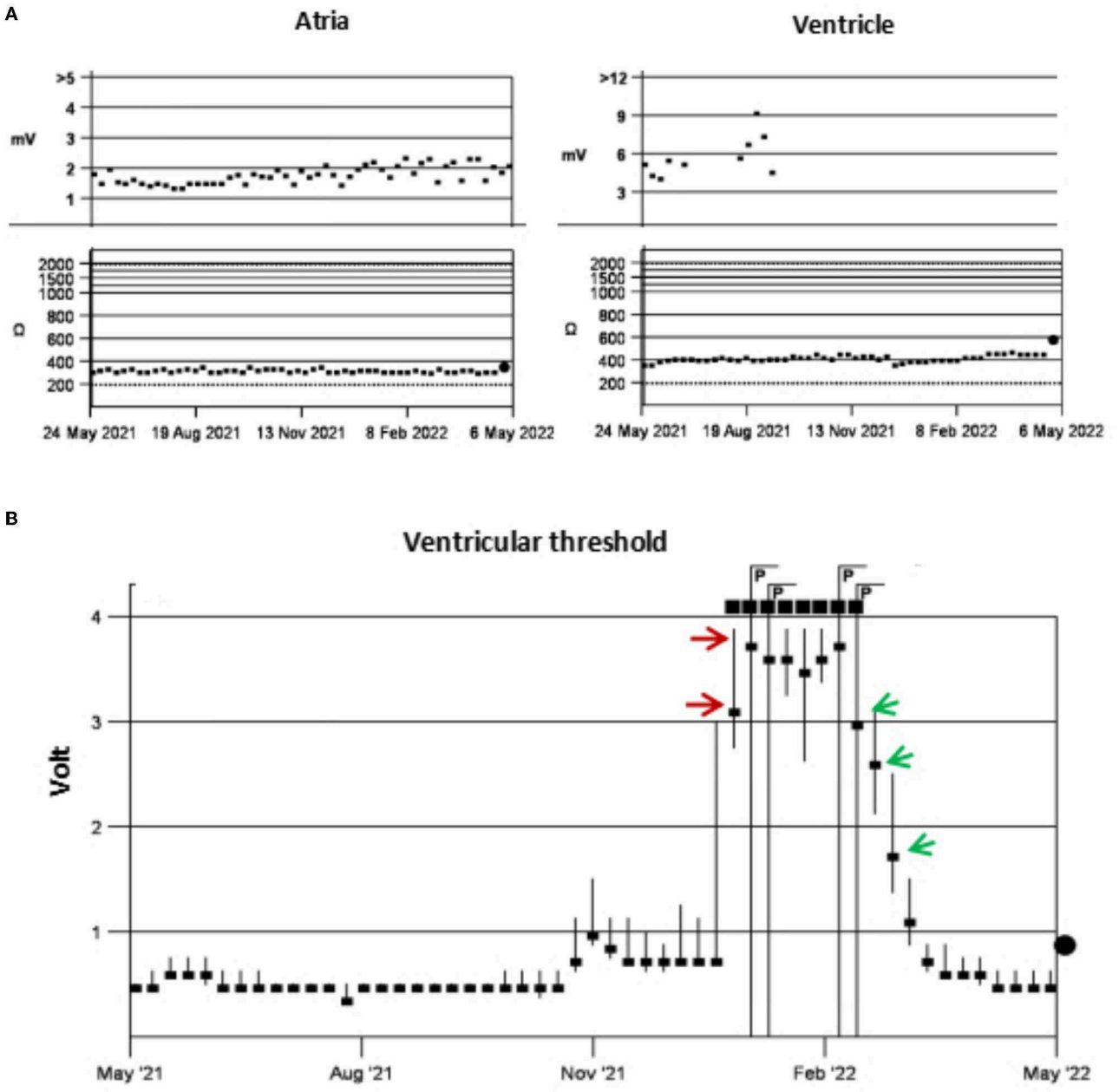

FIGURE 2
Pacing test. Testing showed stable impedance and sensing in the atria and ventricle, with no ventricular sensing data after October 2021 **(A)**. There was a clear increase in the ventricular capture threshold in early January 2022 (red arrows) and this gradually decreased to normal after 1 month of anticoagulant therapy (green arrows) **(B)**.

addition, patients with PE have been found to exhibit conduction block due to neurohumoral mechanisms that regulate pulmonary artery pressure and increase ventricular pressure (8). Bradycardia after PE may occur if a defect is present in the sympathetic reflex or the right atrial pressure receptors or if their function is inadequate to compensate for the elevated parasympathetic activity (9, 10).

Furthermore, experimental data have shown that adenosine 5′-triphiosphate (ATP) could also play a role in bradycardia and syncope in a subset of patients with PE (11). The proposed mechanism involves the activation of platelets in the lungs, which leads to the localized release of ATP, followed by ATP-triggered pulmonary–pulmonary and cardio-cardiac vagal reflexes. The presence of bradycardia indicates that the block is caused by the influence of increased parasympathetic activity on the sinoatrial (SA) and atrioventricular (AV) nodes (12). Further animal studies are needed to confirm the potential role of PE in increasing the pacing threshold.

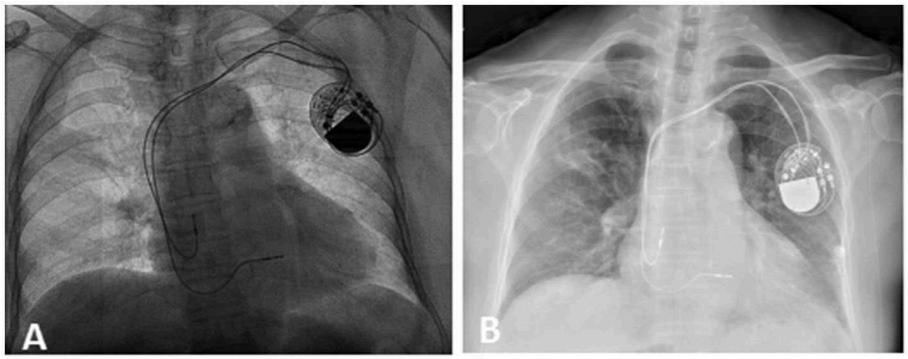

FIGURE 3
Chest x-ray. X-ray after the first pacemaker operation on September 12, 2019 **(A)** and on February 16, 2022 **(B)**. This showed correct lead position on admission.

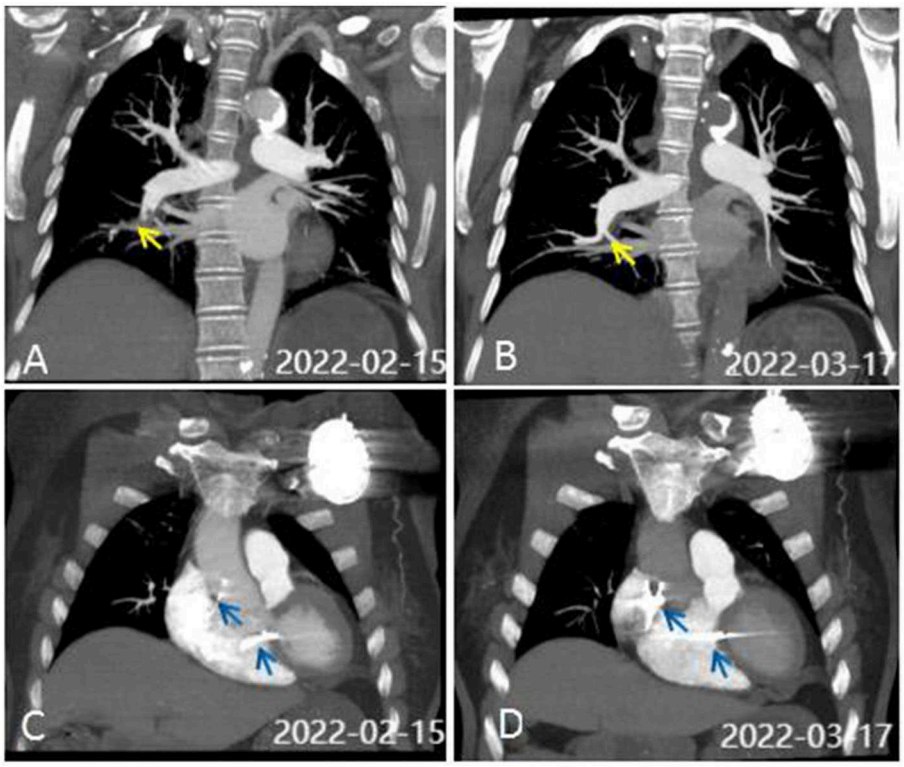

FIGURE 4
CT scan of the pulmonary artery. **(A)** CTA on admission showed pulmonary embolism (PE) in the right lower pulmonary arterial branch. Perfusion defects are identified by yellow arrows. **(B)** CTA was normal after 1 month of anticoagulant therapy. **(C, D)** show the positions of the RV and atrial leads (blue arrows) on PA view on admission **(C)** and 1 month later **(D)**.

Conclusion

PE presenting with bradycardia is rare and is difficult to diagnose. Therefore, physicians must be vigilant with patients who exhibit syncope, especially those with syncope who have an implanted pacemaker, as this symptom may be a "forgotten sign" of life-threatening PE. Missing a diagnosis of PE can increase patient morbidity and mortality. Early diagnosis and appropriate treatment are required to improve clinical outcomes in patients with PE. This case report highlights the correlation between PE and the activation of the parasympathetic nervous system, which raises the cardiac pacing threshold in the SA or VA node. The pacemaker parameters in this patient revealed this electrophysiological phenomenon, supporting previous hypotheses.

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 authors.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YS, LF, XH, and BH: original draft preparation. YS and YY: review and editing. All authors have read and agreed to the published version of the manuscript.

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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, necroptosis and cuproptosis: Novel forms of regulated cell death in diabetic cardiomyopathy

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Diabetes is a common chronic metabolic disease, and its incidence continues to increase year after year. Diabetic patients mainly die from various complications, with the most common being diabetic cardiomyopathy. However, the detection rate of diabetic cardiomyopathy is low in clinical practice, and targeted treatment is lacking. Recently, a large number of studies have confirmed that myocardial cell death in diabetic cardiomyopathy involves pyroptosis, apoptosis, necrosis, ferroptosis, necroptosis, cuproptosis, cellular burial, and other processes. Most importantly, numerous animal studies have shown that the onset and progression of diabetic cardiomyopathy can be mitigated by inhibiting these regulatory cell death processes, such as by utilizing inhibitors, chelators, or genetic manipulation. Therefore, we review the role of ferroptosis, necroptosis, and cuproptosis, three novel forms of cell death in diabetic cardiomyopathy, searching for possible targets, and analyzing the corresponding therapeutic approaches to these targets.

KEYWORDS
diabetic cardiomyopathy, ferroptosis, necroptosis, cuproptosis, regulatory cell death

1. Introduction

In 2021, worldwide, the number of adults with diabetes reached 537 million (10.5%), with more than one-in-ten adults affected by the disease. The number of people in 2021 is 7.4 million more than that in 2019, an increase of 16%, which highlights the prevalence of diabetes worldwide. The international diabetes federation (IDF) estimates that this number will reach 783 million by 2045, a 46% increase that is more than double the projected population growth of 20% over the same period, with one-in-eight adults likely to have the disease (1).

Diabetic cardiomyopathy (DCM), a disease common to most diabetic patients, is based on changes in the cardiac structure and systolic and diastolic functions. When patients have cardiac insufficiency, other clear causes, such as hypertension and structural or ischemic heart disease can be diagnosed (2). However, due to limits in awareness and a lack of diagnostic means, the current clinical diagnosis rate of diabetic cardiomyopathy is low, and the corresponding targeted clinical intervention is insufficient.

In general, the intervention involves conventional sugar control measures such as diet and hypoglycemic drugs, in the early stage. When the disease progresses to the stage of heart failure, the corresponding treatment is given according to the current guidelines for

heart failure (3). However, the pathogenesis of diabetic cardiomyopathy is different from that of ordinary cardiomyopathy, which involves various factors, such as abnormal glucose and lipid metabolism, calcium balance disorder, etc. Therefore, treatment by ordinary means may lead to a poor prognosis (4).

In fact, the major risk factors for the development of diabetic cardiomyopathy are the progressive death of cardiomyocytes, changes in endothelial cells (5), and cardiomyocytes (6) in the high glucose environment. As early as 15 years ago, it was confirmed that myocardial apoptosis in patients with diabetic cardiomyopathy was 85 times more than that in the control group based on clinical biopsies (7). As a recognized form of cell death in diabetic cardiomyopathy, apoptosis has been studied extensively and intensively. However, the pathophysiological and clinical significance of this mode of cell death is inconclusive. Therefore, expanding the study of death modalities beyond apoptosis and pyroptosis will help pinpoint the target of cell death to alleviate or even cure the onset of diabetic cardiomyopathy. Recently, there have been numerous studies on regulatory cell death. More forms of death have been discovered, such as pyroptosis, immunogenic cell death, ferroptosis, necroptosis, cuproptosis, exocytosis, etc. These various forms of cell death have been proven to play a role in many diseases. From these studies, we will mainly discuss ferroptosis, necroptosis, and cuproptosis, three relatively newly identified forms of cell death. We will search for possible targets of these three forms in diabetic cardiomyopathy and analyze the corresponding therapeutic approaches according to these targets.

2. Ferroptosis, necroptosis, and cuproptosis in DCM

Much attention has been paid to the mechanisms related to diabetes. In recent years, oxidative stress, inflammation, and Ca^{2+}-related dysfunction have been identified, as well as changes in substrate metabolism, insulin signal transduction, gene regulation, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, neurohumoral activation, and cardiac cell death (8, 9). Oxidative stress is the production of excessive reactive oxygen species (ROS). In addition to the increased ROS production in diabetic patients, the endogenous antioxidant mechanism of diabetic patients is often impaired (10, 11). In diabetic animal models, intervention with appropriate antioxidants, such as superoxide dismutase (SOD) mimics or coenzyme Q10, has determined the relationship between oxidative stress and diabetic cardiomyopathy, whether as an early preventive measure to limit the progression of diabetic cardiomyopathy or after the heart has been damaged (12, 13). In addition to the direct oxidative damage caused by inappropriate cardiac ROS levels, ROS is also a key trigger for the activation of inflammatory bodies (10, 14). ROS activates a variety of other pathological signaling cascades, such as the protein kinase C (PKC), apoptosis signal-regulated kinase-1 (ASK-1), p38 mitogen-activated protein kinase (p38-MAPK), NH2 terminal JUN kinase (JUNK), and JAK-STAT pathways (8, 15–18). These signals themselves are also related to diabetes-induced cardiac complications (19, 20). Some of these signals, such as PKC, p38-MAPK, and JAK-STAT, can also induce the generation of ROS, thus forming a harmful feedforward loop. Therefore, we believe that diabetic cardiomyocytes are in a high glucose microenvironment for a long time, resulting in the activation of a variety of signaling pathways, that causes cell death. Cell death is a normal cellular phenomenon. Since the ultrastructural characteristics of programmed cell suicide were determined in 1972 (21), programmed cell death with cell apoptosis as the "representative name" has gradually come into the view of scientists, and related research has been a hot spot in the field of life science. A large number of new cells are generated every day along with the death of old cells. The normal development and maintenance of the homeostasis of living organisms as well as

TABLE 1 Comparison of ferroptosis, necroptosis and cuproptosis.

	Ferroptosis	Necroptosis	Cuproptosis
Definition	Iron dependent Different from apoptosis, necrosis and pyroptosis Novel ways of programmed cell death	The death of local tissue cells *in vivo* characterized by changes in enzymatic solubility	Depending on the accumulation of copper in the cell, copper directly bind to the fatty acylated components in the TCA cycle, resulting in the aggregation and imbalance of these proteins, blocking the TCA cycle, triggering protein toxic stress and inducing cell death
Nucleus	Not much has changed	Perinuclear spatial focal expansion	Not much has changed
Chromatin	No chromatin concentration	Condensation of chromatin	No chromatin concentration
Morphological characteristics	Mitochondrial volume decreased, the density of bilayer membrane increased, mitochondrial crest decreased or disappeared, and mitochondrial outer membrane ruptured	Cells and organelles swell, chromatin moderately concentrates, cell membranes rupture, and cell components overflow	No morphological features
Biochemical characteristics	Iron accumulation Lipid peroxidation	ATP levels go down RIP1, RIP3 and MLKL are activated	Copper accumulation
Immune characteristics	Proinflammatory	Proinflammatory	Proinflammatory
Cellular action	Regulates tumor cell growth and cell death	Virus defense mechanism	Cell death targeting the TCA cycle
Precipitating factors	Iron accumulation	Caspase8 inhibited	Copper accumulation
Key indicators	Fe, GSH, MDA, GPX4, ROS, LPO	RIPK1, RIPK3, MLKL	Cu, FDX1, DLAT, LIAS, Pyruvate acid, 2-Ketoglutaricacid, HSP70
Reference	(22, 23)	(24, 25)	(26, 27)

the elimination of damaged, senescent, and infected cells all depend on a closely regulated programmed cell death. With the deepening of research, it was found that apoptosis is not the only form of cell death. According to the different mechanisms of cell death, regulated cell death can be divided into apoptosis, pyroptosis, ferroptosis, necroptosis, cuproptosis, etc. The purpose of this review is to describe the frontier research of ferroptosis, necroptosis, and cuproptosis in DCM, to sort out the research results and current situation, to point out areas for further exploration and possible research directions, and to provide some reference for future research. Ferroptosis, necroptosis, and cuproptosis differ from other forms of cell death based on morphology, biochemistry and immune status (**Table 1**).

2.1. Ferroptosis

Ferroptosis, named in 2012, is a new cell death mode and has become a focus of recent research. It is iron-dependent regulatory cell death that is different from other programmed cell death and is characterized by lipid peroxidation and iron overload (28). Since the concept was first proposed a decade ago, we have seen an explosion of research in this field, with more than 5,000 papers on ferroptosis. As a form of regulatory death, ferroptosis is significantly different from classical regulatory cell death, such as pyroptosis, especially in metabolic heart disease (29–31). Therefore, studying the mechanism of ferroptosis may reveal targets to control the progression of DCM and reduce mortality.

Currently, it is believed that the accumulation of phospholipid high oxides (PLOOHs) directly leads to ferroptosis (22). Spontaneous lipid peroxidation is initiated by ROS, and intracellular soluble ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals. Among these, hydroxyl radicals are the most active and toxic ROS (32), which can rob the hydrogen atoms of phosphorylated PUFAs and form peroxides (PUFA-PL-OOH), directly leading to the death of iron. We will describe this process by splitting and tracing.

2.1.1. Mechanism

2.1.1.1. Source: PUFA-PL

In human cells, Acetyl-CoA is a global currency that mediates carbon trading among the metabolic pathways, including glycolysis, the tricarboxylic acid cycle, amino acid metabolism, gluconeogenesis, and fatty acid synthesis (33). Acetyl-CoA in the mitochondria is catalyzed by ACC to produce unsaturated fatty acid PUFA. In addition, dietary habits also affect the content of free unsaturated fatty acids (34). However, PUFA cannot directly cause ferroptosis (35), which needs to be further catalyzed by ACSL family members into phosphorylated PUFA (PUFA-PL), or lipid metabolism that also produces a small amount of PUFA-PL (36). PUFA-PL then enters the cytoplasm as a prelude to ferroptosis. Starting from the initial Acetyl-CoA, energy stress activates AMPK, directly inhibits ACC, and restricts the synthesis of PUFA, thus inhibiting ferroptosis (37). Correspondingly, proteins that inhibit ACSLs can also inhibit ferroptosis, such as E-cadherin, which plays a role through the Hippo-Yap pathway (38).

2.1.1.2. Poison: hydroxyl free radicals

ROS include a superoxide anion ($O^{2\cdot-}$), H_2O_2, and hydroxyl radical (HO·), which are derived from the four-electron reduction reaction of oxygen in the mitochondrial electron transport chain (39). If oxygen is reduced by only one electron in this reduction process, superoxide will be produced (40). Then, superoxide is reduced to a low-reactive substance hydrogen peroxide under the action of SOD (39). Iron bivalent in cells is an active REDOX metal, which is involved in the formation of free atomic groups and the expansion of lipid peroxidation, which is one of the most destructive effects of iron-catalyzing Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow {+}\bullet OH + OH^- + Fe^{3+}$ producing highly toxic hydroxyl radicals (41–43). Abnormal uptake, excretion, and storage of iron can lead to increased intracellular free iron and Fenton reactions (44).

2.1.1.3. Key: iron

Ferric ions transported by the Tf/TfR1 protein and required by the iron uptake pathway cannot directly participate in the Fenton reaction. Ferric ions are released from Tf and become ferric bivalent after acidification from endocytosis (45). Then, ferric bivalent enters the cytoplasm by endocytosis (46), a process mainly due to ESCRT1 on the membrane (47). Therefore, both Tf/TfR1 and ESCRT are ferroptosis-promoting proteins. The role of iron is not only to promote a Fenton reaction but also to use iron as a cofactor for some key enzymes. These include arachidonic lipoxygenase (ALOX), which triggers the formation of lipid hydroperoxides that are substrates for Fenton reactions. ALOX is also involved in the peroxidation of PUFA-PL (48), and 12-lipoxygenase is required for p53-dependent ferroptosis (49). Cytochrome P450 REDOX reductase (POR) also contributes to lipid peroxidation during ferroptosis (50), suggesting that several enzymes using iron as cofactors can promote lipid peroxidation leading to ferroptosis. Of course, iron is an essential trace element in the human body (51), and the emergence of iron does not mean the death of iron. A part of the iron bivalent will be transformed into iron trivalent and stored in ferritin, and another part of it can become a polyvesicular body containing iron (MVB) mediated through a prominin-2 (prom2) solution (52). The balance of iron homeostasis depends on the expression levels and activities of ferri-carriers, ferri-transporters, and ferri-regulatory and storage proteins (53). The sensitivity of cells to ferroptosis can be regulated by controlling the level of ferritin through iron pyroptosis and further regulating the abundance of free iron because the abundance of ferritin determines the size of the labile iron pool (54).

2.1.1.4. Detoxification: GPX4、DHODH、FSP1

Similar to the regulation of iron, the presence of PUFA-PL-OOH in cells does not directly lead to ferroptosis. In fact, PUFA-PL-OOH can accumulate under the action of iron bivalent, resulting in cell death through damage to cell membranes or the production of lipid-derived electrophilic small molecules (55). This accumulation process can be inhibited by CoQ10, NADPH, and BH4 (56). In addition to the inhibitory process, there is a major

antioxidant defense mechanism: GPX4. GPX4, glutathione peroxidase, is a GSH-dependent enzyme that converts reduced GSH to oxidized GSH and reduces PUFA-PL-OOH to non-toxic PUFA-PL-OH (57). Based on the dependence on GPX4, GSH depletion leads to the inactivation of GPX4, causing lipid peroxide accumulation to trigger ferroptosis. GSH is an important antioxidant in cells, so it is critical to find out where GSH comes from. System Xc- on the cell membrane is an amino acid transporter that can transport extracellular cystine into the cell and intracellular glutamate into the cell, and then the intracellular cystine is reduced to cysteine, which is the rate-limiting precursor of GSH synthesis (58). GSH then serves as a cofactor for GPX4, which acts as a lipid hydroperoxidase to reduce lipid peroxides (PUFA-OOH) to the non-toxic PUFA-OH (28, 59). Therefore, GPX4 is one of the important defense mechanisms for the cellular detoxification of lipid peroxides. Normal GPX4 activity is essential for maintaining membrane lipid homeostasis, preventing the excessive accumulation of toxic lipid peroxides and the formation of free radicals (L-OOH and L-O•), thereby reducing ferroptosis (60, 61) Acyl-CoA synthetase long-chain family member 4 (ACSL4) family is an important gene promoting ferroptosis, but the sensitivity of cancer cells to GPX4 inhibitors varies depending on the cancer type, which indicates that there may be another resistance mechanism similar to GPX4-FSP1-CoQ10-NAD(*P*)H pathway (62). Ferroptosis suppressor protein 1 (FSP1), located on human chromosome 10q22.1, mediates p53-independent apoptosis (63). FSP1 was originally called apoptosis-inducing factor mitochondrial-related gene 2 (AIFM2). It specifically mediates cell death because it is caspase-independent (64). Recently, it was revealed that Ferroptosis is mediated by ubiquinone (CoQ10) (63, 65). FSP1 is enriched in the cell membrane by myristoylation. As an NAD(*P*)H-dependent CoQ10 oxidoreductase, NAD(*P*)H can be used to reduce the oxidized ubiquinone. It is known that the reduced form of ubiquinone can capture the free radicals that mediate lipid peroxidation and limit the occurrence of lipid peroxidation. Thus, FSP1 can inhibit the occurrence of ferroptosis (62). All in all, the FSP1-CoQ10-NAD(*P*) H axis may play an important role in the occurrence of ferroptosis caused by lipid peroxidation.

In a study conducted by Mao C, the effect of a GPX4 inhibitor on ferroptosis in tumor cells was reversed by dihydroorotate dehydrogenase (DHODH), and this reversal was more significant in tumor cells with a low expression of GPX4, suggesting that DHODH may be the third mechanism of inhibiting ferroptosis (66). The anti-ferroptosis mechanism of DHODH is different from that of FSP1. According to research, the function of FSP1 is mainly limited to the cell membrane, and it is not clear whether it affects the mitochondrial membrane. DHODH mainly plays a role in the mitochondria. In the process of mitochondrial pyrimidine synthesis, DHODH oxidizes FMNH2 to FMN and reduces CoQ to CoQ10, thus inhibiting ferroptosis through CoQ10 capture and the scavenging of lipid peroxides (66). In addition, it has been reported that benzene-induced inflammatory anemia is associated with ferroptosis caused by the IRP1-DHODH-ALOX12 axis (67). Sorafenib is a commonly used drug in liver cancer, and its anti-tumor effect is closely related to ferroptosis. However, in recent years, the enrichment of DHODH has been found in some cells and tissues resistant to sorafenib and is related to the poor prognosis of HCC patients (68). The role of DHODH was also found in the occurrence of cervical cancer. In summary, DHODH-mediated ferroptosis in cancer is expected to become a new target for tumor therapy (66), and a nano-platform based on DHODH inhibitors has been reported to block the redox system (69) (**Figure 1**).

2.1.2. Ferroptosis and DCM

As mentioned earlier, the system Xc-/GSH/GPX4 axis is an important pathway for ferroptosis. Many drugs function in the system Xc-/GSH/GPX4 axis, such as the classic Erastin, when ferroptosis was originally associated with this compound. Erastin inactivates GPX4 by inhibiting the system Xc- and reducing the production of GSH, promoting ferroptosis (70–72). Skipping the system Xc-, reagents that promote ferroptosis through the depletion of GSH, including acetaminophen, etc., are commonly used in clinics (73). In addition, ML162 (74) and DPI compounds (75) can also directly inhibit GPX4 and promote ferroptosis. As one of the forms of cell death, ferroptosis is involved in diabetic cardiomyopathy.

The accumulation of advanced glycation end products (AGEs) caused by hyperglycemia increases lipid peroxidation and the ferroptosis of cardiomyocytes (44), which then leads to cardiac inflammation and remodeling (cardiomyocyte hypertrophy, pro-fibrotic response and fibrosis), and ultimately leads to cardiac dysfunction, namely DCM (76). DCM inhibits the expression of SLC7A11 (system Xc- gene) and ferritin, thereby reducing GSH levels and increasing active iron levels, leading to mitochondrial oxidative damage (77). SFN plays a shielding role by activating AMPK to stimulate the downstream expression of SLC7A11 and ferritin, and at the same time, SFN enhances the AMPK dependence of the heart in age-induced ECT dysfunction and diabetic heart remodeling and dysfunction (78). These results suggest that the selective removal of excess iron inhibits ferroptosis, has a protective effect against chronic oxidative stress and that a modest reduction in plasma iron levels may be a viable treatment for the prevention of DCM in diabetic patients. From a clinical point of view, studies can test the combination of SFN with low-dose iron chelating agents, and further explore more targets of iron in the mechanism of ferroptosis depending on the effect and dose of the drug.

Increasing evidence shows that ferroptosis is involved in the onset and progression of DCM, and ferroptosis also affects the pathological consequences of cardiac function (79–82). These findings identify ferroptosis as a promising cellular target for correcting heart function in patients with DCM and possibly also for delaying the degenerative progression of DCM. However, the pathophysiological role of ferroptosis is not fully understood. Because the concept of ferroptosis is only ten years old and the data are limited, the current review does not provide any further insight into the mechanisms associated with ferroptosis and DCM, leaving many questions unanswered. For example, what role does ferroptosis play in the development of DCM, and does it play the same role at different stages in the development of

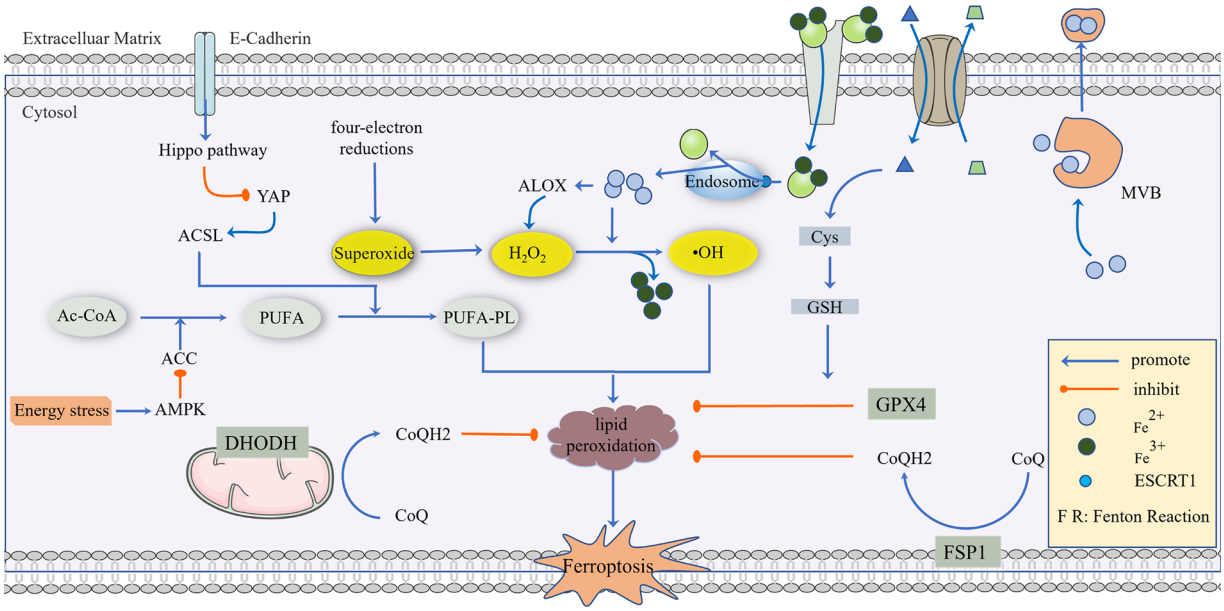

FIGURE 1
The regulatory mechanism of ferroptosis. AC-CoA in the mitochondria catalyzes the formation of PUFA under the action of ACC, which is then phosphorylated to generate PUFA-PL by the ACSL family and transferred into the cytoplasm to mediate the next stage of ferroptosis, which can be inhibited by energy stress and E-Cadherin, respectively. Superoxide produced in a mitochondrial four-electron reaction is reduced to H_2O_2 under the action of SOD, and a Fenton Reaction with the participation of Fe^{2+} produces highly toxic •OH, which mediates PUFA-PL peroxidation. This process can be inhibited by GPX4, CoQ10, NADPH, and BH4. GPX4 is the main antioxidant defense substance, and its inhibition of protein lipidation requires the participation of GSH. Cells transport Fe^{3+} *via* the Tf/TfR1 protein, which enters into the body through ESCRT1 on the endosome and is reduced to Fe^{2+}. As a reducing agent, Fe^{2+} participates in a Fenton Reaction or as a cofactor of some key enzymes (such as ALOX), and part of Fe^{2+} can be excreted to the extracellular space *via* MVB. System Xc- can reverse the transport of Glu out of the cell membrane and Cys-Cys into the cell membrane, and Cys-Cys is further converted into Cys as a rate-limiting precursor of GSH synthesis. When excess lipid peroxides accumulate in cells, this damages to the cell membrane and eventually leads to ferroptosis.

DCM? In addition, all current treatments for diabetic patients are based on the premise of controlling blood sugar levels. However, the effect of controlling blood sugar levels on ferroptosis has not been determined. Whether ferroptosis affects other tissue cells in the presence of DCM has not been studied, and clearly these questions need to be further explored. Elucidating the mechanism of ferroptosis in DCM will help us develop better strategies to precisely target ferroptosis in order to effectively alleviate DCM and prevent its progression to more severe conditions. However, no such studies have been done in the literature to date, and this is a very valuable conclusion and recommendation. But up to now, no such studies have been conducted. Thus, this is a rather valuable field waiting to be explored.

2.2. Necroptosis

Initially, necroptosis was not considered a new form of cell death. It was morphologically similar to necrosis and shared an upstream pathway with apoptosis. Later, researchers gradually distinguished the differences between them, which led to the emergence of necroptosis (83). As a well-known mode of cell death, necrosis has long been found to be an unregulated form of cell death, caused by external physical and chemical stress, and a subsequent reaction of injury to the body (84). In contrast, necroptosis is highly regulated and serves as a defense mechanism or escape route for cells facing viral infection (85). When a viral caspase inhibitor is present, the cell can only choose to commit suicide in a way that is not caspase-dependent. The two cell death pathways, necroptosis and apoptosis, share some upstream signaling elements and ultimately lead to plasma membrane rupture. Thus, at first there was no distinction between the two forms of regulatory death, but as research progressed, it was revealed that there was a distinct difference in the cellular morphology of each process. Necroptosis is characterized by increased cell size, organelle swelling, and membrane perforation, followed by cell disintegration, the release of contents, initiation of innate and adaptive immune responses, and clearance of necrotic cells through giant cytostome (86). Meanwhile, apoptosis is characterized by cell shrinkage, cell membrane blisters, chromatin concentration, apoptotic body formation, and immunogenic protein phagocytosis through phagocytes and macrophages (87). Next, we will introduce the mechanism of necroptosis to draw out the role of necroptosis in DCM, present possible targets, and provide some suggestions for future research.

2.2.1. Mechanism

Necroptosis is a unique cell death mode in vertebrate cells, which is the second line of defense established by cells to resist pathogens. When apoptosis fails, necroptosis becomes a fail-safe

mechanism. Abnormal regulation of necroptosis is associated with these diseases. The expression and activity of necroptosis signaling proteins are low in cancers, such as breast cancer (88) and ovarian cancer (89), whereas the occurrence of certain inflammatory diseases, such as myocardial ischemia-reperfusion injury (90) and DCM (91), is associated with an increased expression of the signaling proteins.

Recently, necroptosis signaling pathways have been extensively studied. These pathways share some upstream signaling elements with apoptosis (92). Here we highlight well-studied tumor necrosis factor receptor 1 (TNFR1). The powerful TNF family has a homeostasis function (93) to defend against pathogens, in which TNF-α binds to the transmembrane protein TNFR1, allowing the TNF receptor-associated death domain (TRADD) to signal RIPK1 and recruit RIPK3 to form bad dead bodies (94). Either in terms of apoptosis or necrosis, caspase is an indissoluble protein. Thus, here, we discuss necroptosis from the caspase protein. In 2019, the journal *Nature* reported that caspase-8 induces and inhibits cell death at the same time. It induces apoptosis through death receptors such as TNFR1, and inhibits necroptosis through lysis and the inactivation of RIPK1 and RIPK3 (95). If caspase-8 is active in cells, the formation of a complex between activated caspase-8 and RIPK1 and FADD could induce apoptosis (96). If caspase-8 is inhibited, RIPK1 and RIPK3 are phosphorylated and bind to the phosphorylated MLKL, eventually leading to necroptosis (97). Caspase-8 acts like a shunt, with different gates opening and different flow directions. As a type of regulatory cell death independent of caspase, necroptosis is due to a series of automatic and cross-phosphorylation interactions between RIPK1 and RIPK3. Upon phosphorylation of RIPK3, MLKL aggregates and subsequently phosphorylates, penetrates the plasma membrane and organelles, and the membrane breakdown results in the cell contents spilling into organs, further leading to inflammatory phenotypes and damage-related DAMP releases, such as IL-1α, IL-β, and IL-33, which induce immune responses (98). DAMP sends signals to the circulatory system to recruit immune cells to damaged tissues, the component in macrophages, macro pinocytosis, and clears necrotic apoptotic cells by pinocytosis (99) (**Figure 2**).

2.2.2. Necroptosis and DCM

The study of the role of necroptosis in DCM is relatively insufficient but still presents a high correlation. According to an animal model established by Song, using STZ-induced type 1 diabetic mice and HG-cultured rat cardiomyocytes, it was confirmed that Sirtuin 3 (SIRT3) deficiency aggravated hyperglycemia-induced mitochondrial damage, increased ROS accumulation, promoted necrosis, and possibly activated NLRP3 inflammatory. Ultimately, these processes aggravated DCM in mice (91). Recently, the correlation of the RIPK3/MLKL signaling pathway, a key pathway of necroptosis, in DCM has been confirmed. Cao et al. revealed that necroptosis of cardiomyocytes plays an essential role in mediating cardiac pathology in type 1 DCM (100). In addition, Chen et al. reduced myocardial damage, improved cardiac function, and inhibited Ca^{2+}-calmodulin-dependent kinase II (CAMK II) activation through RIPK3 defects. Moreover, alleviating necroptosis in DCM mice demonstrates the existence of severe necroptosis in DCM mice (101).

It is certainly not enough to simply state a correlation. Irbesartan is a common clinical ARB drug. Existing studies have explored how irbesartan acts. Irbesartan has a cardioprotective effect on diabetic rats, and its mechanism may be related to the inhibition of the RIP1-RIP3-MLKL pathway, which alleviates necroptosis in cells. When it comes to the RIP1-RIP3 complex, AMPK inhibits the formation of this complex (102). Based on this, Cao et al. demonstrated that the anti-diabetic drug Engliazine and metformin prevented hyperglycemic-induced cardiomyopathy, which may be related to the activation of AMPK. Metformin reduces the inhibition of AMPK activation by cardiomyocytes in hyperglycemic environments (103). Therefore, the inhibition of necroptosis is a means to control DCM. More studies are needed to explore specific targets. We can consider further exploring the pathway proteins related to necroptosis, such as whether the upstream kinase mediating the regulation of caspase phosphorylation can be blocked by corresponding drugs. Do the blocking drugs have dual regulatory effects and can they regulate apoptosis at the same time? Is double regulation synergistic or antagonistic? What effect does this have on the treatment and prognosis of DCM?.

2.3. Cuproptosis

Similar to iron, copper is an indispensable trace element in all organisms, but it can exhibit cytotoxicity if the concentration of copper ions in cells exceeds the threshold for maintaining homeostasis (104, 105). In fact, as early as 2001, researchers found that compared with non-diabetic patients, plasma copper ion levels and urine copper ionized water are increased to varying degrees on average, while the content of copper in cardiomyocytes is decreased (106, 107). In March 2022, Peter Tsvetkov's team named a controlled cell death mode whose mechanism is clearly different from the known apoptosis by pyroptosis, necroptosis, and ferroptosis as "cuproptosis" (108).

2.3.1. Mechanism

To confirm that this form of death is distinct from other regulatory cell death processes, researchers knocked out the key apoptosis factors BAX and BAK1 and used inhibitors of known types of cell death, including caspase inhibitors for apoptosis, and ferrostatin 1 for ferroptosis. When the cells were treated with necrostatin 1 in response to necroptosis and N-pancreatic cysteine in response to oxidative stress, the cell death induced by a copper ion carrier could not be eliminated. The data suggest that cupric ion-carrier-induced cell death is a mechanism quite different from other known modes of cell death. Interestingly, cells that rely more on mitochondrial respiration are about 1,000 times more sensitive to copper inducers than cells that rely on glycolysis. Copper deficiency damages the function of mitochondria and leads to energy reduction, thus leading to myocardial hypertrophy. A clinical investigation by Oster et al.

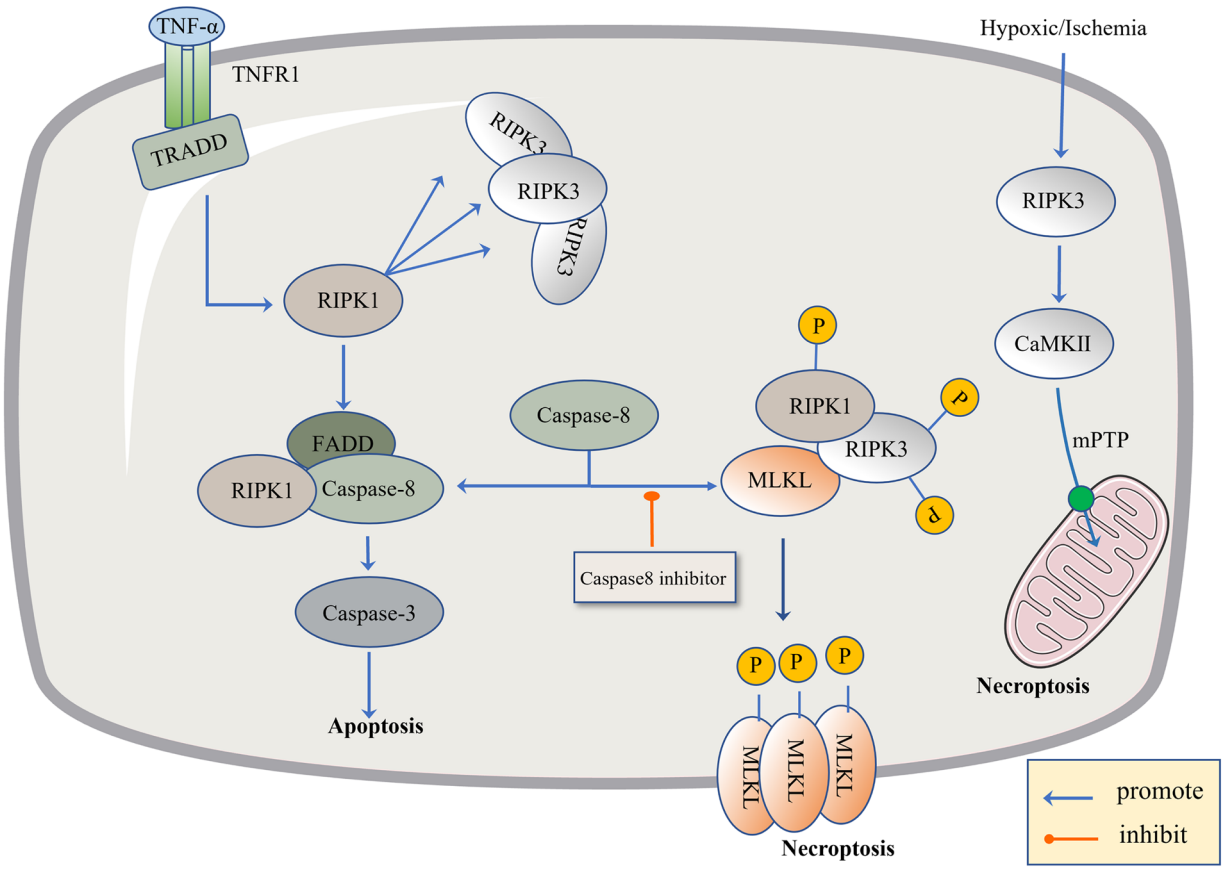

FIGURE 2
The regulatory mechanism of necroptosis. The pathogen inhibits Caspase-8, leading to apoptosis failure and the initiation of necroptosis. The level of TNF-α is up-regulated, TNF-α binds to TNFR1, activates TRADD and RIPK1, and there is the recruitment of RIPK3. When Caspase-8 is active, it promotes the combination of FADD, RIPK1 and Caspase-8, and then activates Caspase-3 to induce apoptosis. When Caspase-8 is inhibited, RIPK1 and RIPK3 are phosphorylated and bind to the phosphorylated MLKL. Phosphorylated MLKL is inserted into organelles and cell membranes, eventually leading to necroptosis. In addition, necroptosis can be activated through RIPK1-independent pathways, where RIPK3 activates CAMK II and the mitochondrial permeability transition pore (MPTP), leading to the loss of membrane potential and integrity, resulting in necroptosis.

showed that the copper ion level in cardiomyocytes was positively correlated with ejection fraction in 27 patients after coronary artery bypass grafting (109). Treatment with mitochondrial antioxidants (110), fatty acids (111) and mitochondrial function inhibitors (112) significantly changed the sensitivity of the cells to copper ions. FDX1 is the upstream regulator of protein-lipid acylation, and the loss of FDX1 leads to the complete loss of protein-lipid acylation function, as well as the accumulation of pyruvate and α-ketoglutaric acid and the consumption of succinic acid in cells, indicating that the loss of protein lipid acylation function blocks the progression of the tricarboxylic acid cycle (TCA) (113). FDX1 and protein-lipid acylation are key factors in the induction of cell death by copper ion carriers. Excessive copper promotes the aggregation and function loss of fatty acylation proteins, triggering the instability of Fe-S cluster proteins, leading to toxic protein stress and ultimately cell death (108) (**Figure 3**).

2.3.2. Cuproptosis and DCM

DCM is characterized by myocardial remodeling, including myocardial fibrosis and hypertrophy. As mentioned above, cuproptosis mainly occurs in the mitochondria, which means that cuproptosis affects the course of DCM by destroying mitochondrial structure and function (78). Abnormal copper ion metabolism in cardiomyocytes is a key pathogenic process of diabetes-induced heart failure (114). Before the concept of cuproptosis was proposed, experiments were conducted to detect metabolite levels in cardiomyocytes treated with copper, and the results showed that the overall downregulation of metabolites mainly affected glycerol phospholipid metabolism, fatty acid degradation and other processes, indicating that copper can induce metabolic pathway disorder and lead to myocardial damage (115). More seriously, excessive oxidative stress is activated after copper accumulation, which disrupts the self-regulation and metabolic dynamics of the mitochondria, and the REDOX reaction cannot maintain balance, forming a vicious cycle (116).

Currently, many studies have shown that an increasing copper level can reduce myocardial hypertrophy (117–119), but for patients with DCM, the problem is not copper intake, but copper transport (120). Thus, we have taken into consideration two aspects: First, how can copper transport be regulated in patients with DCM? Is there a target for that? Second, if excessive copper

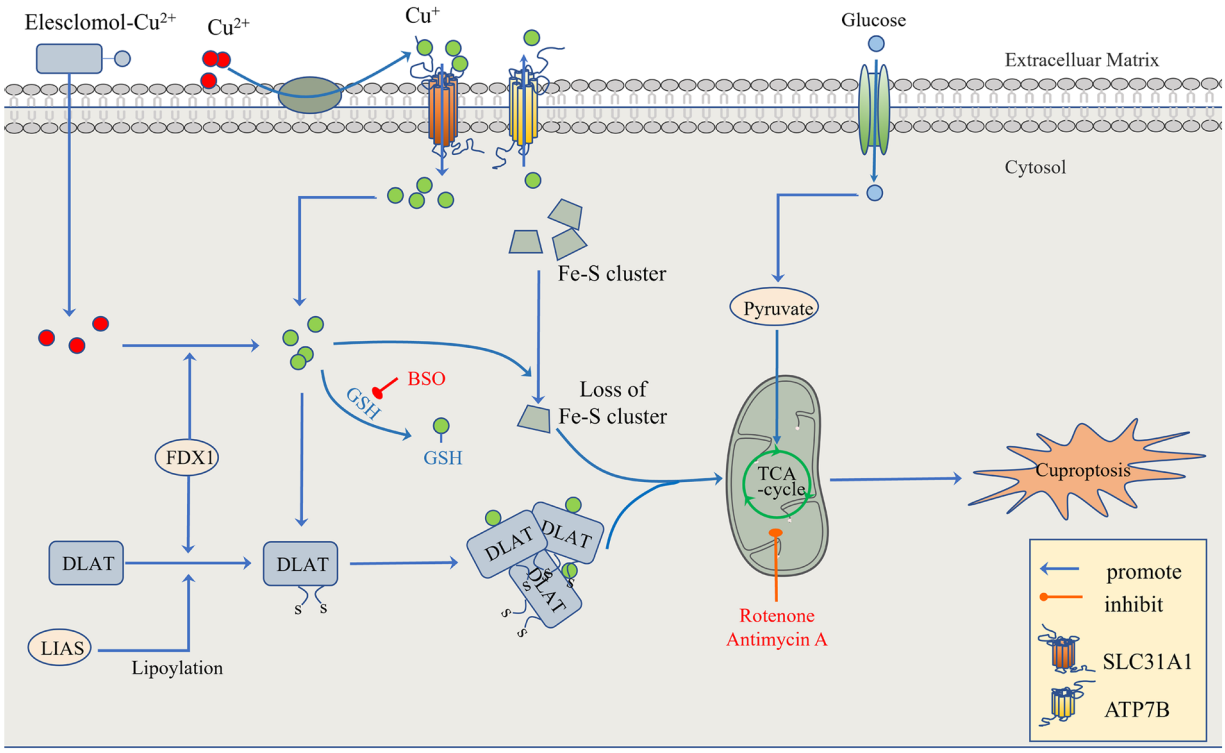

FIGURE 3
The regulatory mechanism of cuproptosis. The mitochondria are the main organelles involved in Cu-induced cell death. When the mitochondrial membrane is damaged by oxidation, the enzyme function in the TCA cycle is impaired, leading to cuproptosis. On the one hand, FDX1 and protein-lipid acylation are the key factors involved in cupric ion carrier-induced cell death. Excessive copper promotes the aggregation and functional loss of fatty acylation proteins, leading to the instability of Fe-S cluster proteins. On the other hand, FDX1 reduces Cu^{2+} to Cu^{+}. GSH, a thiol-containing copper chelator, blocks cuproptosis, whereas buthionine sulfoximine (BSO) promotes cuproptosis by depleting GSH. With the participation of Cu^{+}, the lipid acylation and aggregation of enzymes involved in the regulation of the mitochondrial TCA cycle, such as DLAT, are promoted. Together with the instability of Fe-S cluster proteins, the mitochondrial membrane and its TCA cycle are destroyed, leading to the occurrence of cuproptosis. This process can be inhibited by electron transport chain (ETC) complex I/III inhibitors such as rotenone and antimycin A. In addition to Elesclomol-Cu^{2+}, which transports Cu^{2+} into and out of cells, copper ion channels SLC31A1 and ATP7B regulate the accumulation of copper ions by mediating the entry and exocytosis of copper ions, respectively.

is toxic after all, what is the relationship between copper toxicity and the occurrence and development of DCM? Or does copper toxicity affect the prognosis of heart failure in patients with DCM? Can copper damage be reduced by regulating plasma copper ion levels? TETA, a drug that promotes copper excretion, is currently used as an experimental treatment for diabetes to improve DCM (121) and is undergoing phase II clinical trials (122). Its principle is to promote copper excretion, prevent the excessive deposition of cardiac collagen, and improve cardiac structure and function (123). TETA has been previously approved for the treatment of Wilson disease (124), which to some extent indicates the safety of TETA.

As a newly proposed form of cell death, the current research on cuproptosis still focuses on its intervention in cancer therapy, which destroys the energy system of cancer cells by providing excessive copper to cancer tissues through targeting, leading to the death of cancer cells (108, 125), and its mechanism is still in the stage of exploration. Therefore, regarding the metabolic pathway related to cuproptosis and the influencing mechanism of its related diseases our understanding needs to be improved to find more corresponding targets. In addition, most of the current research is limited to cellular and molecular experiments, and clinical studies are rare. In the future, more studies should be carried out by combining clinical and molecular experiments.

3. Conclusion

Based on the above review, novel regulatory cell death mechanisms are involved in the cell death and progression of DCM, and it is worth noting that inhibition of any form of cell death can significantly restore damaged myocardial cell function or structure in DCM (80, 126–131), suggesting that different cell death types may be potential therapeutic targets for DCM (**Figure 4**). It is exciting to note that there are already regulatory cell death-related drug applications, such as Venetoclax (132), a selective Bcl-2 inhibitor in acute myelogenous leukemia, chronic lymphocytic leukemia, and small lymphocytic lymphoma. In addition, inhibiting the necroptosis of hepatocytes is an effective way to improve drug-induced liver injury. The administration of acetaminophen (300 mg/kg) and followed (1 h later) by hydroxyethidine (100 mg/kg) inhibits apoptosis and necroptosis

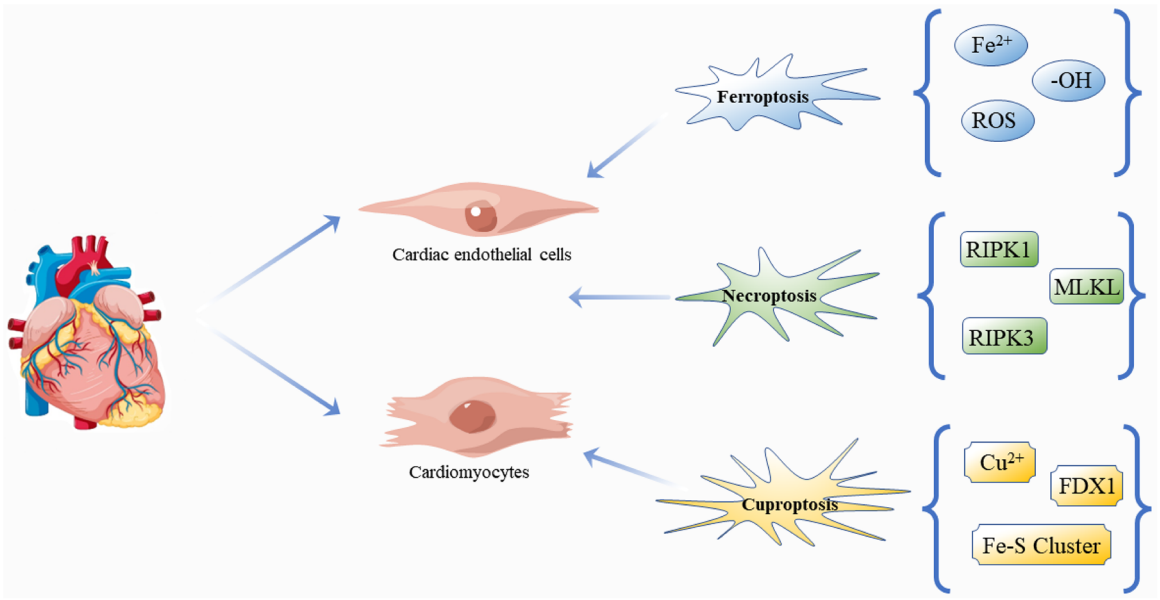

FIGURE 4
Regulatory cell death: ferroptosis, necroptosis and cuproptosis in DCM. The pathogenesis and progression of DCM are closely related to the effects of three kinds of death on cardiomyocytes and cardiac endothelial cells. Free icons were used from https://www.vecteezy.com (leaders: Shawn Rubel, Adam Gamble, Richard Fontenot).

in mice (133). Some anti-cancer drugs appear to target and enhance the ferroptosis process to kill glioma cells, including dihydroartemisinin (134) and ibuprofen (135). Inducing ferroptosis is thought to enhance the effect of traditional anti-cancer treatments that trigger other cell death pathways, such as apoptosis. However, until now, we have not thoroughly studied the mechanism of clearing these regulatory cell deaths, nor understood their pathophysiological effects. The number of studies is small, models are not perfect, and there is no reliable biomarker together with unsolved problems and limited conclusions obtained. Therefore, more studies are needed to answer the relevant questions. For example, we know that the clinical diagnosis and treatment of DM are premised on the control of blood glucose level, and of course, there is a definite relationship between hyperglycemia and DCM (136), but how hyperglycemia affects the various forms of cell death remains unclear. Do these different forms of cell death work independently? If so, what role does each play? If not, what is the connection between these different mechanisms? For example, does oxidation play the same role in each form of death at different stages of the development of DCM, and what effect does it have on the progression of the disease? Unfortunately, we have not found any studies that focus on the proportion of the various forms of death in DCM and their impact on prognosis, which may be one of the directions for further research aiming to improve the effectiveness of the treatments and survival of patients.

As mentioned above, the AMPK pathway is also an important pyroptosis pathway (137). Pyroptosis-related proteins promote mitochondrial apoptosis through ROS (138). Therefore, can one mechanism promote or inhibit other mechanisms? In addition, the associations implied by these similarities may also be targets for genetic intervention. We also noted that ferroptosis has many similarities with cuproptosis, such as the oxidative stress response caused by ROS accumulation and GSH depletion, which are related to the synergistic or antagonistic effects of drugs and may be the focus of drug development. In addition, necroptosis is more closely related to apoptosis and necrosis. The determination of the caspase protein may be a potential drug target, and the phosphorylation and subsequent modification of RIPK family may also be one direction for future drug research. In addition, there are also some similarities between death forms, such as pyroptosis, that are worth exploring.

Of particular interest to us is what these similarities represent. Is there an overlap between some forms of death? If there is overlap, are these overlapping processes an opportunity for the onset of DCM? Shedding light on these issues can help to better determine targets and target diseases. Metabolic disorders caused by diabetes are triggers for many forms of cell death. However, as mentioned above, there is currently no specific clinical treatment plan for DCM, and even treatment after heart failure only follows the guidelines for heart failure. If we can further study these forms of death, we may be able to solve this problem. After all, the incidence of DM is high and rising year by year, and cardiovascular diseases are also the main cause of death and disability at present.

Author contributions

Conceptualization, LL; original draft preparation, DK; collected the literatures, PC, JLi and XS; review and editing, LL, ZZ and JLiu; supervision, YC. 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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The impact of circulating IGF-1 and IGFBP-2 on cardiovascular prognosis in patients with acute coronary syndrome

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Background: While insulin-like growth factor 1 (IGF-1) exerts a cardioprotective effect in the setting of atherosclerosis, insulin-like growth factor binding protein 2 (IGFBP-2) is involved in metabolic syndrome. Although IGF-1 and IGFBP-2 are known to be predictors for mortality in patients with heart failure, their use in clinic as prognostic biomarkers for acute coronary syndrome (ACS) requires investigation. We evaluated the relationship between IGF-1 and IGFBP-2 levels at admission and the risk of major adverse cardiovascular events (MACEs) in patients with ACS.
Methods: A total of 277 ACS patients and 42 healthy controls were included in this prospective cohort study. Plasma samples were obtained and analyzed at admission. Patients were followed for MACEs after hospitalization.
Results: Among patients who suffered acute myocardial infarction, plasma levels of IGF-1 and IGFBP-2 were lower and higher, respectively, as compared to healthy controls (both $p < 0.05$). The mean follow-up period was 5.22 (1.0–6.0) months and MACEs incidence was 22.4% (62 of 277 patients). Kaplan–Meier survival analysis revealed that patients with low IGFBP-2 levels had a greater event-free survival rate than patients with high IGFBP-2 levels ($p < 0.001$). Multivariate Cox proportional hazards analysis revealed IGFBP-2, but not IGF-1, to be a positive predictor of MACEs (hazard ratio 2.412, 95% CI 1.360–4.277; $p = 0.003$).
Conclusion: Our findings suggest that high IGFBP-2 levels are associated with the development of MACEs following ACS. Moreover, IGFBP-2 is likely an independent predictive marker of clinical outcomes in ACS.

KEYWORDS

acute coronary syndrome, insulin-like growth factor-1, insulin-like growth factor binding protein 2, cardiovascular prognosis, IGF-1, IGFBP-2

Introduction

Acute coronary syndrome (ACS) is characterized by a sudden decrease in blood flow to the heart. Worldwide, more than seven million people are annually diagnosed with ACS; approximately 5% of this patient population was reported to die prior to hospital discharge (1, 2). Although scientific advances have greatly facilitated implementation of effective secondary cardiovascular prevention strategies, previously unrecognized mediators of cardiovascular disease (CVD) continue to be discovered.

Insulin-like growth factors (IGFs) are conserved peptide hormones structurally similar to insulin that are expressed universally in multiple tissues (3). Interestingly, IGF-1 is not only

found in the circulation but also in arteries, with studies having reported IGF-1 to exert cardioprotective effects in the setting of atherosclerosis (4, 5). Preclinical model studies reported that administration of IGF-1 suppresses cardiac fibrosis induced by angiotensin II (6), and treatment of sheep fetuses with IGF-1 was reported to stimulate growth of the coronary vasculature and myocardium (7). Furthermore, bone marrow mesenchymal stem cells overexpressing IGF-1 were reported to better resist apoptosis in myocardial infarction (8). In clinical practice, serum IGF-1 levels are decreased in heart failure (HF) patients (9) and serve as a predictor of cardiovascular mortality in this condition (10).

All six members of the IGF-binding protein (IGFBP) family regulate IGF bioavailability (11, 12). As the second most abundant protein of this family (13), IGFBP-2 plays critical roles in several pathological processes including carcinogenesis (14), pulmonary arterial hypertension (PAH) (15), obesity and insulin resistance (13). In addition to strongly predicting mortality in HF patients (16), IGFBP-2 has recently emerged as a novel candidate biomarker for cardiovascular risk assessment in patients with aortic stenosis who undergo transcatheter aortic valve implantation (17) and elderly men (18).

To date, relevant research has been limited to animal experiments, preclinical analyses or patients with HF, PAH or aortic stenosis. As such, the prognostic influences of circulating IGF-1 and IGFBP-2 in ACS patients remain unclear. Here, we evaluated the relationship of circulating IGF-1 and IGFBP-2 with major adverse cardiovascular events (MACEs) in ACS patients who underwent coronary angiography (CAG).

Methods

Study design and population

This study was a prospective cohort study conducted according to regulations set forth by the Declaration of Helsinki. This study was approved by the Ethics Committee of Liaocheng People's Hospital and all subjects enrolled provided informed consent. From June 1 2021 to October 1 2021, 304 ACS patients who underwent CAG and 42 site-matched controls free of clinical heart disease were initially enrolled in this study. All ACS patients enrolled met relevant diagnostic criteria for either ST-elevation myocardial infarction (STEMI) or non-ST-elevation ACS (19, 20). Patients with severe liver disease or renal failure, neoplasms of any kind or infectious or inflammatory conditions were excluded from this study. Patients lost to follow-up were also excluded from analyses. The treatment therapy, including intensive treatment with medicine, percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) was decided by two experienced cardiologists according to the results of CAG and international standards and guidelines (21).

Laboratory measurement

Venous blood samples were draw from study subjects for evaluation prior to administration of any medications. Blood samples were collected using tubes containing ethylenediaminetetraacetic acid and centrifuged at 3,000 rpm for 15 min immediately after collection. Samples were aliquoted and stored at −80°C until use. Plasma levels of IGF-1 and IGFBP-2 were assayed using the enzyme linked immunosorbent assays (ELISA) IGF-1 (DG100B) and IGFBP-2 (DGB 200) (both R&D Systems, USA), according to manufacturer's protocol. No significant cross-reactivity or interference of IGFBP/IGF-1 with IGFBP-2 was found in the immunoassay according to product description. ELISA IGFBP-2 kit did not measure IGFBP in complex with IGF-1.

Definitions

Participants with one major coronary artery ≥50% stenosis were considered as single-vessel disease, whereas multi-vessel disease (MVD) was defined in cases of stenoses ≥50% in 2 or 3 major epicardial coronary arteries. Stenosis of the left main coronary artery ≥50% was regarded as the left main disease (22). Incomplete revascularization (IR) was defined as one or more vessels stenosis ≥50% being left untreated after revascularization (23).

Follow-up and outcomes

The entire cohort was followed up for 6 months starting from date of hospitalization. Data were systematically obtained *via* phone interviews and review of medical records. A total of 27 ACS patients were lost of follow-up and excluded from this study. A total of 277 ACS patients who completed follow-up were finally evaluated and included 93 unstable angina (UA), 89 non-ST-elevation myocardial infarction (NSTEMI) and 95 STEMI patients. Cardiovascular death, angina, new-onset HF, recurrent myocardial infarction (MI) or any revascularization were all defined as MACEs.

Statistical analyses

The Shapiro–Wilk test was used to determine whether continuous data were normally distributed. Normally distributed continuous variables were expressed as mean ± standard deviation; data not normally distributed were expressed as median (interquartile range). Comparisons of continuous variables between two groups were performed using the Mann–Whitney U test or t-test. Comparisons of continuous variables among four groups were performed using one-way analysis of variance or the Kruskal–Wallis H test. The pairwise test for multiple comparisons was used to analyze intergroup differences for IGF-1 and IGFBP-2 levels after Kruskal–Wallis H analysis. Categorical variables were presented as frequency and percentage and compared using the chi-squared test. Spearman correlation analysis was performed to analyze the correlation of plasma IGF-1 and IGFBP-2 levels.

A receiver operator characteristics (ROC) curve was generated and the area under the curve (AUC) was calculated, and Z test were used to compare AUC values. Optimal IGF-1 and IGFBP-2 cutoff

points for MACEs prediction were determined based on maximal Youden's index. Kaplan–Meier survival curves were constructed to analyze the short-term event-free survival (EFS) rate and comparisons were performed using the log-rank test. Cox proportional hazards regression analysis was performed to determine independent factors predictive for MACEs; confounders with unadjusted p-values <0.05 in univariate analysis were included in a multivariate regression model. A two-tailed p-value of <0.05 was considered as statistically significant. Statistical analyses were performed using SPSS 23.0 (IBM, USA).

Results

Clinical characteristics

Median values (interquartile ranges) of IGF-1 and IGFBP-2 concentrations in healthy controls ($n = 42$) were 170.39 (106.14) ng/ml and 199.1 (255.01) ng/ml, respectively. While IGF-1 levels in healthy controls were higher than in patients who suffered NSTEMI or STEMI ($p < 0.05$), IGFBP-2 levels showed the opposite pattern. Although IGFBP-2 levels in STEMI patients were higher than those in UA patients ($p < 0.05$), no significant difference between healthy controls and UA patients was found ($p > 0.05$; Table 1).

In a total of 277 ACS patients, 195 (70.4%) presented with MVD, 37 (13.4%) presented with left main vessel disease, and the number of patients with IR treatment was 32 (11.6%). 89 (32.1%) patients were treated with intensive medication, and 188 (67.9%) patients were treated with PCI or CABG. Patients were divided into two groups based on median IGF-1 concentrations: a high IGF-1 group (IGF-1 levels ≥126.92 ng/ml; $n = 139$) and a low IGF-1 group (IGF-1 levels <126.92 ng/ml; $n = 138$). High IGF-1 group patients were found to have a higher ejection fraction (EF), a lower rate of acute myocardial infarction (AMI) and a higher rate of multi-vessel lesion as compared to those in the low IGF-1 group. No other statistically significant differences in demographic characteristics or laboratory evaluations were noted between the two groups (Table 2).

Patients were divided into two groups based on median IGFBP-2 concentrations: a high IGFBP-2 group (IGFBP-2 ≥ 308.3 ng/ml; $n = 139$) and a low IGFBP-2 group (IGFBP-2 < 308.3 ng/ml; $n = 138$). As shown in Table 3, high IGFBP-2 group patients were older, had lower levels of hemoglobin, triglycerides and EF, higher D-dimer levels, and lower body mass indices (BMI). Moreover, high IGFBP-2 group patients were found to have a higher rate of AMI as compared to low IGFBP-2 group patients.

The correlation of plasma IGF-1 and IGFBP-2 levels was evaluated by Spearman correlation analysis. A significant negative correlation was found among IGF-1 and IGFBP-2 ($r = -0.172$, $p = 0.002$; Figure 1).

TABLE 1 Plasma levels of IGF-1 and IGFBP-2 in studied population.

Variables	HC ($n = 42$)	UA ($n = 93$)	NSTEMI ($n = 89$)	STEMI ($n = 95$)
IGF-1 (ng/ml)	170.39 (106.14)	138.89 (86.53)	117.53 (76.31)[a]	117.95 (96.6)[a]
IGFBP-2 (ng/ml)	199.1 (255.01)	258.74 (245.03)	318.55 (394.59)[a]	364.75 (400.79)[a,b]

[a]$p < 0.05$ when compared NSTEMI or STEMI with HC.
[b]$p < 0.05$ when compared STEMI with UA.
IGF-1, Insulin like growth factor 1; IGFBP-2, Insulin like growth factor binding protein 2; HC, Healthy control; UA, Unstable angina; NSTEMI, Non-ST-segment elevation myocardial infarction; STEMI, ST-segment elevation myocardial infarction.

Clinical outcomes of adverse cardiovascular events

The mean follow-up period was 5.22 (1.0–6.0) months. The incidence of MACEs was 22.4% (62 of 277 patients) and included instances of cardiovascular death ($n = 8$), angina ($n = 21$), HF ($n = 13$) and reinfarction or revascularization ($n = 20$). No patient

TABLE 2 Basic characteristics of studied patients according to plasma IGF-1 levels.

Characteristics	IGF-1 ≧ 126.92 ng/ml	IGF-1 < 126.92 ng/ml	p value
	$n = 139$	$n = 138$	
Age (years)	59 ± 12	62 ± 12	0.491
Male, n (%)	111 (79.9)	102 (73.9)	0.241
Smoking, n (%)	77 (55.4)	73 (52.9)	0.677
Hypertension, n (%)	85 (61.2)	74 (53.6)	0.205
DM, n (%)	37 (26.6)	38 (27.5)	0.864
Heart rate (bpm)	72 (17)	72 (17)	0.584
BMI (kg/m^2)	25.78 ± 3.75	25.25 (4.3)	0.137
Hemoglobin (g/L)	137 ± 16	134 ± 16	0.786
WBC count (×10^9/L)	7.5 (3.6)	7.7 (3.7)	0.671
D-dimer (mg/L)	0.35 (0.45)	0.35 (0.37)	0.912
Creatinine (umol/L)	67.3 (19.5)	69.6 (18.9)	0.576
TG (mmol/L)	1.35 (1.06)	1.28 (0.80)	0.115
LDL (mmol/L)	2.62 ± 0.79	2.63 (0.94)	0.539
TC (mmol/L)	4.27 ± 1.08	4.29 (1.33)	0.761
CRP (mg/L)	3.55 (6.02)	4.26 (7.48)	0.227
EF (%)	58 (14)	54 (14)	0.025
LVEDD (mm)	45 (5)	46 (6)	0.198
Diagnosis, n (%)			
AMI	83 (59.7)	101 (73.2)	0.018
UA	56 (40.1)	37 (26.8)	0.018
Angiography, n (%)			
One-vessel lesion	33 (23.7)	49 (35.5)	0.032
Multi-vessel lesion	106 (76.3)	89 (64.5)	0.032
Left main vessel lesion	22 (15.8)	15 (10.8)	0.225
IR	18 (12.9)	14 (10.1)	0.465
Treatment strategies, n (%)			
Intensive medication	39 (28.1)	50 (36.2)	0.145
PCI/CABG	100 (71.9)	88 (63.8)	0.145
IGF-1 (ng/ml)	173.6 (78.6)	88.9 (37.3)	<0.001
IGFBP-2 (ng/ml)	259.3 (317.9)	326.53 (394.5)	0.112

IGF-1, Insulin like growth factor 1; DM, Diabetes mellitus; BMI, Body mass index; WBC, White blood cell; TG, Triglyceride; LDL, Low-density lipoprotein; TC, Total cholesterol; CRP, C-reactive protein; EF, Ejection fraction; LVEDD, Left ventricular end-diastolic dimension; AMI, Acute myocardial infarction; UA, Unstable angina; IR, Incomplete revascularization; PCI, Percutaneous coronary intervention; CABG, Coronary artery bypass grafting; IGFBP-2, Insulin like growth factor binding protein 2.

TABLE 3 Basic characteristics of studied patients according to plasma IGFBP-2 levels.

Characteristics	IGFBP-2 ≧ 308.3 ng/ml	IGFBP-2 < 308.3 ng/ml	*p* value
	n = 139	*n* = 138	
Age (years)	62 ± 12	58 ± 12	0.006
Male, *n* (%)	108 (77.7)	105 (76.1)	0.750
Smoking, *n* (%)	81 (58.3)	69 (50.0)	0.167
Hypertension, *n* (%)	79 (56.8)	80 (58.0)	0.848
DM, *n* (%)	38 (27.3)	37 (26.8)	0.921
Heart rate (bpm)	74 (18)	70 (16)	0.552
BMI (kg/m^2)	25.1 ± 3.46	25.9 ± 3.49	0.049
Hemoglobin (g/L)	134 ± 16	138 ± 17	0.034
WBC count (×10^9/L)	7.59 (3.59)	7.66 (3.45)	0.561
D-dimer (mg/L)	0.4 (0.57)	0.33 (0.34)	0.014
Creatinine (umol/L)	67 (19)	68 (20)	0.143
TG (mmol/L)	1.24 (0.73)	1.45 (0.96)	0.015
LDL (mmol/L)	2.63 ± 0.74	2.67 ± 0.81	0.682
TC (mmol/L)	4.24 ± 1.01	4.26 ± 1.09	0.871
CRP (mg/L)	3.88 (7.41)	3.84 (6.46)	0.421
EF (%)	54 (15)	59 (14)	0.017
LVEDD (mm)	46 (7)	45 (5)	0.174
Diagnosis, *n* (%)			
AMI	103 (74.1)	81 (58.7)	0.007
UA	36 (25.9)	57 (41.3)	0.007
Angiography, *n* (%)			
One-vessel lesion	40 (28.9)	42 (30.4)	0.762
Multi-vessel lesion	99 (71.1)	96 (69.6)	0.762
Left main vessel lesion	17 (12.2)	20 (14.4)	0.580
IR	18 (12.9)	14 (10.1)	0.465
Treatment strategies, *n* (%)			
Intensive medication	38 (27.3)	51 (37)	0.087
PCI/CABG	101 (72.7)	87 (63)	0.087
IGF-1 (ng/ml)	121.9 (82.4)	129.0 (88.4)	0.328
IGFBP-2 (ng/ml)	518.25 (312.63)	187.89 (123.35)	<0.001

IGFBP-2, Insulin like growth factor binding protein 2; DM, Diabetes mellitus; BMI, Body mass index; WBC, White blood cell; TG, Triglyceride; LDL, Low-density lipoprotein; TC, Total cholesterol; CRP, C-reactive protein; EF, Ejection fraction; LVEDD, Left ventricular end-diastolic dimension; AMI, Acute myocardial infarction; UA, Unstable angina; IR, Incomplete revascularization; PCI, Percutaneous coronary intervention; CABG, Coronary artery bypass grafting; IGF-1, Insulin like growth factor 1.

died of non-cardiovascular causes. No statistical differences in MACEs rates between high and low IGF-1 group patients were found ($p = 0.141$). Total MACEs incidence was higher among high IGFBP-2 group patients as compared to low IGFBP-2 group patients ($p < 0.001$). Furthermore, incidences of angina ($p = 0.003$) and HF ($p = 0.011$) were higher among high IGFBP-2 group patients as compared to low IGFBP-2 group patients (Table 4).

Kaplan-Meier survival curves of circulating IGF-1 and IGFBP-2 in ACS patients during follow-up

Kaplan-Meier survival analysis revealed no statistically significant differences in event-free survival (EFS) between high and low IGF-1 group patients ($p = 0.145$; Figure 2A). Low IGFBP-2 group patients were found to have had a higher EFS as compared to high IGFBP-2 group patients ($p < 0.001$; Figure 2B). As such, patients with low levels of IGFBP-2 were found to have had a more favorable prognosis as compared to those with high levels of IGFBP-2.

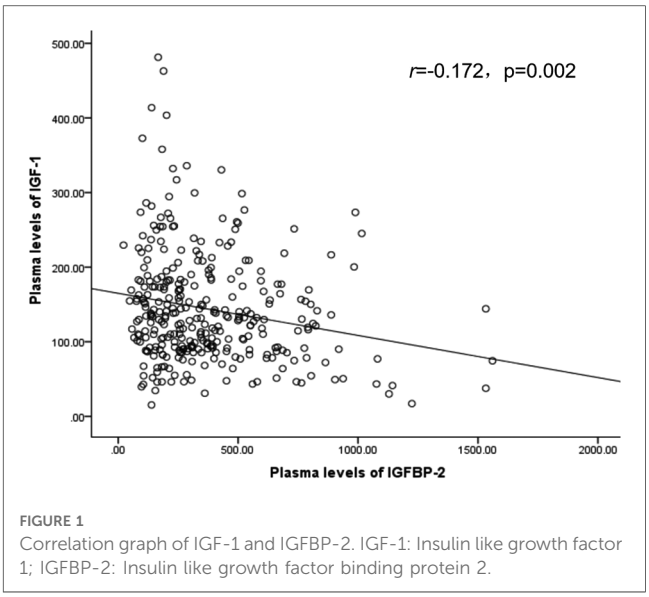

FIGURE 1
Correlation graph of IGF-1 and IGFBP-2. IGF-1: Insulin like growth factor 1; IGFBP-2: Insulin like growth factor binding protein 2.

Independent predictors for MACEs in ACS patients

As shown in Table 5, we analyzed potential confounders for association with short-term MACEs using univariate analysis. Confounders with *p*-values of <0.05 in univariate analysis were included in multivariate Cox regression analysis. After correcting for age, diagnosis of AMI, creatinine level, EF, intensive medication therapy and left main vessel lesion, high IGFBP-2 level was confirmed to have positively predicted value for MACEs [adjusted hazard ratio: 2.412, 95% confidential interval (CI) 1.360–4.277; $p = 0.003$]. Furthermore, EF and left main vessel lesion were found to be independent predictors for MACEs, while IGF-1 level was not.

MACEs prediction in ACS patients using receiver operator characteristics curves of circulating IGFBP-2 and EF

To evaluate the potential prognostic power of circulating IGFBP-2 and EF for MACEs prediction, ROC curves were generated. Analysis revealed that AUC values of plasma IGFBP-2 (Figure 3A) and EF (Figure 3B) for MACEs prediction in ACS patients were 0.722 (95% CI 0.640–0.804; $p < 0.001$) and 0.659 (95% CI 0.570–0.748; $p < 0.001$), respectively. No statistical differences in AUC values of IGFBP-2 and EF for MACEs prediction were found ($Z = 1.02$; $p > 0.05$).

TABLE 4 Major adverse cardiac events according to plasma IGF-1 and IGFBP-2 levels.

Complications	Plasma IGF-1 levels			Plasma IGFBP-2 levels		
	Low ($n = 138$)	High ($n = 139$)	p value	Low ($n = 138$)	High ($n = 139$)	p value
Death, n (%)	5 (3.6)	3 (2.2)	0.467	3 (2.2)	5 (3.6)	0.728
Angina, n (%)	11 (8.0)	10 (7.2)	0.807	4 (2.9)	17 (12.2)	0.003
Heart failure, n (%)	9 (6.5)	4 (2.9)	0.152	2 (1.4)	11 (7.9)	0.011
Reinfarction or Revascularization, n (%)	11 (6.0)	9 (6.5)	0.630	8 (5.8)	12 (8.6)	0.362
Total MACEs, n (%)	36 (25.9)	26 (18.8)	0.141	17 (12.3)	45 (32.4)	<0.001

IGF-1, Insulin like growth factor 1; IGFBP-2, Insulin like growth factor binding protein 2; MACEs, Major adverse cardiac events.

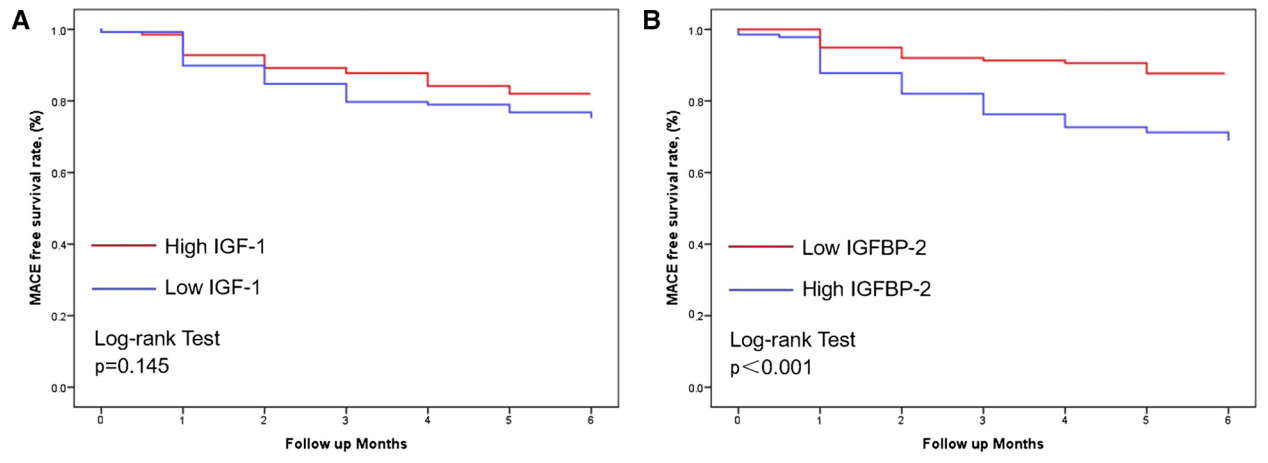

FIGURE 2
Kaplan-Meier curves in patients with ACS with individual levels of IGF-1 (A) and IGFBP-2 (B) during follow-up (p = 0.145 and <0.001, respectively). MACEs, Major adverse cardiovascular events; ACS, Acute coronary syndrome; IGF-1, Insulin like growth factor 1; IGFBP-2, Insulin like growth factor binding protein 2.

Discussion

We evaluated a cohort of 277 ACS patients and 42 healthy controls to investigate the relationship between IGF-1 and IGFBP-2 levels and prognosis for short-term outcomes. Our findings revealed that (1) circulating IGF-1 levels in healthy controls were higher than in NSTEMI or STEMI patients, and IGFBP-2 levels showed an opposite pattern; (2) EFS was poor in patients with high levels of IGFBP-2; and (3) high IGFBP-2 levels, but not low IGF-1 levels, independently predicted for MACEs in ACS patients who underwent CAG.

IGF-1 levels were previously found to be lower in acute MI patients compared to healthy controls (24, 25). To date, however, studies evaluating the influence of IGFBP-2 levels on cardiovascular disease remain scarce. In this study, we not only confirmed IGF-1 levels in healthy controls to have been significantly higher than those in acute MI patients, but also found IGFBP-2 levels to have been higher in acute MI patients than those in healthy controls. Levels of IGF-1 and IGFBP-2 were previously reported to associate with EF and potentially serve as biomarkers for HF (9, 26–28). In agreement with previous studies, we found that IGF-1 levels positively associated with EF, whereas IGFBP-2 negatively associated with EF. Although HF incidence was greater in high IGFBP-2 group patients, no differences between groups were found for IGF-1 levels. We noted that high IGFBP-2 group patients had lower triglyceride levels and BMI. Our findings are in agreement with prior literature that reported IGFBP-2 to be a marker of metabolic syndrome and inversely correlate with BMI and triglyceride levels (29–31).

No differences in incidences of MACEs or death were found between low and high IGF-1 group patients in this study. Although no difference in mortality was noted between high and low IGFBP-2 group patients, the incidence of MACEs in high IGFBP-2 group patients was found significantly higher as compared to low IGFBP-2 group patients. Moreover, after adjusting for age and other variables, we found that IGFBP-2 was an independent predictive factor for MACEs, while IGF-1 was not. Iswandi et al. (32) reported that IGF-1 was not an independent predictor of cardiovascular mortality or morbidity in ACS patients over a 5-year follow-up period. Furthermore, Wallander et al. (33) found that IGF-1 levels at hospital admission were not related to cardiovascular death over a three-year follow-up period in patients with type 2 diabetes who suffered acute MI. Although our findings were in agreement with most previously reported, a study by Bourron and et al. (34) reported that low IGF-1 levels not only associate with increased mortality risk but also with risk of any MACEs in acute MI

TABLE 5 Cox proportional hazard analysis for predictors of MACEs.

Variables	HR	95% CI	*p* value	Adjusted HR	95% CI	*p* value
Male	1.295	0.741–2.264	0.363			
Age	1.037	1.014–1.061	0.001	1.016	0.993–1.040	0.175
Smoking status	1.044	0.634–1.720	0.865			
Hypertension	1.062	0.643–1.874	0.816			
DM	1.563	0.929–2.629	0.093			
Diagnosis of AMI	3.319	1.637–6.731	0.001	1.760	0.787–3.936	0.169
Heart rate	1.012	0.996–1.029	0.145			
BMI	0.971	0.957–0.986	0.052			
WBC count	1.031	0.955–1.113	0.440			
Creatinine	1.012	1.005–1.019	0.001	1.006	0.999–1.014	0.110
TG	0.790	0.578–1.079	0.138			
LDL	0.906	0.658–1.247	0.543			
TC	0.869	0.684–1.105	0.252			
CRP	1.010	1.000–1.021	0.054			
EF	0.935	0.911–0.960	<0.001	0.956	0.927–0.985	0.004
Intensive medication	1.762	1.066–2.911	0.027	0.609	0.356–1.043	0.071
Multi-vessel lesion	1.243	0.704–2.196	0.454			
Left main Vessel lesion	2.134	1.193–3.816	0.011	1.868	1.014–3.440	0.045
IR	1.525	0.752–3.091	0.242			
High IGF-1	0.694	0.419–1.150	0.157			
High IGFBP-2	2.894	1.656–5.057	<0.001	2.412	1.360–4.277	0.003

HR, Hazard ratio; MACEs, Major adverse cardiac events; CI, confidence interval; DM, Diabetes mellitus; AMI, Acute myocardial infarction; BMI, Body mass index; WBC,: White blood cell; TG, Triglyceride; LDL, Low-density lipoprotein; TC, Total cholesterol; CRP, C-reactive protein; EF, Ejection fraction; IR, Incomplete revascularization; IGF-1, Insulin like growth factor 1; IGFBP-2, Insulin like growth factor binding protein 2.

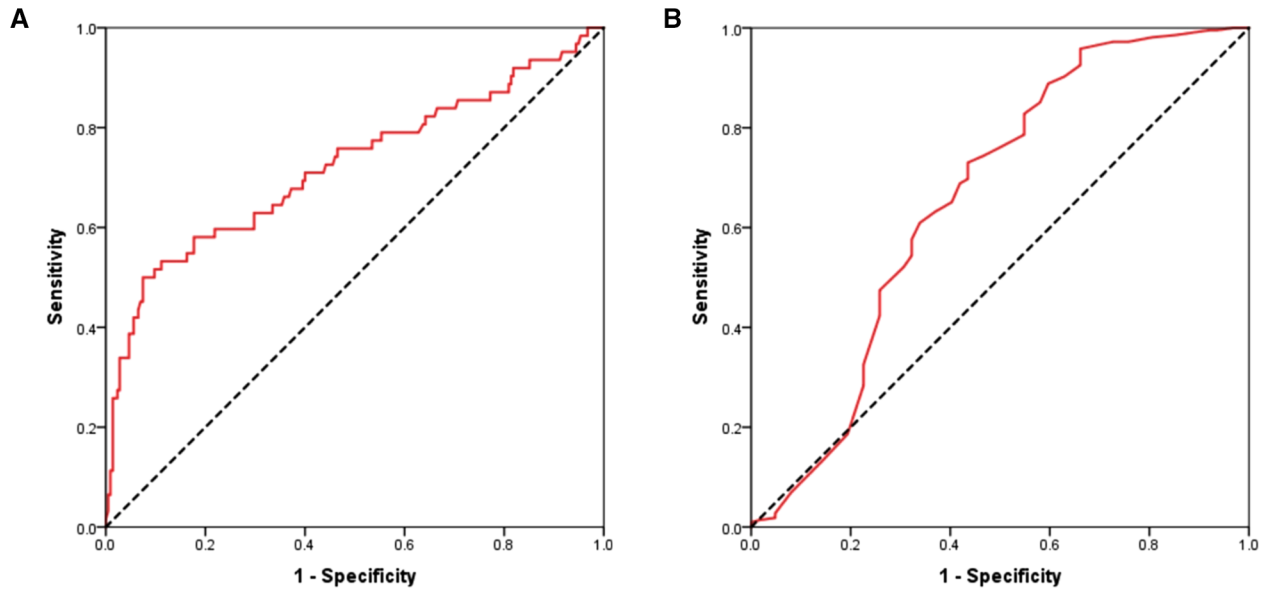

FIGURE 3
Receiver operating characteristic curves of circulating IGFBP-2 (A) and EF (B) for predicting MACEs in patients with ACS. IGFBP-2, Insulin like growth factor binding protein 2; EF, Ejection fraction; MACEs, Major adverse cardiovascular events; ACS, Acute coronary syndrome.

patients over 2 years of follow-up. As various populations may yield disparate findings, future studies should confirm whether IGF-1 is predictive for adverse outcomes in diverse groups.

Increasing evidence has highlighted the cardioprotective effects of IGF-1 in the setting of cardiovascular disease. Numerous *in vivo* and *in vitro* studies reported that IGF-1 facilitates resistance to apoptosis in hypoxic conditions (8), stimulates fetal cardiac growth (7), increases production of circulating angiogenic cytokines (35) and exerts positive inotropic and antioxidant effects (36). Translational research has further revealed that treatment with IGF-1 significantly reduces left ventricular volume, attenuates left ventricular mass and improves stroke volume in STEMI patients (37), and improves EF in HF patients (38). However, Conover et al. (39) found that transgenic

overexpression of pregnancy-associated plasma protein-A increases IGF-1 activity and results in accelerated atherosclerotic lesion development. Hirai et al. (40) reported that IGF-1 promotes atherosclerosis by affecting endothelial function and increasing aging in rabbits fed a cholesterol-rich diet. As such, the roles of IGF-1 in cardiovascular disease remain unclear and warrant further study.

Previously, IGFBP-2 was reported to inversely correlate with BMI (41). Indeed, studies reported that low IGFBP-2 independently associates with an increased risk of metabolic syndrome as well as elevated fasting glucose levels (30). Higher circulating IGFBP-2 concentrations were also longitudinally associated with lower type 2 diabetes risk (42, 43). Although IGFBP-2 is considered to protect against cardiovascular risk factors, high levels of IGFBP-2 were reported to associate with poor prognoses in several diseases. Prior studies reported that IGFBP-2 independently predicts for adverse clinical outcomes in patients with HF (16, 44), severe aortic stenosis (17), dilated cardiomyopathy (45) and PAH (46). To date, studies about IGFBP-2 in CVD is seldom, and the present study first uncovered that IGFBP-2 is an independent predictor of MACEs in ACS patients. Additionally, although no significant differences in prognosis power among IGFBP-2 and EF were noted, the AUC value of circulating IGFBP-2 was found to be greater than that of EF. Studies enrolling more eligible patients may suggest the prognostic power of IGFBP-2 was superior to EF in ACS patients. The seemingly paradoxical influences of IGFBP-2 on cardiovascular risk factors and pathological processes warrant detailed study.

The potential mechanism of IGFBP-2 and MACE is thought to be multifactorial. IGFBP-2 plays a crucial role in regulating mitogen-activated protein kinase (MAPK) pathway, which is a driver of atherosclerosis and involved in inflammatory signaling and oxidative stress (47, 48). Moreover, IGFBP-2 regulates the phosphatidylinositol 3-kinase (PI3K)/alpha serine/threonine-protein kinase (Akt) signaling pathway, which has a fundamental role in the pathological processes of atherosclerosis (49, 50). In addition, IGFBP-2 enhances the migration and proliferation of vascular smooth muscle cells (VSMC), this process is associated with the development of atherosclerosis (51). Further studies about the underlying mechanisms of IGFBP-2 in CVD are certainly warranted.

Our study, although well-designed, was not without limitations. First, the participants in this study were predominantly male, our sample size was relatively small and the follow-up period was short. As such, sex differences were not investigated, and the low incidence of mortality limited the hard endpoint analysis of the study. In addition, a lack of significant findings concerning IGF-1 and MACEs prediction may have occurred due to a type II statistical error. Second, the mechanisms behind the association between circulating levels of IGF-1 and IGFBP-2 and the incidence of MACE were not elucidated. Third, healthy controls were not well-matched with ACS patients for gender or age. Finally, blood samples were collected at admission and lacked data concerning serial fluctuations in IGF-1 and IGFBP-2 levels during follow-up. Therefore, multi-center studies with a greater sample size and animal studies about potential biological mechanisms of IGF-1 and IGFBP-2 are needed to confirm these results.

Conclusion

Plasma IGFBP-2 levels were higher in patients who suffered acute MI compared to healthy controls. A high IGFBP-2 level, but not a low IGF-1 level, likely has clinical use as a prognostic biomarker for MACEs in patients with ACS. Although the underlying mechanisms for our findings remain unclear, we provide a foundation for further study of IGFBP-2 and improvements in clinical management of patients suffering ACS.

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 human participants were reviewed and approved by Ethics Committee of Liaocheng People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HY put forward conception and study design. KY, SZ and LH researched data, tested biomarkers. WW wrote the manuscript and contributed to statistical analysis. TL, DM and JL edited and contributed to the manuscript, data interpretation, and discussion. 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.

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TIMP3 induces gene expression partly through PI3K and their association with vascularization and heart rate

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Background: Tissue inhibitor of metalloproteinase 3 (TIMP3) was recently demonstrated capable to regulate some gene expression in a myocardial infarction model. Here we aim to explore the gene expression profile in TIMP3-treated cardiomyocytes and related potential cardiovascular functions.
Methods: Total RNA extracted from cultured neonatal rat ventricular myocytes (NRVMs) were used for RNA sequencing analysis and real-time PCR. KEGG pathway enrichment assay and Ingenuity Pathway Analysis (IPA) were performed to study the signaling pathways and downstream effects. Western blot was used to detect phosphorylation of protein kinase B (Akt). A Cell Counting Kit-8 assay was employed to evaluate the proliferation of human umbilical vein endothelial cells (HUVECs). Contraction rate of NRVMs was measured with microscopy.
Results: RNA sequencing data showed that expression of 2,526 genes were significantly modulated by recombinant TIMP3 (rTIMP3, 100 ng/ml) in NRVMs. Some differentially expressed genes (DEGs) were validated with real-time PCR. Several KEGG pathways including the phosphoinositide-3-kinase (PI3K)-Akt pathway were significantly regulated by rTIMP3. Phosphorylation of Akt was increased by rTIMP3 and a PI3K inhibitor LY294002 suppressed rTIMP3-induced up-regulation of some genes. Some DEGs were predicted by IPA to increase vascularization, and some to decrease heart rate. RTIMP3 could reduce the contraction rate of NRVMs and its conditioned media increased the proliferation of HUVECs.
Conclusion: TIMP3 can regulate expression of multiple genes partly through PI3K. Some DEGs were associated with activation of vascularization and some with heart rate reduction. This study suggests that TIMP3 can potentially modulate cardiovascular functions *via* DEGs.

KEYWORDS

tissue inhibitor of metalloproteinase 3, angiogenesis, heart rate, cardiovascular system, signal pathway, gene expression, phosphoinositide-3-Kinase (PI3K)

Introduction

Tissue inhibitor of metalloproteinase 3 (TIMP3) is a member of the TIMP family (TIMP1–4). As a physiological inhibitor of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs), TIMP3 can also interact with a variety of molecules or receptors, but the roles of the interactions remain largely unknown (1–5). Deficiency of TIMP3 can worsen cardiac remodeling and dysfunction after myocardial infarction (MI) (6–8), which can be ameliorated by supply of TIMP3 through overexpression or recombinant protein (1, 9–13). Interestingly, there is a dose-dependent

effect for TIMP3 on angiogenesis (9). Recombinant TIMP3 (rTIMP3) at and above 1000 ng/ml can competitively inhibit the binding of vascular endothelial growth factor (VEGF) to its receptor 2 (VEGFR2) and suppress VEGF-induced angiogenesis (2, 9), however, rTIMP3 at 10 and 100 ng/ml can enhance VEGF-induced endothelial sprouting with unknown mechanism (9). TIMP3 can also interact with Type-2 angiotensin II receptor (AGTR2), but silencing AGTR2 did not influence the anti-angiogenic effect of TIMP3 (5). Therefore, the function of the interaction and the pro-angiogenic mechanism of TIMP3 require further investigations.

RTIMP3 can suppress MI-induced mRNA levels of MMP9, monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL8) in porcine MI heart (13). Recently, Boutagy et al. found that a full-length rTIMP3 reduced but an N-terminal TIMP3 enhanced cytokines (mainly interleukins and chemokines) expression in the porcine MI heart (14). RTIMP3 (1000 ng/ml) was also demonstrated capable to reduce expression and phosphorylation of epidermal growth factor receptor (EGFR) and to suppress the proliferation of neonatal mouse cardiomyocytes (15). Compared to apolipoprotein E (ApoE)-knockout mice, double knockout of ApoE and TIMP3 significantly decreased expression of apelin (APLN) and enzymes involved in fatty acid oxidation in the heart (16). Therefore, TIMP3 has capability to regulate gene expression in the heart but the influence varies with different length of TIMP3. So far the whole transcriptional profile in cardiomyocytes regulated by TIMP3 alone remains to be explored.

Therefore, we hypothesized that TIMP3 could affect cardiovascular function through regulating different gene expression. We treated neonatal rat ventricular myocytes (NRVMs) with rTIMP3 at 100 ng/ml and screened mRNA expression with RNA sequencing. Differentially expressed genes (DEGs) were analyzed with KEGG pathway enrichment analysis, as well as Ingenuity Pathway Analysis (IPA) for potential upstream regulators and downstream effects. Contraction rate of NRVMs and the proliferation of human umbilical vein endothelial cells (HUVECs) were also measured. We found that more than 2,000 DEGs were significantly modulated by rTIMP3. Phosphoinositide-3-kinase (PI3K) and AGTR2 were involved partly in rTIMP3-induced gene expression. Some DEGs were predicted to increase vascularization, and some to reduce heart rate. RTIMP3 could reduce the contraction rate of NRVMs, and the conditioned media of NRVMs treated with rTIMP3 could enhance the proliferation of HUVECs. This is the first proof that TIMP3 can regulate expression of multiple genes in cardiomyocytes which may mediate its cardiovascular functions.

Materials and methods

Isolation and culture of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured as described previously (17–19) with minor modifications. In brief, ventricles from neonatal (1–3 days old) Sprague-Dawley rats were minced and digested in Hanks' Balanced Salt Solution (HBSS, Gibco, United States) containing collagenase type II (Worthington, United States) and trypsin (Gibco). After digestion, the cells were pre-incubated in culture media (DMEM with addition of 1% penicillin/streptomycin and 10% FBS) for 90 min to exclude non-myocytes. Unattached myocytes were seeded to new culture dishes or multi-well plates with culture media for 48 h. All myocytes were treated with rTIMP3 (RayBiotech, United States) or phosphate-buffered saline (PBS) after serum-deprivation for 24 h. All experimental procedures involving animals were in accordance with the protocol approved by the Animal Care and Use Committee of Zunyi Medical University.

Real-time polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Invitrogen, United States) according to its manufacturer's instructions. And cDNA was produced from the total RNA by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, United States). Real-time polymerase chain reaction (PCR) was done with the BeyoFast^{TM} SYBR Green qPCR Mix kit (Beyotime, China) using QuantStudio 5 (Applied Biosystems). Relative expression levels of mRNAs were calculated with the $2^{-\Delta\Delta CT}$ method (20, 21). The primers used in the real-time PCR were designed with an online Primer-BLAST tool (NCBI) (18, 22–24) and listed in the **Supplementary Table S1**.

RNA sequencing, upstream regulators and downstream function analysis

The RNA samples were sent to Novogene (China) for RNA sequencing on an Illumina NovaSeq 6,000 platform. The raw sequence reads were deposited in the NCBI Sequence Read Archive (SRA) database and the accession number is PRJNA923808. Differentially expressed genes (DEGs) were those with |fold changes| $\geq$2 and $p.adjust < 0.05$. Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis was performed with the DEGs to identify related signaling pathways. Upstream regulators and downstream functions of the DEGs were evaluated by using Upstream Regulator Analysis and Downstream Effects Analysis *via* Ingenuity Pathway Analysis (IPA, version 81348237, Qiagen) software. Potential KEGG pathway enrichment and downstream cardiovascular system development and function were illustrated with an R package ggplot2 (25). Heat maps showing the changes of related DEGs were generated using TBtools software (26).

Gene set enrichment analysis (GSEA) (27, 28) was also employed to determine the enrichment of rat KEGG pathway gene sets with modest but coordinated changes comparing control and rTIMP3 groups. Enrichment score (ES), normalized ES, nominal p value and false discovery rate (FDR, q value) were calculated by using a GSEA program (v4.3.2) developed by the

Broad Institute and a WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) as reported (27–29).

Western blot

Total protein was extracted from NRVMs with RIPA Lysis Buffer (Beyotime, China). Western blots for phosphorylated protein kinase B (*p*-Akt), total Akt and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with their corresponding antibodies (Cell Signaling Technology, United States) were performed as previously described (17, 30). The band intensity was measured with ImageJ software (National Institutes of Health, United States) and the ratio of *p*-Akt to total Akt was calculated.

Cell area measurement of neonatal rat ventricular myocytes

NRVMs were treated with or without rTIMP3 (100 ng/ml). Then images of NRVMs were captured at 24 and 48 h by using microscopy (Olympus IX73) and Olympus cellSens Dimension software. Cell area was measured by ImageJ software.

Contraction rate of neonatal rat ventricular myocytes

NRVMs were treated with or without rTIMP3 (100 ng/ml) for 48 h and then myocyte contractions were observed and recorded by using microscopy (Olympus IX73) and Olympus cellSens Dimension software. Number of deflections per minute was counted as the rate of contractions as described (31).

Proliferation of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were purchased from iCell Bioscience Inc. (China) and cultured in the Endothelial Cell Medium (Sciencell Research Laboratories, United States). After 24 h of serum deprivation, HUVECs were incubated with the conditioned media from the NRVMs treated with or without rTIMP3 for 48 h. After 24 h, proliferation of HUVECs was measured with a Cell Counting Kit-8 (CCK-8, Beyotime, China) assay according to its manufacturer's instructions.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Student's *t*-test and one-way ANOVA followed by Bonferroni's multiple comparisons test were performed using GraphPad Prism software (V7.0). Statistical significance was considered at $p < 0.05$.

Results

The gene expression profile induced by TIMP3 in neonatal rat ventricular myocytes

To test the hypothesis that TIMP3 could regulate different gene expression in cardiomyocytes, we treated isolated NRVMs with or without 100 ng/ml rTIMP3, and then RNA levels were detected by RNA sequencing. Compared with the control group, 2,526 differentially expressed genes (DEGs) with |fold change| ≥2 and $p < 0.05$ were found in the rTIMP3 group. Among the DEGs, 1,062 genes were up-regulated and 1,464 genes down-regulated, which were shown in a volcano illustration (**Figure 1A**). We randomly chose some DEGs and verified their expression levels with real-time PCR. The results showed that ADAM12, ADAM17, ADAMTS7, APLN, interleukin-1β (IL1β), IL6, IL33, MMP2, MMP3 and MMP9 were significantly up-regulated, while activating transcription factor 3 (ATF3) and dual specificity mitogen-activated protein kinase kinase 6 (MAP2K6) were significantly down-regulated (**Figure 2**), which were consistent with the RNA sequencing data. Therefore, the RNA sequencing data were reliable.

KEGG pathways regulated by TIMP3

The DEGs regulated by rTIMP3 were used for KEGG pathway enrichment analysis. 19 pathways were significantly modified, including adrenergic signaling in cardiomyocytes, cAMP signaling pathway, cardiac muscle contraction, cytokine-cytokine receptor interaction, dilated cardiomyopathy (DCM), extracellular matrix (ECM)-receptor interaction, focal adhesion, hypertrophic cardiomyopathy (HCM), mitogen activated protein kinase (MAPK) signaling pathway, nuclear factor-κB (NF-κB) signaling pathway, and PI3K-Akt signaling pathway (**Figure 1B**). All genes (15,631) with reads higher than zero in RNA sequencing were also analyzed by GSEA with gene sets of rat KEGG pathways, which can detect modest but coordinate alterations comparing two groups (27, 28). Compared to the control group, rTIMP3 significantly up-regulated signaling pathways of chemokine, cytokine-cytokine receptor interaction, MAPK, NF-κB, PI3K-Akt, TNF, and Toll-like receptor, while down-regulated pathways of adrenergic signaling in cardiomyocytes, cardiac muscle contraction, DCM, HCM, and oxidative phosphorylation (**Supplementary Table S2**).

Downstream cardiovascular functions of differentially expressed genes regulated by TIMP3

Those DEGs were also used for Downstream Effects Analysis with IPA software. 129 DEGs were predicted to increase cell movement of endothelial cells in cardiovascular system development and function (activation *z*-score = 3.21, $p < 0.01$,

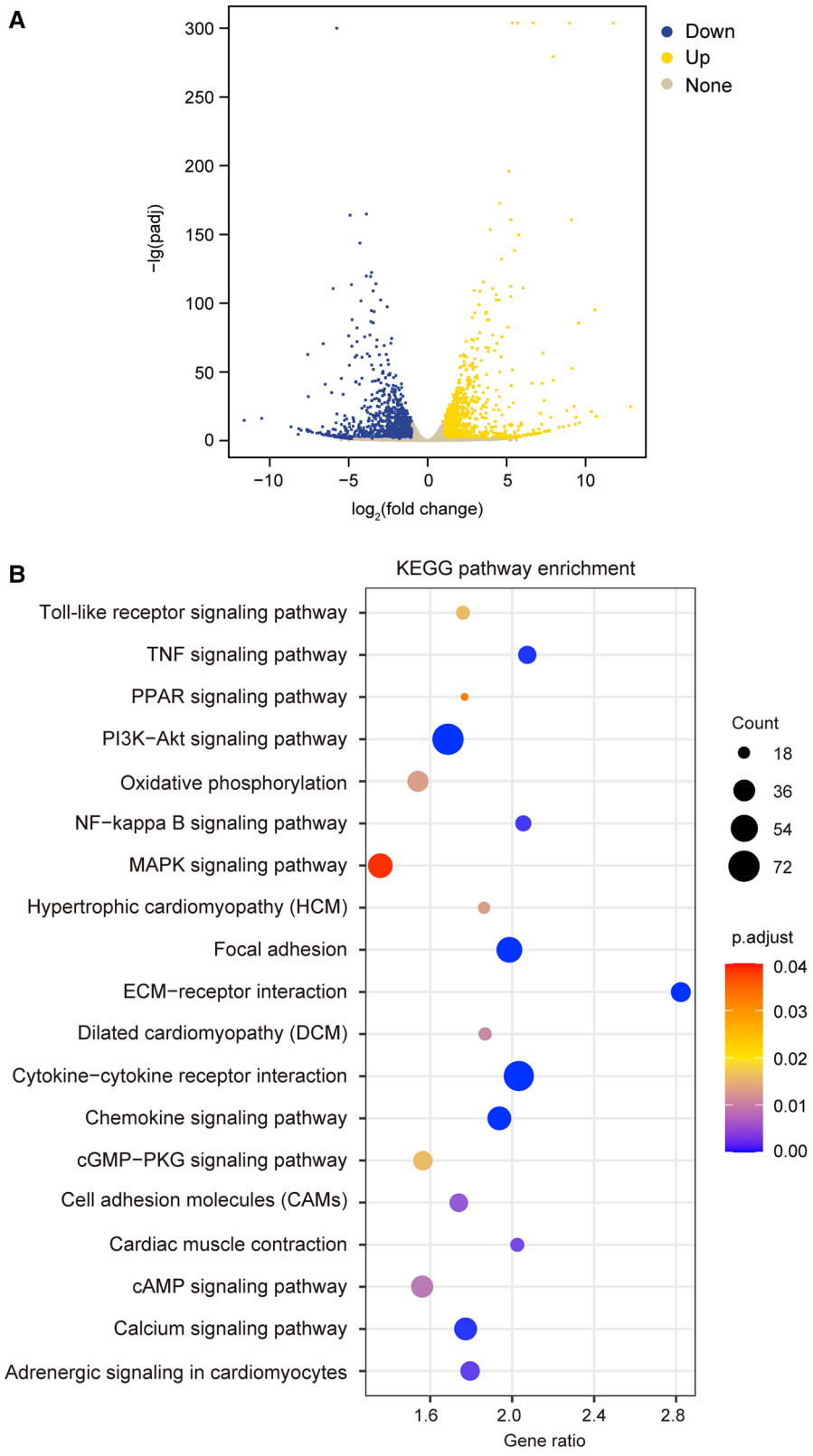

FIGURE 1
Recombinant TIMP3 (rTIMP3) modulated differential gene expression and potential pathways involved. (**A**) A volcano picture shows differentially expressed genes (DEGs) regulated by rTIMP3, including up-regulated (Up) and down-regulated DEGs (Down) with change fold ≥2 and *p.adjust* < 0.05 and other genes without significance (None). (**B**) KEGG pathway enrichment figure shows some pathways that were significantly regulated by rTIMP3 through DEGs in cardiomyocytes.

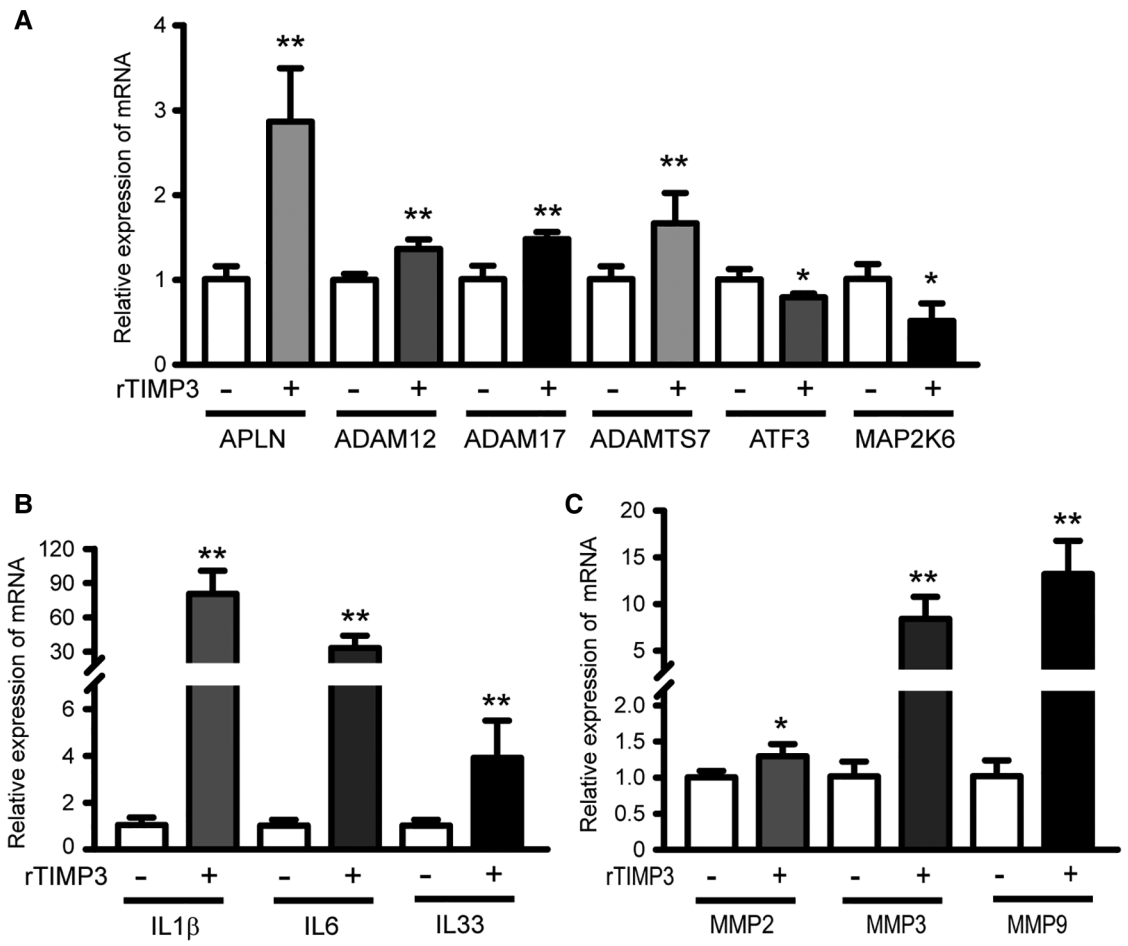

FIGURE 2
Some DEGs regulated by rTIMP3 were verified by real-time PCR. Neonatal rat ventricular myocytes were treated with rTIMP3 (100 ng/ml) and mRNA levels ($n = 3$), were determined with real-time PCR. ADAM, a disintegrin and metalloproteinase; ADAMTS7, ADAM with thrombospondin motifs 7; APLN, apelin; ATF3, activating transcription factor 3; IL, interleukin; MAP2K6, dual specificity mitogen-activated protein kinase kinase 6; MMP, matrix metalloproteinase. *$p < 0.05$, **$p < 0.01$ vs. control.

Supplementary Figure S1). 92 DEGs including IL1β, IL6, MMP2, MMP3 and MMP9, whose expression levels were verified by real-time PCR (Figure 2), were predicted to increase vascularization (activation z-score = 2.04, $p < 0.01$, Supplementary Figure S1), and the DEGs were exhibited in a heat map (Figure 3A). DEGs induced by rTIMP3 also have the potential to positively regulate vasculogenesis (activation z-score = 1.90, $p < 0.01$) and angiogenesis (activation z-score = 1.60, $p < 0.01$, Supplementary Figure S1). However, rTIMP3 significantly decreased VEGF-D expression and did not significantly affect the expression of VEGF-A, -B, -C and VEGFR2 in NRVMs (data not shown). A recent study showed that paracrine molecules thymosin $\beta 4$ (TMSB4) and prothymosin α (PTMA) released from cardiomyocytes can promote angiogenesis in the infarcted heart (32). In our study, TMSB4 (1.6-fold, $p < 0.01$) but not PTMA was up-regulated by rTIMP3 in cardiomyocytes (data not shown). We also tested the paracrine hypothesis in an *in vitro* experiment. Results showed that the conditioned media from NRVMs treated with rTIMP3 could increase the proliferation of HUVECs compared to control conditioned media (Supplementary Figure S2). This suggests that rTIMP3 may promote angiogenesis/vascularization through paracrine molecules from cardiomyocytes.

Meanwhile, 111 DEGs were predicted to decrease heart rate (activation z-score = −2.36, $p < 0.01$) in cardiovascular system development and function (Supplementary Figure S1). The DEGs were illustrated in a heat map (Figure 3B) and some mRNA levels (ADAM17, APLN, ATF3 and MAP2K6) were confirmed with real-time PCR (Figure 2). Then we studied this role with cultured NRVMs. The results demonstrated that rTIMP3 could decrease the contraction rate of NRVMs (Supplementary Figure S3, Supplementary Videos S1–S2). The gene set of cardiac muscle contraction was also significantly down-regulated by rTIMP3 in GSEA analysis (Supplementary Table S2). Therefore, rTIMP3 may reduce heart rate *via* the DEGs found in this project.

As we have found that deficiency of TIMP3 reduced angiotensin II (Ang II)-induced cardiac hypertrophy (33). KEGG enrichment analysis with DEGs regulated by rTIMP3 showed that signaling pathways of HCM and DCM were significantly

FIGURE 3
Some DEGs modulated by rTIMP3 were involved in regulation of vascularization and heart rate. DEGs regulated by rTIMP3 were predicted to increase vascularization (**A**) or to decrease heart rate (**B**) *via* Ingenuity Pathway Analysis (IPA) software, and their alterations were presented with heat maps illustrated by using TBtools.

altered (Figure 1B). GSEA also found that gene sets of HCM and DCM pathways were significantly down-regulated in the rTIMP3 group compared with control (Supplementary Table S2). However, there was no activity pattern available for effects of DEGs on HCM and DCM in the downstream effect analysis *via* IPA software (Supplementary Figure S1). Then we treated NRVMs with rTIMP3 (100 ng/ml) and detected the cell area and mRNA levels of natriuretic peptide A (ANP), BNP, and myosin heavy chain 7 (MYH7). Compared with control, rTIMP3 significantly increased NRVMs area at 24 (Supplementary Figures S4A, B) and 48 h (data not shown), but not the mRNA levels of hypertrophic genes (ANP, BNP and MYH7) at 48 h (Supplementary Figure S4C). It has been reported that physiological cardiac hypertrophy exhibited enlargement of cardiomyocytes with no change or decreased expression of hypertrophic genes (34). Thus, rTIMP3 may trigger physiological hypertrophy in NRVMs.

Upstream regulators involved in TIMP3-induced gene expression

The DEGs were further used for the Upstream Regulator Analysis in IPA. A variety of molecules or proteins were predicted as upstream regulators, such as IL6, Toll-like receptor 2 (TLR2), NF-κB1, p38 MAPK, ERK1/2, JNK, TLR7, transforming growth factor α (TGFA), mothers against decapentaplegic homolog 3 (SMAD3), signal transducer and activator of transcription 3 (STAT3), prostaglandin G/H synthase 2 (PTGS2), PI3K, which were predicted to increase gene expression. Some regulators such as versican core protein (VCAN), B-cell lymphoma 6 protein homolog (BCL6), secreted frizzled-related protein 1 (SFRP1), cbp/p300-interacting transactivator 2 (CITED2), Peroxisome proliferator-activated receptor γ coactivator 1-α (PPARGC1A), glucocorticoid receptor (NR3C1) were predicted to inhibit gene expression. Some of these regulators/molecules themselves were also regulated by rTIMP3.

As the PI3K-Akt signaling pathway was also found as one of the top KEGG pathways and up-regulated in GSEA analysis, we studied the role of PI3K-Akt in TIMP3-induced gene expression. The phosphorylation of Akt was significantly increased by rTIMP3 in NRVMs (Supplementary Figure S5). And a PI3K inhibitor LY294002 significantly suppressed TIMP3-induced up-regulation of APLN, ADAM12, ADAM17, ADAMTS7, IL6, IL33, MMP2, MMP3 and MMP9, while enhanced TIMP3-induced up-regulation of IL1β (Figure 4). Therefore, PI3K mediated some gene expression induced by rTIMP3. However, the detailed signaling pathway demands further experimental investigations.

AGTR2 has been found to be able to interact with TIMP3 (5). Therefore, we also checked its role in TIMP3-induced gene express by using AGTR2 specific inhibitor PD123319 (P186, Sigma-Aldrich). Results showed that PD123319 did not affect TIMP3-induced up-regulation of APLN, ADAM12 and MMP3. PD123319 suppressed TIMP3-induced up-regulation of ADAM17, ADAMTS7, MMP2 and MMP9, while enhanced the up-regulation of IL1β, IL6 and IL33 (Figure 4). Therefore, the roles of AGTR2 in TIMP3-induced gene expression vary with different genes.

Discussion

TIMP3 has been found capable to interact with multiple molecules and receptors (1, 3–5), but whether the interactions have impact on gene expression or cardiovascular functions remain largely unknown. So far, most functions of TIMP3 in cardiovascular diseases are mediated primarily by its MMP-inhibitive effect and partly by its anti-inflammatory role (1, 10, 11, 13, 14, 35, 36). Here we demonstrated that rTIMP3 could significantly regulate expression of more than 2,000 genes in cultured NRVMs. Multiple upstream regulators including PI3K were involved in rTIMP3-induced differential gene expression in NRVMs, and rTIMP3 might activate vascularization and reduce heart rate through some DEGs.

TIMP3 has been demonstrated to influence several signal pathways. RTIMP3 (1000 ng/ml) can reduce expression and phosphorylation of epidermal growth factor receptor (EGFR) (15). TIMP3 can competitively bind to VEGFR2 and inhibit its activation (2, 9). TIMP3 can also interact with AGTR2 (5), but the role of the interaction demands further investigation. It has been demonstrated that activation of AGTR2 has negative or no effects on the expression of different genes in the brain tissue (37). Here we found that inhibition of AGTR2 with PD123319 had diverse roles in TIMP3-induced DEGs, including positive (IL1β, IL6 and IL33), negative (ADAM17, ADAMTS7, MMP2 and MMP9), and no effects (ADAM12, APLN and MMP3). Therefore, AGTR2 is partly involved in TIMP3-induced gene expression and its roles depend on specific genes, whereas the detailed mechanisms remain to be further studied.

TIMP3 could inhibit VEGF-induced activation of PI3K in endothelial cells (5) but whether TIMP3 alone can influence the activity of PI3K remain unclear. Recently, Yang et al. found that the PI3K-Akt pathway was promoted by TIMP3 silencing in cervical cancer cells and osteosarcoma cells (38, 39). TIMP3 may suppress the PI3K-Akt pathway *via* up-regulation of phosphatase and tensin homolog (PTEN) (39). Here we demonstrated that the PI3K-Akt pathway was significantly up-regulated by rTIMP3 in GSEA analysis and the phosphorylation of Akt was significantly increased by rTIMP3 in NRVMs. Meanwhile, expression of PTEN was not significantly affected by rTIMP3 in NRVMs (data not shown). Inhibition of PI3K suppressed rTIMP3-induced up-regulation of APLN, ADAM12, ADAM17, ADAMTS7, IL6, IL33, MMP2, MMP3 and MMP9 in NRVMs. Therefore, rTIMP3 can promote expression of some genes through the PI3K-Akt pathway in cardiomyocytes. However, LY294002 further enhanced rTIMP3-induced expression of IL1β. Thus, the role of PI3K in TIMP3-mediated gene expression may vary with different genes and in different cells. IPA also predicted other upstream regulators mediating TIMP3-regulated gene expression, such as MAPK family members, STAT3, TGFA, CITED2, PPARGC1A, and NR3C1, which require further experimental investigations.

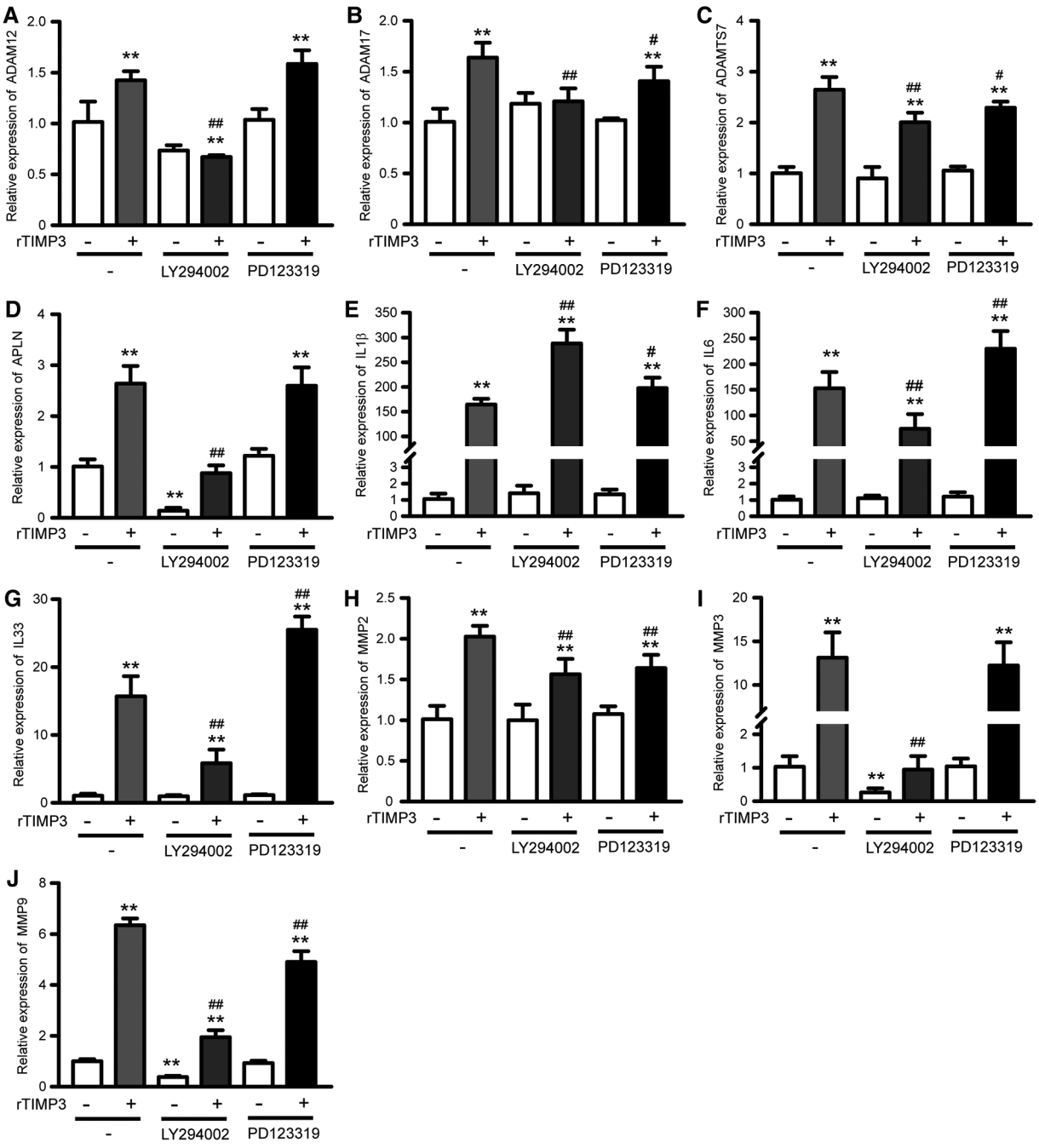

FIGURE 4
Pi3k and AGTR2 participated in rTIMP3-induced gene expression. Neonatal rat ventricular myocytes were treated with rTIMP3 (100 ng/ml) for 48 h with or without pre-incubation of specific inhibitors for phosphoinositide-3-kinase (PI3K, LY294002, 10 μM) or Type-2 angiotensin II receptor (AGTR2, PD123319, 1 μM) for 30 min. Total RNAs were collected and mRNA levels of ADAM12, ADAM17, ADAMTS7, APLN, IL1β, IL6, IL33, MMP2, MMP3, and MMP9 were detected by using real-time PCR ($n = 3$). **$p < 0.01$ vs. control, # $p < 0.05$, ## $p < 0.01$, vs. rTIMP3.

KEGG Signaling in HCM and DCM was significantly regulated by rTIMP3, and GSEA revealed that gene sets of both cardiomyopathy were significantly down-regulated, although specific activation pattern could not be found for HCM or DCM with the downstream effect analysis in IPA software. Compared with pathological cardiac hypertrophy, physiological hypertrophy exhibits enlargement of cardiomyocytes with no change or decreased expression of hypertrophic (fetal) genes (34). Here we found that rTIMP3 could induce physiological hypertrophy in NRVMs as the cell area but not the hypertrophic genes was significantly enhanced. It has been demonstrated that exercise-induced physiological cardiac hypertrophy can protect against pathological cardiac hypertrophy and heart failure (40–42). Therefore, it is worthy to study whether rTIMP3-induced physiological cardiomyocyte hypertrophy had such similar cardioprotective effects. However, the response of NRVMs may

not be the same as adult cardiomyocytes or the *in vivo* status. Therefore, effects of rTIMP3 on these two types of cardiomyopathy require further experimental studies. Meanwhile, deficiency of TIMP3 has been found capable to suppress Ang II-induced cardiac hypertrophy (33), but to enhance pressure overload-induced DCM primarily due to increased activity of ADAM17, TNFα and MMPs (43). Lack of TIMP3 also result in enhanced activation of MMPs and DCM in MI mice (8), which could be suppressed by MMP inhibition or addition of TIMP3 (8, 10, 12, 13). Therefore, the mechanism of TIMP3-modulating cardiac morphology may primarily relate to its MMP inhibitive role.

Angiogenesis or vascularization play a very important role in cardiac repair post MI (9, 44, 45). RTIMP3 at concentration of 1000 ng/ml and above can restrain VEGF-induced angiogenesis through binding to and inhibiting VEGFR2 (2, 9), however, rTIMP3 at 10 and 100 ng/ml can promote VEGF-induced endothelial sprouting with unknown mechanism (9). Overexpression of TIMP3 to some extent can also improve cardiac structure and function post MI by ameliorating adverse cardiac remodeling and promoting angiogenesis (9). These studies indicate that TIMP3 may regulate angiogenesis in a concentration-dependent way. Recent studies showed that paracrine molecules such as VEGF, TMSB4, PTMA, MMP2 and MMP9 released from cardiomyocytes can stimulate angiogenesis during cardiac repair post-MI (32, 46–48). We found that TMSB4 but not PTMA was increased by rTIMP3 in NRVMs. 92 DEGs induced by rTIMP3 (100 ng/ml) in this study were also predicted to promote vascularization and angiogenesis by IPA software. The conditioned media of NRVMs treated with rTIMP3 could increase the proliferation of HUVECs. Therefore, rTIMP3 has the potential to trigger angiogenesis *via* a paracrine way. The DEGs include IL1β, IL6, MMP2, MMP3 and MMP9, but rTIMP3 did not affect the expression of most VEGF family members and even significantly decreased expression of VEGFD. Therefore, TIMP3 may not promote vascularization through VEGF-VEGFR system directly but *via* enhanced expression of inflammatory cytokines and matrix-degrading proteins. On the other hand, TIMP3 can inhibit the enzyme activity of MMPs, which makes the roles of MMPs in vascularization complicated and further investigations are required.

Increased heart rate has been related to poor cardiac function and in-hospital death after MI, and heart rate can be used as a therapeutic target for heart failure (49–51). Recently, reducing heart rate moderately has been proved capable to stimulate cardiomyocyte proliferation under physiological conditions (52). Heart rate reduction can also promote cardiac regenerative repair and angiogenesis after myocardial damage including MI (52). The heart rate was not significantly influenced by deficiency or overexpression of TIMP3 in animal experiments (8, 9), which may be related to that mice were under anesthesia when the heart rate was measured by ultrasound equipment. Therefore, the role of heart rate reduction in TIMP3-mediated protection against cardiac injury remain unclear. It was demonstrated that apelin can reduce heart rate and inducibility of atrial fibrillation (53, 54). Apelin mRNA was reduced in mice with double knockout of ApoE and TIMP3, compared with ApoE-knockout mice (16). And 32-weeks old double knockout mice had more arrhythmic episodes which was detected by using invasive telemetry 24 hour electrocardiography in freely moving mice (16). Therefore, apelin may play an important role in heart rate reduction potentially mediated by TIMP3. In our study, apelin (APLN) was found increased by rTIMP3 and it was included in 111 DEGs that were predicted to reduce heart rate. GSEA also found that the gene set of cardiac muscle contraction was significantly down-regulated by rTIMP3. Meanwhile, the contraction rate of NRVMs was decreased by rTIMP3. Therefore, the 111 DEGs provide potential molecular mechanisms for possible reduction of heart rate by TIMP3.

This study has several limitations or some perspectives to research in future. We used isolated and cultured NRVMs for the gene expression profile, which may be different from the profile in adult cardiomyocytes or the heart *in vivo*. MRNA levels were determined by RNA sequencing and some were confirmed with real-time PCR, but protein levels of the DEGs were to be confirmed. Expression levels of some metalloproteinases, such as MMP-2, −3, −9, ADAM17, were significantly up-regulated, but their activities were not measured. As TIMP3 can also inhibit these metalloproteinases, their precise roles in TIMP3-modulated cardiovascular functions remain to be investigated. We did some *in vitro* experiments to study potential effects of rTIMP3 on vascularization and heart rate, but further *in vivo* experiments are required.

In conclusion, our results demonstrate that rTIMP3 can regulate differential gene expression at least partly through PI3K and AGTR2 in NRVMs. Some DEGs are predicted to activate vascularization and some to decrease heart rate. Meanwhile, rTIMP3 can reduce the contraction rate of NRVMs and its conditioned medium can increase the proliferation of HUVECs. Therefore, our study suggests that TIMP3 can potentially modulate cardiovascular functions through regulation of different gene expression.

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**.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Zunyi Medical University.

Author contributions

ZX performed the experiments. ZX, MS, YC, GC and DF analyzed the data. ZX, MS and DF interpreted results. ZX and DF prepared and revised all figures. DF conceived and designed

research. DF wrote 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

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1130388/full#supplementary-material.

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Early atrial remodeling predicts the risk of cardiovascular events in patients with metabolic syndrome: a retrospective cohort study

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Background: This study aims to assess the prevalence of atrial cardiomyopathy (ACM) in patients with new-onset metabolic syndrome (MetS) and investigate whether ACM could be a predictor of hospital admission for cardiovascular (CV) events.

Methods: Patients with MetS who were free of clinically proven atrial fibrillation and other CV diseases (CVDs) at baseline were included in the present study. The prevalence of ACM was compared between MetS patients with and without left ventricular hypertrophy (LVH). The time to first hospital admission for a CV event between subgroups was assessed using the Cox proportional hazard model.

Results: A total of 15,528 MetS patients were included in the final analysis. Overall, LVH patients accounted for 25.6% of all newly diagnosed MetS patients. ACM occurred in 52.9% of the cohort and involved 74.8% of LVH patients. Interestingly, a significant percentage of ACM patients (45.4%) experienced MetS without LVH. After 33.2 ± 20.6 months of follow-up, 7,468 (48.1%) patients had a history of readmission due to CV events. Multivariable Cox regression analysis revealed that ACM was associated with an increased risk of admission for CVDs in the MetS patients with LVH [hazard ratio (HR), 1.29; 95% confidence interval (CI), 1.142–1.458; $P < 0.001$]. Likewise, ACM was found to be independently associated with hospital readmission due to CVD-related events in MetS patients without LVH (HR, 1.175; 95% CI, 1.105–1.250; $P < 0.001$).

Conclusion: ACM is a marker of early myocardial remodeling and predicts hospitalization for CV events in patients with MetS.

KEYWORDS

atrial myopathy, metabolic syndrome, left ventricular hypertrophy, cardiovascular diseases, atrial remodeling

Abbreviations

MetS, metabolic syndrome; LVH, left ventricular hypertrophy; BMI, body mass index; HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ACM, atrial myopathy; LA, left atrium; PTFV1, P-wave terminal force in V1; CV, cardiovascular; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; LVEF, left ventricular ejection fraction; HR, hazard ratio; CI, confidence interval.

Introduction

The burden of cardiovascular disease (CVD) is expected to more than double in the next three decades because of the increased average global life expectancy (1). Consequently, the rate of hospitalization due to CVD in healthcare facilities is equally expected to increase. This demands intensive efforts from the scientific community to identify predictors or indicators of the risk of hospitalization due to CVD.

Metabolic syndrome (MetS) is a cluster of metabolic disorders including glucose intolerance, low levels of high-density lipoprotein cholesterol (HDL-C), high levels of triglycerides (TG), obesity, and hypertension (HTN) (2, 3). MetS, frequently combined with other cardiovascular (CV) risk factors, increases CV morbidity and mortality (4). There is emerging evidence that MetS is associated with left ventricular hypertrophy (LVH), a hallmark of preclinical CV diseases (CVDs) (5). However, LVH is not a sensitive marker for myocardial damage.

Atrial cardiomyopathy (ACM), the structural and pathophysiologic changes in the atria, can lead to sustained cardiac arrhythmia. Such dysrhythmia is denoted as atrial fibrillation (AF) (6). The underlying mechanisms involving ACM are atrial dilation (7), fibrosis (8), endothelial cell dysfunction (9), and impaired myocyte function (10). Various electrocardiographic (ECG), echocardiographic, and serum markers have been found to be associated with ACM, such as increased P-wave terminal force in V1 (PTFV1) (11), paroxysmal supraventricular tachycardia (12), premature atrial contraction (13), increased PR interval (14), increased left atrial (LA) size (15) or volume (16), and elevated N-terminal pro-B-type natriuretic peptide (16).

Recently, there has been an increased interest in the diagnostic value of LA remodeling in myocardial injury. This may be attributed to the fact that LA enlargement (LAE) occurs earlier than LVH and is regarded as an independent risk factor for CV events (17). Understanding the prevalence and impact of ACM in MetS may shed light on the risk of hospitalization due to CVDs. Thus, here, we aimed to determine the prevalence of ACM and its effect on the risk of hospitalization for CV events in patients with MetS.

Methods

Study design and participants

This retrospective cohort study was conducted on the basis of data obtained from the Electronic Medical Record Research Database (EMRRD) of the First Affiliated Hospital of Dalian Medical University (FAHDM). The EMRRD was developed to establish a computerized clinical database, and the clinical records are continuously updated (18). A total of 37,764 patients who experienced MetS and were hospitalized at the FAHDM between 1 January 2011 and 31 June 2021 were initially recruited. Patients with a history of AF, secondary HTN, coronary heart disease (including a history of angina pectoris, myocardial infarction, coronary revascularization, or more than 50% narrowing of one of the epicardial coronary arteries on coronary computed angiography), heart failure, cardiac valvular stenosis, moderate or severe valvular regurgitation, cardiomyopathy, severe hepatic and renal dysfunction, and malignant tumor, and/or whose data were missing or contained errors were excluded from the study. After excluding patients who fulfilled the exclusion criteria, a total of 15,528 patients were included in the analysis (**Figure 1**).

Evaluation of the metabolic syndrome

Using the National Cholesterol Education Program Third Adult Treatment Panel (NCEP-ATP III) guidelines, MetS was defined on the basis of the presence of three or more of the following: increased waist circumference [≥90 cm in men or ≥85 cm in women], elevated TG [≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated TG], low HDL-C [<40 mg/dL (1 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women or medical treatment for low HDL-C], elevated blood pressure [systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥85 mmHg or current use of antihypertensive medication], and impaired fasting glucose [fasting plasma glucose ≥100 mg/dL (5.6 mmol/L) or current use of antidiabetic medication] (19).

Echocardiographic assessment

All subjects underwent transthoracic echocardiography at rest in the left lateral decubitus position using the Vivid 7 ultrasound system (GE Vingmed Ultrasound, Horten, Norway). Standard two-dimensional echocardiography with Doppler examination was performed, and measurements were obtained according to the guidelines of the American Society of Echocardiography (20). The left atrium diameter was obtained as previously reported (21), and LAE was defined on the basis of a posteroanterior dimension >35 mm. The left ventricular mass was measured by echocardiography, and the left ventricular mass index was calculated (22). LVH was defined on the basis of the following parameters: LVMI >115 g/m^2 for men and >95 g/m^2 for women (23). Experienced ultrasound experts who were blinded to the clinical data reviewed the echocardiography results.

Covariates, follow-up, and clinical outcomes

PTFV1 was calculated for all patients using standard digital 12-lead ECGs on admission and was obtained using digital calipers, measuring the absolute value of the depth (μV) times the duration (ms) of the downward deflection (terminal portion) of the P-wave in lead V_1. ACM was defined as PTFV1 >4,000 μV·ms or severe LAE (24, 25). A patient was considered

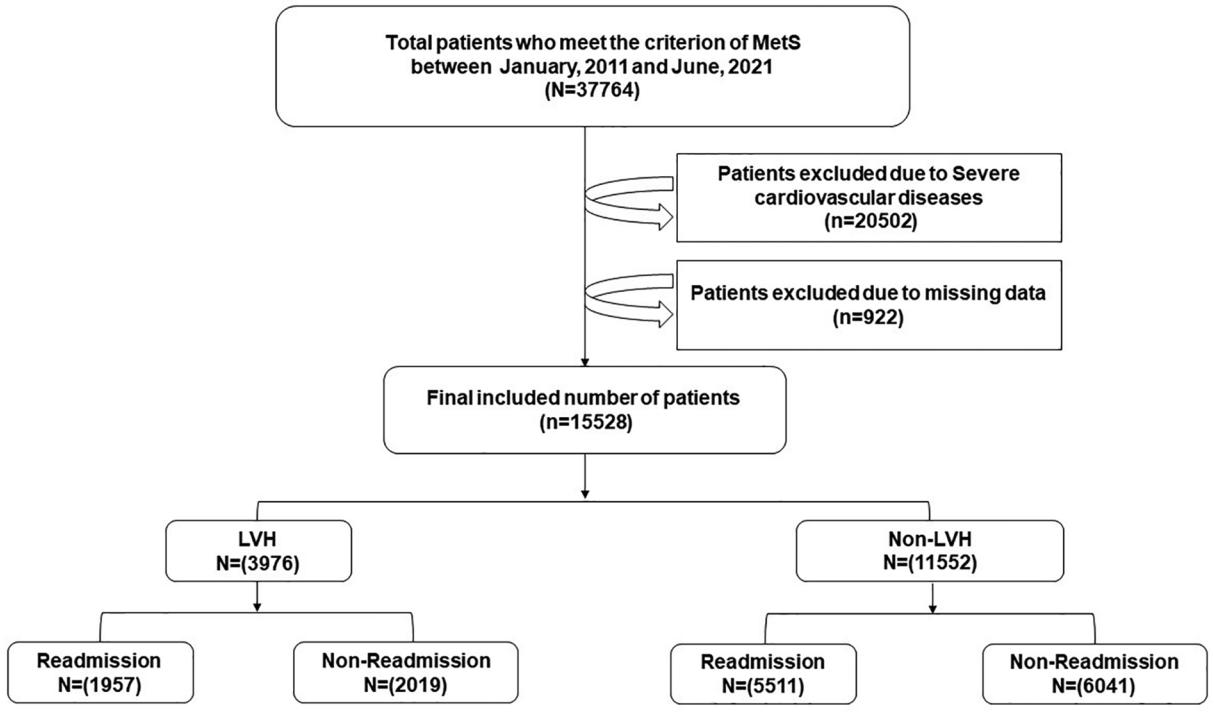

FIGURE 1
A brief overview of the selection of study participants. MetS, metabolism syndrome; LVH, left ventricular hypertrophy.

to have HTN if a systolic SBP ≥140 mmHg, a mean DBP ≥90 mmHg, and/or current use of an antihypertensive drug were shown in their medical history (26). According to the 2016 Chinese guidelines for the management of dyslipidemia in adults, dyslipidemia was defined on the basis of the following parameters: total cholesterol ≥6.22 mmol/L, low-density lipoprotein cholesterol (LDL-C) ≥4.14 mmol/L, HDL-C <1.04 mmol/L, TG ≥2.26 mmol/L, or an indication of the use of lipid-lowering drugs (27). Diabetes mellitus (DM) was defined in terms of fasting plasma glucose ≥126 mg/dL or treatment with insulin or oral hypoglycemic medication (28). Smoking was defined in terms of current smoking status or a lifetime consumption of >100 cigarettes.

The study endpoint was hospitalization for any CV event (acute myocardial infarction, congestive heart failure, ischemic stroke, or atrial fibrillation) during the follow-up period. Data were analyzed according to the number of hospitalizations for each CV event. The follow-up period was the time starting from the index date to the occurrence of hospitalization for a CV event or to the end of this period (31 October 2021), whichever came first.

Statistical analysis

Continuous variables with a normal distribution pattern are expressed as means ± standard deviations, whereas variables with a non-normal distribution pattern are presented as medians with 25th and 75th percentiles. For categorical variables, the chi-square test (χ^2) was used for comparison analysis, and data were presented using frequency and percentage. A comparison between continuous data for two independent groups was conducted using the Mann–Whitney *U*-test or independent-samples *T*-test. Cox proportional models were analyzed for determining the predictors of hospitalization for CV events. The findings were reported as a hazard ratio (HR) [95% confidence interval (CI)]. Statistically significant predictors in the univariate analysis were selected for multivariate analysis. Kaplan–Meier analysis with a log-rank test was performed to determine the effect of ACM related to the cumulative risk of hospitalization for CV events. The restricted mean survival time was the parameter used to estimate the expected value of time for patients to be free from CV events.

Results

Clinical characteristics of the study participants

A comparison of demographic and clinical variables between MetS patients with and without LVH is presented in **Table 1**. Overall, LVH accounted for 25.6% of patients hospitalized with MetS. The rate of prevalence of ACM in hospitalized patients who experienced MetS was 52.9%. The rates of ACM in hospitalized patients who presented with a normal LV size and

TABLE 1 Baseline characteristics.

Variables	All (15,528)	With LVH (3,976)	Without LVH (11,552)	*P*-value
Age, years	64.4 (57.5, 71.8)	62.3 (53.6, 69.5)	59.8 (51.2, 66.5)	<0.001
Male patients, *n* (%)	9,052 (58.3)	2,688 (67.6)	6,365 (55.1)	<0.001
BMI, kg/m^2	26.24 (25.39, 28.43)	26.47 (25.53, 28.43)	26.16 (25.36, 27.81)	<0.001
Smokers, *n* (%)	4,281 (29.9)	1,272 (35.0)	3,009 (28.1)	<0.001
Alcohol consumption, *n* (%)	2,862 (20.7)	867 (24.8)	1,995 (19.3)	<0.001
HTN, *n* (%)	11,611 (74.8)	3,295 (82.9)	8,312 (72.0)	<0.001
SBP, mmHg	140 (130, 156)	149 (133,165)	140 (129, 151)	<0.001
DBP, mmHg	83 (76, 91)	86 (78, 97)	81 (75, 90)	<0.001
DM, *n* (%)	5,353 (34.5)	1,605 (40.4)	3,748 (32.4)	<0.001
Dyslipidemia, *n* (%)	12,776 (82.3)	3,321 (83.5)	9,455 (81.8)	0.017
TC, mmol/L	4.87 (4.18, 5.62)	4.84 (4.14, 5.63)	4.88 (4.19, 5.62)	0.270
TG, mmol/L	1.76 (1.24, 2.42)	1.80 (1.29, 2.54)	1.75 (1.23, 2.38)	<0.001
HDL, mmol/L	1.14 (0.96, 1.36)	1.11 (0.94, 1.35)	1.14 (0.96, 1.37)	0.001
LDL, mmol/L	2.82 (2.35, 3.34)	2.79 (2.34, 3.32)	2.83 (2.35, 3.35)	0.355
Atrial myopathy, *n* (%)	8,222 (52.9)	2,976 (74.8)	5,246 (45.4)	<0.001
PTFV1 < −4,000 uV*ms, *n* (%)	3,735 (24.1)	1,324 (33.3)	2,411 (20.9)	<0.001
LA enlargement, *n* (%)	6,687 (43.1)	2,644 (66.5)	4,043 (35.0)	<0.001
Ptfv1, 4,000 uV*ms	−2,418 (−3,906, −1,015)	−2,961 (−4,640, −1,368)	−2,256 (−3,654, −936)	<0.001
LA diameter, mm	37 (35, 39)	38 (36, 40)	36 (34, 38)	<0.001
LVEF, %	60.59 ± 3.35	60.44 ± 3.54	60.64 ± 3.28	0.002
Lp(a), mg/L	124 (65, 237)	131 (67, 244)	123 (65, 233)	0.029
Creatinine, μmol/L	67 (56, 78)	71 (59, 84)	65 (55, 76)	<0.001
Medications, *n* (%)				
Antihypertension, *n* (%)	9,426 (60.7)	2,734 (68.8)	6,692 (57.9)	<0.001
ACEIs/ARBs	2,068 (13.3)	666 (16.8)	1,402 (12.1)	<0.001
β-blockers	6,117 (39.4)	1,788 (45.0)	4,329 (37.5)	<0.001
Calcium antagonists	6,205 (40.0)	2,108 (53.0)	4,097 (35.5)	<0.001
Diuretics	2,122 (13.7)	827 (20.8)	1,295 (11.2)	<0.001
Antidiabetic drugs, *n* (%)	4,707 (30.3)	1,413 (35.6)	3,294 (28.5)	<0.001
Lipid-lowering drugs, *n* (%)	9,892 (63.7)	2,726 (67.3)	7,216 (62.5)	<0.001

LVH, left ventricular hypertrophy; BMI, body mass index; HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ACM, atrial myopathy; LA, left atrium; PTFV1, P-wave terminal force in V1; LVEF, left ventricular ejection fraction; Lp(a), Lipoprotein(a); CV, cardiovascular; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker.

LVH were 45.4% and 74.8%, respectively, suggesting that ACM was also found in a large proportion of MetS patients without LVH. Those with LVH had an increased burden of CVD-related risk factors compared with their non-LVH counterparts. The median age (62.3 vs. 59.8, $P < 0.001$), body mass index (26.47 vs. 26.16, $P < 0.001$), SBP (149 vs. 140, $P < 0.001$), DBP (86 vs. 81, $P < 0.001$), and creatinine (71 vs. 65, $P < 0.001$) were higher in MetS patients with LVH than in MetS patients with a normal left ventricle ($P < 0.001$). Likewise, the proportion of smokers (35.0% vs. 28.1%, $P < 0.001$) and alcohol consumers (24.8% vs. 19.3%, $P < 0.001$) was higher in MetS patients with LVH than in those without LVH. In addition, the rates of HTN (82.9% vs. 72.0%, $P < 0.001$), DM (40.4% vs. 32.4%, $P < 0.001$), and dyslipidemia (83.5% vs. 81.8%, $P = 0.017$) were significantly higher in the LVH group than in the non-LVH group ($P < 0.05$).

As shown in **Table 2**, the incidence of admission for CV events is slightly higher in the LVH group (49.3%) than in patients with a normal left ventricle size (47.1%). Among the LVH group, hospital admission due to CV events was more common among the older patients (63.05 vs. 61.55, $P < 0.001$). Admitted patients with LVH also had an increased likelihood of having ACM (79.2% vs. 70.6%, $P < 0.001$). Moreover, the use of antihypertensive (57.0% vs. 80.2%, $P < 0.001$), antidiabetic (32.3% vs. 38.8%, $P < 0.001$), and lipid-lowering medications (58.9% vs. 75.7%, $P < 0.001$) was relatively less common in hospitalized patients with LVH. Similarly, the prevalence of HTN (78.1% vs., 87.5%, $P < 0.001$), DM (38.2% vs. 42.4%, $P < 0.001$), and dyslipidemia (80.4% vs. 86.5%, $P < 0.001$) was significantly lower among the hospitalized patients experiencing MetS with LVH.

Among patients who had MetS with LVH, CVD-related risk factors were more common among the admission group. For example, patients who were admitted were older (60.68 vs. 58.70, $P < 0.001$) and presented with ACM (49.9% vs. 41.3%, $P < 0.001$). Surprisingly, patients with admission for CV events were less likely to have HTN (76.7% vs. 66.8%, $P < 0.001$), DM (33.9% vs. 30.9%, $P < 0.001$), and dyslipidemia (85.0% vs. 78.4%, $P < 0.001$), which may be attributed to the greater use of antihypertensive (45.4% vs. 69.3%, $P < 0.001$), antidiabetic (26.7% vs. 30.2%, $P < 0.001$), and lipid-lowering (53.0% vs. 71.1%, $P < 0.001$) treatment in the admission group than in their counterparts.

Atrial cardiomyopathy for the prediction of cardiovascular disease-related readmissions

After a follow-up period of 33.2 ± 20.6 months, 7,468 (48.1%) patients were readmitted for CV events. The total duration of follow-up accounted for 43,049 person-years. Table 3 shows the results from the Cox proportional hazards model, revealing that ACM (HR, 1.29; 95% CI, 1.142–1.458) was associated with an increased risk of admission for CV events in patients with MetS and LVH. Also, ACM was found to be independently associated with the incidence of readmission due to CVD-related events in patients with MetS but with a normal LV size (HR, 1.175; 95% CI, 1.105–1.250). Moreover, factors such as older age, HTN, dyslipidemia, increased creatine levels, and poor adherence to antihypertensive drugs were associated with an increased likelihood of hospital admission. Among these variables, HTN accounted for the highest risk of admission in those patients experiencing MetS with a normal left ventricle size (HR, 1.634; 95% CI, 1.499–1.778) and LVH (HR, 1.521; 95% CI, 1.29–1.784). In addition, non-compliance with lipid-lowering and antidiabetic drugs significantly increased the risk of hospital admission due to CV events among those with Mets but with a normal left ventricle size.

Figures 2 shows the Kaplan–Meier curves for freedom for hospital admission in MetS patients with and without ACM, respectively. This result indicates that these individuals with ACM were more often rehospitalized for CVD compared with those without ACM. Also, the ACM group had a shorter free duration from hospitalization due to CVD events. Over the 5-year follow-up period, patients with MetS and LVH were expected to be free from hospital admission for 33.93 months if they suffered from ACM (95% CI, 33.26–34.60) and for 37.08 months (95% CI, 35.96–38.20) if they did not suffer from ACM (Figures 3). Also, patients with MetS but no evidence of LVH were expected to be free from hospital admission for 34.27 months (95% CI, 33.77–34.77) if they presented with ACM and for 37.01 months (95% CI, 36.57–37.45) if they were free from ACM.

TABLE 2 Baseline characteristics in patients with and without atrial myopathy grouped by those with and without LVH.

Variables	With LVH			Without LVH		
	No readmission	Readmission	*P*-value	No readmission	Readmission	*P*-value
	(2,019)	(1,957)		(6,041)	(5,511)	
Age, years	61.55 (52.60, 68.49)	63.05 (54.59, 70.52)	<0.001	58.70 (49.56, 65.89)	60.68 (52.87, 67.21)	<0.001
Male patients, *n* (%)	1,390 (68.8)	1,298 (66.3)	0.090	3,411 (56.5)	2,954 (53.6)	0.002
Smoker, *n* (%)	666 (37.1)	606 (32.9)	0.007	1,569 (28.8)	1,440 (27.5)	0.127
Alcohol consumption, *n* (%)	447 (26.1)	420 (23.6)	0.095	1,033 (19.7)	962 (19.0)	0.385
BMI, kg/m^2	26.47 (25.52, 28.41)	26.47 (25.54, 28.46)	0.828	26.17 (25.37, 27.81)	26.16 (25.35, 27.81)	0.390
HTN, *n* (%)	1,767 (87.5)	1,528 (78.1)	<0.001	4,633 (76.7)	3,683 (66.8)	<0.001
DBP, mmHg	89 (80, 99)	89 (80, 99)	0.078	82 (79, 90)	84 (78, 92)	0.001
SBP, mmHg	150 (138,165)	150 (139, 168)	0.017	140 (130, 152)	140 (130, 154)	0.005
DM, *n* (%)	857 (42.4)	748 (38.2)	0.007	2,047 (33.9)	1,701 (30.9)	0.001
Dyslipidemia, *n* (%)	1,747 (86.5)	1,574 (80.4)	<0.001	5,136 (85.0)	4,319 (78.4)	<0.001
TC, mmol/L	4.81 (4.12, 5.64)	4.86 (4.17, 5.62)	0.493	4.87 (4.15, 5.59)	4.89 (4.21, 5.66)	0.087
TG, mmol/L	1.72 (1.23, 2.37)	1.85 (1.35, 2.67)	0.001	1.66 (1.19, 2.30)	1.83 (1.28, 2.46)	<0.001
HDLC, mmol/L	1.16 (0.98, 1.38)	1.07 (0.91, 1.29)	<0.001	1.19 (1.00, 1.40)	1.10 (0.93, 1.32)	<0.001
LDLC, mmol/L	2.76 (2.28, 3.29)	2.81 (2.38, 3.35)	0.146	2.80 (2.33, 3.32)	2.85 (2.38, 3.37)	0.003
ACM, *n* (%)	1,426 (70.6)	1,550 (79.2)	<0.001	2,497 (41.3)	2,749 (49.9)	<0.001
LA enlargement, *n* (%)	1,280 (63.4)	1,364 (69.7)	<0.001	1,984 (32.8)	2,059 (37.4)	<0.001
PTFV1 < −4,000 uV*ms, *n* (%)	730 (37.3)	1,324 (33.3)	0.001	1,114 (18.4)	1,297 (23.5)	<0.001
LA diameter, mm	38 (36,40)	38 (36, 41)	<0.001	36 (34, 38)	36 (34, 39)	<0.001
PTFV1, 4,000 uV*ms	−2,666 (−4,399,−1,014)	−3,182 (−4,891, −1,833)	<0.001	−2,006 (−3,408, −720)	−2,535 (−3,886, −1,248)	<0.001
LVEF, %	60.41 ± 3.55	60.48 ± 3.53	0.523	60.67 ± 3.27	60.61 ± 3.29	0.356
Lp(a), mg/L	127 (66,244)	134 (68, 246)	0.530	121 (64,233)	127 (65, 235)	0.428
Creatinine, μmol/L	71 (60,83)	70 (59, 85)	0.832	65 (55,76)	65 (54, 75)	0.009
Medication, *n* (%)						
Antihypertriton, *n* (%)	1,619 (80.2)	1,115 (57.0)	<0.001	4,185 (69.3)	2,507 (45.5)	<0.001
ACEI	396 (19.6)	270 (13.8)	<0.001	857 (14.2)	545 (9.9)	<0.001
β-blockers	1,129 (55.9)	659 (33.7)	<0.001	2,796 (46.3)	1,533 (27.8)	<0.001
CCB	1,233 (61.1)	875 (44.7)	<0.001	2,505 (41.5)	1,592 (28.9)	<0.001
Diuretics	453 (22.4)	374 (19.1)	0.010	743 (12.3%)	552 (10.0)	<0.001
Antidiabetic drugs	783 (38.8)	630 (32.2)	<0.001	1,824 (30.2)	1,470 (26.7)	<0.001
Lipid-lowering drugs	1,524 (75.5)	1,152 (58.9)	<0.001	4,297 (71.1)	2,919 (53.0)	<0.001

LVH, left ventricular hypertrophy; BMI, body mass index; HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ACM, atrial myopathy; LA, left atrium; PTFV1, P-wave terminal force in V1; LVEF, left ventricular ejection fraction; Lp(a), Lipoprotein(a); CV, cardiovascular; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker.

TABLE 3 Univariate and multivariate COX analysis predictors of admission for cardiovascular events.

Variables	Univariate analysis						Multivariate analysis					
	With LVH			Without LVH			With LVH			Without LVH		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.009	1.005–1.013	<0.001	1.013	1.011–1.015	<0.001	1.009	1.005–1.014	<0.001	1.016	1.013–1.019	<0.001
Gender	0.959	0.873–1.054	0.384	0.974	0.924–1.027	0.332	1.010	1.005–1.014	0.873	1.006	0.933–1.086	0.871
Smoker	0.893	0.810–0.984	0.023	0.965	0.909–1.026	0.256	0.959	0.830–1.108	0.569	1.039	0.945–1.142	0.427
Alcohol	0.917	0.822–1.023	0.120	0.971	0.905–1.041	0.405	0.992	0.853–1.153	0.917	1.108	0.992–1.123	0.725
BMI	1.001	0.982–1.020	0.908	0.990	0.977–1.004	0.151	1.015	0.993–1.038	0.189	1.003	0.987–1.018	0.739
ACM	1.374	1.232–1.533	<0.001	1.283	1.217–1.352	<0.001	1.290	1.142–1.458	<0.001	1.175	1.105–1.250	<0.001
LVEF	0.996	0.984–1.009	0.566	0.994	0.986–1.003	0.176	1.007	0.992–1.021	0.355	0.993	0.984–1.002	0.150
HTN	0.789	0.709–0.878	<0.001	0.806	0.752–0.853	<0.001	1.521	1.297–1.784	<0.001	1.634	1.499–1.778	<0.001
DM	0.879	0.803–0.963	<0.001	0.872	0.824–0.924	<0.001	1.089	0.873–1.357	0.450	0.852	0.725–1.001	0.051
Dyslipidemia	0.850	0.760–0.950	0.004	0.797	0.748–0.850	<0.001	1.223	1.041–1.438	0.015	1.147	1.045–1.259	0.004
Creatine	1.001	1.000–1.001	0.001	1.002	1.001–1.003	<0.001	1.001	1.001–1.002	<0.001	1.002	1.001–1.003	0.003
Antihypertension	0.595	0.544–0.650	<0.001	0.578	0.548–0.609	<0.001	0.484	0.417–0.562	<0.001	0.453	0.416–0.493	<0.001
Lipid-lowering	0.737	0.674–0.807	<0.001	0.687	0.651–0.724	<0.001	0.881	0.753–1.032	0.113	0.774	0.712–0.841	<0.001
Antidiabetics	0.824	0.750–0.906	<0.001	0.856	0.807–0.909	<0.001	0.889	0.713–1.135	0.371	1.242	1.049–1.470	0.012

LVH, left ventricular hypertrophy; BMI, body mass index; LVEF, left ventricular ejection fraction; ACM, atrial myopathy; LVH, left ventricular hypertrophy; HTN, hypertension; DM, diabetes mellitus; HR, hazard ratio; CI, confidence interval.

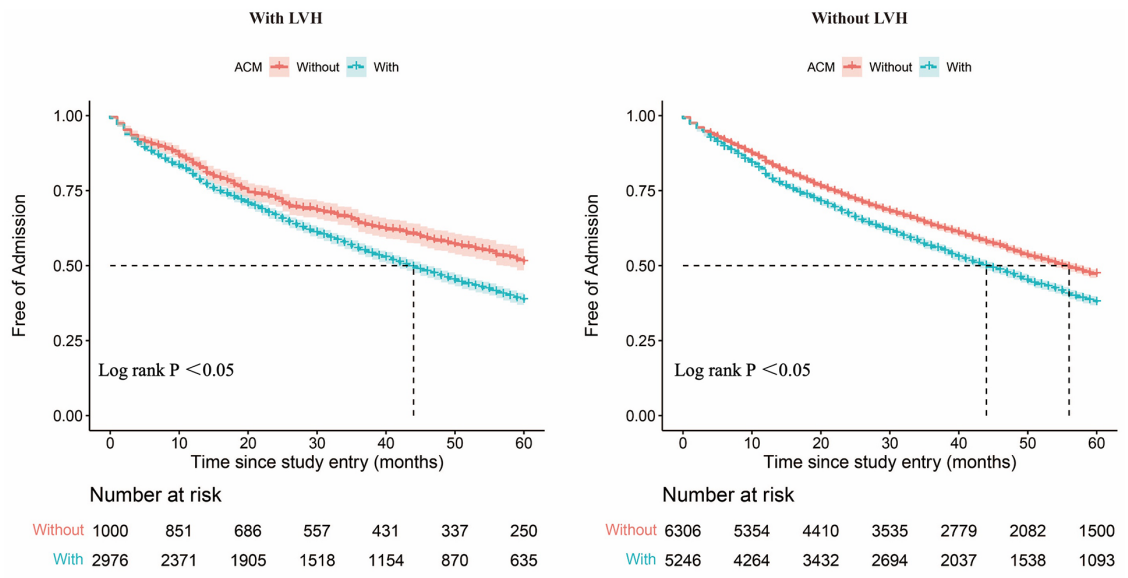

FIGURE 2
Admission-free survival curves. Admission-free survival curves for patients with and without the ACM group among patients with LVH. Admission-free survival curves for patients with and without the ACM group among patients without LVH. ACM, atrial myopathy; LVH, left ventricular hypertrophy.

Discussion

Our study demonstrates that the prevalence of ACM is high in individuals with MetS. Notably, ACM was also significant in MetS patients without LVH (45.4%), which implied that ACM may be a marker of early myocardial remodeling in patients with MetS. In particular, we found that patients with ACM and MetS had an increased risk of hospitalization for CV events.

Historically, AF has been observed under prolonged hemodynamic stress, including HTN and valvular heart disease (29). Conversely, a recent study proposed a different theory and stated that metabolic diseases such as obesity, DM, and systemic inflammatory disorders associated with adipose tissue may be the most prominent antecedents of AF. In addition, recent studies have indicated that ACM may exist in the absence of AF and may facilitate the development of AF (30). In addition, ACM may be an underlying mechanism in the development of systemic thromboembolism (31). The combination of these contributing factors may explain the high prevalence of ACM in patients with MetS. Interestingly, 45.4% of the ACM patients with MetS were free of LVH, suggesting that ACM can be a marker of early myocardial remodeling in patients with MetS.

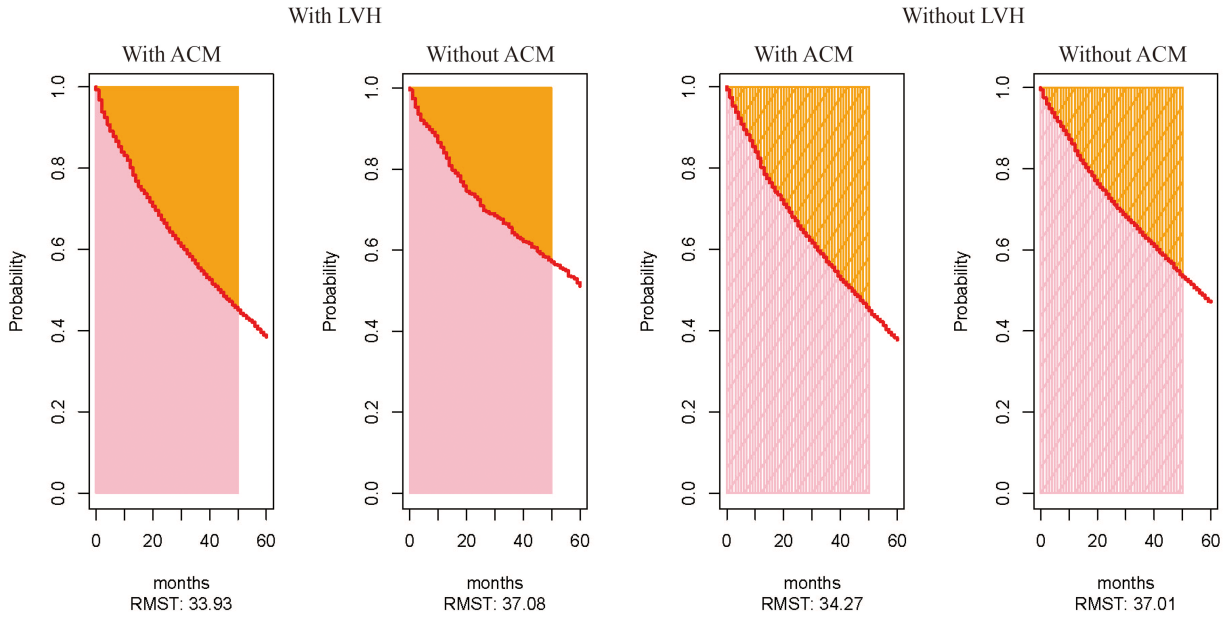

FIGURE 3
Expected values of time for patients to be free from CV events, calculated by using the parameter of restricted mean survival time. The expected value of time to be free from CV events for patients with and without ACM among those with LVH. The expected value of time to be free from CV events for patients with and without ACM among those without LVH.

ACM is exclusively defined in terms of PTFV1 >4,000 uV·ms and the presence of severe LAE. It has been well established that abnormal PTFV1 is associated with LA abnormalities on the ECG (32). In the past, the diagnosis of ACM depended on the presence of structural or functional abnormalities of the LA on cardiac imaging (33). Although increased PTFV1 is thought to be a sign of LAE, it is more reliably a sign of delayed interarterial conduction (34). In the present study, some patients had an increase in PTFV1 without LAE, whereas some patients had an enlarged LA without an increase in PTFV1. Since both increased PTFV1 and LA diameter (LAD) have been correlated with elevated LA pressure, systemic HTN, ischemic heart disease, and prolonged interatrial conduction (31, 35), the interaction between PTFV1 and LAD needs to be elucidated.

In the past, a large Asianpopulation–based cohort reported a positive correlation between the components of MetS diagnostic criteria and the risk of AF (36). The association between the cumulative number of total MetS components and the risk of AF may suggest that upgrading efforts to identify and correct metabolic derangements even before the development of MetS could be of crucial importance to preventing ACM and related CVDs. However, in our study, we found that almost 26% of hospitalized patients with HTN and MetS did not undergo any antihypertensive therapy. Moreover, over 34% of individuals with MetS did not meet the recommended target SBP of less than 140 mmHg and target DBP pressure of less than 90 mmHg. In addition, the optimized management targeting DM and dyslipidemia was also found to be limited. Low awareness of the importance of lipid control strategies among non-cardiac departments may be a contributing factor. This finding highlights that there is still a need for optimizing blood pressure and lipid control in the inpatient management of hospitalized populations who are at risk for ACM.

The use of the early cardiac remodeling technique may be a reasonable proposition in patients with ACM. However, the association between increased instances of early cardiac remodeling and CV events requires further elucidation. Although it is beyond the scope of the present study to investigate the underlying biological mechanisms, it can be hypothesized that chronic inflammation may represent a triggering factor in the development of MetS, and recently, ACM in ischemic stroke has been demonstrated to correlate with the degree of chronic inflammation (37), which represents a possible pathogenic factor. It is well known that both obesity and diabetes promote a state of systemic inflammation that can lead to the expansion of epicardial adipose tissue, which becomes a source of proinflammatory secretory products that cause structural and functional abnormalities in the underlying myocardium (38). The expansion of epicardial fat in the LA, resulting in electroanatomic remodeling, could lead to ACM (39), which further predisposes to blood stasis, spontaneous thrombus formation, and stroke (40). Additionally, altered autonomic nervous system activity may be involved in the development of both MetS and ACM. It has been proven that there is a close link between the autonomic nervous system (41) and MetS. The alterations in the autonomic nervous system also play an important role in atrial cardiopathy (42). Last but not least, other forms of adipose tissue inflammation and insulin resistance in Mets are accompanied by an increased risk of atrial remodeling (43). All in all, the relevant roles of many factors contribute to the development of both ACM and MetS.

Limitations

There are several limitations to this study. Firstly, this retrospective study was carried out in a single center, and therefore, collection and registration bias may be present. Secondly, no implantable loop monitoring was performed in MetS patients, which may lead to an underestimation of AF occurrence. Lastly, only recorded indicators and variables are included; unregistered significant variables may have been omitted. Despite these limitations, our study was the first to our knowledge to investigate the prevalence of ACM in hospitalized patients with MetS and demonstrated that ACM is a common condition that could predict hospital admissions for CV events.

Conclusion

To conclude, ACM may be a marker of early myocardial remodeling in patients with MetS and predicts CV-related hospital admissions. Therefore, there is a need for further optimization in the management of ACM in a hospital setting.

Data availability statement

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

Ethics statement

This retrospective cohort study was based on data obtained from the EMRRD of the first affiliated hospital of the FAHDM, which was developed to establish a computerized clinical database, and the clinical records are continuously updated. The study was approved by the Ethics Committee of the FAHDM with reference number PJ-KS-KY-2020-127(X). Informed consent was not required for this study as the data were retrieved from the hospital system itself.

Author contributions

RW contributed to the concept and design of the study, acquisition of data, and interpretation of data. XW and CL contributed to data acquisition and interpretation. HY, FL, SS, LB, SL, and TH contributed to the acquisition of data. XY contributed to the study concept and design, interpretation of data, and study supervision. YX contributed to the study concept and design, interpretation of data, and critical revision of the manuscript for intellectual content. All authors contributed to the writing of the article and approved the submitted version.

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Conflict of interest

LB was employed by Yidu Cloud Technology, Ltd.

The remaining 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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Mendelian randomization study reveals a causal relationship between serum iron status and coronary heart disease and related cardiovascular diseases

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Background: Growing observational studies have shown that abnormal systemic iron status is associated with Coronary heart disease (CHD). However, these results from observational studies was not entirely consistent.It remains unclear whether this relationship represents causality.It is necessary to explore the causal relationship between iron status and CHD and related cardiovascular diseases (CVD).
Objective: We aimed to investigate the potential casual relationship between serum iron status and CHD and related CVD using a two-sample Mendelian randomization (MR) approach.
Methods: Genetic statistics for single nucleotide polymorphisms (SNPs) between four iron status parameters were identified in a large-scale genome-wide association study (GWAS) conducted by the Iron Status Genetics organization. Three independent single nucleotide polymorphisms (SNPs) (rs1800562, rs1799945, and rs855791) aligned with four iron status biomarkers were used as instrumental variables. CHD and related CVD genetic statistics We used publicly available summary-level GWAS data. Five different MR methods random effects inverse variance weighting (IVW), MR Egger, weighted median, weighted mode, and Wald ratio were used to explore the causal relationship between serum iron status and CHD and related CVD.
Results: In the MR analysis, we found that the causal effect of serum iron (OR = 0.995, 95% CI = 0.992–0.998, $p = 0.002$) was negatively associated with the odds of coronary atherosclerosis (AS). Transferrin saturation (TS) (OR = 0.885, 95% CI = 0.797–0.982, $p = 0.02$) was negatively associated with the odds of Myocardial infarction (MI).
Conclusion: This MR analysis provides evidence for a causal relationship between whole-body iron status and CHD development. Our study suggests that a high iron status may be associated with a reduced risk of developing CHD.

KEYWORDS

iron status, cardiovascular disease, causal effect, genome-wide association studies, Mendelian randomization

1. Introduction

Due to an aging population and declining fertility rates, cardiovascular disease mortality continues to rise and imposes a considerable economic and health burden on society (1). As research progresses, more and more studies show the correlation between systemic iron status and heart disease and related CVD (2).

CHD remains one of the major diseases threatening the health of the entire human population (3). The development of CHD involves many associated CVD. Among CVD, MI, a type of coronary heart disease, is a serious consequence of coronary heart disease. Hypertension (HP) is not only a CVD but also one of the risk factors for CHD, while heart failure (HF) is the end-stage disease of most CVD including CHD.

Iron, an essential mineral for maintaining homeostasis in the body, plays a key role in oxygen transport and utilization as well as in mitochondrial function (4). Iron deficiency (ID) is associated with morbidity and mortality in CHD, M I and HF (5–8). Studies have shown that ID is one of the most common complications of HF. Iron supplementation via intravenous can reduce the number of hospitalizations for HF (9). It has been shown that ID impairs the contractility of human cardiomyocytes by reducing mitochondrial function and decreasing energy production, which leads to impaired cardiac function (10). When uncontrolled elevation of iron concentration leads to iron overload, the basic cellular mechanisms and functional composition are disrupted and changed (11). The redox properties of iron enable the generation of reactive oxygen species (ROS), and iron (Fe^{2+}) and iron (Fe^{3+}) mediate lipid peroxidation, leading to the formation of alkoxyl (RO) and peroxyl (RO^2) radicals (12, 13). Studies have displayed that in animal models of ischemia/reperfusion (I/R) cardiac tissue samples it can be observed that increased mitochondrial iron-related reactive oxygen species (ROS) production leads to myocardial injury (14). However, the physiopathological mechanisms of ID and iron overload participating in CHD and associated CVD remain unclear. In conclusion both opposite factors, ID and iron overload, can have an impact on CHD and related CVD. However, even in observational studies it is difficult to distinguish which specific association exists between iron status and CHD and associated CVD, as selective bias or other biases inherent in observational studies can still influence the results. Therefore, further studies are necessary to elucidate whether there is a causal or other relationship between iron status and CHD and associated CVD.

MR analysis is a novel method of epidemiological analysis that strengthens causal inferences by using genetic variation as an instrumental variable (IV) such as SNPs for exposure. This method minimizes the effects of residual confounding and strengthens causal inferences about the effects of specific exposure factors on outcomes while overcoming the limitations of traditional epidemiological studies (15). Here, we performed a 2-sample MR study to examine the association of iron status with HP, AS, CHD, MI, and HF based on the effect of systemic iron status and CHD and associated CVD using GWAS data, aiming to provide new evidence on the relationship between iron status and disease progression of CHD.

2. Materials and methods

2.1. Study design

A genetic tool of four iron status biomarkers: ferritin, iron, transferrin, and transferrin saturation (TS), was selected for a two-sample MR analysis as a way to investigate the association of iron status biomarkers with the chain of cardiovascular disease events including HP, AS, CHD, MI and HF. The screening flowsheet is shown in **Figure 1**.

2.2. Selection of instrumental SNPs

Genetic variants associated with serum iron status were identified through a meta-analysis of 19 GWAS, which included 48,972 Europeans (16). A higher level of systemic iron was associated with higher iron levels, higher transferrin saturation, and higher ferritin levels, but decreased transferrin levels (4, 16, 17). Thus, genetic tools for iron status should be consistently related to each of these four markers, and thus three loci (rs1800562 and rs1799945 in the HFE gene, and rs855791 in TMPRSS6) could be identified in the meta-analysis performed by the GIS Consortium as being significantly associated with all four iron status markers genome-wide ($p < 5 \times 10^{-8}$) in a pattern consistent with effects on systemic iron status (i.e., increased serum iron, transferrin saturation and ferritin levels and decreased transferrin levels) (16), and these three were suggested as tools for systemic iron status in our MR analysis. Three SNPs had $p < 5 \times 10^{-8}$ and $r^2 \leq 0.01$, and their F-statistics were calculated to quantify the intensity of the selected instrument (F-value > 10) (18).

Characteristics and summary data of the SNPs and iron status parameters shown in **Table 1** and **Supplementary Table S1**, respectively.

2.3. Outcome data

GWAS statistics related CHD and related CVD can be extracted from the corresponding authoritative consortium or cohort studies.

Data for both HP and HF were obtained from UKBiobank, with a final sample of 462,933 people of European descent for HP (119,731 HP cases and 343,202 non-cases), and a final sample of 361,194 people of European descent for HF (1,405 HF cases and 359,789 non-cases) (19). GWAS resources for coronary atherosclerosis are based on data from the UKBiobank consortium for a total of 361,194 individuals of European descent including 14,334 AS patients and 346,860 healthy controls (20), and FinnGen for a total of 211,203 individuals of European descent including 23,363 AS patients and 187,840

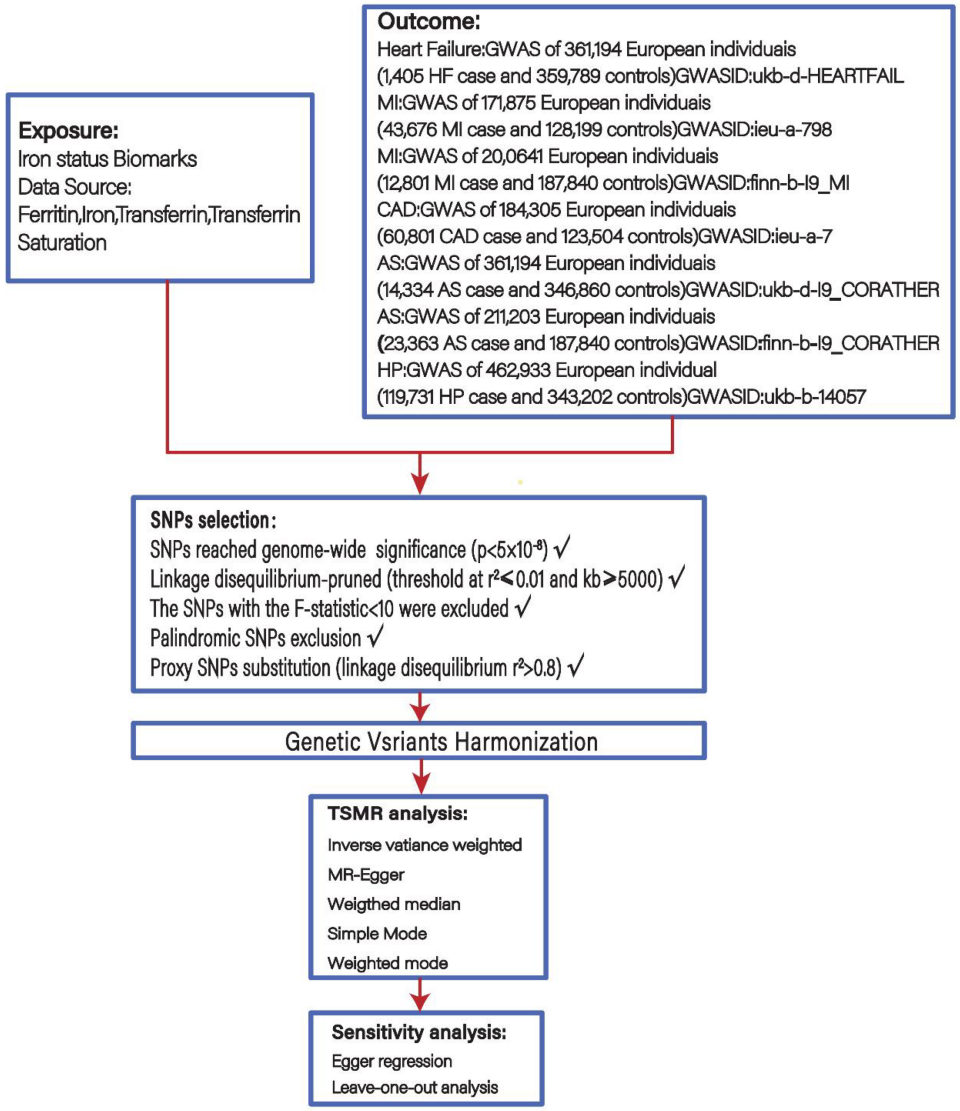

FIGURE 1
Flowsheet of Mendelian randomisation in this study.

TABLE 1 The characteristics and summary data of the exposed SNPs and iron status parameters.

	SNP	Nearest gene	Effect allele	Other allele	Eaf	F	Beta	SE	*P*
Iron	rs1799945	HFE (H63D)	C	G	0.85	450	−0.189	0.01	1.10×10^{-81}
	rs1800562	HFE (C282Y)	A	G	0.067	668	0.328	0.016	2.72×10^{-97}
	rs855791	TMPRSS6 (V736A)	A	G	0.446	806	−0.181	0.007	1.32×10^{-139}
Ferritin	rs1799945	HFE (H63D)	C	G	0.85	52	−0.065	0.01	1.71×10^{-10}
	rs1800562	HFE (C282Y)	A	G	0.067	256	0.204	0.016	1.54×10^{-38}
	rs855791	TMPRSS6 (V736A)	A	G	0.446	73	−0.055	0.007	1.38×10^{-14}
Transferrin	rs1799945	HFE (H63D)	C	G	0.85	676	0.114	0.01	9.36×10^{-30}
	rs1800562	HFE (C282Y)	A	G	0.067	1446	−0.479	0.016	8.90×10^{-196}
	rs855791	TMPRSS6 (V736A)	A	G	0.446	47	−0.055	0.007	1.38×10^{-14}
Transferrin saturation	rs1799945	HFE (H63D)	C	G	0.85	162	−0.231	0.01	5.13×10^{-109}
	rs1800562	HFE (C282Y)	A	G	0.067	2126	−0.479	0.016	2.19×10^{-270}
	rs855791	TMPRSS6 (V736A)	A	G	0.446	889	−0.19	0.008	6.41×10^{-137}

healthy controls. The second is based on FinnGen data for 211,203 individuals of European descent, including 23,363 AS patients and 187,840 healthy controls, available on the FinnGen study website (https://finngen.gitbook.io/documentation/) (20, 21). CHD Statistical data were obtained from the coronary artery genome-wide replication and meta-analysis (CARDIoGRAM) and coronary

artery disease genetics (CARDIoGRAMplusC4D) GWASmeta-analysis, which included 60,801 cases and 123,504 controls (22). Statistics for MI were also obtained from the Coronary Artery Whole Genome Replication and Meta-Analysis (CARDIoGRAM) and Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) GWASmeta-analysis, which included 43,678 cases and 128,199 controls (22), and FinnGen which included 12,801 cases and 187,840 controls (21). We used aggregated data from published GWAS that referenced the original definitions of the diseases in their GWAS without any modifications. The specific data sources used are in **Supplementary Table S4**.

2.4. Statistical analysis

MR analysis was performed using five methods, inverse variance weighting (IVW) under a multiplicative random effects model, MR-Egger, Weighted median, Simple mode, and Weighted mode. IVW was performed by combining the Wald ratio estimates for each individual SNP will be one causal estimate for each risk factor (23). A sensitivity analysis is required to test the validity and robustness of the IVW estimates due to invalid instrumental bias and polymorphism.

Sensitivity analyses include heterogeneity tests, genetic pleiotropy tests, and the "leave-one-out" method (19). In sensitivity analysis, we can use the weighted median method to check for invalid instrumental bias to estimate the multiplicity of potential causal effects or the inclusion of invalid instruments (24), while the use of MR-Egger regression can explain both the dilution bias of the skewed regression, with the mean level of multiplicity consisting of the intercept term (24, 25). Subsequently, symmetries can be visualized using funnel plots, and if they are skewed in one direction, they indicate a potential multiplicative effect (26). Cochran's Q test was also used to estimate the heterogeneity between the Wald ratio estimated for the different SNPs (27). Finally, to identify all genetic variants potentially affecting SNPs, we performed a "leave-one-out" analysis, whose fluctuations in results before and after removal of SNPs may reflect unstable associations.

To further investigate the relationship between iron status biomarkers and CHD and related CVD, we separately selected the iron status biomarkers with positive MR analysis results as described above and each SNP of the corresponding disease for two-sample MR analysis again, respectively, to obtain a more accurate estimate of the causal effect of each iron status biomarker and disease. The characteristics and summary data of the separately selected SNPs are shown in **Supplementary Table S2**.

"Two SampleMR" (version 0.5.6) of R software (version 4.2.1) was used for all analyses. *P* values less than 0.05 were considered statistically significant.

3. Result

Genetically determined higher serum iron was negatively associated with higher odds of AS (OR = 0.995, 95% CI = 0.992–0.998, $p = 0.002$). The same results were obtained again using each SNP (OR = 0.996,95% CI = 0.992–0.998, $p = 0.0009$). Higher TS was negatively associated with higher odds of MI(Finn) (OR = 0.885, 95% CI = 0.797–0.982, $p = 0.02$). A repeat MR analysis using each SNP yielded no causal relationship between TS and MI (Finn) ($p = 0.657$) while higher TS was negatively associated with higher odds of MI (OR = 0.939, 95% CI = 0.886–0,996, $p = 0.037$), using the inverse variance-weighted approach. Moreover, the sensitivity analysis revealed that the selected instruments did not differ horizontally (p-values >0.05 for MR-Egger intercepts) or heterogeneously (p-values >0.05 for Cochran's Q statistic). In addition, MR-Egger regression and funnel plot appearance analyses showed a poor possibility of horizontal polymorphism (all p-values for MR-Egger intercept > 0.05) and visually, the leave-one-out analysis plot proved that the results were not altered by the removal of any SNPs and the results remained quite robust. The remaining MR analyses were negative that iron status markers were not causally associated with HP, CHD or HF.

Complete results are presented in **Supplementary Figure S2**. And the positive results were presented in **Figure 2**. The IVW results are shown in **Table 2**. Results of MR Analysis are expressed as the ORs for a positive result per one standard deviation (SD) increase for each iron biomarker, as shown in **Figure 3** and **Supplementary Figure S4**. The leave-one-out analysis plot proves that removing any SNP does not change the results and is quite robust. The leave-one-out analysis graph of positive results are presented in **Figure 4**, and all results are presented in **Supplementary Figure S3**. The results of MR Analysis of AS and MI with the separately selected SNPs associated with Iron and TS are presented in **Supplementary Table S3** and **Supplementary Figure S3**. The results of the positive results reanalysis are expressed as ORs per one standard deviation (SD) increase in positive results for each iron biomarker, as shown **Supplementary Figure S4**.

4. Discussion

We applied MR to analyze the causal relationship between four biomarkers of iron status and CHD and related CVD and concluded that there is a partial causal relationship between systemic iron status and CHD and related CVD. In this MR analysis, there was a negative correlation between serum iron levels and coronary AS and between TS and MI. The same conclusion was reached when the MR analysis was repeated for AS and MI using the individual SNPs for serum iron and TS, respectively. It implies that a genetically determined increase in serum iron decreases the risk of AS and a genetically determined increase in TS decreases the risk of MI. Based on the fact that TS is one of the biomarkers reflecting systemic iron status, we can speculate that genetically determined ID may lead to an increased risk of CHD.

In a study of patients on hemodialysis, it was found that high doses of intravenous iron reduced the risk of MI compared to lower doses (28). More noteworthy is the finding in another study that patients who were iron deficient at the time of acute

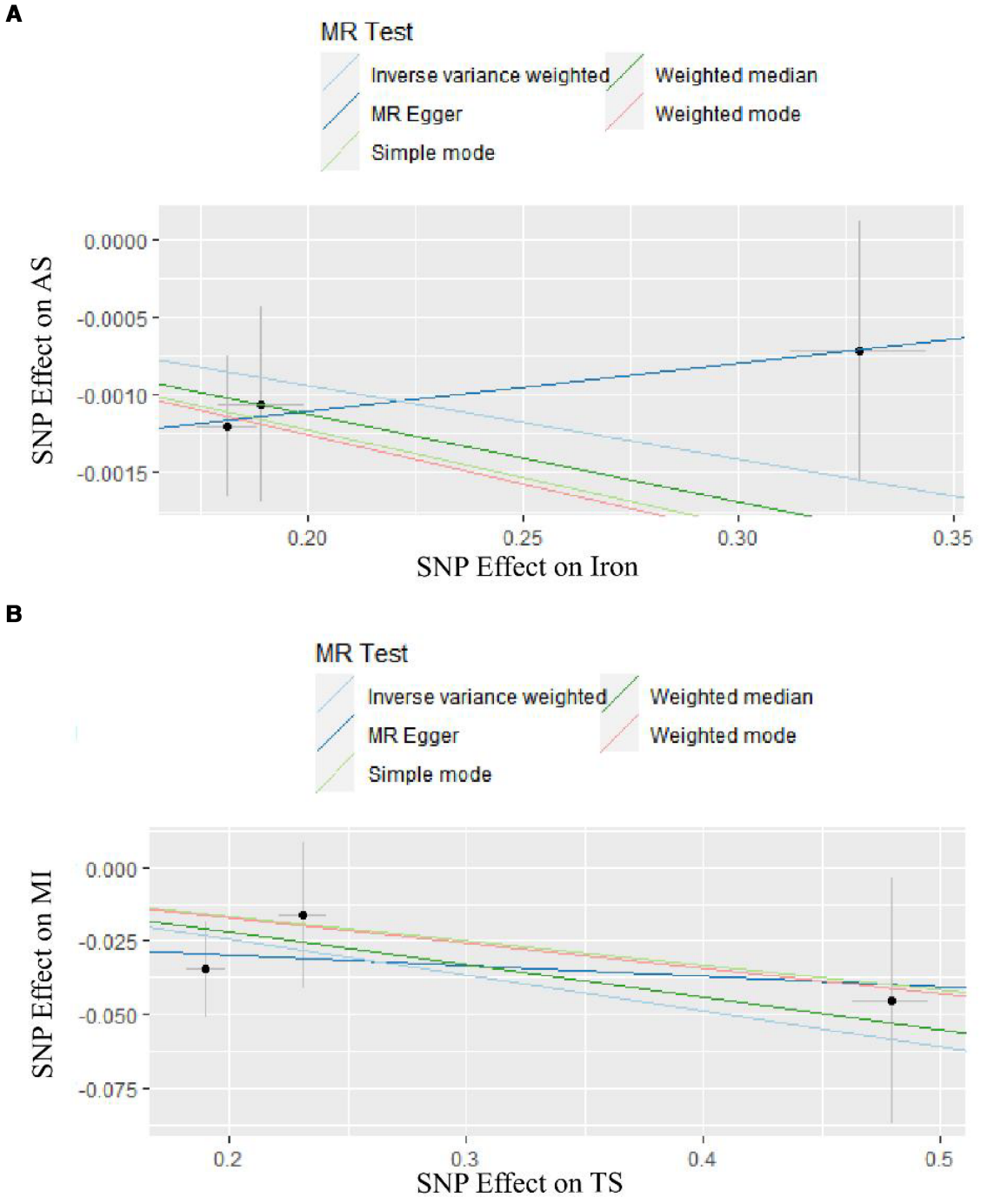

FIGURE 2
The result of two-sample MR analyses. (**A**) is the result of two samples MR analyses of Iron. (**B**) is the result of two samples MR analyses of TS.

coronary syndrome had a significantly higher risk of cardiovascular mortality and nonfatal myocardial infarction over 4 years (6), results that are consistent with those obtained in this paper. However, in patients with st-segment elevation myocardial infarction treated with percutaneous coronary intervention, patients with ID were found to have a better in-hospital prognosis. The investigators speculate that this may be related to the ability of ID to reduce myocardial ischemia-reperfusion injury (29). Based on the above, we hypothesize that the effect of iron on MI is not only that higher iron levels reduce the risk of MI, but also that ID increases the long-term effects of a worse incidence of MI. Considering the small causal effect, these MR-based analyses should be referenced with caution. Previous MR analyses on iron status and carotid plaque have also shown that genetically determined iron levels increase carotid plaque with a protective effect (30). This laterally supports the results of the MR of serum iron with AS in this paper. However, some studies have yielded contradictory results, suggesting that high iron promotes

TABLE 2 The results of IVW.

		HP			AS			AS(Finn)			CAD		
Exposure	nSNP	Beta	SE	*P*	Beta	SE	*P*	Beta	SE	*P*	Beta	SE	*P*
Iron	3	0.028	0.0194	0.147	−0.005	0.0016	0.002	−0.031	0.0805	0.704	−0.075	0.0461	0.104
Ferritin	3	0.064	0.0402	0.110	−0.008	0.0053	0.116	0.003	0.1998	0.989	−0.189	0.1008	0.060
Transferrin	3	−0.032	0.0182	0.081	0.001	0.0036	0.726	−0.105	0.0687	0.127	0.042	0.0791	0.595
Transferrin saturation	3	−0.001	0.0221	0.959	−0.002	0.0025	0.446	−0.071	0.0474	0.134	0.006	0.0579	0.920

		MI(Finn)			MI			HF		
Exposure	nSNP	Beta	SE	*P*	Beta	SE	*P*	Beta	SE	*P*
Iron	3	−0.082	0.0958	0.390	−0.062	0.0426	0.145	-1.43×10^{-4}	0.0005	0.779
Ferritin	3	−0.084	0.2624	0.746	−0.176	0.0988	0.074	-4.66×10^{-4}	0.0011	0.676
Transferrin	3	−0.101	0.1160	0.382	0.063	0.0593	0.287	4.20×10^{-4}	0.0005	0.429
Transferrin saturation	3	−0.122	0.0532	0.022	0.021	0.0484	0.668	1.16×10^{-4}	0.0004	0.776

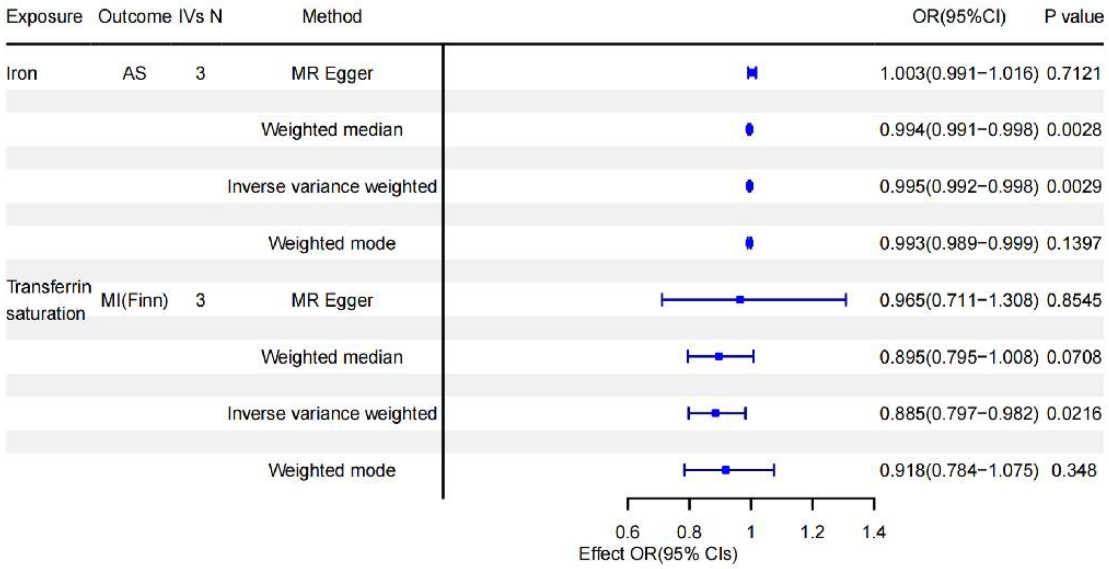

FIGURE 3
Forest plot summarizing causality of positive results.

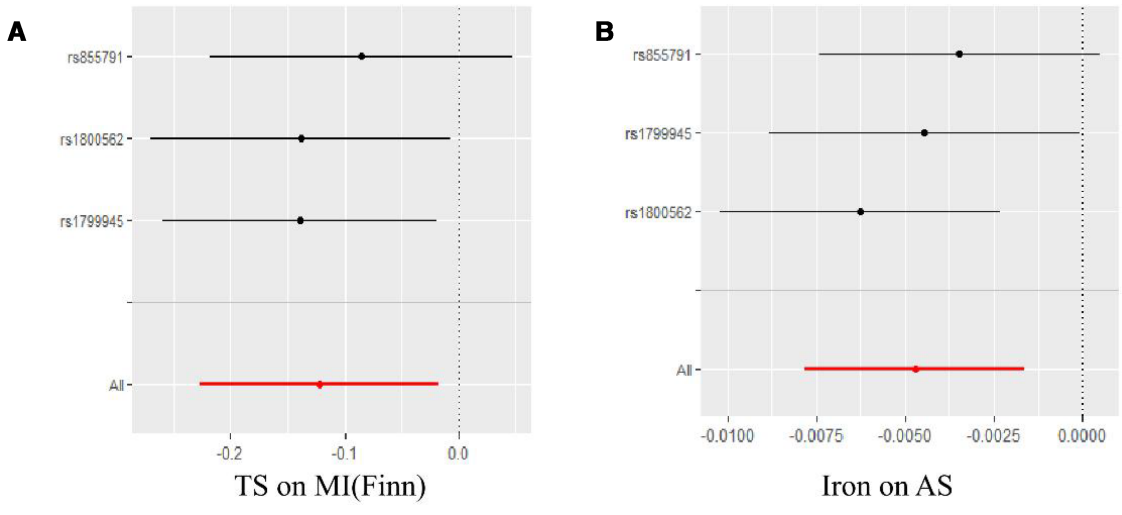

FIGURE 4
Leave-one-outanalyses of positive results in the causal relationship between iron status and CHD and related CVD. (A) is the result of TS and MI(Finn), and (B) is the result of Iron and AS.

the progression of atherosclerosis and increases its severity (31). This discrepancy may stem from the fact that the occurrence of AS is influenced by genetic and environmental factors, and we analyzed the relationship from a genetic perspective, focusing on lifetime effects rather than short-term effects.

Some of these results contradict the results of the reported papers, which may be explained by the fact that no correction for disease subtyping population stratification and population pedigree was performed when processing GWAS data for CHD, in which cases of acute coronary syndrome, coronary artery bypass grafting, and percutaneous coronary revascularization were included in the CHD cases used (17). As part of the current MR study, three SNPs were selected from a recent large-scale GWAS. Among them, mutations in the gene HFE include rs1800562 (also known as C282Y) and rs1799945 (also known as H63D). The missense mutation in rs1800562 retains the dysfunctional HFE in the endoplasmic reticulum instead of being transported to the plasma membrane, resulting in the parenchymal iron overload disease of hereditary hemochromatosis (HH) (32). A recent study of 2890 European patients with C282Y-pure HH found a significantly lower risk of cardiovascular disease in C282Y-pure HH patients compared to age-matched subjects (33). Moreover, compared to HFE wild-type study participants, C282Y-positive participants had lower total cholesterol and LDL-C levels (34). In addition, a large GWAS study showed that genetic variants on H63D are associated with the prevalence of hypertension (35). It has been hypothesized that H63D causes toxic damage to the vascular endothelium by increasing iron stores and producing oxidative stress (36). However, the exact mechanism by which H63D causes hypertension remains unclear. Hepcidin, a key role of iron homeostasis, is a peptide hormone synthesized in hepatocytes that regulates cellular iron output by interacting with iron transport proteins (37, 38). Mutations in TMPRSS6 result in excessive iron uptake by encoding a type II plasma membrane serine protease, called matriptase-2, which inhibits hepcidin (39). Moreover, rs1800562 and rs1799945 have been reported to affect hepcidin (40). In conclusion, the above indicates that all three SNPs above can be involved in the occurrence and development of CVD by regulating iron metabolism.

Iron, one of the most essential nutrients, has been shown in many previous studies to be associated with abnormal iron status and CVD. In a cohort of 12,164 individuals from three European populations ID was associated with a 24% increased risk of CHD, a 26% increased risk of CVD death and a 12% increased risk of all-cause mortality, with 5.4% of deaths, 11.7% of CVD deaths and 10.7% of CHD events attributable to iron deficiency(ID) (41). ID is also an observable indicator of HF, about half of HF patients having ID according to the definition of ID (42, 43), with a specific prevalence of ID in chronic HF of about 47%–68%, and the more severe the HF, the more likely it is to occur (44). A double-blind randomized trial showed that intravenous iron carboxymaltose supplementation reduced the risk of HF hospitalization in iron-deficient patients with stable left ventricular ejection fraction below 50% after an acute HF episode, but had no significant effect on their risk of cardiovascular death (45). However, although observational studies and MR analyses have indicated that ID increases the risk of CHD, there are no relevant experimental results indicating that improving ID reduces the risk of developing CHD. In an epic-Heidelberg study,serum ferritin concentrations were associated with IM risk and cardiovascular disease mortality, but were not statistically significant after adjustment (46). Thus the effect of iron deficiency on CHD and CVD remains questionable. Therefore, the hypothesis has been put forward that the effect of iron on the heart lies in the increased oxidative stress due to iron overload (47).

Ferroptosis, in which the key factors of iron toxicity are Fe^{2+} accumulation and lipid peroxidation, is a novel form of cell death with unique genetic, biochemical, morphological and metabolic characteristics in contrast to apoptosis, necroptosis and scorch death (13). As iron plays a key role in catalyzing phospholipid peroxidation in ferroptosis, unrestricted lipid peroxidation is exactly one of the hallmark symptoms of ferroptosis (48) Lipid peroxidation is subject to molecular oxidation reactions that generate peroxyl radicals, and if not eventually reduced to the corresponding alcohols, the propagation of the radical-mediated reactions leads to the formation of numerous secondary products that disrupt cell membrane integrity and eventually lead to cell death (49). On the other hand, excessive intracellular iron accumulation is associated with an overproduction of ROS, which leads to extensive oxidation of polyunsaturated fatty acids and disruption of cell membrane structure, ultimately leading to cell death (50). Iron overload has also been suggested as one of the potential mechanisms of myocardial I/R injury (51). An increase in Fe^{2+} concentration in cardiomyocytes has been reported to be observed in I/R-treated rats (52, 53). The upregulation of TfR1 in I/R-treated rat hearts was associated with elevated iron content, and the inhibiting of TfR1 expression accompanied a decrease in iron content and reduced I/R injury, so hypoxia may be responsible for causing I/R iron overload (54). Consistently, iron death is observed during atherosclerosis. The likelihood of atherosclerosis can be reduced by inhibiting iron death in aortic endothelial cells to attenuate lipid peroxidation and endothelial dysfunction (55).

Morever, it has been shown that systemic iron satuts are not equal to cardiac iron levels, so systemic iron disorders do not directly affect cardiac iron satuts (56, 57), and further studies are needed to determine whether systemic iron supplements have a beneficial effect on CVD. Studies have shown that a low-iron diet fed to rats results in reduced levels of iron transport proteins in the rat heart, resulting in reduced iron output from heart cells (58), suggesting that iron levels in the heart are not necessarily affected by a low-iron diet. Consistently, in another study of a mild cardiomyopathy model in FthMCK/MCK mice both showed reduced cardiac iron levels without changes in serum or skeletal muscle iron levels, but after 4 weeks on a high iron diet, cardiac GSH levels in mice were reduced due to increased cardiac iron levels, resulting in cardiomyocyte ferroptosis.

Herein, the decrease in serum iron does not correlate positively with the decrease in cardiac iron levels,which cellular iron levels may be increased and be caused ferroptosis in cardiomyocytes while iron supplementation (59). Combined with the results of the Mendelian study in this paper, we can propose the hypothesis that the effect of decreased systemic iron status on CVD lies in overall cardiomyocyte function. Systemic iron status is negatively correlated with diseasewhen there are more normal cardiomyocytes than damaged cardiomyocytes in a cardiovascular disease event, whereas systemic iron status is not correlated with disease development when the situation is reversed. It is possible that iron supplementation at this point will lead to cellular iron overload, which will promote the development of cellular ferroptosis as a result. Therefore, considering the potentially deleterious effects of ID and iron overload, iron status intervention strategies may not be beneficial for patients in the CHD and related CVD without ID. In conclusion, based on the effect of systemic iron status on cardiac cellular iron levels, future studies should aim to identify CHD and related CVD phenotypes that would benefit from improved ID and to investigate their specific pathophysiological mechanisms.

The study we conducted has several limitations. First, because individual data were not available and we only performed summary statistics, the CHD and related CVD data used in this study were not stratified by disease subtype, such as dividing CHD into stable angina pectoris and acute non-ST-segment elevation myocardial infarction. Indeed, iron status markers may have a stronger association with specific subtypes of CHD or at acute onset. Therefore, further studies are needed to investigate whether similar results exist in patients of different races, different subtypes of CHD and CVD, and different degrees of disease severity. Second, for some exposures, the body may have mechanisms to respond to this exposure level, such as systemic iron status that is not synchronized with cellular iron levels, which hinders our study of iron metabolism and CHD and related CVD from macroscopic regulators to microscopic pathophysiological changes in the present study. Considering the small causal effect of this analysis, the MR Analysis estimates of this study should be interpreted with caution, and the inferences and assumptions in this paper should be referred to with caution. Nevertheless, the present study provides some clues to the pathophysiology and therapeutic exploration of CHD and related CVD. We expect future studies to delve into the relationship between CHD and related CVD and iron status, which may provide new insights into the prevention and treatment of CHD.

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 authors.

Author contributions

FL and YL: Are joint first co-authors, and oversaw investigation, Resources, Data Curation and Writing—Original Draft; YL and FX: Conceptualization, Methodology and Writing—Review & Editing; QW and SX: Data Curation and Visualization. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1152201/full#supplementary-material

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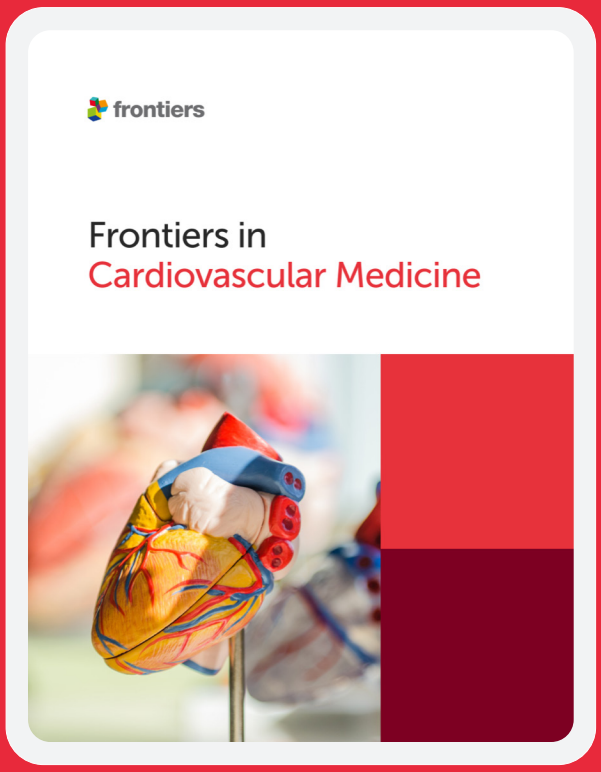

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