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Inflammatory factors in coronary heart disease: Mechanism, diagnosis and therapy

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Inflammatory factors in coronary heart disease: Mechanism, diagnosis and therapy

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Editorial: Inflammatory factors in coronary heart disease: mechanism, diagnosis, and therapy

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Editorial on the Research Topic
Inflammatory factors in coronary heart disease: mechanism, diagnosis and therapy

Coronary heart disease (CHD), one of the main causes of death in humans, is a group of clinical syndromes caused by the progression of coronary atherosclerosis. The effectiveness of conventional drugs or invasive treatments has reached a bottleneck. In recent years, studies have defined coronary heart disease as an inflammatory disease. Inflammatory factors, including anti-inflammatory factors and pro-inflammatory factors, have gradually become important biomarkers and effective targets for the treatment of coronary heart disease. In this Research Topic, titled "Inflammatory Factors in Coronary Heart Disease: Mechanism, Diagnosis, and Therapy", we received 24 submissions and accepted 11 manuscripts. In addition, two abstracts were also included. Among the articles, two of the research papers explored the role and molecular mechanisms of drug or inflammation-related genes in animal models, four studies are about the correlation analysis between inflammation-related factors and ischemic cardiomyopathy or CAD, one study is about the bifurcation strategies using second-generation drug-eluting stents, and three articles are meta-analysis or reviews.

Wei et al. found that edgeworthia gardneri (Wall.) Meisn. (EG) extract protects against myocardial infarction by inhibiting NF-κB-and MAPK-mediated endothelial inflammation, indicating EG as a potential therapeutic agent in ischemic cardiovascular disease (Wei et al.). Furthermore, Zuo et al. established a remote ischemic postconditioning (RIPostC) rat model and revealed by transcriptome analysis that RIPostC could markedly reduce infarct size and decrease the level of myocardial pro-inflammatory factors, including IL-1β and IL-6, but that it can increase the level of IL-10, which was negatively correlated with ADAMTS15 (Zuo et al.). The authors point out that the role and the mechanism of the inflammation-related gene ADAMTS15 should be investigated. In another study, Wang et al. identified five potential biomarkers (SERPINA3, FCN3, PTN, CD163, and SCUBE2) in human ischemic cardiomyopathy (ICM) via datasets, and performed the functional experiment on ICM rats. These studies are expected to provide clinicians with useful tools for ICM diagnosis and treatment from the perspective of inflammation (Wang et al.).

Another study calculated the mean platelet volume lymphocyte ratio (MPVLR) in patients with chronic total occlusion (CTO) and analyzed the relationship between MPVLR and coronary collateral circulation (CCC) formation (Niu et al.). The results

revealed that MPVLR was negatively correlated with CCC, and a high MPVLR level was an independent predictor of poorly formed CCC, which constitutes a simple and practical method for diagnosis. Xiong et al. revealed that sLAG3 levels in patients with coronary heart disease were significantly lower than those in the control group, and sLAG3 levels were negatively correlated with the occurrence of coronary heart disease but not with the severity of coronary heart disease. At the same time, sLAG3 was negatively associated with BMI and diabetes, suggesting that sLAG3 reduction may be a new risk factor for CAD (Xiong et al.). Another clinical study included all cardiovascular disease (CVD) patients who participated in the National Health and Nutrition Examination Survey (NHANES 2011–2014); serum albumin was measured, revealing a J-shaped association between low serum albumin levels and increased long-term mortality of CVD, which may provide a simple method for assessing the risks of CVD in the general population of the United States (Li et al.).

Cha et al. conducted a cohort study of clinical outcomes in Korean patients with diabetes using a bifurcation strategy with a second-generation drug-eluting stent; a total of 905 patients with DM and marked bifurcation lesions were enrolled in the study. The primary outcome was the 5-year incidence of target lesion failure (TLF), which was defined as a composite indicator of cardiac death, target vessel myocardial infarction, and target lesion revascularization. The results demonstrated that the T- or V-stenting technique but not the crush or culotte technique in patients with DM resulted in increased TLF compared to the one-stent strategy (Cha et al.).

In addition, research hotspots, frontiers, and development trends in anti-inflammatory studies for coronary heart disease over the past 30 years were summarized, showing that a total of 5,818 articles focused on anti-inflammatory studies in CHD (Zhang et al.), which is of great significance for future studies. Another meta-analysis, by Liu et al. includes a total of 23 studies about CRP and MACE and suggests that CRP is a prospective predictor of prognosis in patients with AMI undergoing PCI, especially in those who are hospitalized, have a short-term prognosis, and those from Asian descent (Liu et al.). In China, Danhong injection (DHI) is recommended by expert consensus and is widely used in the perioperative management of patients with acute coronary syndrome (ACS). A systematic review and meta-analysis by Li et al. demonstrated that Danhong injection combined with conventional treatment has a better therapeutic effect on patients with ACS than conventional treatment alone by inhibiting inflammation (Li et al.). Kawasaki disease (KD) is an acute, inflammation-mediated vasculitis that primarily affects children under 5 years old and is considered the most common coronary artery disease in children. A series of studies have identified vascular endothelial cell damage and dysfunction in patients with KD. Qiu et al. systematically described the role of endothelial cells in the pathogenesis of KD and the therapeutic methods of endothelial cells (Qiu et al.).

In summary, the 11 articles included in this Research Topic cover multiple themes, such as the cellular and molecular mechanisms of inflammatory factors in CHD, the influence of inflammatory factors on CHD, and inflammatory factors as biomarkers of CHD, as well as a review about the clinical outcomes targeting inflammatory factors in the treatment of coronary heart disease.

Author contributions

QD and KY were devoted to writing for this article. QJ is editor of this topic and contributed to editing several publised article in this topic. All authors contributed to the article and approved the submitted version.

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Molecular mechanisms of endothelial dysfunction in Kawasaki-disease-associated vasculitis

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Kawasaki disease (KD) is an acute, inflammation mediated vasculitis, mainly affecting in children under five, which is consider as the most common coronary artery disease in children. The injuries of coronary arteries would result in dilation or thrombus formation, bringing great threaten to patients. Endothelium, located in the inner surface of coronary artery, serves as the interface between the circulating inflammatory cells and vascular media or adventitia, which is the first target of inflammatory attacks during early stage of KD. A series of studies have determined vascular endothelial cells damages and dysfunction in KD patients. However, current therapeutic strategy is still challenging. So that it is critical to underline the mechanisms of endothelium injuries. In this review, the role of endothelial cells in the pathogenesis of KD and the therapeutic methods for endothelial cells were systematically described.

KEYWORDS

Kawasaki disease, coronary artery disease, inflammation, endothelial cells, molecular mechanisms

Introduction

Kawasaki disease (KD), also known as mucosa-cutaneous lymph node syndrome, was first reported by Dr. Tomisaku Kawasaki in 1967. Generally, KD mainly attacks children aged under 5 years and demonstrates the highest prevalence among children in Asia. Moreover, the largest patient groups were identified in Japan, South Korea, and China, with a relatively even distribution elsewhere in the world (1). KD has now replaced rheumatic fever as the most common acquired heart disease in pediatric patients worldwide. It is described as an acute febrile extraneous disease that would induce systemic vasculitis mainly targeting small- and medium-sized arteries, which is considered the dominant pathological change accounting for the major adverse effects secondary to KD (2). Acute vasculitis associated with KD may lead to the development of a complex set of coronary artery abnormalities (CAAs). Coronary artery injuries would result in dilation or thrombus formation, both of which endanger patients (3). Dr. Takahashi figured out coronary vasculitis begins 6–8 days after the onset of KD from histopathological examinations, and then the inflammation rapidly invades all layers of the artery, causing significant damage to the structure of the arterial wall,

which is the essential cause of CAA development. Monocytes and macrophages have been identified as the major cell types involved in KD-associated arterial inflammatory infiltration. Besides, activated neutrophils were also considered to participate in the initial phase of coronary arteritis. Inflammatory cell infiltration persists for more than 25 days. After that, the accumulation of inflammatory cells gradually reduces, and the pathophysiological process of KD moves to the chronic phase. However, once CAA is initiated in medium- to large-sized arteries, the possibility of recovery diminishes. Arterial dysfunction would persist for a long period in the presence of a large aneurysm or in the event of recanalization after aneurysm embolization (4). As such, it is critical to underline the mechanisms of CAA formation, and several clinical attempts had been made to reduce or avoid the severe situation during the acute phase of KD. Therefore, a series of hypotheses had been raised to explore the association between inflammation and cardiovascular damage.

The endothelium, located in the inner surface of coronary arteries, serves as the interface between the circulation and the vascular media or adventitia, the first target of inflammatory cells during the early stages of inflammatory attacks. Moreover, vascular endothelial cells can secret a variety of pro-inflammatory cytokines, which participate in inducing systemic immune responses and inflammatory activities (5). Several inflammation activation genes, such as NOD1 and NLRP1, contribute to the development of KD and are involved in the regulation of autophagy (6). Endothelial dysfunction due to KD and its association with CAA formation has highlighted the need for further research in recent years (7–9). Endothelial cells also play an important role in the pathogenesis of KD in many aspects as had been pointed out in a consensus of studies. Systemic endothelial dysfunction had been identified in children with a history of KD. Besides, several parameters were identified as independent risk factors for predicting coronary artery lesions, including prolonged febrile periods and increased neutrophil-to-lymphocyte ratios (10). Moreover, some molecular mechanisms were also found to be involved in coronary endothelial dysfunction, such as low-density lipoprotein oxidation and its receptor-mediated signaling activation (11). Qin et al. reported the critical role of autophagy in mediating endothelial dysfunction in KD *via* the co-culture of peripheral blood mononuclear cells (PBMCs) and human coronary artery endothelial cells (HCAECs) (12). Also, non-coding RNAs would always participate in the maintenance of endothelial homeostasis, and miR-197-3p had been detected in KD-associated endothelial damage *via* the regulation of TIMP3 expression (13). In an experimental study, human umbilical cord mesenchymal stem cells could regulate the expression levels of CD54 and CD105 in vascular endothelial cells in KD to suppress the responding inflammation process and reduce endothelial damage, providing a new basis for stem cell therapy for KD (14). Thus, endothelial cells in coronary arteries play an essential role in the outcomes of KD. It is critical to understand the molecular mechanisms of KD-induced endothelial dysfunction and the potential therapeutic strategies that would help maintain endothelial homeostasis. In this review, we focus on the molecular mechanisms of endothelial function in KD and summarize the cutting-edge evidence on endothelial cell therapeutic targets in recent years.

Endothelial cell dysfunction in Kawasaki disease

The most common but threatening complication associated with KD should be coronary artery lesions (CALs), including coronary artery aneurysm (CAA) or coronary artery dilation (CAD), which would induce coronary artery thrombosis. In severe cases, patients may suffer myocardial infarctions or progress to coronary artery diseases when they get older (15). A study of KD specimens from autopsies ($n = 32$), explanted hearts ($n = 8$), and an incidentally-detected CAA resection by light microscopy (LM) and transmission electron microscopy (TEM) identified three distinct but linked basic vascular pathophysiological processes of KD, including necrotizing arteritis (NA), subacute/chronic (SA/C) vasculitis, and luminal myofibroblast proliferation (16). NA was a synchronous neutrophil process of endothelial cells that occurred within the first 2 weeks of KD. It was considered a self-limiting process that gradually destroyed the homeostasis of the intima, media, and part of the outer membrane of the coronary artery, leading to the development of cystic aneurysms, which would, in turn, lead to aneurysm rupture or thrombosis. It was the dominant cause of KD-induced early death. An increasing number of studies demonstrated that the inflammatory factors induced by KD are accumulated in coronary artery endothelial cells by targeting particular receptors and regulating responding pathways to initiate vasculitis. It was believed that the heterogeneities in the recruitment of chemokines, adhesion molecules, or other molecules on endothelial cells between coronary arteries and other similar-sized arteries should be responsible for the differences in the types of injuries, which decided the specific coronary endothelial dysfunction in KD (17). Li et al. found a significant association between rs2069952, rs9574, and rs1415774 of the endothelial protein C receptor (EPCR) gene and a higher probability of the occurrence of KD in Chinese children (18). The ectodomain of SyndecanSDC-1 could be shed from the cell surface and released into serum, and the shed SDC-1 in serum is regarded as a biomarker for endothelial activation or damage. Serum levels of SDC-1 were significantly higher in patients with KD than in healthy controls and febrile controls (19). As such, the coronary artery endothelial cells serve as the first interface between inflammatory molecules and the vascular wall, and it was important to identify the molecular

TABLE 1 Non-coding RNAs regulates vascular endothelial cell injury and maintain in KD.

miRNA	Alteration	Target	Function
miR-233	Up	IL6ST	Inhibit STAT3 signal pathway, induce cell injury
miR-233	Up	N/A	Promote cell apoptosis
miR-197-3p	Up	TIMP3	Regulate MMP9, induce cell damage
miR-483	Down	CTGF	Maintain cell homeostasis
miR-27b	Up	SMAD7	Regulate TGF pathway, effect cell migration and proliferation
SOCS2-AS1	Up	MiR-324-5p	Increase cell proliferation and decrease cell apoptosis
miR-324-5p	Down	CUEDC2	Decrease cell proliferation and facilitate cell apoptosis
miR-320a	Up	BMPR1A	Modulate TNF-α production
miR-145-5p	Up	TMEM9B	Regulate the expression of inflammatory cytokines
PINC	Up	N/A	Induce cell apoptosis and inhibit cell proliferation
miR-125a-5p	Up	MKK7	Regulate the Bax/Bcl2 pathway and activates Caspase-3, induced cell apoptosis
miR-186	Up	SMAD6	Induce cell apoptosis
miR-93	Down	VEGF-A	Regulate cell mitogenesis and cell migration

mechanisms of the role of endothelial cells in KD-associated coronary artery disease.

Molecular mechanism of vascular endothelial cell injury and dysfunction in KD

Expression of non-coding RNAs

MicroRNA (miRNA, miR) is a kind of small-molecule RNA with a length of approximately 21 nucleotides, which is involved in the regulation of cell proliferation, apoptosis, inflammation, autoimmunity, and functional maintenance. MiRNAs regulate protein expression *via* the post-transcriptional level by targeting mRNA molecules, mainly affecting the 3′ untranslated regions (3′UTR) (20). In recent years, miRNAs in serum exosomes or coronary artery tissues, including miR-93, miR-186, miR-223, miR-483, and miR-23a have been found to be associated with KD. The downstream signaling of miRNAs provides clues to identifying the molecular mechanisms of KD-induced coronary artery lesions (Table 1).

MiRs induce pro-inflammation activities in vascular endothelial cells. Serum miR-223-3p levels were found to be higher in KD patients than in healthy controls. The expression changes in interleukin (IL)-6, intercellular adhesion molecule 1 (ICAM-1), and E-selectin were similar to those of miR-223-3p expression, which increased in the acute stage and reduced in the subacute stage. Also, IL6 was confirmed to be the target gene of miR-223-3p in HCAECs and mouse models, and IL6 would suppress the STAT3 signaling pathway to induce vascular endothelial injury (21). Endothelial microparticles (EMPs) are abundant in circulating blood during inflammation. MiRNAs encapsulated within EMPs had been proven to be involved in the pathogenesis of inflammatory diseases (22). Both miR-320a and miR-145-5p were encapsulated in KD patients with CAL. MiR-320a interacted with *BMPR1A* and correlated with TNF-α expression while miR-145-5p targeted *TMEM9B*, which stimulated IL-6 expression (23). TNF-α was elevated in KD patients, and it induced human umbilical vein endothelial cell (HUVEC) apoptosis. Serum levels of miR-223 were significantly higher in KD patients with CAL than in patients without severe vascular injury, and the serum miR-223 level would decrease after immunoglobulin treatment. However, the miR-233 in serum was mainly secreted by bone marrow-derived blood cells and not transcript in endothelial cells. It was confirmed that miR-233 could be made to enter endothelial cells and promote their apoptosis by co-culturing endothelial cells and macrophages (24).

Besides, miRs had been proven to be involved in several cellular biological processes in endothelial cells subjected to KD. The proliferation of HCAECs could be inhibited by KD serum supplements, and miR-197-3p levels were significantly higher in KD. *TIMP3*, a regulator of matrix metallopeptidase 9 (MMP9), was confirmed as the target of miR-197-3p. Accordingly, the miR-197-3p/TIMP3 axis played a critical role in KD-induced endothelial damage *in vitro* and *in vivo* (13). MMP9 secreted by endothelial cells was thought to take part in CAL formation (25). Endothelial-to-mesenchymal transition (EndoMT) describes the process by which endothelial cells differentiate into mesenchymal cells under various stimulations, and EndoMT was found to be essential for cardiac valve development and involved in a series of cardiovascular diseases such as myocardial infarction, cardiac fibrosis, valve calcification, endocardial elastic fibrosis, atherosclerosis, and pulmonary hypertension (26). A set of spindle-like cells with a high expression of alpha-smooth muscle actin (α-SMA) are thought to be transdifferentiated from endothelial cells to mesenchymal conditions, and the

translated cells have great potential in the recruitment of pro-inflammatory cells and induce arterial wall injuries by secreting IL-17, MMPs, and connective tissue growth factor (CTGF). The cells in this category produce disordered collagen, which reduces the structural integrity of the media layer of arteries and contributes to aneurysm formation in KD (27). Also, CTGF was elevated in the coronary arterial wall and serum of KD patients and found to be regulated by miR-483 *via* experiments based on KD serum-treated HUVECs. He et al. revealed that the transcription factor, Kruppel-like factor 4 (KLF4), binds to the promoter region of IGF2-miR-483, up-regulated the expression of miR-483, and maintained coronary artery endothelial cell homeostasis by inhibiting the expression of CTGF (28). While miR-27b was found to be significantly up-regulated in KD serum and HUVECs exposed to KD serum, miR-27b would target *SMAD7* and the Transforming Growth Factor (TGF) pathway, leading to HUVEC migration and proliferation (29). MiR-125a-5p, which is highly expressed in KD, regulates the Bax/Bcl2 pathway and activates Caspase-3 by inhibiting MKK7, resulting in the initiation of HUVEC apoptosis (30). There is a high abundance of miR-186 in serum during KD's acute phase, and the application of KD serum would up-regulate the expression of miR-186 in HUVECs and induce cell apoptosis by targeting *SMAD6* (31). MiR-93 was dysregulated and may be involved in the regulation of vascular endothelial growth factor A (VEGF-A) expression in the pathogenesis of acute KD-induced arteritis (32).

Beyond miRs, long non-coding RNAs (lncRNAs) also contribute to the maintenance of the biological function of coronary artery endothelial cells. Zhao et al. found that lncRNA SOCS2 antisense 1 (SOCS2-AS1) is highly expressed in KD patients' serum and coronary artery tissue, especially in patients with coronary aneurysms. The depletion of SOCS2-AS1 in HUVECs could attenuate cell proliferation and facilitate cell apoptosis. SOCS2-AS1 competitively binds to miR-324-5p to elevate its target gene (*CUEDC2*) expression in the progression of HUVECs in KD (33). lncRNA pregnancy-induced non-coding RNA (PINC) was also involved in the pathogenesis of KD-associated vascular injury (34).

Inflammatory cell activation

During the acute and subacute phases of KD, significant changes occur among inflammatory cells. A series of studies demonstrated that activated monocytes, neutrophils, and natural killer (NK) cells were significantly associated with coronary artery injuries. So that, the interplay between inflammatory cells and vascular endothelial cells contributes to the formation of CAAs and other related complications. A single-cell RNA-seq study revealed that a subset group of monocytes was involved in KD.

Cell count and neutrophil ratio elevations were major clinical features of KD; thus, the relationship between neutrophils and vascular injuries was a hot topic. Serum Semaphorin 4D (Sema4D) levels were significantly increased in KD patients with CALs. Sema4D had been identified as a pro-inflammatory factor influencing vascular endothelial cell function. Besides, the expression of Sema4D disturbs the cellular function of CD15+ neutrophils and correlates with the shedding proteinase, ADAM17, which interacts with endothelial cells and is associated with cardiovascular disease. Sema4D also promotes the production of cytokines in HCAECs in a dose-dependent manner through the Sema4D-plexin B axis (35) (Figure 1).

Moreover, monocytes had been confirmed to be recruited to the coronary artery and demonstrated more adhesion with endothelial cells. The expression of monocyte chemoattractant protein-1 (MCP1) enhanced the adhesion between monocytes and endothelial cells, which exerts its action by interacting with the C-C chemokine receptor type 2 (CCR2) on the surfaces of monocytes (36, 37) (Figure 1). The expression level of E-selectin was also increased when incubating HCAECs with serum samples of KD with CAL, which led to more endothelium-monocyte interactions. The co-culture of HCAECs with the supernatant of S100A12-activated monocytes induced the expressions of IL6, IL8, ICAM1, and VCAM1 in HCAECs, and this process was mediated by IL1β (38). TNFα or MRP-8/MRP-14, which was secreted by granulocytes, would induce thrombosis and inflammatory responses in patients with acute KD as the consequence of endothelial damage (39) (Figure 1). Neutrophils produced VEGF during the early stages of acute KD while monocytes mainly expressed VEGF from the second week and continued to the fourth week after the onset of KD (Figure 1). The interaction between monocytes and endothelial cells, as well as the secretion of VEGF, influence the proliferation and migration of endothelial cells, resulting in pathological changes in KD-affected coronary artery tissues (40). In a *Lactobacillus casei* cell wall extract (LCWE)-induced macrophages model of KD, the calcium-activated potassium channel, KCa3.1, was highly activated, causing inflammatory reactions in murine coronary artery endothelial cells (MCAECs) (41). DICER1 had been identified to promote the maturation of pre-miRNA in KD-affected platelets after vascular injury, and it contributes to the binding of these platelets to vascular smooth muscle cells (VSMCs), initiating the transport of miRNAs from platelets to VSMCs *via* phagocytosis. Platelet-generated miRNAs inhibited the expression of PDGFRβ in VSMCs after internalization, which prevented vascular smooth muscle cell dedifferentiation and attenuated endothelial repair and damaged tissue healing (42). In another mouse vasculitis model of KD induced by a *Candida albicans* water-soluble extract (CAWS), Miyabe et al. found that Dectin-2 signaling in residual macrophages in the aortic root of the heart induced early CCL2 production and the initial recruitment of $CCR2^{+}$ inflammatory

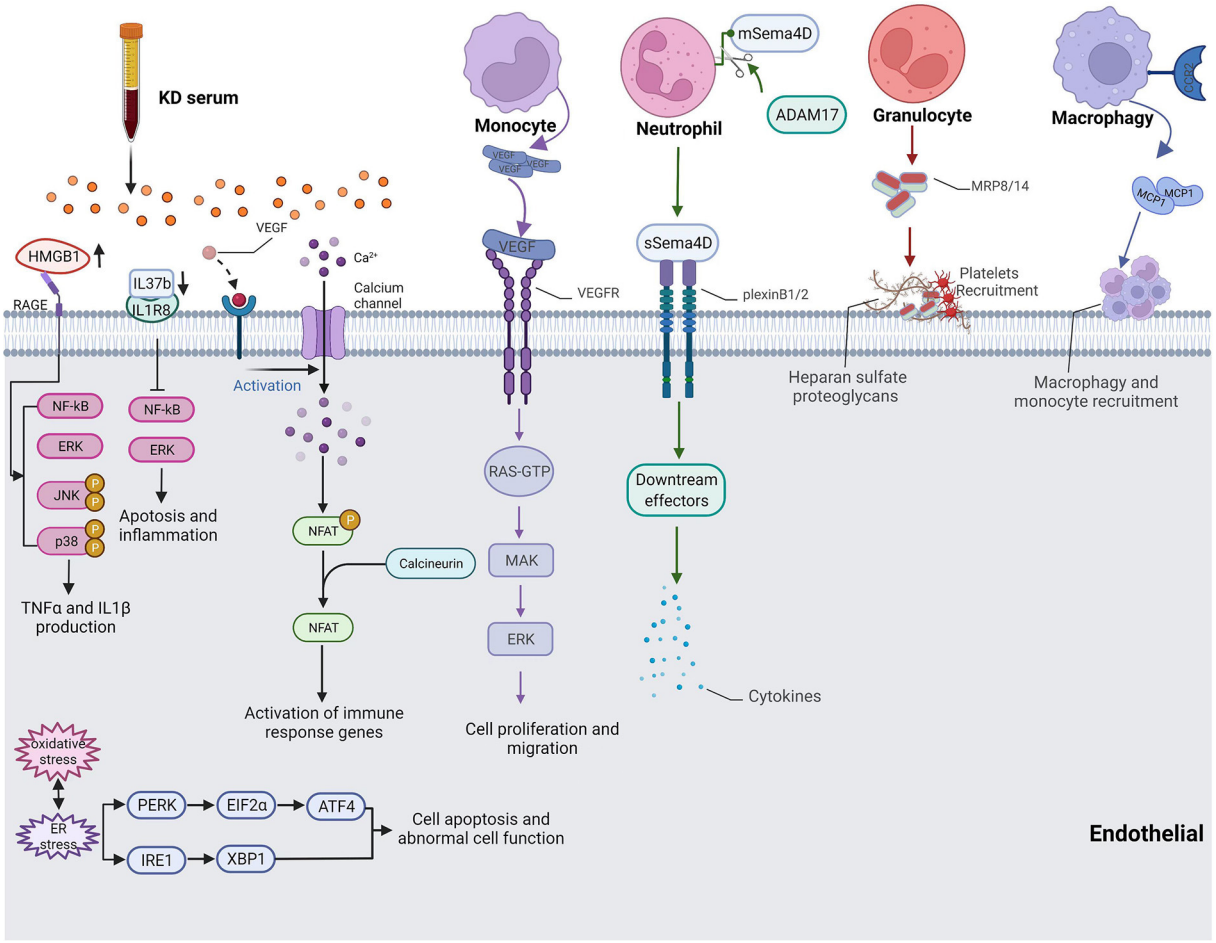

FIGURE 1
Pathway of cytokine production and inflammatory cell-endothelial cell interaction in KD. HMGB1 up-regulated NF-κB-mediated inflammatory responses and promoted IL1β and TNFα production. IL37 induced apoptosis and inflammation of endothelial cells through the IL-1R8 pathway. VEGF activated Ca2+/NFAT pathway induced inflammation activity by regulating E-selectin, VCAM-1 and MCP-1. Monocyte-produced VEGF also took part in cell migration. Sema4D released by Neutrophil affected endothelial cell cytokine production. Monocytes, macrophage, platelets, and granulocytes recruited to coronary artery and adhesion endothelial cells.

monocytes (iMos) into the aortic root and coronary arteries (43). An elevation in the nuclear factor of activated T cells (NFAT) was recorded in the KD group, and KD serum would activate the signaling of Ca+/NFAT in HCAECs. Then, NFAT induced inflammatory activity by regulating E-selectin, VCAM-1, and MCP-1 (44) (Figure 1).

Cytokine production

After the activation of inflammatory cells in KD, cytokines would be produced by inflammatory cells and coronary artery endothelial cells that were stimulated by targeting monocytes or neutrophils. Serum-treated HCAECs showed high mitochondrial membrane potentials, increased mitochondrial gene transcription levels, and mitochondrial complex I activity, indicating that oxidative phosphorylation (OXPHOS) had been regulated by cytokines associated with inflammatory cells (45). IL-37 is a member of the IL-1 family and plays an anti-inflammation role in endothelial cells and cardiovascular disease (46). The expression of IL37 decreased in KD serum-treated HUVECs, and exogenous supplementary of IL-37b alleviated KD serum-induced apoptosis and inflammation of endothelial cells through the IL-1R8 pathway. Besides, IL-37b injections remarkably decreased VCAM-1 expression and the infiltration of macrophages and neutrophils in a CAWS-induced KD mouse model (47) (Figure 1). Anzai et al. found that CAWS mediates IL-1β and NLRP3 inflammasome activation through the Dectin-2/Syk/JNK/NF-κB pathway and the Dectin-2/Syk/JNK/mitochondrial Reactive oxygen species (mtROS) pathway, which both participate in KD vasculitis (48). High mobility group box B1 (HMGB1) is a molecular

pattern molecule associated with extracellular damage and a key regulator of autophagy (49). HMGB1 is released by endothelial cells when exposed to microorganisms, pathogens, and endogenous inflammatory factors (50). HMGB1 and its receptors, RAGE, TLR2, and TLR4, were up-regulated in KD serum-treated HCAECs; however, only RAGE could decrease after prednisolone administration. They also up-regulated NF-κB-mediated inflammatory responses in KD and promoted IL1β and TNFα production to cause endothelial cell injury (51) (Figure 1).

Reactive oxygen species accumulation

Excessive reactive oxygen species (ROS) represent endothelial dysfunction, which leads to the progression of coronary artery inflammation or dysfunction. Once prolonged or excessive perturbations exist, the unfolded protein response might trigger intracellular signal cascades and induce oxidative stress, inflammation, and apoptotic responses (52). Bollmann et al. found that systemic inflammation caused by tristetraprolin deficiency leads to endothelial dysfunction. Oxidative stress, especially increased Nox2 activity, promoted the development of atherosclerosis in models of systemic inflammatory diseases (53). Oxidative and ER stress was identified to be involved in KD-induced damages in HCAECs via KD serum stimulation. ROS production and its related molecules, including ATF4, p-EIF2α, p-PERK, XBP1, p-IRE1, HSP90B1, HSPG2, DNAJC3, P4HB, and VCP, were increased in KD patients and decreased by berberine treatment (52) (Figure 1). In giant cell arteritis, immature neutrophils migrate from both lumen and capillaries to adhere to the elastic lamina and release ROS in an inflammatory microenvironment for a prolonged duration, leading to aggressive protein oxidation and the disruption of the permeability of the endothelial barrier in an *in vitro* experimental system (54). Martin et al. found that endothelial cell-derived microparticles expressing VCAM-1 or C4d increase in KD patients and cause endothelial dysfunction by releasing substances such as ROS and cytokines (55). Hypoxia-inducible factor (HIF) signaling had been considered a protective mechanism against ROS in endothelial cells. Ehling et al. found that the B55α/PP2A complex restrained PHD-2's activity to promote endothelial cell survival in a HIF-dependent manner and dephosphorylated p38 (56). During angiogenesis, endothelial progenitor cells are recruited from the bone marrow and differentiate *in situ* into mature endothelial cells under the influence of NO produced by eNOS activation (57). NADPH oxidase (NOX), a primary cause of oxidative stress in the vasculature, contributed significantly to endothelial dysfunction in the microcirculation under excessive ROS exposure, subsequently disrupting nitric oxide (NO) signaling in KD. The product of this reaction, peroxynitrite, was a kind of powerful oxidant and could exacerbate vascular dysfunction by causing further damage to lipids, proteins, and DNA, uncoupling endothelial nitric oxide synthase (eNOS), and diminishing smooth muscle responses to NO (58).

Lipid oxidation

Oxidized low-density lipoprotein (oxLDL), which is derived from low-density lipoprotein (LDL), is a specific lipid metabolite produced under oxidative stress and the most active component of lipoproteins that promotes the development of atherosclerosis. As a specific receptor of oxLDL, lectin-like-oxLDL receptor-1 (LOX-1) was the only receptor that could be released from the cell surface to form a soluble scavenger molecule. LOX-1 is mainly expressed in vascular endothelial cells of coronary arteries, macrophages, lymphocytes, and dendritic cells. Previous studies showed that the interaction between oxLDL and LOX-1 was a key mechanism for endothelial cell injury (59). A study conducted on 80 children with KD, 20 febrile children, and 20 healthy children revealed that the plasma oxLDL concentration and LOX-1 mRNA expression in PBMCs were significantly higher in children with KD in the acute phase, especially when associated with CAL formation (11).

Targeting endothelial cell treatment in KD

As endothelial cells serve as a major target during KD, endothelial cell injuries play a critical role in the development of adverse complications, especially for KD-associated coronary artery disease. Thus, attempts have been made to attenuate or reverse endothelial cell damage in response to excessive inflammatory activity. Atorvastatin has been shown to help maintain endothelial cell homeostasis and suppress vascular inflammation and has been identified as a potential new candidate treatment for KD. Atorvastatin could activate KLF4, reducing CTGF production of KD-injured endothelial cells. Besides, atorvastatin showed great potential to be an additional alternative to the standard treatment by significantly reducing CTGF levels in patients (28). A phase I/IIa dose-escalation study of atorvastatin in KD patients with CAA validated the safety and pharmacokinetic data of atorvastatin (60). Intravenous immunoglobulin (IVIG), which is somehow efficient in most patients, has been used as the first-line standard treatment of KD. The addition of prednisolone to intravenous immunoglobulin for acute KD may ameliorate HMGB-1-mediated inflammation in KD-induced vasculitis (51). In a HCAEC cell model of KD, the addition of corticosteroids to standard IVIG therapy suppressed cellular Caspase3/7 activity and inhibited cell apoptosis. It can also inhibit the release of HMGB1 and reduce the expression of three HMGB1-mediated inflammatory cytokines, TNF-α,

IL-1α, and IL-1β, which is of great benefit to the clinical treatment of patients with severe KD (61). Resveratrol inhibits TNF-α-induced ICAM-1 expression via the activation of autophagy (62). 1α,25-dihydroxy vitamin D3 (1-25$(OH)_2$-VitD3) has an inhibitory effect on TNF-α-induced E-selectin expression, inhibits TNF-α-induced NF-κB activation in HUVECs, and modulates the inflammatory response in KD vasculitis (63). 1-25$(OH)_2$-VitD3 also inhibits TNF-induced ICAM-1, VCAM-1, IL-6, and IL-8 expression in HCAECs (64). Cyclosporine is an immunosuppressant that blocks calcineurin, a downstream molecule of the Ca^{2+}/NFAT pathway that inhibits nuclear translocation of NFAT-regulating genes, thereby mediating immunosuppression. The researchers studied the efficacy of cyclosporine A in the treatment of IVIG resistant and refractory Kawasaki disease. It is speculated that from a pharmacogenomics perspective, the study of new Ca^{2+}/NFAT pathway inhibitors may be promising in treatment (44).

Moreover, with the rapid development of gene therapy, it allows to generate specific RNAi, gene overexpression, knock-down strategies for endothelial cells with adeno-associated virus (AAV) or particular nanoparticles. The current gene therapy process much shorten the develop duration for a newly invented medication, and expanded the capability to handle specific genes expression. As the above content mentioned, several miRs have involved in KD related coronary artery disease, the AAV vector presented great efficient to target abnormal miRs and provide protective role in reducing endothelial injuries (65).

Conclusion

It has been 50 years since KD proposed for the first time. Although the etiology and the initiating factor is always not clear, but for KD pathology and coronary arterial vasculitis of research to improve our essential understanding of KD. At present, it is generally accepted that KD may be one or more uncertain infection factor in genetically susceptible individuals induced intense inflammation host response. Vascular endothelial cell inflammation caused by KD has been persistent since the beginning of the disease. Therefore, it is of great significance to discover serum biomarkers with diagnostic significance for endothelial cell injury in the early stage of the disease and to carry out treatment for vascular endothelial cell injury in the near future. Further research should focus on cytogenetics and molecular biology, so that more effective targeted drugs can be used in clinic to improve the prognosis of children with KD.

Author contributions

YueZ and YL conceived of the presented idea. YQ, YulZ, YL, and YueZ summarized the reference and draft the manuscript. YueZ organized the figure with online free material. YueZ and YH supervised the project and contributed equally to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Identification of potential biomarkers of inflammation-related genes for ischemic cardiomyopathy

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Objective: Inflammation plays an important role in the pathophysiology of ischemic cardiomyopathy (ICM). We aimed to identify potential biomarkers of inflammation-related genes for ICM and build a model based on the potential biomarkers for the diagnosis of ICM.

Materials and methods: The microarray datasets and RNA-Sequencing datasets of human ICM were downloaded from the Gene Expression Omnibus database. We integrated 8 microarray datasets *via* the SVA package to screen the differentially expressed genes (DEGs) between ICM and non-failing control samples, then the differentially expressed inflammation-related genes (DEIRGs) were identified. The least absolute shrinkage and selection operator, support vector machine recursive feature elimination, and random forest were utilized to screen the potential diagnostic biomarkers from the DEIRGs. The potential biomarkers were validated in the RNA-Sequencing datasets and the functional experiment of the ICM rat, respectively. A nomogram was established based on the potential biomarkers and evaluated *via* the area under the receiver operating characteristic curve (AUC), calibration curve, decision curve analysis (DCA), and Clinical impact curve (CIC).

Results: 64 DEGs and 19 DEIRGs were identified, respectively. 5 potential biomarkers (SERPINA3, FCN3, PTN, CD163, and SCUBE2) were ultimately selected. The validation results showed that each of these five potential biomarkers showed good discriminant power for ICM, and their expression trends were consistent with the bioinformatics results. The results of AUC, calibration curve, DCA, and CIC showed that the nomogram demonstrated good performance, calibration, and clinical utility.

Conclusion: SERPINA3, FCN3, PTN, CD163, and SCUBE2 were identified as potential biomarkers associated with the inflammatory response to ICM. The proposed nomogram could potentially provide clinicians with a helpful tool to the diagnosis and treatment of ICM from an inflammatory perspective.

KEYWORDS

ischemic cardiomyopathy, inflammation-related genes, biomarker, nomogram, bioinformatics analyses, heart failure

Introduction

Heart failure (HF) is generally considered a complex clinical syndrome with symptoms and/or signs caused by structural and/or functional cardiac abnormalities, leading to considerable morbidity and mortality (1). Data from 2020 showed that there were an estimated 23 million HF patients worldwide, with a 1-year mortality rate of 7.2% and a 5-year survival rate of 25% after hospitalization (2). There were 12.1 million patients with HF and 3.0 million patients with incident HF $\geq$ 25 years old in China (3). HF imposes a significant burden on global health systems. Therefore, it is socially important to actively study countermeasures to prevent and treat HF.

Over the past few decades, the aetiologies of HF have shifted from valvular heart disease and hypertension to coronary artery disease (4). The term ischemic cardiomyopathy (ICM) is also known as ischemic heart disease in many cases and manifests as the syndrome of HF due to chronic left ventricle systolic dysfunction resulting from underlying coronary artery disease (4, 5). ICM is the most common but underestimated manifestation and cause of HF and also the main cause of mortality in patients with HF. The evidence from clinical, angiographic, and autoptic findings demonstrates a more complex pathophysiological process in ICM (6). Although massive studies have been made in exploring the pathological mechanisms of ICM, it remains poorly understood. There is no doubt that an in-depth exploration of the pathological mechanisms of ICM will bring novel insights and ideas for its diagnosis and treatment.

Inflammation plays an important role in the pathophysiology of many cardiovascular diseases. Strong correlations were observed between the elevated level of inflammation and the several stages of ICM (7). Clinically, there are some limitations in determining the severity of inflammation in ICM. In recent decades, the studies on biomarkers of cardiovascular disease and their clinical application have increased exponentially (8). As is well known, early diagnosis is extremely important for effective treatment and prognosis of the disease. Several inflammation-related factors including C-reactive protein, interleukin (IL) 6, galectin-3, etc., are considered biomarkers for ICM (9). However, the limited number of important and specific inflammatory biomarkers for ICM has become a growing problem in its diagnosis and treatment. Against this background, it is significant to optimize the diagnosis and treatment of ICM from the perspective of inflammation.

With the continuous development and popularization of high-throughput technologies such as biochips and second-generation sequencing, data information such as transcriptomics and epigenetics of many disease pathological processes have been accessed by researchers, and these massive data provide important support for researchers to deeply explore and reveal the mechanisms and patterns of the occurrence, development, and regression of human diseases. Machine learning (ML), a subset of artificial intelligence, has been used in several medical fields (10, 11). ML algorithms can discover complex patterns and powerful evidence in large volumes of medical data to support clinical decision-making (10–12). There is a trend that multiple ML algorithms such as least absolute shrinkage and selection operator (LASSO), support vector machine recursive feature elimination (SVM-RFE), random forest (RF) are used to screen disease biomarkers and therapeutic targets, explore pathogenesis and predict clinical outcomes, which will allow for more rigorous and standardized processes. Many studies have also attempted to explore the possible pathological progression mechanisms of ICM by combining data from the public database, bioinformatics analysis, and ML algorithms (13–15). However, few studies have been conducted to identify potential inflammation-related diagnostic genes for ICM. Hence, it is very important to explore the immune-related diagnostic biomarkers that can make the early diagnosis of ICM possible.

In this study, we integrated 8 microarray datasets to screen the differentially expressed genes (DEGs) between ICM and non-failing control (NFC) myocardial tissue. Then, the differentially expressed inflammation-related genes (DEIRGs) were identified by intersecting the inflammation-related genes (IRGs) with DEGs. Subsequently, three ML algorithms were used to screen the promising diagnostic biomarkers of ICM from DEIRGs. The expression levels of these potential biomarkers were validated in additional RNA-Sequencing datasets and the functional experiment of the ICM rat. Finally, a nomogram model based on the potential biomarkers was

established to predict ICM (**Figure 1**). We hope that our results can further strengthen the understanding of the role of inflammation in ICM and contribute to the development of promising diagnostic and therapeutic strategies.

Materials and methods

Data acquisition and preprocessing

The ICM-associated datasets were extracted from the Gene Expression Omnibus (GEO) database.[1] The relevant information of the datasets included in the current study were shown in **Supplementary Table 1A**. The research population in this study included 315 patients with ICM and 232 subjects in the NFC group. The characteristics of the population were listed in **Supplementary Table 1B**. The probe names in the microarray datasets were converted to corresponding gene symbols using Perl script. The microarray datasets, GSE5406, GSE16499, GSE21610, GSE42955, GSE52601, GSE1869, GSE57338, and GSE76701, were merged into one dataset (the merged dataset) and the batch effects among microarrays were removed using the SVA package (16). The RNA-Sequencing datasets, GSE116250, GSE48166, and GSE46224, were used as external validation datasets.

Identification and functional enrichment analyses of differentially expressed inflammation-related genes

The DEGs between NFC and ICM samples in the merged dataset were screened by limma package (17) with the threshold criteria of $|\log_2$ fold change (FC)$| > 0.585$ and P-adj < 0.05. The IRGs were extracted from DisGeNET[2] and the Molecular Signature database.[3] 467 IRGs were obtained from the DisGeNET. 4 gene sets (M5932, M15877, M13807, and M38152) were downloaded from the MSigDB database. Ultimately, 1746 IRGs were yielded after eliminating the duplicates. DEIRGs were identified by intersecting the IRGs with DEGs identified in the merged dataset. The functional enrichment analyses of DEIRGs were performed in Metascape[4] (18), using Gene ontology (GO), KEGG Pathway, Reactome Gene Sets, and WikiPathways. $P < 0.01$, a minimum count of 3, and the enrichment factor > 1.5 were set as the thresholds.

1 http://www.ncbi.nlm.nih.gov/geo

2 http://www.disgenet.org

3 http://www.gsea-msigdb.org/gsea/index.jsp

4 http://metascape.org

Screening potential biomarkers based on machine learning

We used LASSO *via* glmnet package (19, 20), SVM-RFE *via* e1071 package (21), and RF *via* "randomForest" package (22) to screen vital biomarkers for ICM from DEIRGs, respectively. The ML algorithms parameters were set as follows: LASSO, cvfit = cv.glmnet (nfold = 10, family = "binomial," type.measure = "class"); SVM = rfe (functions = caretFuncs, method = "cv," methods = "svmRadial"); randomForest (ntree = 500). Characteristic genes with the minimum cross-validation error were used as output files. The potential diagnostic biomarkers were yielded by intersecting the vital biomarkers identified by three algorithms. The diagnostic value of the biomarker was evaluated by the receiver operating curves (ROC) curve in the merged dataset. Concurrently, their expressions and diagnostic values were also validated in the external validation datasets.

Validation of the biomarkers expressions in the rat ischemic cardiomyopathy model

Male SD rats aged 6–8 weeks (purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd., production license: SCXK (Jing)-2016-0011) were randomly divided into sham and ICM groups (n = 6/group). The rats were fed under a 12 h cycle of light/dark in IVC condition and had free access to food and water. A rat ICM model was constructed by permanent ligating the left anterior descending coronary artery, as previously described (23). At 8 weeks, M-mode echocardiography in the left ventricular parasternal long-axis view was performed using a Vivid E9 (GE Vingmed, Horten, Norway). Serum B-type natriuretic peptide (BNP) levels in sham and ICM rats were measured with rat BNP ELISA kits (Elabscience Biotechnology, China). Meanwhile, wheat germ agglutinin (WGA, Sigma, L4895) staining was performed to evaluate the size of the cardiomyocytes, as previously described (24). The myocardial pathological changes were also detected by conventional hematoxylin-eosin (HE) staining kit (G1120, Solarbio, Beijing, China) and Masson staining kit (G1340, Solarbio, Beijing, China). Proteins were extracted from ischemic left ventricular regions of rats. After separation *via* SDS-PAGE, the proteins were transferred to the PVDF membranes (Millipore, Darmstadt, Germany). The membranes were blocked with 5% BSA and incubated with the primary antibodies at 4°C overnight. Then the secondary antibody was added and incubated for 1 h. Finally, the blots were visualized using the ECL Plus kit (Solarbio, PE0010, Beijing, China) and exposed to a ChemiDoc MP Imaging System (Bio-Rad, CA, United States). Protein density was measured using Image Lab

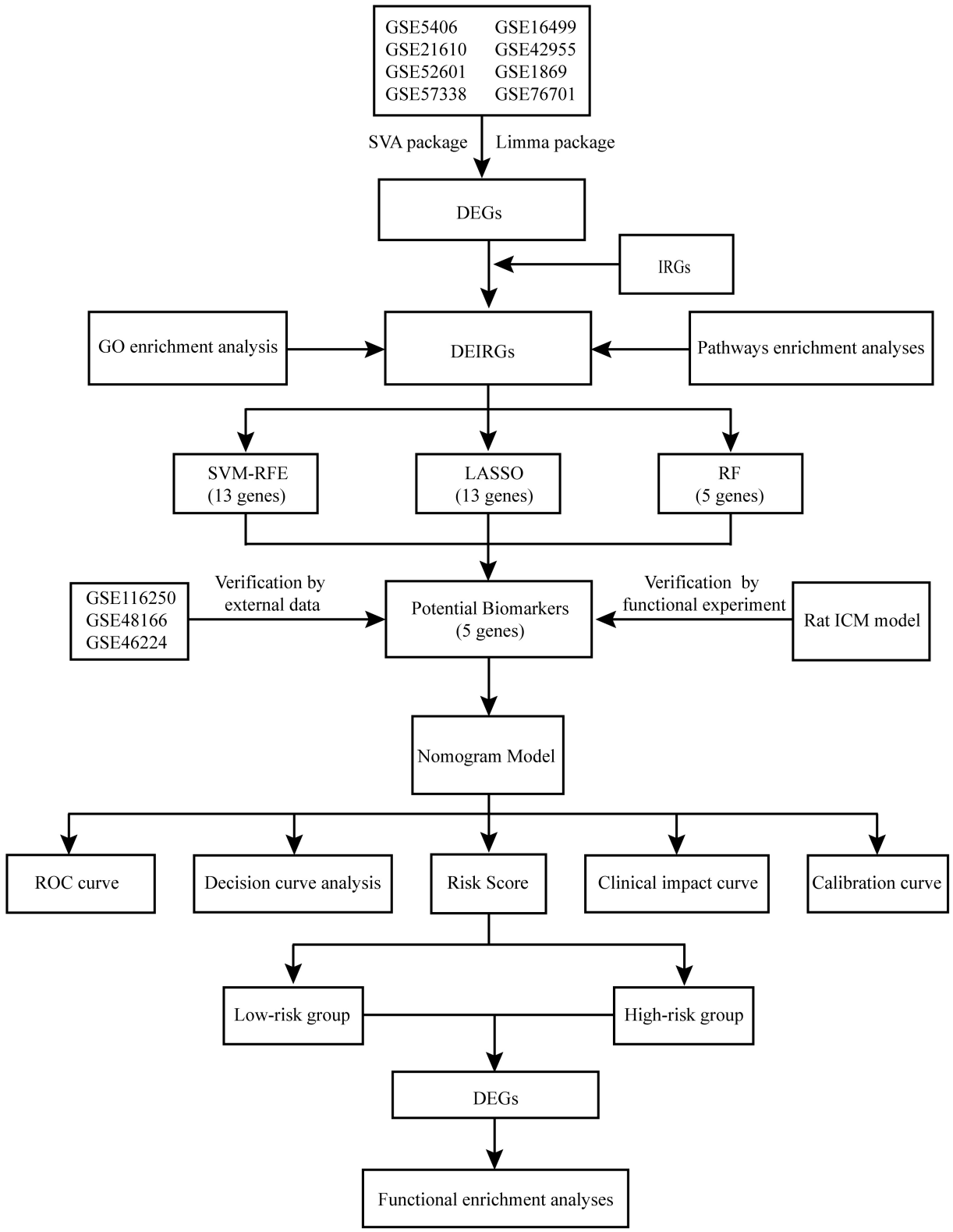

FIGURE 1
Flowchart of the study design.

software. The variation of protein density was expressed as fold changes compared to the sham in the blot. The antibodies used in this study were as follows: CD163 (Proteintech, Cat#16646-1-AP; 1:600), PTN (Immunoway, Cat#YT5519; 1:1000), FCN3 (Immunoway, Cat#YN2366; 1:1000), SCUBE2 (Abcepta, Cat#ALS13965; 1:500), SERPINA3 (Immunoway, Cat#YT5391; 1:1000), GAPDH (Immunoway, Cat#YM3029; 1:10000).

Establishment and evaluation of a nomogram model

The "rms" package was employed to build a nomogram model based on the potential biomarkers selected by three ML algorithms. The calibration curve and ROC curve were employed to estimate the predictive power of the nomogram model. Decision curve analysis (DCA) and clinical impact curve (CIC) were used to evaluate the clinical value of the nomogram model. The risk score for each sample was derived from the nomogram model.

Functional enrichment analyses of the differentially expressed genes grouped by risk score

All samples in the merged dataset were dichotomized into the low-risk and high-risk groups according to the median risk score. The DEGs between the low-risk and high-risk groups were screened by limma package with the cutoff criteria of $|\log_2 FC| > 0.585$ and $P\text{-adj} < 0.05$. The functional enrichment analyses of DEGs were implemented in Metascape *via* the reference gene set and screening conditions mentioned above.

Statistical analysis

R software (version 4.1.0) was used in our study. The data were shown as mean ± SD and analyzed using IBM SPSS statistics 21.0 software. Student's *t*-test or Wilcoxon test was used to compare the data between the 2 groups. A P value < 0.05 was accepted as statistically significant.

Results

Screening of differentially expressed inflammation-related genes in the merged dataset

In our research, 8 microarray datasets were merged into a merged dataset, which contained 194 NFC and 270 ICM samples. **Figures 2A,B** indicated that batch-to-batch variation was removed. According to the screening method and criteria mentioned above, a total of 64 DEGs between NFC and ICM samples in the merged dataset were obtained, including 34 up-regulated and 30 down-regulated genes (**Figure 2C**). Finally, we obtained 19 DEIRGs by crossing the 64 DEGs with 1746 IRGs (**Figure 2D**).

Functional enrichment analyses of differentially expressed inflammation-related genes

We performed functional enrichment analyses to elucidate the roles of the DEIRGs during ICM. 75 biological processes, 9 cellular components, and 15 molecular functions were identified. We enriched 3, 20, and 11 pathways from the KEGG, Reactome, and Wiki databases, respectively. By manually curation, the GO terms and pathways that had an adjusted *p*-value of below 0.05 were shown in **Supplementary Tables 2, 3**. The significantly enriched GO terms related to ICM included inflammatory response, cell chemotaxis, extracellular matrix, antioxidant activity, and so forth. The pathways closely associated with ICM were mainly the apelin signaling pathway, toll-like receptors (TLRs) cascades, IL-18 and IL-17 signaling pathway, neutrophil degranulation, and so forth.

Screening potential diagnostic biomarkers for ischemic cardiomyopathy

We identified 13 genes from the DEIRGs as biomarkers for ICM using the LASSO algorithm under lambda.min = 0.0021 (**Supplementary Figures 1A,B**). Meanwhile, 13 DEIRGs were recognized as vital biomarkers using the SVM-RFE algorithm (**Supplementary Figure 1C**). The RF algorithm was adopted to rank the importance of the DEIRGs according to MeanDecreaseGini (**Supplementary Figures 1D,E**). The top 5 DEIRGs based on MeanDecreaseGini values were used as important biomarkers for subsequent analysis. To obtain the robust potential biomarkers in ICM, the vital biomarkers from three ML algorithms were overlapped. 5 potential biomarkers were ultimately selected (**Figure 3A**), including SERPINA3, FCN3, PTN, CD163, and SCUBE2. **Figure 3B** showed the chromosomal positions of the 5 potential biomarkers. A powerful diagnostic capacity was confirmed in the merged dataset with an AUC of 0.921 (95% CI 0.895–0.947) in SERPINA3, AUC of 0.923 (95% CI 0.898–0.948) in FCN3, AUC of 0.864 (95% CI 0.829–0.899) in PTN, AUC of 0.843 (95% CI 0.804–0.882) in CD163, and AUC of 0.835 (95% CI 0.798–0.872) in SCUBE2 (**Figure 3C**). Correlation between biomarkers was assessed using Spearman correlation analysis.

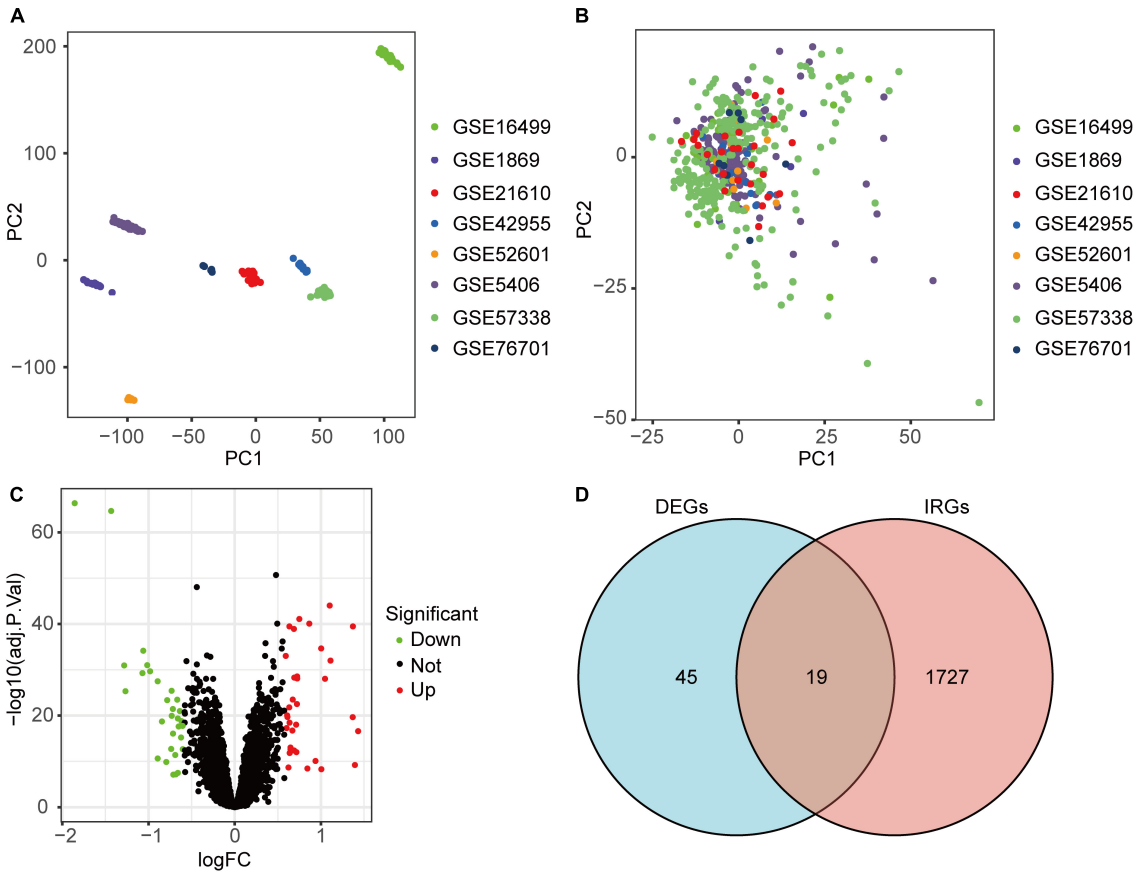

FIGURE 2
Identification of DEIRGs in ICM. **(A)** Two-dimensional principal component analysis cluster plot before merging of 8 datasets *via* SVA package. **(B)** Two-dimensional principal component analysis cluster plot after merging of 8 datasets *via* SVA package. **(C)** Volcano plot of the DEGs in the merged dataset. **(D)** Venn diagram of 19 DEIRGs shared by 64 DEGs and 1746 IRGs.

There was a significant positive correlation between SERPINA3 and CD163, and a significant negative correlation between FCN3 and SCUBE2 (Figure 3D). Moreover, the expression levels of SERPINA3, FCN3, and CD163 were lower in ICM samples compared with NFC samples in the merged dataset, while the opposite was true for PTN and SCUBE2 (Figure 3E).

External validation of the potential biomarkers in the external validation datasets

To obtain more accurate and reliable results, we performed ROC analysis and verified the expression levels of 5 potential biomarkers in the three external validation datasets, respectively. The results showed that their expression trends in the external validation datasets were consistent with those in the merged dataset (Supplementary Figures 2A–C), and they also had good discriminant power in distinguishing between NFC and ICM samples, as evidenced by AUC > 0.6 (Supplementary Figures 2D–F).

Further validation of the biomarkers in the rat ischemic cardiomyopathy model

As illustrated in Figures 4A,B, the deterioration of cardiac morphology and function was observed in the ICM group. The serum BNP levels were significantly increased in the ICM group (Figure 4C). The results of HE and Masson staining showed that compared to the sham group, disorganized myocardial tissue, disrupted cell structure with an enlarged or dissolved nucleus, and massive deposited collagen fibres were observed in the ICM group (Figures 4D,E). Moreover, cardiomyocyte hypertrophy was also observed in the ICM group (Figure 4F). Taken together, the results above showed that the rat ICM model was successfully established. To further verify the results of bioinformatics analysis, we detected the protein expression

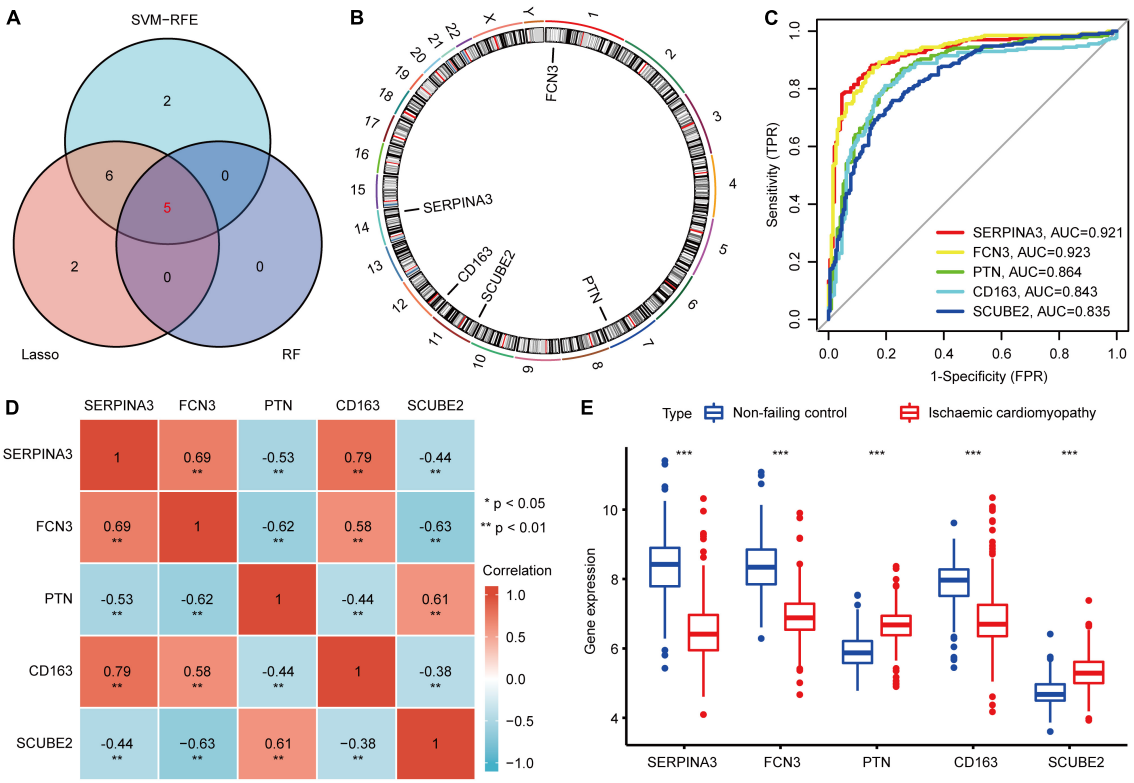

FIGURE 3
Identification of potential diagnostic biomarkers for ICM. **(A)** Venn diagram of the biomarkers extracted from LASSO, RF, and SVM-RFE algorithms. **(B)** The chromosomal positions of the 5 biomarkers. **(C)** ROC curve of the diagnostic effectiveness of the biomarkers. **(D)** Heatmap of correlations between the 5 biomarkers. **(E)** The differential expression histogram of the 5 biomarkers between the NFC and ICM samples from the merged dataset. ***$P < 0.001$.

levels of the 5 potential biomarkers in the myocardial tissues. Compared with the sham group, the protein expression levels of SERPINA3, FCN3, and CD163 were lower in the ICM group and the opposite was true for PTN and SCUBE2 (Figures 4G,H). Altogether, their changing trends were consistent with the results of bioinformatics analysis.

Establishment and evaluation of the nomogram model

The nomogram model constructed based on the 5 potential diagnostic biomarkers was shown in Figure 5A. The ROC analysis yielded the AUC values of 0.959 (95% CI, 0.941–0.977), which suggested that the prediction efficiency of the nomogram model was good (Figure 5B). The calibration curve indicated brilliant agreement among the apparent curve, bias-corrected curve, and ideal curve (Figure 5C). DCA and CIC showed that patients could benefit from the nomogram at threshold probabilities of 0.01–0.98 (Figures 5D,E). The risk score of each individual in the merged dataset was obtained using the nomogram model (Supplementary Table 4).

Functional enrichment analyses of the differentially expressed genes grouped by risk score

The principal component analysis showed that the clusters of low- and high-risk groups significantly showed no overlap, which suggested that these 5 potential diagnostic biomarkers could be well differentiated for the presence or absence of ICM in individuals (Figure 6A). After screening with the threshold, 72 DEGs between the low- and high-risk groups were identified (Figure 6B). To facilitate the understanding of the functional mechanisms of 5 potential biomarkers regulating ICM, functional enrichment analyses were implemented. The results of the GO enrichment analysis showed 23 terms enriched for cellular components, 32 for molecular function, and 146 for biological processes. The GO terms closely associated with ICM were mainly collagen binding, extracellular matrix, inflammatory response, innate immune response, etc. (Figure 6C). Based on the pre-set screening criteria, we enriched 11, 40, and 17 pathways from the KEGG, Reactome, and Wiki databases, respectively. The enriched KEGG pathways closely associated with ICM were mainly the IL-17 signaling

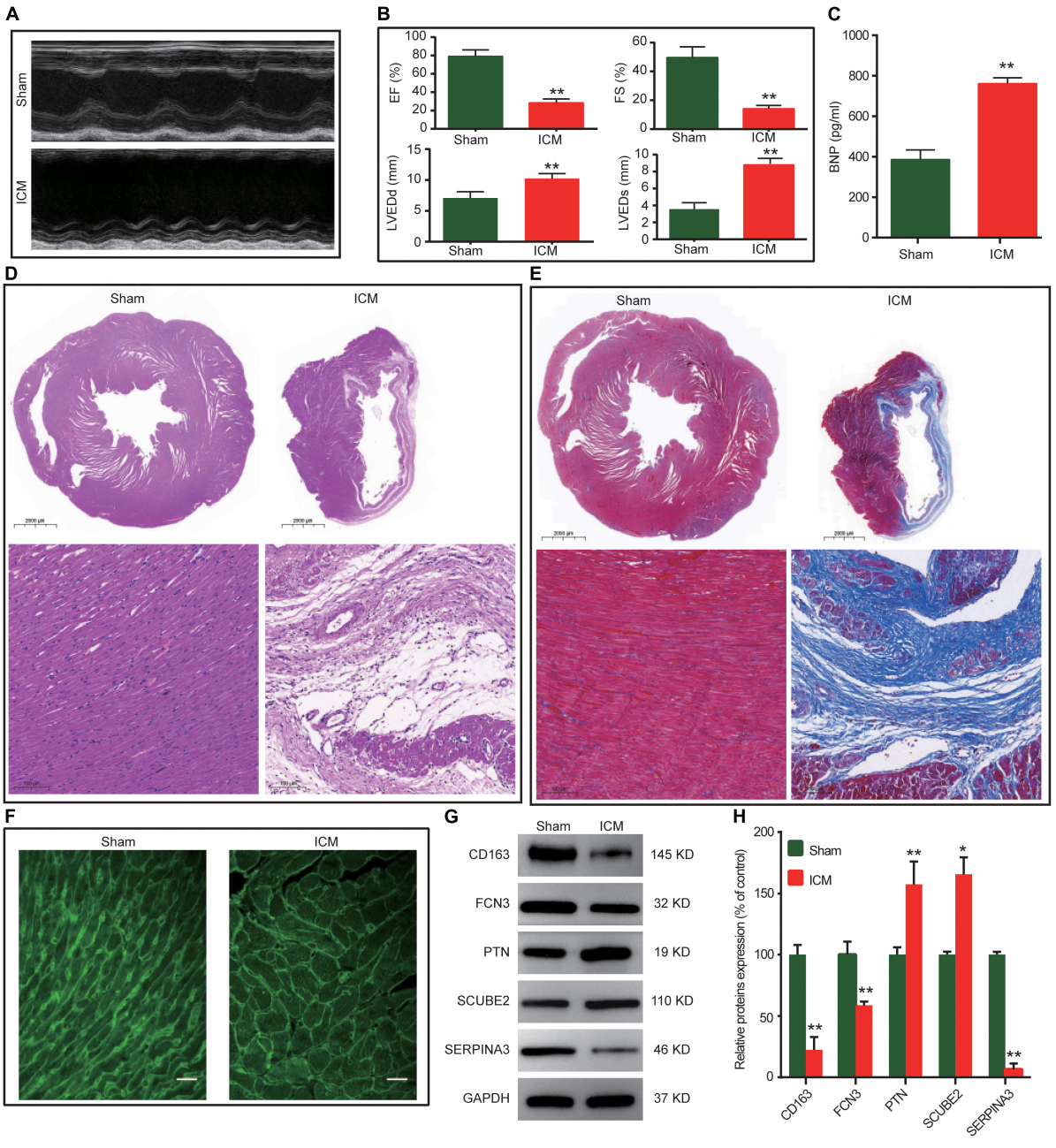

FIGURE 4
The validation of the biomarkers in the rat ICM model. **(A)** Representative M-mode echocardiographic images for each group. **(B)** Echocardiographic parameters for each group. (n = 6). **(C)** Serum BNP levels by ELISA (n = 6). **(D)** Representative images of HE staining. **(E)** Representative images of Masson staining. **(F)** Representative images of WGA staining. **(G)** Representative western blot results of the 5 biomarkers. **(H)** Relative protein expression levels of the 5 biomarkers (n = 3). *P < 0.05, **P < 0.01 vs the sham group.

pathway, Apelin signaling pathway, PPAR signaling pathway, etc. (Figure 6D). The enriched Wiki pathways closely associated with ICM were mainly the VEGFA-VEGFR2 signaling pathway, IL-18 signaling pathway, complement, coagulation cascades, etc. (Figure 6E). The enriched Reactome pathways closely associated with ICM were mainly TLRs cascades, cytokine signaling in the immune system, neutrophil degranulation, etc. (Figure 6F).

Discussion

Atherosclerosis, a chronic inflammatory disease of the arterial wall, is the common pathophysiological mechanism of many atherosclerotic diseases, such as coronary arterial disease, ischemic stroke, and ICM. There is ample evidence that inflammation has been identified as an important cardiovascular

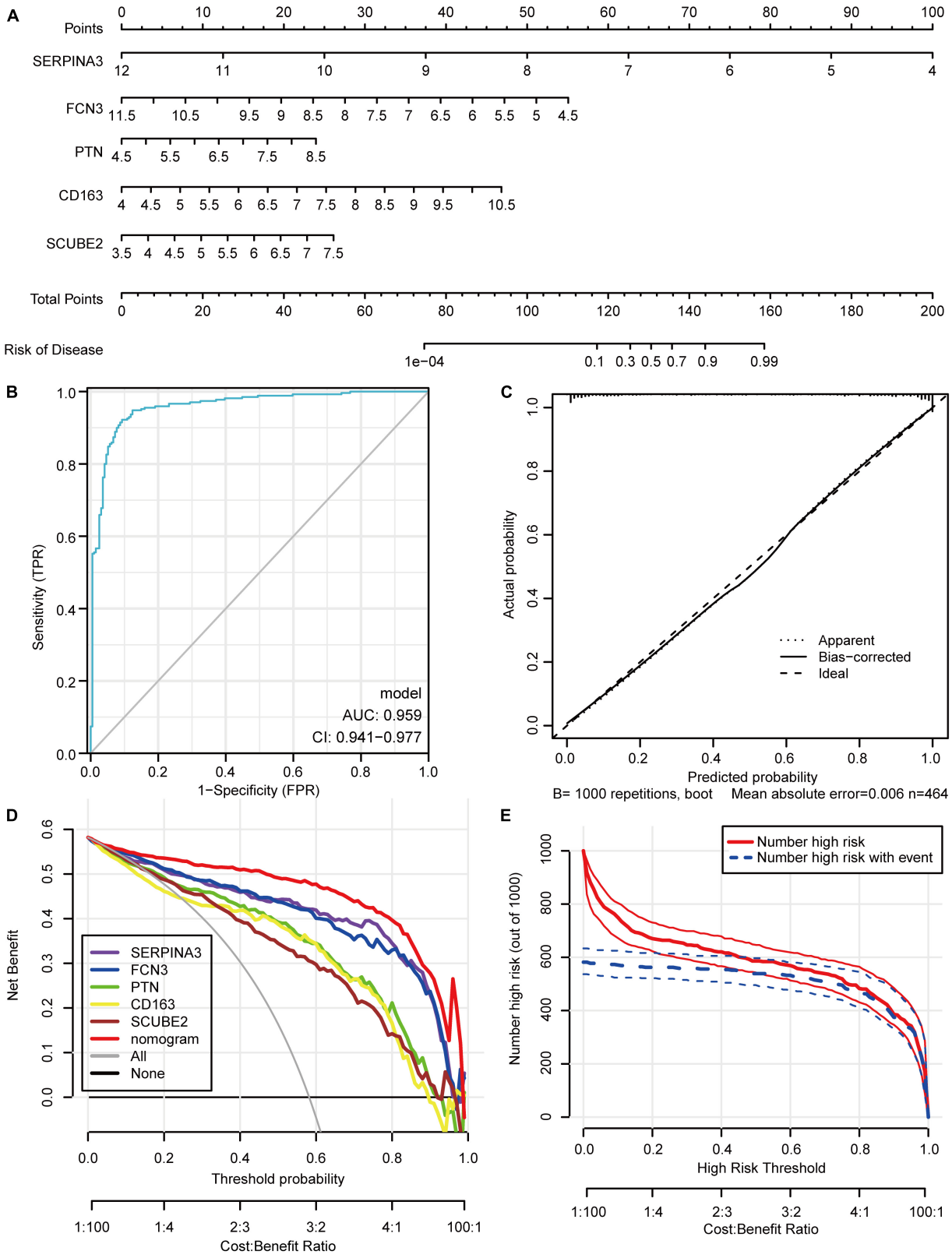

FIGURE 5
Construction and Evaluation of the nomogram model. **(A)** Construction of the nomogram model based on the 5 potential diagnostic biomarkers. **(B)** Receiver operating characteristic curve. **(C)** Calibration curve. **(D)** Decision curve analysis. **(E)** Clinical impact curve.

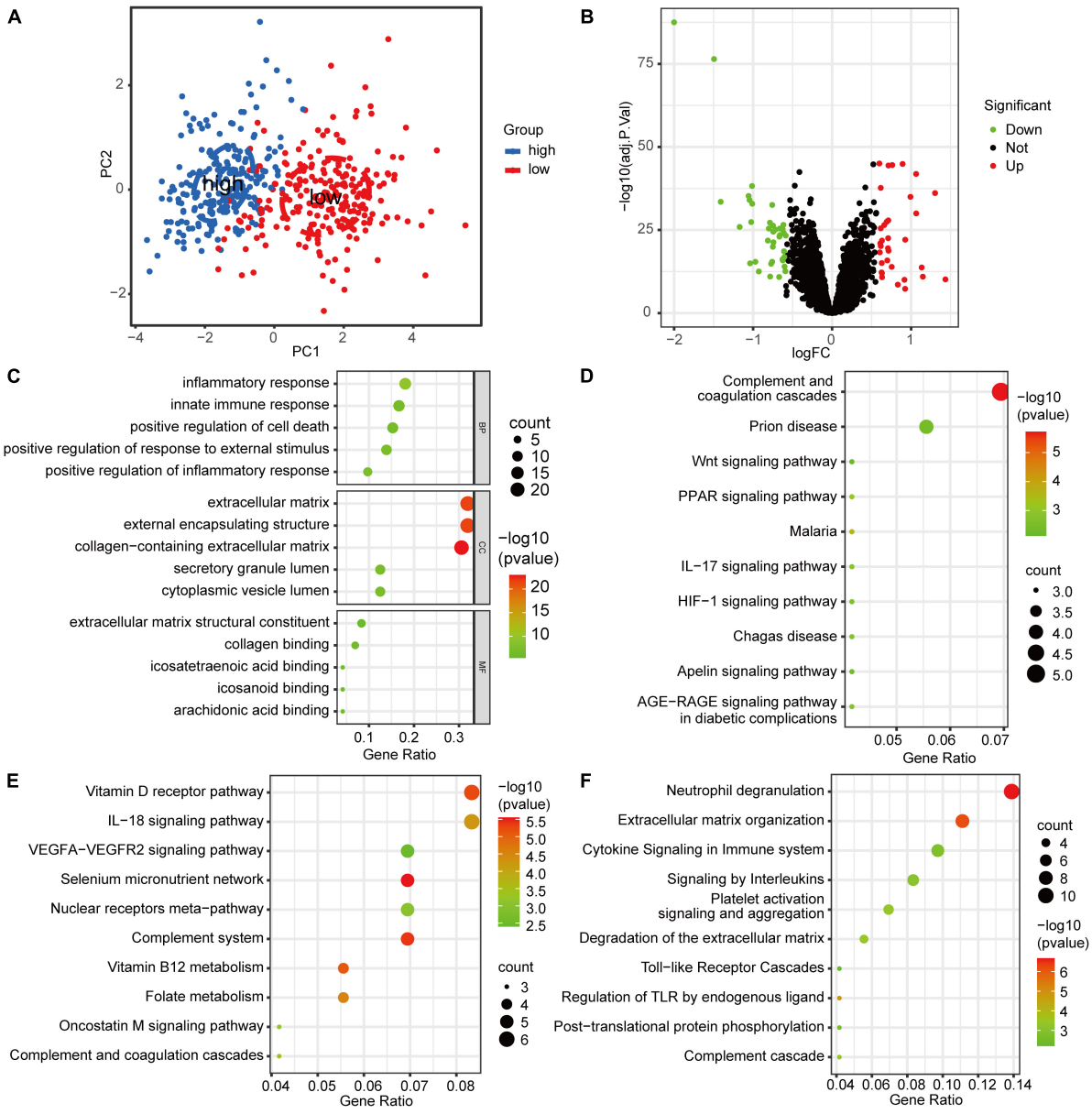

FIGURE 6
The Functional enrichment analyses of the DEGs grouped by risk score. **(A)** The principal component analysis cluster plot of low- and high-risk samples in the merged dataset. **(B)** Volcano plot of the DEGs between low- and high-risk groups. **(C)** The top five enriched GO terms for the DEGs between low- and high-risk groups in biological process, molecular function, and cellular component categories. **(D)** The ten KEGG pathways were closely related to ICM enriched by DEGs between the low- and high-risk groups. **(E)** The ten Wiki pathways were closely related to ICM enriched by DEGs between the low- and high-risk groups. **(F)** The ten Reactome pathways were closely related to ICM enriched by DEGs between the low- and high-risk groups.

risk factor for atherosclerosis, which in turn leads to ICM (25). The accumulated evidence has highlighted the link between inflammatory burden and residual risk in ICM, which provides novel ideas and entry points in risk mitigation (25). The concept of management against residual inflammatory risk among patients with ICM is gaining increasing attention (25). Therefore, anti-inflammatory strategies represent attractive approaches for treating ICM (26). In sum, exploring the inflammatory mechanism associated with the occurrence and progression of ICM is of great significance for a deeper understanding of the pathological mechanism of ICM, early diagnosis, and identifying new therapeutic targets from the perspective of inflammation.

In our study, we combined 8 microarray datasets using the SVA package and identified 19 DEIRGs. Then, we annotated these DEIRGs and found that they were mainly involved in the

inflammatory response, leukocyte chemotaxis and migration, response to bacterium and other processes, and enriched in the apelin signaling pathway, IL-17 and IL-18 signaling pathway, neutrophil degranulation, TLRs cascades, and other pathways. Neutrophils, the most plentiful kind of leukocytes, play an important role during key cellular and molecular events of ICM (27, 28). Neutrophil migration, chemotaxis, and degranulation are necessary for different pathological stages of ICM (27, 28). IL-17 and 18, as pro-inflammatory cytokines, are up-regulated in patients with ICM and related to the New York Heart Association (NYHA) class (29, 30). They play a pathogenic role in HF through several pathways including NF-κB, and MAPK signaling pathways (31, 32). TLRs, a family of pattern recognition receptors, play a pivotal role in immune responses and are closely associated with several cardiovascular diseases (33). In ICM, TLRs can induce activation of NF-κB and inflammatory responses, regulate cardiomyocyte apoptosis and alter cardiac function, with TLR2 and TLR4 playing the most important role (34). According to the above studies, the regulatory mechanisms of DEIRGs during ICM were related to inflammation-related biological processes and pathways, which might contribute to a better understanding of the pathologic mechanism of ICM.

Next, we used SVM-RFE, LASSO, and RF algorithms to identify 5 robust potential biomarkers (including SERPINA3, FCN3, PTN, CD163, and SCUBE2) from DEIRGs. The verified results of the functional experiment of the ICM rat showed that the protein expression levels of SERPINA3, FCN3, and CD163 were decreased in the ICM rat and the opposite was true for PTN and SCUBE2. Meanwhile, the validation results of RNA-Sequencing datasets showed that each of the 5 potential biomarkers showed good discriminant power, and their expression trends in RNA-Sequencing datasets were also consistent with the bioinformatics results. In addition, we established one risk prediction nomogram of ICM based on the 5 potential biomarkers and further tested its efficacy with moderate discrimination and good calibration. The results indicated that the nomogram may assist clinicians in effectively evaluating the risk and precise treatment of patients in ICM.

Next, the 5 potential biomarkers were analyzed and discussed for their association with ICM along with the literature. SERPINA3 belonging to the superfamily of serine protease inhibitors plays a significant role in the pathogenesis of atherosclerosis (35). SERPINA3 not only was a novel diagnostic and pharmacological target for HF but also associated with major adverse cardiovascular events in patients with acute myocardial infarction (36, 37). FCN3 is the most abundant and potent recognition molecule in the complement system lectin pathway and mediates autoimmune system and inflammation and other diseases (38). FCN3 can be used as a new biomarker for the prognosis of HF and has nothing to do with the etiology of HF (38, 39). In our study, SERPINA3 and FCN3 were down-regulated in bioinformatics analysis, the external validation data, and the functional experiment of the ICM rat. Jing et al. (40) found that SERPINA3 and FCN3 were identified as the potential biomarkers in ICM using bioinformatics analysis. The validation results of SERPINA3 and FCN3 in myocardial tissue of ICM patients were consistent with the results of our study.

PTN, a growth factor, plays an important role in nervous system development, tumor angiogenesis and growth, wound repair, and other disease phenotypes (41, 42). PTN may be a novel potential diagnostic and therapeutic target for dilated cardiomyopathy (36). Some studies have reported that PTN increased apoptosis of cardiomyocytes and enhanced neovasculature formation in ICM (42–44). SCUBE2, as a member of the SCUBE family, is expressed in a wide range of human tissues including cardiovascular tissues. SCUBE2 is involved in vascular endothelial function changes and vascular complications, particularly in diabetes and atherosclerosis (45). The results of our study suggested that SCUBE2 might be a new target that played an important role in ICM. CD163 mainly expressed in monocytes/macrophages is critical in inflammation. CD163 is involved in the progression of acute HF and also contributed to the pathogenesis of acute decompensated HF (46). Furthermore, it was reported that CD163-expressing macrophages not only regulated tissue regeneration after ischaemic injury induced by unilateral femoral artery ligation but also promoted ventricular functional recovery after myocardial infarction (47, 48). In sum, the unique findings of our study were that PTN, SCUBE2, and CD163 played an important role in the pathology of ICM and might be potential diagnostic and therapeutic targets for ICM. Meanwhile, we also innovatively integrated the 5 potential biomarkers (including SERPINA3, FCN3, PTN, CD163, and SCUBE2) for a multiple indicator co-diagnosis of ICM from an inflammatory perspective and constructed a nomogram that might facilitate accurate disease assessment of ICM patients by clinicians or researchers.

To facilitate an understanding of the functional mechanisms of 5 potential biomarkers regulating ICM, we next divided all samples into high and low-risk groups according to the nomogram model and carried out functional enrichment analyses of the DEGs between the two groups. The results revealed that these genes principally participated in the inflammatory response, cell death, and other processes, and enriched in the HIF-1 signaling pathway, neutrophil degranulation, TLRs cascades, signaling by interleukins, and other signaling pathways. The inflammatory response has been considered the major pathophysiological contributor to HF (49). Interleukins are a group of cytokines that are produced by and interact with a variety of cells and play an important role in the transmission of information, activation and regulation of immune cells, and inflammatory response. Interleukins, as major inflammatory mediators, have been reported to be closely associated with HF (49). In addition to IL-17 and 18 mentioned

above, there are also IL-1, 6, 8, 10, and 33 (31). IL-1 cytokine family has several members, including IL-1, IL-33, ST2, IL-18, and others. The CANTOS study showed that canakinumab, a human monoclonal anti-human IL-1β antibody, could reduce the risk of major cardiovascular disease events in patients with a history of myocardial infarction without any effect on lipid levels, which demonstrated that anti-inflammatory therapy inhibiting the IL-1 pathway might indeed be beneficial for patients with coronary heart disease (50). During HF, IL-1 causes cardiac dysfunction and remodeling by stimulating cardiomyocyte apoptosis, favoring fibrosis, promoting arterial stiffness and microvascular inflammation, and other multiple mechanisms (49). Meanwhile, IL-1 also induces the activation of leukocytes and endothelial cells, which in turn promotes their interaction and increases the mobilization of inflammatory cells to the myocardium (49). The above studies suggest that the IL-1 pathway may be an important contributor to the progression of ICM and has emerged as an attractive target for the anti-inflammatory treatment of cardiovascular disease. The IL-33/ST2 signaling pathway mediated the inflammatory response in ICM patients (51). The IL-6 concentration was elevated in patients with ICM and proportional to the NYHA functional class (52, 53). Studies had shown that IL-6 mediated the pathological process of HF through JAK/STAT3, MAPKs, and PI3K/Akt pathway and other signaling pathways (32). In summary, the enriched pathway and process of the DEGs between high and low-risk groups were to some extent consistent with the pathological process of ICM shown in prior research. In the future, in-depth research should be done to verify the above findings.

Our study had the following strengths. First, a small sample size was an issue generally encountered in research for bioinformatics analysis, especially for non-oncological diseases. We attempted to mend the limitation by integrating multiple independent databases using the SVA package to guarantee that the results were convincing. Second, three ML algorithms were used to screen the potential inflammation-related biomarkers of ICM. Third, multiple RNA-Sequencing datasets and the functional experiment of the ICM rat were implemented to validate the obtained 5 potential inflammatory biomarkers, which could prevent the bias from the results of pure bioinformatics analysis. Finally, the inflammatory biomarker-based nomogram described in the study has not been reported previously and provides a new diagnostic predictive tool for ICM. However, there were several limitations in our research, which should be considered in drawing the conclusions. First, our findings are derived from currently available gene expression data from both microarray and RNA-seq technologies. Some genes, although playing potential roles in ICM, were overlooked in our study because they were not detected by existing assays. Second, the samples in the datasets used for our study were human myocardial tissue rather than peripheral blood. Therefore, it will be interesting to observe whether the potential inflammation-related diagnostic biomarkers mined with ML algorithms are amenable to measurement in blood samples. Third, the 5 potential inflammatory biomarkers had been validated by multiple independent datasets and an ICM animal model, which makes our results more convincing and accurate. However, it is important and urgent to validate these results using cardiac tissue from ICM patients. Finally, although internal validation was executed to test the validity of the nomogram, future studies are needed to externally validate the proposed nomograms. In addition, due to imperfect information in the included datasets, the clinical information in the nomogram was excluded. If clinical information closely related to ICM was added to the nomogram, this will improve the accurate screening and precise diagnosis of ICM.

Conclusion

In conclusion, we have innovatively identified SERPINA3, FCN3, PTN, CD163, and SCUBE2 as potential biomarkers for ICM by combining bioinformatics analysis, ML algorithms, and the experimental verification strategy. Meanwhile, we also developed a nomogram model based on potential inflammation-related biomarkers to predict ICM. However, further studies should be performed in the future to verify the above findings. Our work may provide new and valuable insights into the mechanisms of ICM and its treatments from an inflammatory perspective.

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 experiment was approved by the Experimental Animal Welfare Ethics Review Committee of The First Affiliated Hospital of Henan University of Chinese Medicine (approval number, YFYDW2017005).

Author contributions

MZ, JW, and JC: conceptualization. JW, XL, and JC: formal analysis and data curation. JW and SX: funding acquisition. SX,

XL, YC, and JW: investigation. SX, JW, and YC: resources. XL and JW: visualization. JW and SX: writing – original draft. MZ and JC: writing – review and editing. All authors approved the final 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.972274/full#supplementary-material

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The relationship between soluble lymphocyte activation gene-3 and coronary artery disease

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Background: Soluble lymphocyte activation gene 3 (sLAG3) may be used for diagnosis or prognosis in various diseases. However, the relationship between sLAG3 and coronary artery disease (CAD) are still unclear. This study aimed to investigate the levels of sLAG3 in patients with CAD, and its potential clinical association with the disease.

Methods: A total of 66 subjects (49 patients with CAD and 17 control subjects without CAD) were enrolled. The sLAG3 level was measured using enzyme-linked immunosorbent assay (ELISA) kits. Clinical variables included demographics, biochemical markers, coronary angiography status, and ejection fraction of the heart (EF) were collected, and Gensini scores were calculated. LAG3 gene data was extracted from three datasets (GSE23561, GSE61144, GSE60993) in Gene Expression Omnibus (GEO) to compare differential expression between CAD and control subjects.

Results: The sLAG3 level was significantly lower in the CAD vs. the controls ($P < 0.05$), and negatively associated with CAD [odds ratio (OR): 0.212, 95% confidential interval (CI): 0.060–0.746, $P < 0.05$]. Furthermore, the area under the curve (AUC) of sLAG3 level was significant ($P < 0.05$). The sLAG3 level in subjects with body mass index (BMI) ≥ 24 kg/m^2 was lower compared to those with BMI < 24 kg/m^2 ($P < 0.05$). The sLAG3 level was also negatively associated with BMI and diabetes mellitus ($P < 0.05$), though not associated with the Gensini scores or EF ($P > 0.05$). Lastly, the LAG3 gene expression in peripheral whole blood of patients with CAD were down-regulated compared to healthy controls ($P < 0.05$).

Conclusion: The sLAG3 level was negatively associated with the occurrence but not severity of CAD. Meanwhile, the sLAG3 was negatively associated with BMI and diabetes mellitus, suggesting the reduced sLAG3 might be a novel risk factor for developing CAD.

KEYWORDS

lymphocyte activation gene-3, coronary artery disease, risk factor, immune, inflammation

Introduction

Coronary artery disease (CAD), a chronic inflammatory immune disease, is one of the leading causes of heart failure and death globally (1, 2). It is characterized by plaque accumulation with the involvement of immune cells and cytokines (3, 4). The mechanism of CAD has been extensively researched. The underlying mechanism for initiation and progression of atherosclerotic plaques involves multifactorial gene expression, and the alteration of inflammatory factors (5, 6). Thus, the association between inflammation and CAD status is well established. Multiple studies have identified circulating biomarkers in peripheral blood for diagnosis, evaluation, or prognosis of CAD (7–10). For example, soluble suppression of tumorigenicity 2 was associated with all-cause mortality in patients with the chronic coronary syndrome (CCS) (7). Cystatin C may also be related to the severity of CAD in patients with diabetes mellitus (8). Lastly, red blood cell distribution width is independently associated with myocardial scar burden and left ventricular function in patients with CAD (9). These markers may provide significant therapeutic insight for treating CAD in clinical practice. Moreover, clinicians can greatly benefit from the discovery of novel markers to elucidate their understanding of disease progression.

Lymphocyte activation gene 3 (LAG3) is an immune checkpoint and transmembrane protein, which binds to major histocompatibility complex class II (MHC-II) and fibrinogen-like protein-1 (FGL1) as ligands for LAG3 (11–13). As a transmembrane protein, under the action of two metalloproteases (ADAM10, ADAM17), the extracellular segment of LAG3 can be released into the plasma to become soluble LAG3 (sLAG3) (14). It was reported that LAG3 was mainly expressed in lymphocytes including activated T cells and NK cells, which had been elaborated in tumors (15–18), hepatitis (19), and inflammatory bowel disease (20). Recently, a study found high expression of LAG3 on type 1 regulatory T cells (Tr1) in both patients with CAD and healthy participants (21). however, the expression correlated with disease severity as patients with three diseased vessels had significantly lower expression compared to those with only one diseased vessel, which indicated that LAG3 expression in Tr1 may be involved in CAD.

To date, the use of sLAG3 has been confirmed in the diagnosis or prognosis of a variety of diseases including cancer (22–24). Golden et al. found that the sLAG3 level could indicate a predisposition for CAD development (25). Nevertheless, the level of sLAG3 in the patients with CAD and the relationship between sLAG3 and CAD are still unclear. Therefore, in this study, we investigated the levels of sLAG3 in patients with CAD, and its potential clinical association with the disease.

Patients and methods

Participants

In this study, 66 subjects were enrolled from February 2021 to July 2021. Informed consent was obtained from all participants. All enrolled subjects were categorized into two groups: CAD (n = 49; 38 men and 11 women, mean age 63.61 ± 11.42 years) and control (n = 17; 9 men and 8 women, mean age 58.94 ± 9.37 years). The diagnostic criteria of CAD were based on literature (26–28). The patients with CAD had at least one main coronary artery stenosis with a luminal diameter of ≥ 50%, confirmed by coronary angiography. The inclusion criteria for patients with CAD were as follows: lumen stenosis of any major epicardial coronary artery 50% with or without clinical manifestation of ischemic symptoms, electrocardiogram changes, or cardiac troponins T changes (26). The control group consisted of healthy volunteers without atherosclerotic cardiovascular disease history or abnormal coronary angiography. Exclusion criteria included the presence of any of the following: malignant tumor, severe chronic heart failure, severe chronic liver and kidney disease, serious infection. Ethical approval for the research was got from the Ethics Committee of Guizhou Medical University Affiliated Hospital. This study was carried out in accordance with the Declaration of Helsinki.

Gensini score of coronary artery stenosis

Gensini scores were adopted to quantify the severity of coronary artery lesion. The Gensini score is based on the extent and position of the stenosis of the coronary artery (29). The higher the score, the more severe the lesion.

Detection of soluble lymphocyte activation gene 3 protein level and clinical data collection

Whole blood without anticoagulants was left for 2 h at room temperature or overnight at 4°C, followed by centrifugation for 15 min at 1,000 g. The supernatant was stored at −80°C until further analysis. The sLAG3 protein level was examined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instruction. The ejection fraction of the heart (EF) and clinical baseline characteristics were collected during hospitalization. The clinical baseline characteristics included age, gender, body mass index (BMI), lipids levels, the history of smoking, hypertension, and diabetes mellitus.

Lymphocyte activation gene 3 gene differential expression analysis in peripheral whole blood

Three datasets (GSE23561, GSE60993, and GSE61144) from the Gene Expression Omnibus (GEO) database[1] were retrieved. The raw data were downloaded as MINiML files. The microarray data were normalized by the normalize quantiles function of the preprocessCore package in R software. Box plots were drawn using the "boxplot" R package, and the "ggord" package was used to draw the principal component analysis (PCA) plot. We extracted the LAG3 gene data from the three datasets and compared the difference in LAG3 gene expression in peripheral whole blood between the CAD and healthy control groups through the Wilcox test.

Statistical analysis

Normally distributed variables are presented as mean ± standard deviation (SD), whereas non-normally distributed variables were expressed as medians with the interquartile range (IQR). Normally distributed variables were compared by student's *t*-tests between the two groups. Non-normally distributed variables were compared by Mann-Whitney *U*-tests between the two groups. The categorical variables were expressed as percentages and compared by the chi-square test or Fisher's exact test. Logistic regression analyses were performed to identify associations of sLAG3 with CAD in different regression models. The receiver operating characteristic (ROC) curve analysis was used to evaluate the area under the curve (AUC) of sLAG3 for discriminating CAD.

Spearman's correlation analysis was applied to determine the correlation between sLAG3 and the variables. Multiple line regression analysis was applied to assess the independent association between sLAG3 and clinical variables. Statistical analysis was performed by SPSS v26.0. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of subjects

Baseline clinical characteristics of the CAD and control groups are presented in **Table 1**. Hypertension and tobacco use were more prevalent in patients with CAD than in the control group ($P < 0.05$). The BMI of patients with CAD was higher compared to the control group ($P < 0.05$). There were no significant differences in age, gender, or history of diabetes between the patient with CAD and control groups ($P > 0.05$). The levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were comparable between patients with CAD and the control group ($P > 0.05$).

Reduced soluble lymphocyte activation gene 3 level in patients with coronary artery disease

We compared the sLAG3 level in patients with CAD and control subjects. As shown in **Figure 1A**, the sLAG3 level was significantly lower in patients with CAD than in the control group [288 (258.5–403) vs. 367 (323–491) ng/ml, $P < 0.05$]. Moreover, we divided patients with CAD into acute and CCS groups, and found no significant differences in sLAG3 protein level between the two groups ($P > 0.05$, **Figure 1B**).

Correlation of soluble lymphocyte activation gene 3 level with coronary artery disease

The sLAG3 was transformed into dichotomous variables according to median of the sLAG3. Univariable analysis showed that the sLAG3 was inversely correlated to the occurrence of CAD [odds ratio (OR) = 0.212, $P < 0.05$]. After adjusting for age and sex, a significant negative association was observed between the sLAG3 level and CAD (OR = 0.143, $P < 0.05$). After

TABLE 1 Clinical baseline characteristics of study participants.

Characteristics	CAD ($n = 49$)	Control ($n = 17$)	*P*
Age (years)	63.61 ± 11.42	58.94 ± 9.37	0.134
BMI (kg/m^2)	24.90 ± 3.33	22.85 ± 2.81	0.027
Male (%)	38 (77.60%)	9 (52.90%)	0.105
Hypertension (%)	23 (46.9%)	3 (17.6%)	0.033
Diabetes mellitus (%)	13 (26.5%)	1 (5.90%)	0.147
Smoking (%)	30 (61.2%)	5 (29.4%)	0.024
TG (mmol/L)	1.47 (1.01, 2.03)	1.77 (0.99, 2.89)	0.253
TC (mmol/L)	4.17 ± 1.12	4.66 ± 0.85	0.105
HDL-C (mmol/L)	1.01 (0.84, 1.23)	1.05 (0.93, 1.44)	0.157
LDL-C (mmol/L)	2.71 ± 1.03	2.77 ± 0.90	0.842
ACS/CCS (*n*)	36/13		

CAD, coronary artery disease; ACS, acute coronary syndrome; CCS, chronic coronary syndrome; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL- C, low-density lipoprotein cholesterol.

1 https://www.ncbi.nlm.nih.gov/geo/

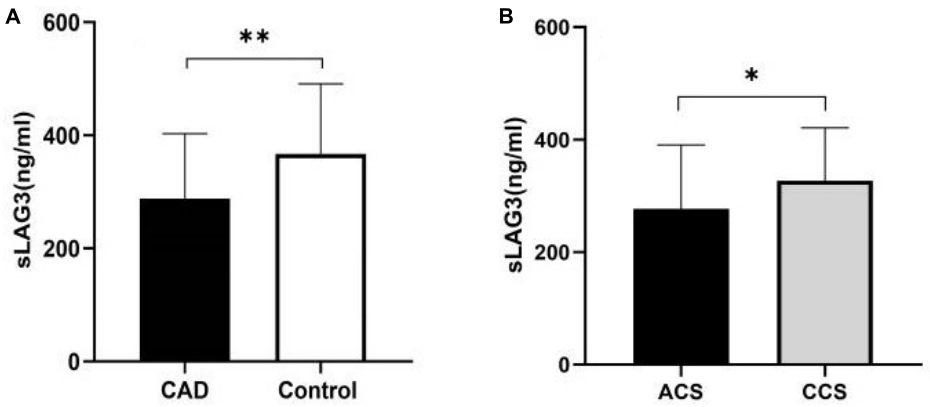

FIGURE 1
The level of the soluble lymphocyte activation gene-3 (sLAG3) in patients with coronary artery disease (CAD). **(A)** The sLAG3 was markedly reduced in patients with CAD. **(B)** The similar Level of sLAG3 between acute coronary syndrome (ACS) subgroup and chronic coronary syndrome (CCS) subgroup. The sLAG3 was compared using Mann-Whitney *U*-tests. $^{**}P < 0.05$, $^{*}P > 0.05$.

adjusting for hypertension and BMI, the sLAG3 was inversely associated with CAD (OR = 0.221, $P < 0.05$). Additionally, after controlling for other variables including hypertension, diabetes, age, smoking, LDL-C, and HDL-C in different models, a lower sLAG3 level was also related to an increased risk of CAD (Table 2).

Discriminative power of soluble lymphocyte activation gene 3 in patients with coronary artery disease

The ROC curve was used to evaluate the diagnostic value of the sLAG3 level for CAD. As presented in Figure 2, the AUC value was 0.733 for CAD ($P < 0.05$), the cut-off value for sLAG3 was 280 ng/mL, whereas the corresponding sensitivity and specificity were 0.941 and 0.490, respectively.

TABLE 2 The correlation of sLAG3 with coronary artery disease (CAD) in different models.

Variables	OR	95% CI	*P*
sLAG3	0.212	0.060–0.746	0.016
sLAG3 + Age + Sex	0.143	0.033–0.610	0.009
sLAG3 + Age + Smoking	0.196	0.046–0.830	0.027
sLAG3 + Hypertension + BMI	0.221	0.056–0.877	0.032
sLAG3 + Hypertension + Diabetes	0.211	0.056–0.802	0.022
sLAG3 + LDL-C + HDL-C	0.192	0.052–0.708	0.013

Multivariable logistic regression analyses were performed to identify the correlation of sLAG3 with CAD. The sLAG3 was transformed into dichotomous variables according to median of sLAG3. sLAG3, soluble lymphocyte activation gene-3; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Soluble lymphocyte activation gene 3 is not associated with the progression of coronary artery lesions and cardiac function

We used Gensini scores to evaluate the severity of coronary artery lesions. Spearman' s correlation analysis showed that the sLAG3 level did not correlate with the Gensini scores ($P > 0.05$). Spearman's correlation analysis was used to determine the correlation between sLAG3 and

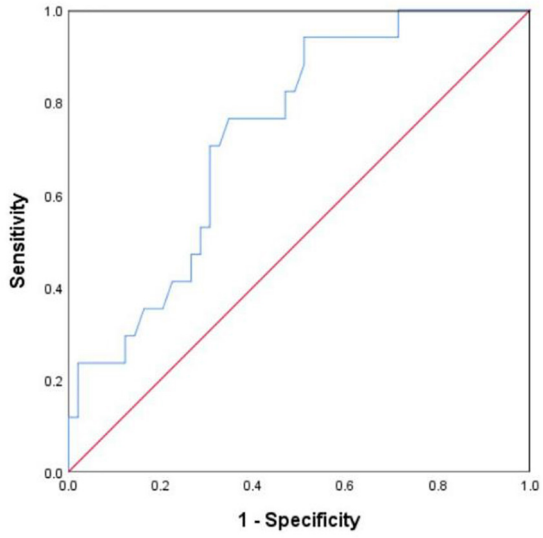

FIGURE 2
Receiver operating characteristic (ROC) curve of soluble lymphocyte activation gene- 3 (sLAG3) for identifying coronary artery disease.

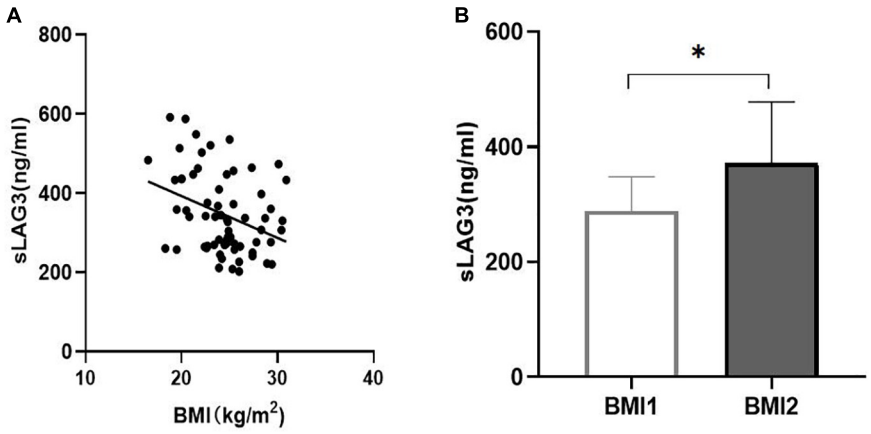

FIGURE 3
The correlation between soluble lymphocyte activation gene-3 (sLAG3) and body mass index (BMI). **(A)** sLAG3 was negatively correlated with BMI ($r = -0.314$, $P < 0.05$). **(B)** The sLAG3 level according to BMI 24 kg/m^2, BMI1 $\geq$ 24 kg/m^2, BMI2 $<$ 24 kg/m^2. sLAG3 between two groups was compared using Mann-Whitney *U*-tests. *$P < 0.05$.

EF from the ultrasound data of 43 subjects. There was no significant correlation between the sLAG3 level and EF ($P > 0.05$).

Association of soluble lymphocyte activation gene 3 level with body mass index and diabetes mellitus

We analyzed the correlation of sLAG3 with risk factors for CAD, and found a negative correlation between sLAG3 and BMI ($P < 0.05$, Figure 3A). All subjects were classified into two groups based on their BMI 24 kg/m^2: BMI1 $\geq$ 24 kg/m^2 and BMI2 $<$ 24 kg/m^2. The level of sLAG3 in BMI1 subjects was lower compared to BMI2 subjects ($P < 0.05$, Figure 3B). After additional consideration of independent variables, including sex, age, BMI, hypertension, diabetes, smoking, LDL-C, and HDL- C, multivariable regression analysis revealed an independent association of sLAG3 with BMI and diabetes mellitus (see Table 3). However, we did not observe the association between sLAG3 with other clinical variables including the lipid profiles (TG, TC, HDL-C, LDL-C), age, hypertension, smoking, and sex.

Reduced expression of lymphocyte activation gene 3 gene of peripheral blood of patients with coronary artery disease

We retrieved LAG3 gene expression data from three datasets in GEO database containing patients with CAD ($n = 39$) and healthy controls ($n = 26$). We found that LAG3 gene expression in the peripheral blood of patients with CAD was down-regulated compared to the control group ($P < 0.05$, Figures 4A–D).

Discussion

We revealed that sLAG3 level is reduced in CAD, accompanied by down-regulation of LAG3 gene in peripheral blood. Moreover, the sLAG3 level was negatively associated with BMI and diabetes mellitus. LAG3 exists mainly in two forms: transmembrane-cytoplasmic domains and extracellular domains (30). As a transmembrane protein, the expression of LAG3 in lymphocytes potentially has a negative immunomodulatory effect on immune cells (12, 31–34). In patients with systemic lupus erythematosus (SLE), human immunodeficiency virus (HIV), or carotid artery stenosis, membrane LAG3 expression was also associated with the disease activity and disease progression (35–37). sLAG3 is formed after part of the transmembrane protein of LAG3 is removed by ADAM10 and ADAM17 (14, 38).

TABLE 3 The association of sLAG3 with BMI and diabetes mellitus.

Variables	Standardized coefficient β	*P*
BMI	−0.448	0.002
Diabetes mellitus	−0.260	0.042

Multivariable regression analysis were performed to identify the association of sLAG3 with BMI and diabetes mellitus. The sLAG3 was dependent variable; the independent variables included sex, age, BMI, hypertension, diabetes, smoking, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol. sLAG3, soluble lymphocyte activation gene-3; BMI, body mass index.

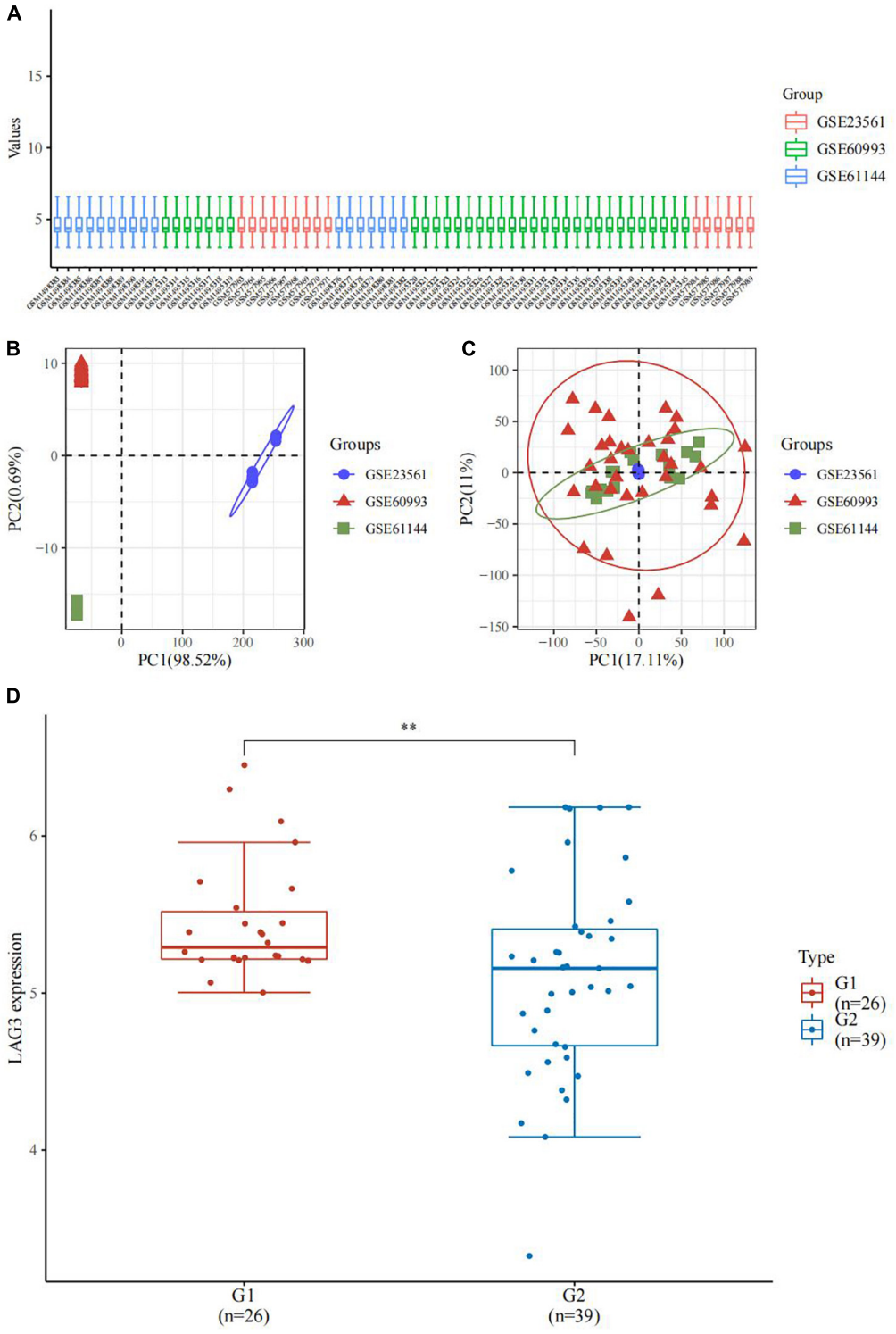

FIGURE 4
LAG3 gene in coronary artery disease (CAD). **(A)** The box plot of the normalized data. Different colors represent different datasets. Rows represent samples, and columns represent the gene expression values in the samples. **(B)** Principal component analysis (PCA) results before batch removal for three data sets. Different colors represent different data sets. As shown in the schematic diagram, the three data sets are separated without any intersection. **(C)** PCA results after batch removal. The schematic diagram shows the intersection of three data sets which can be used as a batch of data for subsequent analysis. **(D)** The expression distribution of the LAG3 gene in peripheral blood. The horizontal axis represents different groups of samples: G1, normal control; G2, CAD group. $^{**}P < 0.05$.

The level and function of sLAG3 have been investigated in certain diseases. Studies have shown that sLAG3 was reduced in patients with gastric cancer with significant diagnostic and prognostic potential (22), and increased in non-small cell lung cancer (39). Furthermore, sLAG3 level has also been shown to be progressively decreased with the severity of lung cancer stages (40). Another study found that sLAG3 level was decreased in patients suffering from severe aplastic anemia (24). In the current study, we revealed that the sLAG3 level was reduced in patients with CAD compared to the control group, and a lower sLAG3 level was associated with CAD. Furthermore, the sLAG3 level by itself could discriminate CAD from normal controls with high sensitivity. Collectively, our findings indicated that sLAG3 correlated with the occurrence of CAD. Our findings for reduced LAG3 in patients with CAD were similar to previous reports by Golden et al. (25), that found initial plasma sLAG3 levels were lower in patients suffering from CAD during follow-up compared to those without CAD, and negatively correlated with the occurrence of future CAD. Nevertheless, the physiological function of sLAG3 remains unclear. Li et al. reported that sLAG3 might be a byproduct without physiological function produced on the T cell surface (14). However, evidence from another study also showed that differentiation of macrophages and dendritic cells was negatively regulated by sLAG3, which could attenuate the antigen-presentation function of dendritic cells (41). In the Multi-Ethnic Study of Atherosclerosis (MESA) (25), the sLAG3 level of participants was positively correlated with anti-inflammatory cytokine IL-10 level. These above mentioned findings indicated that decreased sLAG3 levels may be associated with pro-inflammatory effects in CAD or atherosclerosis. However, further investigation of the *in vivo* effects of sLAG3 in atherosclerosis is required.

Obesity is well known as an important risk factor for CAD. For instance, a higher BMI increases the risk of developing CAD and type 2 diabetes (42, 43). Diabetes mellitus is also a major contributor to CAD (44). After controlling for variables, the sLAG3 was independently associated with BMI and diabetes mellitus. These results suggested that the reduced sLAG3 level could be a novel risk factor for CAD.

Our results demonstrated no association between sLAG3 levels and Gensini scores, suggesting that sLAG3 did not correlate with the severity of coronary artery lesions in CAD. Besides, we also did not find an association of sLAG3 with EF in the current study. This finding appeared to be supported by a report by Pallikkuth et al. (36). The reason for the decrease of sLAG3 in patients with CAD is still unknown. Many factors such as race, and inflammatory factors may influence the sLAG3 level (25, 45). Besides, LAG3 expression may be also regulated by methylation, T cell receptor pathway activation, cytokines, and metalloproteinase (46). Additionally, The genotype of lipoprotein scavenger receptor BI (SCARB1) rs10846744 associated with atherosclerosis and atherosclerotic cardiovascular disease was also related to alteration of sLAG3 level (25), suggesting that sLAG3 level was susceptible to alteration of gene. In the present study, by analyzing LAG3 gene expression data from three GEO datasets, we found LAG3 gene expression was down-regulated in the peripheral blood of patients with CAD. Meanwhile, sLAG3 was reduced in CAD, in alignment with the down-regulation of LAG3 gene expression in peripheral blood, suggesting reduced LAG3 gene expression may contribute to reduction of sLAG3 in peripheral blood in CAD. In addition, the present study indicated that both diabetes mellitus and higher BMI could have an independent impact on reducing the sLAG3 level.

However, as this was a cross-sectional exploratory study, the mechanism by which sLAG3 decreased in patients with CAD needs to be further researched. The association of sLAG3 and LAG3 gene with CAD indicates LAG3 regulation as a potential therapeutic target for CAD.

Several important limitations need to be highlighted in this study. First, the sample size was relatively small, although the sample size met the statistical requirements, as a result of the sample size and conditions of logistic regression, we only used a combination of three variables to investigate the association of sLAG3 with CAD between different models by logistic regression. Moreover, we did not assess the difference among LAG3 proteins including total protein, membrane proteins, and intracellular protein levels of lymphocytes in peripheral blood of the CAD and control groups. Whether these proteins levels of LAG3 were changed may improve our understanding of LAG3 function in CAD. In addition, the mechanism of sLAG3 in CAD has not been elucidated. Therefore, future research needs to explore the pathophysiological mechanism of LAG3 in immune cells in both CAD and atherosclerosis. Finally, the causal relationship of alteration of LAG3 protein and gene expression with CAD should be investigated in the future.

Conclusion

Our study demonstrates that sLAG3 level is reduced in patients with CAD, accompanied by down-regulation of LAG3 gene in peripheral blood. The reduced sLAG3 correlated with the occurrence of CAD. Meanwhile, the reduced sLAG3 might be a novel risk factor for CAD. The sLAG3 level was not associated with the severity of CAD. The definitive role of sLAG3 in CAD requires further investigation in future experiments.

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 below: https://www.ncbi.nlm.nih.gov/geo/, GSE23561, GSE60993, and GSE61144.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Guizhou Medical University Affiliated Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XX and HZ draft the manuscript. XX and ZD carried out the statistical analysis. LN, XX, and ZD participated in data collection and interpretation of results. ZL and WL contributed to discussion, edited the manuscript, designed, and supervised the project. 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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The relationship between mean platelet volume lymphocyte ratio and collateral circulation in patients with chronic total coronary occlusion

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Objective: To correlate mean platelet volume lymphocyte ratio (MPVLR) and coronary collateral circulation (CCC) in patients with chronic total occlusion (CTO).

Materials and methods: A total of 643 patients who were hospitalized at a single large academic medical center from January 2020 to October 2021 and had CTO lesions in at least one major coronary artery confirmed by coronary angiography were retrospectively analyzed. Patients were divided according to the Rentrop criteria into poorly formed CCC (Rentrop grade 0–1, n = 235) and well-formed CCC (Rentrop grade 2–3, n = 408) groups. Mean platelet volume lymphocyte ratio (MPVLR) was calculated from routine laboratory data (MPV divided by lymphocyte count). The clinical data of the two groups were compared, and relationships between MPVLR and CCC formation were analyzed.

Results: The MPVLR of patients with poorly formed CCC was significantly higher than that of patients with well-formed CCC (7.82 ± 3.80 vs. 4.84 ± 1.42, $P < 0.01$). The prevalence of diabetes mellitus and C-reactive protein levels were significantly higher in the poor CCC group than in the good CCC group ($P < 0.01$), while the proportions of patients with CTO or multivessel lesions in the right coronary artery were significantly lower in the poor CCC group than in the good CCC group ($P < 0.01$). Multivariate logistic regression analysis identified MPVLR (OR: 2.101, 95% CI: 1.840–2.399, $P < 0.01$), C-reactive protein level (OR: 1.036, 95% CI: 1.008–1.064, $P < 0.05$), a history of diabetes mellitus (OR: 2.355, 95% CI: 1.532–3.621, $P < 0.01$), and right coronary CTO ratio (OR: 0.313, 95% CI: 0.202–0.485, $P < 0.01$) as independent risk factors for CCC formation. The area under the ROC curve of MPVLR for predicting poorly formed CCC was 0.82 (95% CI: 0.784–0.855, $P < 0.01$), the best cut-off point

was 6.02 and the sensitivity and specificity of MPVLR for predicting poorly formed CCC were 72.3 and 82.4%, respectively.

Conclusion: In patients with coronary CTO, MPVLR was negatively correlated with CCC and a high MPVLR level was an independent predictor of poorly formed CCC.

KEYWORDS

chronic total occlusion, collateral circulation, mean platelet volume, lymphocyte, inflammation

Introduction

Chronic total occlusion (CTO) is a serious and complex coronary artery disease. The formation of good coronary collateral circulation is particularly important for those CTO patients who would be difficult to revascularize (1). Good collateral circulation can increase myocardial perfusion to the ischemic area, improve cardiac systolic function, reduce future cardiovascular events (2–5). Methods for evaluating collateral circulation currently include coronary angiography and myocardial perfusion imaging, etc. These procedures are relatively complex and expensive. It is therefore necessary to find a simple and feasible method for evaluating and predicting the formation of CCC.

Inflammatory response plays a key role in all stages of atherosclerosis and collateral angiogenesis (6). Mean platelet volume to lymphocyte ratio (MPVLR) is a newly discovered inflammatory index that has been studied in a variety of diseases such as strokes and hepatitis (7, 8). Mean platelet volume (MPV) is an indicator of platelet function and activation (9). Hadadi et al. (10) showed that MPV and platelet-to-lymphocyte ratio can predict the presence of CTO in patients presenting with ST-segment elevation myocardial infarction (STEMI). Hudzik et al. (11) showed that acute coronary syndrome patients with an elevated MPVLR had a higher coronary thrombosis burden, and that increased MPVLR was an independent risk factor for early and late death in patients with STEMI. Kurtul et al. (12) found that MPVLR was an independent predictor of no-reflow after percutaneous coronary intervention in patients with STEMI.

The formation of coronary collateral circulation is closely related to the inflammatory response. MPVLR as a comprehensive indicator reflecting platelet function and inflammatory cells may be related to the formation of coronary collateral circulation. Ornek et al. (13) preliminarily studied the correlation between MPVLR and coronary collateral circulation in patients with stable angina pectoris. There are no relevant reports of MPVLR in China. These prior studies also had small sample sizes, and therefore require further confirmation. This study therefore aimed to investigate the viability of MPVLR as a new and simple predictor of the semi quantity of compensatory collateral circulation in CTO patients.

Materials and methods

Study population

A total of 643 hospitalized patients who underwent coronary angiography at the Department of Cardiology of the First Affiliated Hospital of Zhengzhou University from January 2020 to October 2021 were enrolled. CTO lesions were confirmed in at least one major coronary artery (left anterior descending artery, LAD; left circumflex artery, LCX; and right coronary artery, RCA) *via* coronary angiography.

The diagnosis of CTO was based on the guidelines established by the 2019 European CTO Club consensus document for recanalization of CTO, which were the complete occlusion of a coronary artery for more than 3 months with TIMI grade 0 distal blood flow (14). Exclusion criteria were: (1) an acute myocardial infarction within the past 3 months; (2) percutaneous coronary intervention and/or coronary artery bypass grafting within the previous 3 months; (3) coronary myocardial bridging and/or congenital coronary malformation; (4) severe heart failure (left ventricular ejection fraction $< 30\%$), severe heart valve disease or cardiomyopathy; (5) severe liver or renal [estimated glomerular filtration rate (eGFR) < 30 ml/min/1.73 m^2] impairment; and (6) severe infectious disease, systemic inflammatory diseases, malignancy or hematological diseases. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University, and written informed consent was obtained from all patients.

Laboratory analysis

Clinical data collection

For cases that met the inclusion criteria, the patient's name, gender, age, cardiovascular risk factors (hypertension, diabetes, smoking history, family history), medication history (aspirin, clopidogrel, statins, ACEI/ARB, β-blockers, calcium channel blockers), and coronary angiography data were collected. Left ventricular ejection fraction (LVEF) measured by echocardiography and the systolic blood pressure (SBP)

and diastolic blood pressure (DBP) of the right upper arm at the time of admission were collected. Venous blood samples were routinely collected on the morning of the day after admission after at least 8 h after fasting. Collection included a routine basic complete blood count (white blood cell count, red blood cell count, platelet count, neutrophil, lymphocyte absolute value, and absolute value average platelet volume), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, c-reactive protein (CRP), and creatinine. MPVLR was calculated by dividing the MPV by the lymphocyte count.

Hypertension was defined according to the 2018 ESC/ESH Hypertension guidelines (15) as an office systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg, 24-h ambulatory blood pressure monitoring $\geq$ 130/80 mmHg, or home blood pressure monitoring $\geq$ 135/85 mmHg. The diagnostic criteria for diabetes were based on the International Diabetes Federation's 2012 global guidelines for type 2 diabetes (16): a fasting blood glucose $\geq$ 7 mmol/L 2 h after glucose loading, a random blood glucose $\geq$ 11.1 mmol/L or a glycated hemoglobin level $\geq$ 6.5%. Smoking history was defined as continuous or cumulative smoking for 6 months or more over a lifetime. Familial CHD was described as being younger than 55 years of age in men and older than 65 years of age in women at the time of diagnosis with a first-degree relative with a history of CHD or sudden cardiac death.

Coronary angiography methods

All patients underwent coronary angiography *via* the radial or femoral artery using the standard Judkins technique. Results were evaluated by two experienced interventional cardiologists, and CCC was graded according to the Cohen-Rentrop criteria (17): Grade 0: no CCC formation (no contrast agent filling at the distal end of the occlusion); Grade 1: collateral perfusion beside the occluded vessels, but the vascular development was very weak; Grade 2: the distal collateral branches of the occluded vessels were developed at a lower density and slower filling rate than the feeding vessels; and Grade 3: the occluded distal vessels were fully developed, with the same density and feeding collateral branches and a faster filling rate. In patients with multiple coronary lesions or when multiple coronary collateral branches were present, the highest Rentrop grade was selected. Polyvascular disease was defined as the presence of lesions in two or more large epicardial arteries. Patients were further divided into poorly formed CCC (Rentrop grade 0–1) and well-formed CCC (Rentrop grade 2–3) groups.

Statistical analysis

SPSS 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The Kolmogorov-Smirnov test was used to test the normality of the measurement data. Normally distributed data was expressed as mean ± standard deviation and independent sample *t*-tests were used for comparisons between these groups. Non-normally distributed data were expressed as median and interquartile range, and Mann–Whitney *U* tests were used. Enumeration data were expressed as frequency (rate), the χ^2 test was used for comparisons between groups, and a one-way analysis of variance (ANOVA) was performed to compare Rentrop grade categories. The Spearman test was performed to describe correlations of research indicators with the Rentrop grade. Possible influencing factors for CCC formation were analyzed using logistic univariate analysis and multivariate regression analysis. Variables with $P < 0.05$ after univariate analysis were included in the multivariate regression analysis. A receiver operating characteristic (ROC) curve was used to describe the predictive value of these factors. MedCalc version 20.111 (MedCalc software Ltd., Ostend, Belgium) was used to compare the area under the

TABLE 1 Comparison of baseline data between the two groups.

Variables	Poor CCC (n = 235)	Good CCC (n = 408)	P-value
[&]Age (years)	60.9 ± 10.3	59.1 ± 11.3	0.051[a]
[&]BMI (kg/m^2)	24.7 ± 2.9	25.1 ± 3.0	0.133[a]
[†]Men (n, %)	175 (74.5)	318 (77.9)	0.316[b]
[†]Smoking history (n, %)	88 (37.4)	178 (43.6)	0.125[b]
[†]History of drinking (n, %)	58 (24.7)	102 (25.0)	0.928[b]
[†]Diabetes mellitus (n, %)	107 (45.5)	131 (32.1)	0.001[b]
[†]History of hypertension (n, %)	148 (63.0)	230 (56.4)	0.101[b]
[†]Family history (n, %)	37 (15.7)	54 (13.2)	0.379[b]
[&]Systolic blood pressure (mmHg)	134.4 ± 19.5	132.4 ± 17.1	0.173[a]
[&]Diastolic blood pressure (mmHg)	82.2 ± 34.0	78.6 ± 11.2	0.053[a]
Previous medications (n, %)			
[†]Aspirin (n, %)	130 (55.3)	215 (52.7)	0.521[b]
[†]Clopidogrel (n, %)	98 (41.7)	156 (38.2)	0.386[b]
[†]Statins (n, %)	112 (47.7)	204 (50.0)	0.568[b]
[†]ACEI/ARB (n, %)	61 (26.0)	104 (25.5)	0.896[b]
[†]β-blocker (n, %)	71 (30.2)	111 (27.2)	0.415[b]
[†]Calcium channel blocker (n, %)	59 (25.1)	99 (24.3)	0.811[b]

[&]Data are expressed as the mean ± SD. [†]Data are expressed as n (%). [a]p-value by independent sample t-test. [b]p-value by χ^2 test. CCC, coronary collateral circulation; BMI, body mass index; ACEI, angiotension converting enzyme inhibitors; ARB, angiotensin receptor blocker.

ROC curves of two indicators. $P < 0.05$ was considered statistically significant.

Results

Baseline comparison of patients with poor and good collateral circulation

A total of 643 patients met our inclusion and exclusion criteria, including 235 patients in the poor CCC group and 408 patients in the good CCC group. There were no significant differences in age, gender, body mass index (BMI), history of hypertension, smoking, drinking, family history of coronary heart disease, systolic and diastolic blood pressure at admission, left ventricular ejection fraction and medication for coronary heart disease between the two groups (all $P > 0.05$). The proportion of patients with diabetes in the poor CCC group was significantly higher than that of the good CCC group (45.5% vs. 32.1%, $P < 0.05$, Table 1).

Biochemical indicators in the poor and good collateral circulation groups

White blood cell count, absolute neutrophil count, platelet count, absolute monocyte count, red blood cell count, hemoglobin, red blood cell distribution width, fasting glucose, D-Dimer, fibrinogen, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, creatinine, glomerular filtration rate, albumin, troponin and B-type natriuretic peptide levels were not significantly different between the two groups (all $P > 0.05$). The mean MPV of the poor CCC group (9.56 ± 1.49) was significantly higher than that of the good CCC group (8.52 ± 1.07, $P < 0.01$), while the mean lymphocyte count of the poor CCC group was significantly lower [$(1.40 \pm 0.50) \times 10^9$/L] than that of the good CCC group

TABLE 2 Comparison of biochemical parameters between the two groups.

Variables	Poor CCC($n = 235$)	Good CCC($n = 408$)	*P*-value
&White blood cells/ ($\times 10^9$/L)	6.59 ± 2.16	6.89 ± 1.78	0.054[a]
&Red blood cell/ ($\times 10^{12}$/L)	4.36 ± 0.56	4.45 ± 0.53	0.057[a]
&Platelets ($\times 10^9$/L)	211.03 ± 66.06	217.83 ± 52.89	0.153[a]
&Hemoglobin (g/L)	133.78 ± 17.64	136.37 ± 16.06	0.064[a]
&Absolute neutrophil count ($\times 10^9$/L)	4.47 ± 1.92	4.37 ± 1.47	0.479[a]
&Absolute lymphocyte count $(\times 10)^9$/L	1.40 ± 0.50	1.89 ± 0.54	<0.001[a]
&Monocytes ($\times 10^9$/L)	0.48 ± 0.20	0.51 ± 0.18	0.080[a]
&Hematocrit (L/L)	0.40 ± 0.0496	0.41 ± 0.0462	0.056[a]
&Mean Corpuscular Volume (fL)	92.48 ± 4.87	92.96 ± 4.83	0.227[a]
&Red blood cell distribution width (%)	13.40 ± 1.03	13.35 ± 0.86	0.451[a]
&Platelet hematocrit (%)	0.19 ± 0.053	0.19 ± 0.048	0.347[a]
&Platelet distribution width (fL)	16.58 ± 0.55	16.51 ± 0.51	0.088[a]
&Mean Platelet Volume (fL)	9.56 ± 1.49	8.52 ± 1.07	<0.001[a]
‡Troponin I (ng/mL)	0.012 (0.010–0.030)	0.012 (0.010–0.040)	0.889[c]
‡B-type natriuretic peptide (pg/mL)	337.06 (116.0–844.0)	325.07 (137.5–676.5)	0.554[c]
&Creatinine (μmol/L)	75.62 ± 20.28	74.93 ± 18.77	0.661[a]
&Glomerular filtration rate (ml/min/1.73 m^2)	89.09 ± 16.70	91.33 ± 16.95	0.106[a]
&Fasting blood glucose (mmol/L)	6.08 ± 2.10	5.82 ± 2.01	0.119[a]
&High density lipoprotein (mmol/L)	0.97 ± 0.20	0.93 ± 0.23	0.061[a]
&Low density lipoprotein (mmol/L)	2.16 ± 0.82	2.22 ± 0.81	0.369[a]
&Total cholesterol (mmol/L)	3.66 ± 0.96	3.67 ± 0.94	0.854[a]
‡Triglyceride (mmol/L)	1.38 (1.06–1.89)	3.10 (1.38–7.33)	0.496[c]
&Albumin (g/L)	41.31 ± 4.63	41.89 ± 3.42	0.068[a]
&D-Dimer (mg/L)	0.238 ± 0.056	0.240 ± 0.059	0.557[a]
&Fibrinogen (g/L)	3.01 ± 0.70	2.97 ± 0.63	0.412[a]
&Left ventricular ejection fraction (%)	58.32 ± 8.3	58.08 ± 8.3	0.715[a]
‡C-reactive protein (mg/L)	3.57 (1.50–8.65)	1.60 (0.90–4.088)	<0.001[c]
&Mean platelet volume-to-lymphocyte ratio	7.82 ± 3.80	4.84 ± 1.42	<0.001[a]

&Data are expressed as the mean ± SD. ‡Data are expressed as median (interquartile range). [a]*p*-value by independent sample *t*-test. [c]*p*-value by Mann–Whitney *U* test. CCC, coronary collateral circulation.

TABLE 3 Comparison of coronary angiographic characteristics between the two groups.

	Poor CCC (n = 235)	Good CCC (n = 408)	P-value
Occlusion of blood vessels			
Left anterior descending branch	118 (50.2%)	175 (42.9%)	0.073
Left circumflex branch	63 (26.8%)	129 (31.6%)	0.199
Right coronary artery	71 (30.2%)	235 (57.6%)	<0.001
Number of diseased vessels			
One-vessel disease	52 (22.1%).	54 (13.2%).	0.003
Two-vessel disease	91 (38.7%)	134 (32.8%)	0.132
Three-vessel disease	92 (39.1%)	220 (53.9%)	<0.01
Rentrop collateral grading			
0	104 (44.3%)		
1	131 (55.7%)		
2		218 (53.4%)	
3		190 (46.6%)	
Multivessel lesions	183 (77.9%)	354 (86.8%)	0.003

Data are expressed as n (%), p-value by χ^2 test. CCC, coronary collateral circulation.

[(1.89 ± 0.54) × 10^9/L, $P < 0.01$]. The MPVLR of the poor CCC group (7.82 ± 3.80) was significantly higher than that of the good CCC group (4.84 ± 1.42, $P < 0.01$). Finally, the CRP of the poor CCC group was significantly higher than that of the good CCC group ($P < 0.01$, Table 2).

Spearman correlation analysis demonstrated that MPVLR was negatively correlated with CCC grade, and decreased with increasing Rentrop grade (r = –0.560, $P < 0.01$). The MPVLR of patients with Rentrop grade 0 (10.02 ± 4.50) was significantly higher than that of patients with Rentrop grade 1 (6.08 ± 1.73, $P < 0.05$) and the MPVLR of patients with Rentrop grade 1 (6.08 ± 1.73) was significantly higher than that of patients with Rentrop grade 2 (4.96 ± 1.35, $P < 0.05$), but the MPVLR of patients with Rentrop grade 2 (4.96 ± 1.35) was not significantly different than that of patients with Rentrop grade 3 (4.70 ± 1.49, $P > 0.05$).

Coronary angiographic characteristics of patients with poor and good collateral circulation

The proportions of patients with multi-vessel coronary artery disease and right coronary artery occlusion in the good CCC group were significantly higher than those of the poor CCC group (both $P < 0.01$, Table 3).

Multivariate analysis of factors related to coronary collateral circulation formation

With Rentrop classification group as the dependent variable and factors with statistical significance ($P < 0.05$) in univariate comparisons as the independent variables, MPVLR (OR = 2.101, $P < 0.01$), diabetes (OR = 2.355, $P < 0.01$), C-reactive protein level (OR = 1.036, $P < 0.05$), and right coronary artery occlusion (OR = 0.313, $P < 0.01$) were independently related to CCC formation (Table 4).

Receiver operating curve analysis

The Table 5 showed the ROC curve parameters of MPVLR, MPV, lymphocyte count, and CRP for predicting CCC formation, with the area under the curve (AUC) of 0.820, 0.731, 0.767, and 0.670, respectively (all $p < 0.01$); The AUC of MPVLR was compared with that of MPV, lymphocyte count and CRP. All comparisons had P-values < 0.01, indicating that the AUC of MPVLR was significantly different from that of MPV, lymphocyte count and CRP (Figure 1). The four variables (X1: MPVLR; X2: lymphocyte count; X3: MPV; X4: CRP) and their regression coefficients were used to establish the regression equation: Y (the probability of poorly formed

TABLE 4 Univariate and multivariate logistic regression analyses of poor coronary collateral circulation in CTO patients.

Variables	Univariate analysis			Multivariate analysis		
	b	Odds ratio, 95% CI	P-value	b	Odds ratio, 95% CI	P-value
Diabetes mellitus	0.570	1.768 (1.270–2.459)	0.001	0.857	2.355 (1.532–3.621)	<0.001
RCA occlusion	−1.143	0.319 (0.227–0.448)	<0.001	−1.161	0.313 (0.202–0.485)	<0.001
MPVLR	0.695	2.004 (1.775–2.262)	<0.001	0.742	2.101 (1.840–2.399)	<0.001
C-reactive protein	0.039	1.040 (1.016–1.064)	0.001	0.035	1.036 (1.008–1.064)	0.011
Multivessel lesions	−0.644	0.525 (0.344–0.801)	0.003	−0.425	0.654 (0.374–1.144)	0.137

CTO, chronic total occlusion; RCA, right coronary artery; MPVLR, mean platelet volume-to-lymphocyte ratio; CI, confidence interval.

TABLE 5 Receiver-operating characteristic (ROC) curve parameters of MPVLR, MPV, lymphocyte count, and CRP for predicting CCC formation.

Variable	Cutoff point	AUC	95% CI	Sensitivity	Specificity	*P*-value
MPVLR	6.02	0.820	0.784–0.855	72.3%	82.4%	<0.001
Lymphocyte count	1.57	0.767	0.728–0.806	72.1%	69.4%	<0.001
MPV	8.70	0.731	0.691–0.772	71.1%	66.4%	<0.001
CRP	2.29	0.670	0.627–0.713	63.8%	62.5%	<0.001

ROC, receiver-operating characteristics; MPVLR, mean platelet volume to lymphocyte ratio; MPV, mean platelet volume; CRP: c-reactive protein; CCC, coronary collateral circulation; AUC, area under the curve.

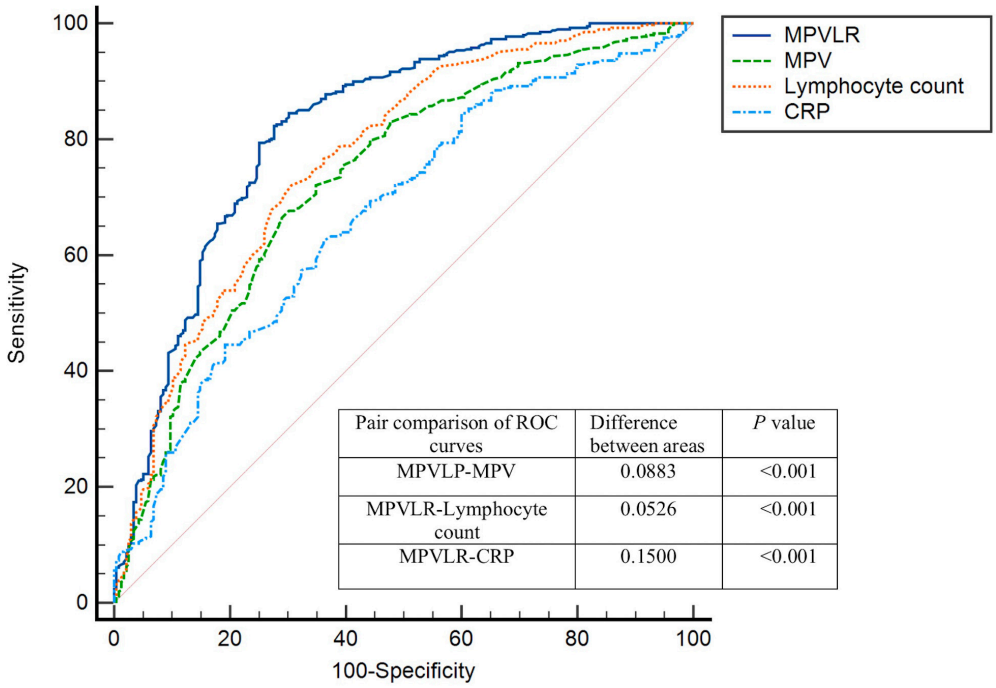

FIGURE 1
Receiver-operating characteristics (ROC) curves for MPVLR, MPV, CRP, and lymphocyte count value in the prediction of impaired coronary collateral circulation. MPVLR, mean platelet volume to lymphocyte ratio; MPV, mean platelet volume; CRP, C-reactive protein.

CCC) = $1/[1 + e^{-(-8.025 + 0.731 * X1 + 0.442 * X2 + 0.254 * X3 + 0.033 * X4)}]$. The Omnibus test of the regression equation ($\chi^2 = 244.843$, $P < 0.001$) showed the regression equation was statistically significant. Hosmer Lemeshow Test showed that the regression equation had a good fitting degree ($\chi^2 = 9.541$, $P = 0.299$). The above results showed that the predictive value of MPVLR for poor CCC in CTO patients was better than that of MPV, CRP, and lymphocyte count.

Discussion

Coronary artery collateral vessel formation is a complex process that includes both angiogenesis and arteriogenesis (18). Angiogenesis is a complex and orderly process that involves a variety of growth factors and adhesion molecules that are secreted and expressed by a variety of different types of cells (such as endothelial cells and smooth muscle cells), in particular endothelial cells (19–22). Chronic inflammation can lead to endothelial dysfunction in a variety of ways, most prominently through increased production of reactive oxygen species (23), which affects the formation of CCC. Lymphocytes are important to innate immunity. Regulatory T cells in particular have significant anti-inflammatory effects. Lymphocyte counts can be reduced in response to inflammation, possibly by increased steroid levels due to stress or apoptosis (24). This decrease in lymphocytes, especially T lymphocytes, leads to decreased vascular infiltration and reductions in vascular endothelial growth factor (VEGF) and other factors related to collateral angiogenesis, thereby inhibiting CCC production (25). Mean platelet volume represents the average volume of a single platelet. It can not only reflect the proliferation and metabolism of megakaryocytes in the bone marrow and platelet production, but also the life span of platelets in the peripheral circulation

(26). Large platelets contain more vasoactive substances and prothrombotic factors, such as platelet factor 4, P-selectin, platelet-derived growth factor, dense particles and thromboxane A2, which can regulate the inflammatory response and endothelial permeability and thereby inhibit the formation of CCC. The higher the MPV, the more active the metabolic response and the faster the thrombosis and inflammation process (27). Hadadi et al. (10) showed that MPV and platelet-to-lymphocyte ratio can predict the presence of CTO in patients presenting with STEMI.

Mean platelet volume lymphocyte ratio is an inflammatory marker that combines mean platelet volume and lymphocyte count. MPVLR is therefore more stable and objective than a single index, and can reflect both the inflammatory response and thrombosis levels. Ornek et al. (13) found that MPVLR is associated with coronary collateral circulation formation in stable angina patients. However, these reports did not utilize a Chinese population. This validative study of 643 patients with CTO found that the MPVLR level in CTO patients decreased with increased coronary collateral grade, and a high MPVLR level could independently predict poor CCC formation in CTO patients. The ROC curve showed that the AUC of MPVLR in the prediction of poor CCC formation was 0.820. The optimal cut-off point was 6.02, with a sensitivity of 72.3% and a specificity of 82.4%. These results suggest that MPVLR, a simple, feasible and inexpensive non-invasive biomarker, may be a clinical predictor of poor CCC in CTO patients.

C-reactive protein, right coronary CTO and diabetes mellitus were independent risk factors for poor coronary collateral circulation. Vascular endothelial cells and nitric oxide (NO) play a crucial role in the formation of coronary collateral circulation. NO not only regulates the functional activity of CCC by relaxing small vessels, but also mediates the angiogenesis of VEGF (28). Fan et al. (29) showed that CRP was significantly associated with poor CCC formation, and therefore could be used as an independent predictor of poor CCC. The results of our study showed that the CRP levels of patients with poor CCC formation were higher than in those with good CCC formation, and high CRP level was negatively correlated with CCC formation in CTO patients. The mechanism behind CRP inhibition may be that CRP inhibits the biological activity and expression of endothelial nitric oxide synthase in endothelial progenitor cells, thereby inhibiting the synthesis of NO and leading to endothelial dysfunction (30, 31). CRP can also inhibit VEGF-induced endothelial cell migration (32). Both CRP and MPVLR, as inflammatory markers, are associated with poor CCC formation. It may therefore be possible to improve the condition and prognosis of CTO patients by reducing the inflammatory response and promoting CCC formation.

Similar to prior work (33), the present study showed that right coronary artery occlusion was more likely to form better collateral circulation than left anterior descending and left circumflex artery occlusion. The mechanism behind this may be related to the formation of collateral circulation and the increase of shear stress. The pressure differential caused by vascular occlusion increased fluid shear stress in arterioles, which leads to upregulation of adhesion molecules in endothelial cells and VEGF production by activated monocytes. At the same time, shear stress stimulates endothelial cells to produce basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), leading to the mitosis of endothelial and smooth muscle cells. Shear stress may also directly stimulate the production of growth factors such as PDGF, bFGF, and transforming growth factor β in endothelial cells (20). The association between right coronary artery occlusion and good collateral circulation formation may be due to the higher driving pressure of the right coronary artery and lower right ventricular tension during ventricular systole, which results in a larger coronary pressure gradient and promotes the development of collateral circulation (34).

The results of this study also showed that the proportion of diabetic patients with poor CCC was higher than that of patients with good CCC, suggesting that diabetes is related to poor CCC establishment and opening. Elevated blood glucose level and insulin resistance can lead to endothelial cell dysfunction, resulting in reduced NO and pro-angiogenic factor secretion and the inhibition of pro-angiogenic factor activity (35, 36). Blood glucose control and reduced insulin resistance in diabetic patients may avoid reduced collateral circulation in these patients. Our study showed that multivascular lesions was more likely to form better collateral circulation than single vessel disease. The mechanism may be that CCC is caused by remodeling process (arteriogenesis) which is triggered by shear stress acting on endothelial cells, and the sprouting of new vessels (angiogenesis) induced by ischemic tissue (37). In the multivascular lesions patients, the severity of ischemia and coronary stenosis was significantly more serious than single vessel disease patients, thus promoting a better collateral circulation formation.

Study limitations

Our study has several limitations. CCC was assessed using coronary angiography alone, without the use of intravascular ultrasound. Second, this was a single-center retrospective study, and further large sample studies are needed to confirm our results.

Conclusion

In conclusion, elevated MPVLR level was independently associated with CCC dysplasia in patients with coronary CTO. MPVLR level may therefore be helpful in the prediction of CCC formation in patients with coronary CTO.

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 the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FH and M-HN conceived and designed the study. M-HN performed the statistical analysis. M-HN and RG interpreted results. M-HN, FH, and RG drafted the report. RG, P-HL, Z-HL, and J-WZ provided critical suggestions for improving the manuscript. All authors contributed to data acquisition and 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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Evaluation of C-reactive protein as predictor of adverse prognosis in acute myocardial infarction after percutaneous coronary intervention: A systematic review and meta-analysis from 18,715 individuals

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Background: Proper prognostic biomarker is of great importance for clinical decision-making in patients with acute myocardial infarction (AMI) undergoing percutaneous coronary intervention (PCI). Although recently emerges plenty of novel inflammatory biomarkers, the canonical inflammatory mediator C-reactive protein still plays an important role in prognosing adverse post-infarction complications.

Methods: PubMed, Embase, and Medline were systematically searched from the establishment of databases up to December 2021, conforming with standards set forth by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.

Results: A total of 23 studies were eventually eligible for this meta-analysis, including 18,715 individuals. Our findings showed that elevated C-reactive protein (CRP) had a statistically significant superiority in predicting all-cause mortality (OR: 3.22, 95% CI: [2.71, 3.84], $p < 0.00001$), cardiovascular death (OR: 3.26, 95% CI: [2.30, 4.61], $p < 0.00001$), major adverse cardiovascular events (MACEs) (OR: 2.85, 95% CI [2.08, 3.90], $p < 0.00001$), heart failure (OR: 2.29, 95% CI: [1.48, 3.54], $p = 0.0002$), recurrent myocardial infarction (OR: 1.76, 95% CI: [1.28, 2.43], $p < 0.001$), and restenosis (OR: 1.71, 95% CI: [1.18, 2.47], $p = 0.004$). Subgroup analysis implies that CRP had better performance in predicting plenty of hospitalization and short-term (<12 months) adverse prognosis than long-term prognosis and Asian patients with elevated CRP were under more risk in adverse prognosis after PCI than Europeans.

Conclusion: Our meta-analysis suggests that CRP is a prospective predictor of the prognosis in patients with AMI undergoing PCI, especially in hospitalization and short-term and in the Asian group.

KEYWORDS

acute myocardial infarction, percutaneous coronary intervention (PCI), C-reactive protein (CRP), adverse prognosis, inflammatory/anti-inflammatory factors, coronary arterial disease

Introduction

Despite advances in the therapies for coronary artery disease, such as percutaneous coronary intervention (PCI) which has saved a lot of lives since its application (1), myocardial infarction is still the main cause of morbidity and mortality among all cardiovascular diseases worldwide (2, 3). For patients with acute myocardial infarction (AMI) undergoing PCI, inflammatory response plays a pivotal role in the whole pathophysiological process. Since inflammation can promote endothelial cell injury (4), vascular remodeling (5), and plaque destabilization (6), it is now regarded as another risk factor for AMI besides traditionally acknowledged risk factors like hypertension and dyslipidemia (7). In addition, the pro-inflammatory response occurs in the early stage of AMI and may exacerbate after PCI, thus contributing to the death of myocardial cells (8).

During AMI, the release of the intracellular content and destruction of the extracellular matrix (9) leads to the generation of a cascade of inflammatory infiltration and mediators (10). Biomarkers from inflammatory infiltration are based on the accumulation of plasma inflammatory cells (11) [e.g., neutrophil-to-lymphocyte ratio (NLR) (12), systemic immune-inflammation index (SII) (13), and novel subclasses of Tregs (14)], which are easily influenced by heterogeneity under ethnicity and individuals. With respect to inflammatory mediators, plasma molecules consisting of common inflammatory pathways are routinely measured in clinical practice and have been studied as prognostic biomarkers, such as IL-1b (15) or cytokine concentrations and proinflammatory to anti-inflammatory cytokine ratios (16). However, some limitations like a small sample size are unneglectable in all these studies, requiring for in-depth investigation. It is noteworthy that C-reactive protein (CRP), among plenty of canonical inflammatory mediators, has exhibited excellent capacity in predicting post-STEMI adverse prognoses in comprehensive comparison studies (16–19). It might result from that CRP, as a canonical downstream of multiple inflammatory pathways (8, 17, 18, 20), actively participating in the inflammatory process (21), stimulating the secretion of various pro-inflammatory cytokines, promoting the release of reactive oxygen, and inducing the switch of quiescent macrophage to pro-inflammatory M1 subtype (22). Even the relationship between the prognosis of AMI and other novel biomarkers has been extensively explored, such as plasma long pentraxin-3 (23) and YKL-40 (24), but none have been proven as practically useful as hsCRP.

Hence, CRP is a typically and frequently used biomarker of inflammation, which may give rise to an increased cardiovascular risk (6). The relationship between the increase of plasma CRP concentration and the prognosis of patients with AMI, such as mortality (25, 26), cardiovascular mortality (27, 28), the rate of major adverse cardiovascular events (MACEs) (29, 30), heart failure (5), recurrent myocardial infarction (31, 32), and restenosis (33, 34), has been thoroughly studied in the past decades. A single study alone can only partially depict the whole profile of the association between CRP and poor prognosis in AMI after CRP. Besides, although Mincu et al. (35) had made a persuasive meta-analysis in 2016 including seven studies and 6,993 patients, the detailed subgroup analyses remain to be in-depth investigated. Therefore, we conducted this meta-analysis in an attempt to elucidate the relationship between the elevation of CRP and the prognosis of patients with AMI undergoing PCI and figure out its special traits in clinical implication.

Methods

A meta-analysis conformed with standards set forth by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (36).

Search strategy

PubMed, Embase, and Medline were systematically searched from the establishment of databases up to December 2021. The search strategy was edited following the principle of each database and included keywords related to CRP, AMI, and PCI. Previous meta-analyses and other reviews

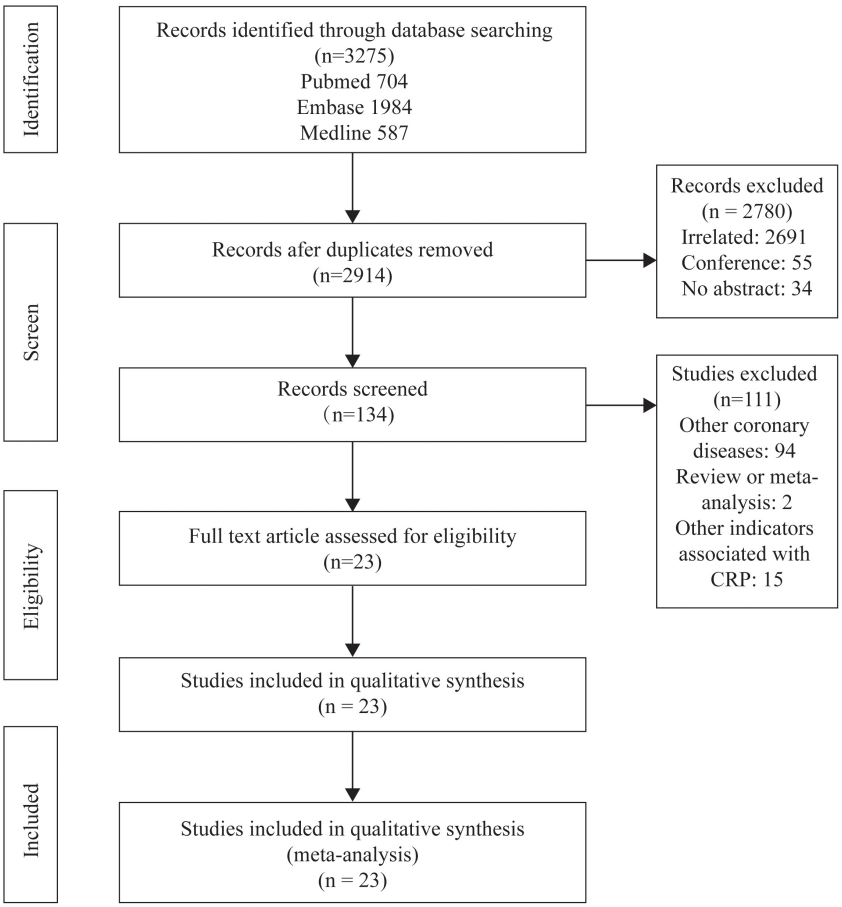

FIGURE 1
Flowcharts showing relevant studies.

related to the topic were reviewed to identify studies not included in this search strategy. We also scanned the bibliographies of the included articles and relevant reviews for further reference.

Inclusion and exclusion criteria

After removing duplicates, titles and abstracts were screened to identify potentially relevant studies. All potentially relevant studies proceeded to full-text review by either reviewer independently and studies that met all the following criteria were included as follows: (1) the study was designed as randomized studies, prospective, or retrospective observational design studies; (2) the study population was patients with AMI (including STEMI and NSTEMI) treated with PCI; (3) the target variant was CRP and concrete information of CRP including cut-off, patient number in different groups, measuring methodology and time was available; and (4) the study reported follow-up duration in detail and outcomes of patients.

Exclusion criteria as follows: (1) articles were reviews, animal studies, laboratory studies, conference documents, and letters; (2) the population of studies included patients who suffered from other coronary diseases like stable or unstable angina; (3) patients were treated with other therapies like conservative medication, thrombolysis, or coronary artery bypass graft; (4) patients were not divided into groups by CRP or cut-off was unavailable; (5) incidence of outcomes in each group could not be directly attained or indirectly calculated; and (6) studies were without access to full text for quality assessment or data extraction.

Any discrepancy was settled by getting through full texture to reach a consensus.

Data extraction

Two of the authors independently performed data extraction, using a standard data extraction form that contained publication details (name of the first author, year of publication, and region), characteristics of the studied

TABLE 1 Characteristics of studies included in the meta-analysis.

First author	Year	Region	Number of patients included (age, %males)	Types of MI	Follw-up*	hs-CRP	Cut-off (mg/L)	Measuring time	Endpoints	Results
Bicciré, F. G.	2021	Europe	220 (68 ± 12.1, 58%)	STEMI	In-hospital	N/A	17	Peak value	MACEs	Low SA is not infrequent in patients admitted for STEMI treatment and is associated with worse outcomes independently from troponin and CRP levels.
Wang, Y.	2021	Asian	2318 (58.8 ± 11.9, 79.8%)	STEMI	30 months	Yes	2.0	After PCI	MACEs	Systemic inflammation (hsCRP≥2mg/L) can modulate Lp(a)-associated MACE risk in STEMI-PCI patients.
Świątkiewicz, I.	2021	Europe	204 (56.17, 76.5%)	STEMI	6 months	Yes	2.0	1 month after discharge	HF	Persistent elevation in CRP concentration post-STEMI can serve as a risk marker and aid in identifying patients at increased risk of HF and HF-related mortality in multi-year period.
Jiang, H.	2021	Asian	203 (60.35 ± 10.24, 66.0%)	STEMI	12 months	Yes	2.0	Within 72 h inhospitalization	All-cause death, cardiovascular death, HF, reMI	The presence of hs-CRP was not only significantly associated with platelet inhibition function, but was also a prognostic marker in STEMI.
Kang, D. O.	2019	Asian	4410 (62.7 ± 12.4, 77.3%)	STEMI and NSTEMI	36 months	Yes	3.0	On admission	All-cause death, cardiovascular death, MACEs, reMI	The prognostic impact of elevated hs-CRP at baseline was most evident during the first 6 months after AMI.
Her, A. Y.	2017	Asian	146 (57.1 ± 12.4, 81.4%)	STEMI	24 months	Yes	2.7	On admission	MACEs	Elevated hs-CRP and NLR levels were significantly associated with MACE in STEMI patients successfully treated with DES and had incremental predictive values over conventional risk factors.
Shin, H. C.	2017	Asian	381 (61.64 ± 11.0, 76.1%)	STEMI and NSTEMI	24 months	No	7.6	Before PCI	All-cause death, cardiovascular death, MACEs, reMI, restonisis	Elevated levels of both NLR and CRP are associated with increased risk of long-term mortality in AMI patients with PCI.
Wang, C. H.	2015	Asian	241 (63.70 ± 11.96, 77.6%)	STEMI	48.3 months	Yes	3.0	After PCI	All-cause death, cardiovascular death, MACEs, reMI,restonisis	Renal dysfunction and elevated hsCRP predict a high long-term incidence of MACE in patients with acute STEMI with primary PCI, with the combination being of prognostic significance for long-term mortality and MI in these patients.
Shacham, Y.	2015	Asian	562(62 ± 13, 80%)	STEMI	1 month	Yes	9.0	Before PCI	All-cause death and HF	Admission serum hs-CRP level (>9 mg/l) is an independent predictor for AKI following primary PCI in STEMI patients.
Jian-Wei, Z.	2014	Asian	1452(64.50 ± 13.29, 81.68%)	STEMI	In-hospital	Yes	3.0	Before PCI	All-cause death and MACEs	Higher baseline hsCRP level (>6.50 mg/L) was an independent and significant predictor of CIN after p-PCI. A high level of hsCRP was strongly associated with inhospital mortality and composite MACE.

(Continued)

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TABLE 1 (Continued)

First author	Year	Region	Number of patients included (age, %males)	Types of MI	Follw-up*	hs-CRP	Cut-off (mg/L)	Measuring time	Endpoints	Results
He, Y. T.	2013	Asian	220(62.38 ± 12.28, 82.73%)	STEMI	In-hospital	Yes	6.26	On admission	All-cause death	hs-CRP is positively correlated with CIN incidence.
Kim, K. H.	2013	Asian	5123(62.94 ± 13.38, 74.12%)	STEMI	24 months	Yes	3.0	On admission	MACEs	For STEMI patients with a long ischemic time (≥6 hours), an elevated level of hs-CRP is a poor prognostic factor of long-term cardiovascular outcomes.
Ahmed, K.	2012	Asian	5647(60.4 ± 12.2, 75.2%)	STEMI and NSTEMI	12 months	Yes	2.0	On admission	All-cause death and MACEs	Higher baseline hs-CRP level (≥4.08 mg/dL) in overweight/obese AMI patients showed significant association with 12-month all-cause mortality independent of other prognostic markers.
Schoos, M. M.	2011	Europe	258(60.83, 75.97%)	STEMI	36 months	Yes	2.0	Before pPCI	All-cause death, cardiovascular death, reMI and restenosis	BMS implantation should be preferred when hs-CRP is <2 mg/L and DES when hs-CRP is >2 mg/L to decrease long-term adverse outcomes including stent thrombosis in patients with STEMI treated with pPCI.
Damman, P.	2011	Europe	1034(62 ± 13, 73%)	STEMI	30 months	No	7.0	Before pPCI	All-cause death	The sole use or addition of a multimarker to a model including established risk factors improves the prediction of mortality in STEMI patients undergoing PPCI.
L. I. Gui-Hua	2009	Asian	84(58 ± 11, 65.48%)	STEMI	3–12 months	No	5.0	Within 6h onset	Cardiovascular death, MACEs and HF	The CRP levels within 6h after attack of AMI can be taken as one of the indexes to predict the prognosis of PCI.
Ortolani, P.	2008	Europe	758(68.00, 70.45%)	STEMI	in-hospital	Yes	3.1	On admission	All-cause death, MACEs, reMI and restenosis	hs-CRP levels at admission independently predict in-hospital and long-term clinical outcome, potentially negatively influencing survival.
Jeong, Y. H.	2008	Asian	207(57.3 ± 12.0, 81.6%)	STEMI	12 months	Yes	5.0	On admission	All-cause death, MACEs, HF and reMI	cTnI and hs-CRP levels on admission give no addictive information on classic TIMI risk score for predicting long-term cardiovascular outcomes in STEMI patients treated with primary DES implantation and intensive medical therapy.
Yip, H. K.	2005	Asian	146(60.0 ± 10.8, 86.3%)	STEMI	1 months	Yes	2.37	Before PCI	All-cause death, MACEs and restenosis	Prospective evaluation ofthe hsCRP in STEMI of onset < 6 h allows accurate risk stratification of individuals at risk of 30-day MACE after primary PCI.
Liu, Jun	2004	Asian	76(N/A)	STEMI	6 months	No	3.0	Within 6 h onset	All-cause death, MACEs and reMI	CRP levels within six hours after the onset of AMI might predict early and late outcome after primary PCI.

(Continued)

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TABLE 1 (Continued)

First author	Year	Region	Number of patients included (age, %males)	Types of MI	Follw-up*	hs-CRP	Cut-off (mg/L)	Measuring time	Endpoints	Results
Magadle, R.	2004	Asian	230(63.58 ± 8.80, 75.22%)	STEMI	12 months	Yes	5.0	Before PCI	All-cause death, MACEs, reMI and restenosis	Preprocedural serum CRP level might be considered a powerful predictor of early but not late complications in patients undergoing PTCA/stent procedures.
Hong, Y. J.	2003	Asian	208(59.43 ± 9.96, 79.81%)	STEMI	12 months	No	1.0	On admission	All-cause death, MACEs, reMI and restenosis	An leevated CRP is an independent prognostic marker in patients with acute myocaridal infarction after primary or rescue PCI.
Tomoda, H.	2000	Asian	234(62.42 ± 10, 77.35%)	STEMI	6 months	No	3.0	Within 6 h onset	All-cause death, MACEs, reMI and restenosis	CRP levels within 6 h after the onset of AMI reflect the vulnerability of culprit coronary lesions and predict adverse coronary events after primary PTCA/stenting.

*The longest follow time of studies.

population (sample size, age, and gender distribution), and traits of CRP (high-sensitivity, cut-off, and time of blood taking), the follow-up, and outcomes.

Outcomes

The end points were as follows: all-cause mortality, cardiovascular death, MACE, heart failure, recurrent myocardial infarction, restenosis, and revascularization. All-cause mortality was defined as the death without specific causes. MACE was a composite definition usually consisting of death, cardiovascular death, recurrent MI, revascularization, stroke, and heart failure. In some studies, more adverse events were composited into MACEs like cardiopulmonary resuscitation and malignant arrhythmia; on the contrary, some selected only few events as MACE. Restenosis is defined as a narrowing of vessel diameter greater than 50% to that of the reference vessel (37), consisting of stent restenosis and in-stent restenosis (38). The former is defined as the presence of an acute coronary syndrome with angiographic or autopsy evidence of thrombus or occlusion. In this meta-analysis, we did not conduct the subgroup analysis between different types of restenosis due to the limitation of research quantity.

Data analysis

All analyses were conducted using the Review Manager 5.4. The results of our meta-analysis were present as adjusted ORs with 95% confidence intervals (CI). The heterogeneity was presented with estimation using the I^2 statistic. When I^2 statistic is less than 50%, the measured data were pooled in the study and analyzed using a fixed-effects meta-analysis model with inverse variance weighting. On the contrary, a random-effects model is selected. All p-values were two-tailed with the statistical significance set at 0.05.

Results

Study selection

The study selection process is shown in **Figure 1**. After selection and evaluation, 23 studies were eventually eligible for this meta-analysis (5, 25–34, 39–50) (**Table 1**). Overall, there were 18,715 patients involved in our analysis, 12,109 in the elevated CRP group and 6,606 in the normal CRP group. The follow-up period varied from in-hospital to 48.3 months. CRP in 17 (5, 27, 28, 30–34, 39, 41, 43–47, 49, 50) studies were measured with high-sensitivity methodology and in one (29) not mentioned. The cut-off values in different experiments were not exactly the same, while 2 mg/L (5, 28, 30, 32, 43), 3 mg/L

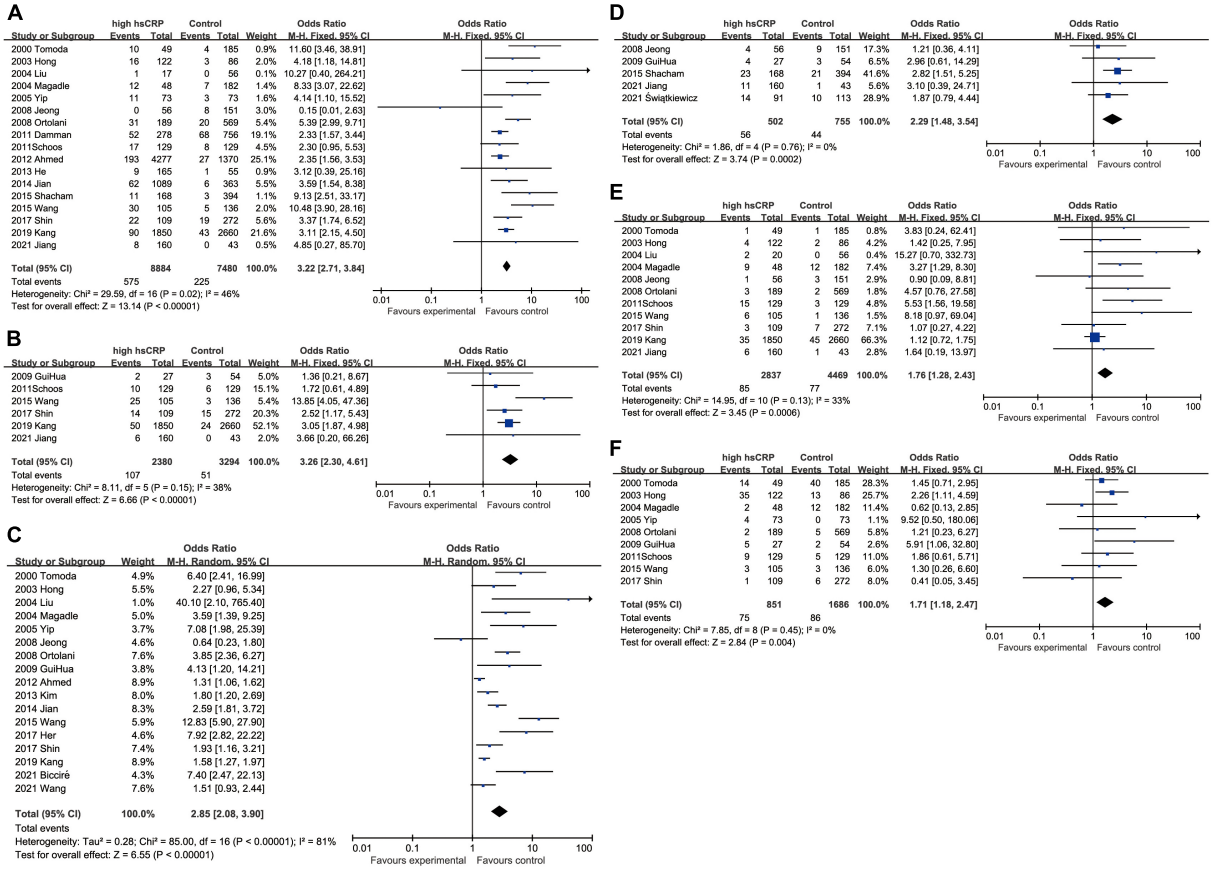

FIGURE 2
Forest plots of studies assessing the association between elevated CRP and different outcomes among patients with AMI with PCI: **(A)** CRP and all-cause mortality; **(B)** CRP and cardiovascular mortality; **(C)** CRP and MACEs; **(D)** CRP and heart failure; **(E)** CRP and recurrent myocardial infarction; and **(F)** CRP and restenosis.

(25, 27, 33, 40, 41, 45, 49), and 5 mg/L (31, 39, 42) were the most common cut-off values in included experiments. Instead of presupposed cut-off, some studies divided patients by points of bisection (34, 46–48, 50), trisection (28, 30), quadrisection (41, 44) according to CRP value. The diversion point ranged from 2 to 9 mg/L (46) and was selected as the cut-off. The majority of population was originated from Europe (5, 29, 32, 41, 48) and Asian (25–28, 30, 31, 33, 34, 39, 40, 42–47, 49, 50).

C-reactive protein and outcomes

Elevated CRP was associated with increased all-cause mortality (OR: 3.22, 95% CI [2.71, 3.84], $p < 0.00001$, $I^2 = 46\%$) (25–28, 31–34, 39–41, 43, 44, 46–49) and cardiovascular mortality (OR: 3.26, 95% CI [2.30, 4.61], $p < 0.00001$, $I^2 = 34\%$) (27, 28, 32–34, 42). In addition to mortality, elevated CRP were also related to higher incidence of various malignant cardiovascular events, such as MACEs (OR: 2.85, 95% CI [2.08, 3.90], $p < 0.00001$, $I^2 = 81\%$) (25–27, 29–31, 33, 34, 39–43, 45, 47, 49, 50), heart failure (OR: 2.29, 95% CI [1.48, 3.54], $p = 0.0002$, $I^2 = 0\%$) (5, 28, 31, 42, 46), recurrent myocardial infarction (OR: 1.76, 95% CI [1.28, 2.43], $p < 0.001$, $I^2 = 33\%$) (25–28, 31–34, 39–41), and restenosis (OR: 1.71, 95% CI [1.18, 2.47], $p = 0.004$, $I^2 = 0\%$) (25, 26, 32–34, 39, 41, 42, 47). All these results have shown the potential of CRP as the prognostic biomarker for multiple outcomes in patients with AMI after PCI (**Figure 2**). With respect to the situation that different variants like duration of follow-up, traits of CRP, and racial distributions may lead to different outcomes, we processed the data in the trials and conducted subgroup analyses to shrink heterogeneity, while sensitivity analysis verified the stability of results (**Supplementary Table 1**).

Subgroup analysis

C-reactive protein and all-cause mortality

All-cause mortality was one of the most concerning outcomes of AMI. Increased CRP was invariably a powerful

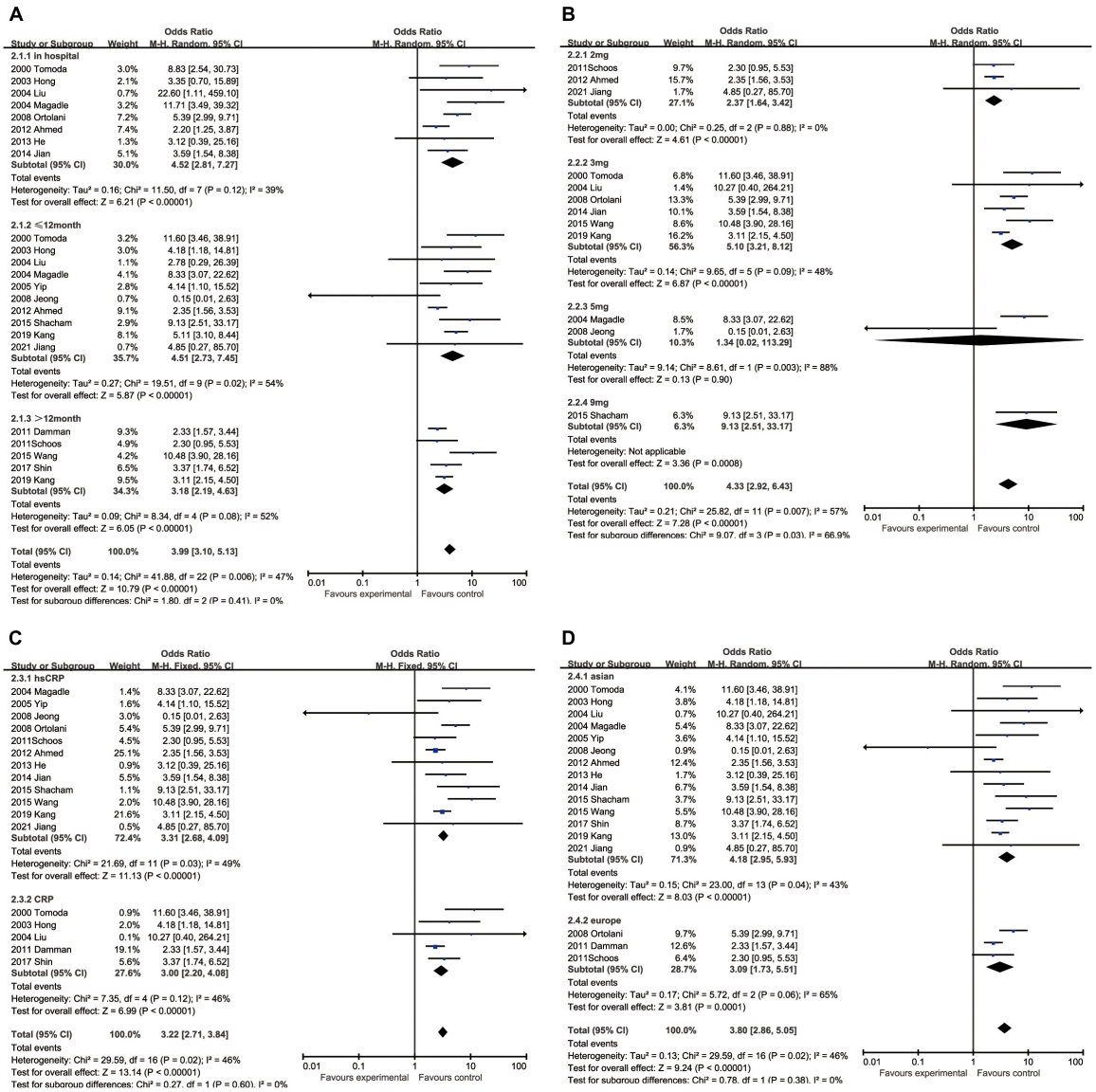

FIGURE 3
Forrest plots of subgroup analysis assessing all-cause mortality associated with increased CRP: **(A)** subgroups based on follow-up; **(B)** subgroups based on cut-off value of hsCRP; **(C)** subgroups based on CRP and hsCRP; and **(D)** subgroups based on ethnicities.

predictor to all-cause mortality in hospital (OR: 4.52, 95% CI [2.81, 7.27], $p < 0.00001$, $I^2 = 39\%$), <12 months (OR: 4.51, 95% CI [2.73, 7.45], $p < 0.00001$, $I^2 = 54\%$) and ≥12 months (OR: 3.18, 95% CI [2.19, 4.63], $p < 0.00001$, $I^2 = 52\%$) (**Figure 3A**). When we took the marginal value of CRP into subgroup analysis, 3 mg/L (OR: 5.10, 95% CI [3.21, 8.12], $p < 0.00001$, $I^2 = 48\%$) was the most frequently chosen cut-off value and had been shown a more powerful capacity than 2 mg/L (OR: 2.37, 95% CI [1.64, 3.42], $p < 0.00001$, $I^2 = 0$) and 5 mg/L (OR: 1.34, 95% CI [0.02, 113.29], $p = 0.90$, $I^2 = 88\%$) to predict the mortality. Five milligrams per liter exhibited no statistical significance. Although 9 mg/L had shown to be the best predictor (OR: 9.13, 95% CI [2.51, 33.17], $p = 0.008$), only one study was included. Besides, elevated hsCRP (OR: 3.31, 95% CI [2.68, 4.09], $p < 0.00001$, $I^2 = 49\%$) had slight advantage than CRP (OR: 3.00, 95% CI [2.20, 4.08], $p < 0.00001$, $I^2 = 46\%$). In addition, the upregulated CRP was shown to be less associated with mortality in European (OR: 3.09, 95% CI [1.73, 5.51], $p < 0.00001$, $I^2 = 43\%$) than in Asian (OR: 4.18, 95% CI [2.95, 5.93], $p < 0.00001$, $I^2 = 65\%$) (**Figure 3**).

C-reactive protein and cardiovascular mortality

The augment of CRP amplified the possibility of cardiovascular death within 12 months after hospitalization

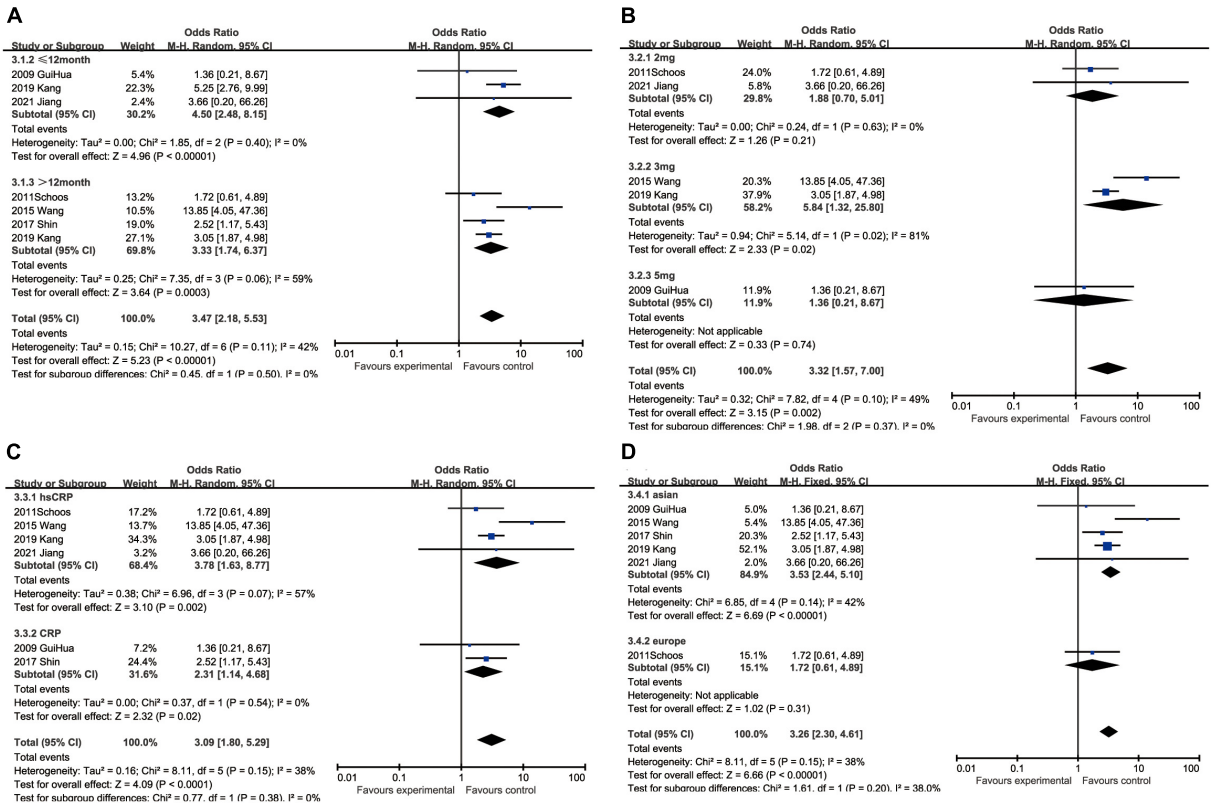

FIGURE 4
Forrest plots of subgroup analysis assessing cardiovascular mortality associated with increased CRP: **(A)** subgroups based on follow-up; **(B)** subgroups based on cut-off value of hsCRP; **(C)** subgroups based on CRP and hsCRP; and **(D)** subgroups based on ethnicities.

(OR: 4.50, 95% CI [2.48, 8.15], $p < 0.00001$, $I^2 = 0$) and the influence still existed when the follow-up was prolonged to more than 12 months (OR: 3.33, 95% CI [1.74, 6.37], $p < 0.00001$, $I^2 = 59\%$). With respect to the critical value of CRP, 3 mg/L (OR: 5.84, 95% CI [1.32, 25.80], $p = 0.02$, $I^2 = 81\%$) was usually the most recommended, while the others like 2 mg/L (OR: 1.88, 95% CI [0.70, 5.01], $p = 0.21$, $I^2 = 0$) and 5 mg/L (OR: 1.36, 95% CI [0.21, 8.67], $p = 0.74$) had no statistical significance. The measuring methodologies did not exert obvious impact on the incidence of cardiovascular death, although hsCRP (OR: 3.78, 95% CI [1.63, 8.77], $p = 0.002$, $I^2 = 57\%$) was presumed to perform better than CRP (OR: 2.31, 95% CI [1.14, 4.68], $p = 0.02$, $I^2 = 0$). The ethnicity had also an effect on cardiovascular death, while Asian (OR 3.53, 95% CI [2.44, 5.10], $p < 0.00001$, $I^2 = 42\%$) were at higher risk than European (OR: 1.72 [0.61, 4.89], $p = 0.20$) and the latter had no statistical significance (**Figure 4**).

C-reactive protein and major adverse cardiovascular events

In regard to MACEs, elevated CRP might result in increased hospitalized malignant events (OR: 3.66, 95% CI [2.54, 5.28], $p < 0.00001$, $I^2 = 37\%$) with medium heterogeneity. We also analyzed the association between ascending CRP and incidence of MACEs within 12 months (OR: 1.76, 95% CI [1.24, 2.51], $p = 0.002$, $I^2 = 69\%$) or over 12 months (OR: 2.68, 95% CI [1.62, 4.46], $p = 0.0001$, $I^2 = 84\%$), but greater heterogeneity was accompanied with the elongation of follow-up. Three milligrams per liter (OR: 3.51, 95% CI [2.10, 5.87], $p < 0.00001$, $I^2 = 86\%$) was a superior threshold in dividing patients with AMI with higher or lower risk of MACEs, while mediocre performances exhibited in other cut-off such as 2 mg/L (OR: 1.34, 95% CI [1.11, 1.63], $p = 0.003$, $I^2 = 0$) and 5 mg/L (OR: 2.09, 95% CI [0.63, 6.89], $p = 0.23$, $I^2 = 73\%$) which had no statistical significance. As for CRP measuring methodology, CRP (OR: 3.37, 95% CI [1.75, 6.46], $p = 0.0003$, $I^2 = 53\%$) was more efficient to predict MACEs than hsCRP (OR: 2.55, 95% CI [1.78, 3.65], $p < 0.00001$, $I^2 = 85\%$). Considering ethnics, the subgroup analysis implied that Caucasian patients (OR: 4.43, 95% CI [2.59, 7.57], $p < 0.00001$, $I^2 = 15\%$) were more susceptible to MACEs than Asian (OR: 2.62, 95% CI [1.90, 3.61], $p < 0.00001$, $I^2 = 80\%$) (**Figure 5**).

C-reactive protein and heart failure

Although heart failure was reported in only five studies containing 1,257 patients, the marginal values of hsCRP were

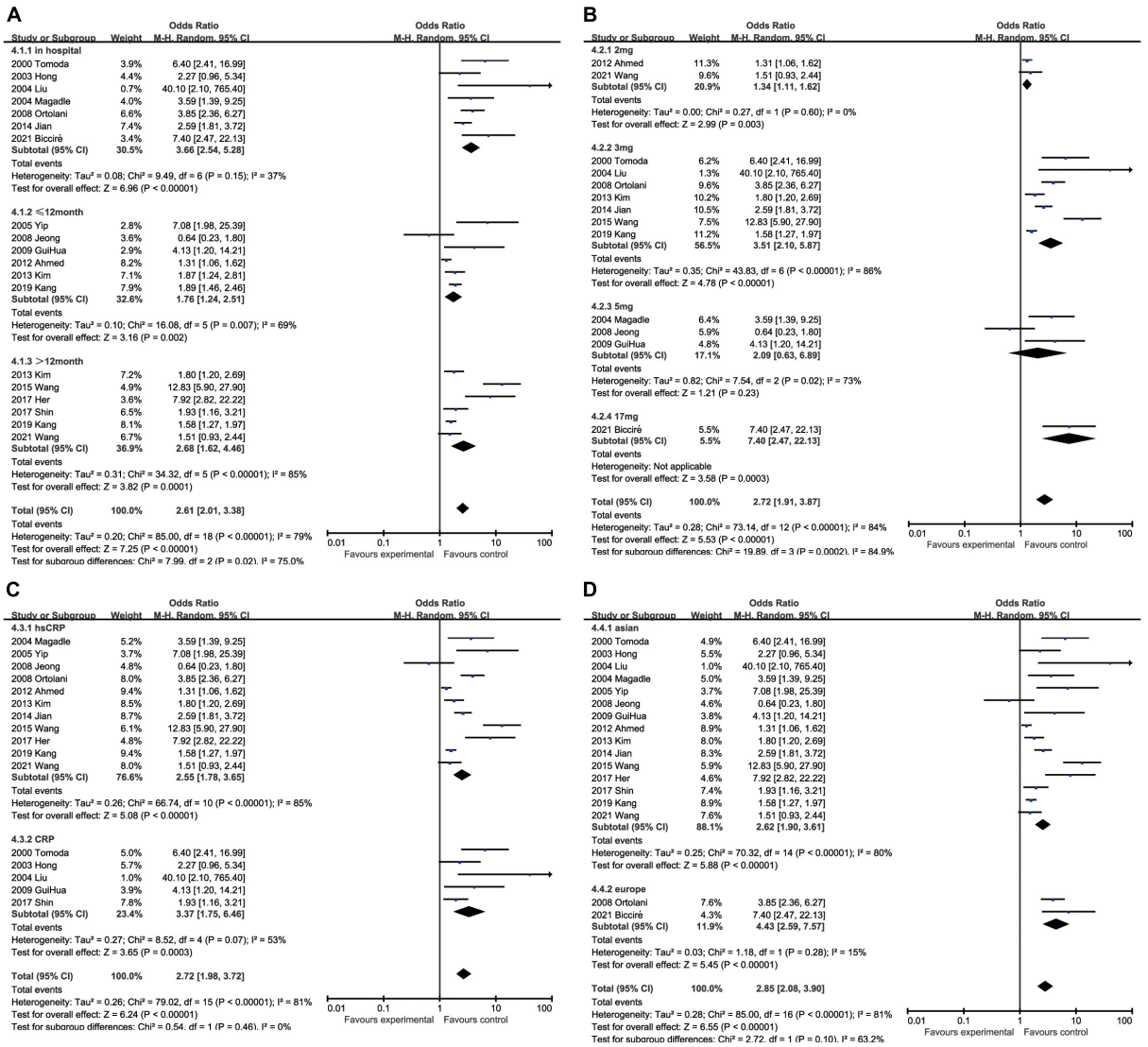

FIGURE 5
Forrest plots of subgroup analysis assessing MACEs associated with increased CRP: **(A)** subgroups based on follow-up; **(B)** subgroups based on cut-off value of hsCRP; **(C)** subgroups based on CRP and hsCRP; and **(D)** subgroups based on ethnicities.

variably selected. When we put different cut-off values into different subgroups for analysis, no statistical significance could be found in some subgroups, namely 2 mg/L group (OR: 2.07, 95% CI [0.94, 4.58], p = 0.07, I^2 = 0) and 5 mg/L group (OR: 1.69, 95% CI [0.66, 4.33], p = 0.27, I^2 = 0). As the critical value was upregulated to 9 mg/L (OR: 2.82, 95% CI [1.51, 5.25], p = 0.001), statistical significance was eventually exhibited. In addition, hsCRP (OR: 2.24, 95% CI [1.43, 3.53], p = 0.0005, I^2 = 0) could be a predictor of incidence of heart failure, while CRP (OR: 2.96, 95% CI [0.61, 14.29], p = 0.18) had no statistical significance. Asian patients (OR: 2.46, 95% CI [1.49, 4.06], p = 0.0004, I^2 = 0) were under statistically significant risk to heart failure, comparing with European (OR: 1.87, 95% CI [0.79, 4.44], p = 0.15) whose subgroup analysis result had no statistical significance (**Figure 6**).

C-reactive protein and recurrent myocardial infarction

With regard to recurrent myocardial infarction, subgroup analysis indicated that enhanced CRP could foresee soaring incidence of recurrent myocardial infarction in hospitalization (OR: 5.32, 95% CI [1.10, 25.60], p = 0.04, I^2 = 0), within 12 months (OR: 1.90, 95% CI [1.24, 2.92], p = 0.003, I^2 = 0), while CRP showed no statistical significance over 12 months (OR: 2.17, 95% CI [0.81, 5.80], p = 0.12, I^2 = 64%). Three milligrams per liter (OR: 6.69, 95% CI [2.16, 20.75], p = 0.0010, I^2 = 0) had a satisfactory performance as a cut-off value of

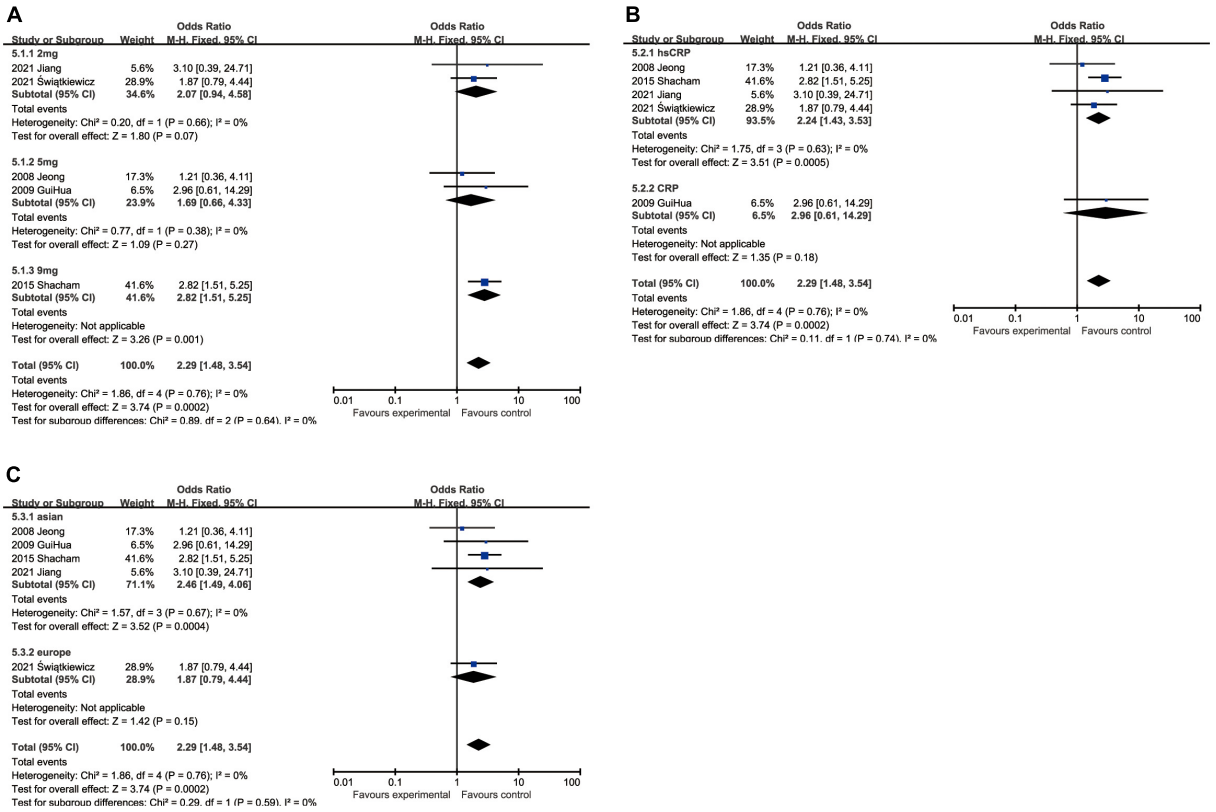

FIGURE 6
Forrest plots of subgroup analysis assessing heart failure associated with increased CRP: **(A)** subgroups based on cut-off value of hsCRP; **(B)** subgroups based on CRP and hsCRP; and **(C)** subgroups based on ethnicities.

CRP, whereas 2 mg/L (OR: 4.11, 95% CI [1.38, 12.24], $p = 0.01$, $I^2 = 0$) and 5 mg/L (OR: 2.60, 95% CI [1.12, 6.06], $p = 0.03$, $I^2 = 6\%$) were also equal to predicting recurrent myocardial infarction. Elevated hsCRP (OR: 2.49, 95% CI [1.24, 5.01], $p = 0.01$, $I^2 = 51\%$) was considered to be a potential risk factor, especially comparing to CRP (OR: 1.75, 95% CI [0.67, 4.53], $p = 0.25$, $I^2 = 0$) measured by normal sensitivity methodology which had no statistical significance. When we focused on the impact of ethnics on heterogeneity, European (OR: 5.27, 95% CI [1.86, 14.96], $p = 0.002$, $I^2 = 0$) were more susceptible to recurrent myocardial infarction than Asian (OR: 1.51, 95% CI [1.07, 2.14], $p = 0.02$, $I^2 = 19\%$) (**Figure 7**).

C-reactive protein and restenosis

The subgroup analysis demonstrated that the predicting capacity of increased CRP had a relatively narrow time window within 12 months (OR: 1.91, 95% CI [1.24, 2.94], $p = 0.003$, $I^2 = 30\%$), whereas neither in hospitalization (OR: 1.21, 95% CI [0.23, 6.27], $p = 0.82$) nor over 12 months (OR: 1.26, 95% CI [0.57, 2.81], $p = 0.57$, $I^2 = 0$) had statistical significance. None of the cut-off performed statistically significant, namely 2 mg/L group (OR: 1.86, 95% CI [0.61, 5.71], $p = 0.28$), 3 mg/L (OR: 1.39, 95% CI [0.76, 2.55], $p = 0.28$, $I^2 = 0$), 5 mg/L (OR: 1.59, 95% CI [0.60, 4.22], $p = 0.35$, $I^2 = 73\%$) and 7.6 mg/L (OR: 0.41, 95% CI [0.05, 3.45], $p = 0.54$). The subgroup analysis could help us make the decision between CRP (OR: 1.82, 95% CI [1.16, 2.86], $p = 0.009$, $I^2 = 32\%$) and hsCRP (OR: 1.50, 95% CI [0.79, 2.84], $p = 0.22$, $I^2 = 0$) as a prognostic factor for restenosis for reason of that the latter had no statistical significance. Ethnically, CRP could predict restenosis in Asians ((OR = 1.72, 95% CI [1.15, 2.57], $p = 0.008$, $I^2 = 22\%$), but not in Europeans (OR: 1.63, 95% CI [0.66, 4.07], $p = 0.29$, $I^2 = 0$) (**Figure 8**).

Discussion

Briefly summarizing the results of our meta-analysis, the elevation of CRP is significantly associated with the boost in multiple adverse prognoses for the patients with AMI undergoing PCI, especially in all-cause mortality and cardiovascular death. Furthermore, it is noteworthy that increased CRP has more prognostic value in hospitalization and short-term outcomes and the Asian group.

Recent years have emerged plenty of studies investigating diverse post-infarction prognostic inflammatory biomarkers originating from either inflammation infiltration or plasma

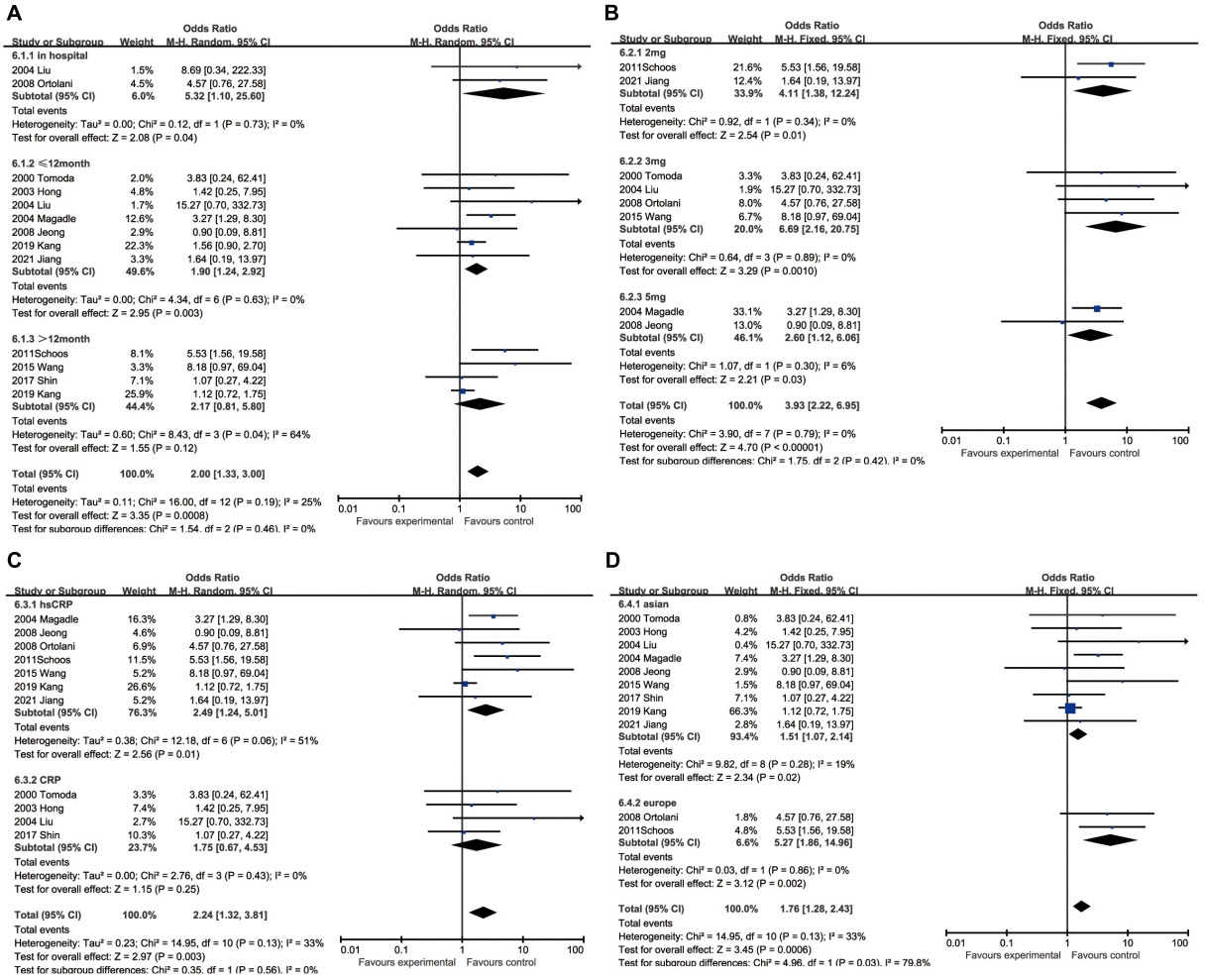

FIGURE 7
Forrest plots of subgroup analysis assessing recurrent myocardial infarction associated with increased CRP: **(A)** subgroups based on cut-off value of hsCRP; **(B)** subgroups based on CRP and hsCRP; **(C)** subgroups based on ethnicities; and **(D)** subgroups based on ethnicities.

inflammatory factors. Biomarkers based on inflammatory infiltration have been verified from simple white blood cell counts (51) to counts (8) or ratios (52, 53) of a different specific subclass of innate immune cells. NLR was most reported to be related to the hospital and long-term prognosis of patients with STEMI after PCI (12). Scoring systems have also been developed from white blood cell counts, such as SII (54) foreseeing no-reflow phenomenon in patients with STEMI after PCI (13). Nevertheless, these biomarkers cannot get rid of the disadvantages of WBC counts that are easily influenced by race and sex and it is difficult to establish a reference range for the whole population (52) and are in need of further verification. Though single-cell proteomic and transcriptomic analyses uncovered distinct features of novel subclasses in atherosclerotic lesions (55), it is promising but remains to be explored in clinical transformation looking forward to high-quality randomized controlled trials.

Comparing to inflammatory infiltration, conventional plasma inflammatory mediators are routinely measured in clinical practice and have been verified as prospective prognostic biomarkers. For example, IL-1b was associated with 90-day all-cause mortality, cardiovascular mortality, and MACEs but defected in predicting nonfatal cardiovascular events (15), while Kilic et al. (16) suggested cytokine concentrations and proinflammatory to anti-inflammatory cytokine ratios as markers of a high risk of new coronary events. Moreover, comprehensive studies or reviews (16, 19, 56–58) were conducted comparing the prognostic performance of multiple conventional inflammatory factors like IL-6, IL-10, TNF-α, and CRP. Among these mediators, CRP has exhibited a predominant power. Lippi et al. (56) compared prognostic significance of 12 cytokines or growth factors in patients with myocardial ischemia and among these biomarkers, CRP displayed the most notable risk estimates identifying potential post-infarction heart failure

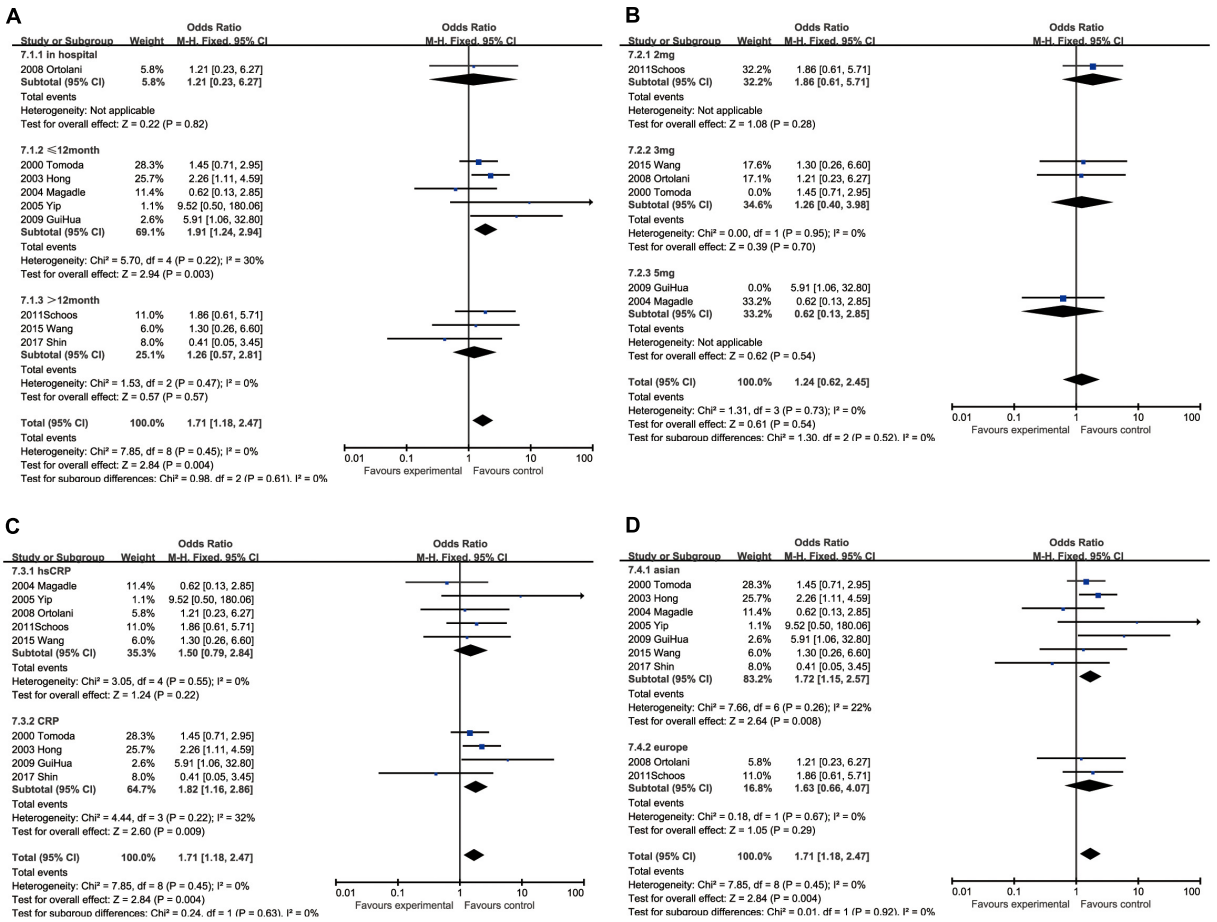

FIGURE 8
Forrest plots of subgroup analysis assessing restenosis associated with increased CRP: **(A)** subgroups based on cut-off value of hsCRP; **(B)** subgroups based on CRP and hsCRP; **(C)** subgroups based on ethnicities; and **(D)** subgroups based on ethnicities.

victims. Ritschel et al. (57) found that neither IL-6 nor sgp130 levels were related to future events by cox regression models, but patients with elevated CRP levels had a higher risk of death. Furthermore, compared with part of inflammatory factors, CRP has its own advantages such as no diurnal variation, insensitivity to external factors like diet and stable measured value (17). In the last decades, elevated CRP has been studied and verified in plenty of cohorts and is widely acknowledged as a cardiovascular risk factor. In the previous meta-analysis by Mincu et al. (35), high preprocedural CRP levels prognosed a significant increase of the incidence of in-hospital and follow-up adverse end points. However, recent years novel cohorts have been reported and associated CRP with more post-infarction events, such as heart failure (5).

In the current study analysis, the subgroup provided us new prospective on the clinical application of the relationship between elevated CRP and poor AMI prognosis. First, though CRP displayed a favorable predicting capacity of hospitalization, short-term (<12 months) or long-term (>12 months) prognosis, it differed between end points. Comparing with all outcomes, CRP can better foresee hospitalization incidence of MACEs and recurrent myocardial infarction and short-term restenosis while both all-term all-cause mortality and cardiovascular mortality. Świątkiewicz et al. (5) had reported that persistent elevation in CRP was associated with increased risk of hospitalization for HF and HF-related mortality in long-term (>6 years) follow-up, which further completed the function of CRP as a predictor. Second, as the most adopted thresholds, 2 and 3 mg/L can both serve as an optimal cut-off value for post-infarction adverse prognosis. In general, standard clinical assays for CRP with the normal sensitivity range from 3 to 8 mg/L (59), which limits its effective application to vascular risk prediction. To solve the limitation of CRP, high-sensitive CRP with excellent fidelity and reproducibility was developed and its comparability was proven (60). In the current study, hsCRP has better performance in predicting all-cause mortality, cardiovascular mortality, and recurrent myocardial infarction. However, CRP had its superiority in MACEs, heart failure, and

restenosis, which implies unknown underlying impact factors in need of further investigation. Third, elevated CRP is a more important sign of adverse prognosis in Asian patients than in European. With respect to ethnic groups, we found that CRP had better performance in predicting the all-cause mortality, cardiovascular mortality, the incidence of heart failure and restenosis in Asians, and the incidence of MACEs and recurrent myocardial infarction in Europeans, while it was not statistically significant to predict the cardiovascular mortality, the incidence of heart failure and restenosis in Europeans. The diversity might originate from the differences in genetic backgrounds and environments, different including criteria, and selection biases (61). A meta-analysis of 7,703 subjects has shown CRP gene polymorphisms associated with decreased risk of MI among Asian populations, while no statistical significance was found among Caucasian populations (62).

There are also limitations in our study. First, as is known, there are two types of AMI (i.e., STEMI and NSTEMI). The majority of our including studies focused on the relationship between CRP and STEMI. Although three studies (27, 34, 43) incorporate patients with NSTEMI, the detailed information could not be extracted from the source articles. In consideration of different inflammation conditions (58) and outcome profiles (63) under STEMI or NSTEMI, these diversities may lead our results to be less appropriate for NSTEMI. Second, in-depth studies have been conducted in an attempt to improve the sensitivity and specificity of CRP, attempting to adjust or combine hsCRP with its change velocity (64, 65), cTnT (66), albumin (67–69), and fibrinogen (70). These studies extended the practicability of CRP but are not included in our study due to lack of enough high-quality cohorts. Finally, there is publication bias existing in all-cause mortality and MACEs. As these studies were carefully selected following PRISMA and the results remain stable after sensitivity analysis, we preferred not to delete studies.

In brief, the current meta-analysis suggests that CRP is a prospective predictor of the diverse adverse prognoses in patients with AMI undergoing PCI. CRP had better performance in predicting plenty of hospitalization and short-term (<12 months) adverse prognoses than long-term prognoses and it is noteworthy that elevated CRP in Asian patients had more predictive value than in European.

Data availability statement

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

Author contributions

HZ conceptually designed the work. LD and YJ critically reviewed the manuscript. MD provided constructive recommendations in data processing and article polishing. SL and HJ conducted the main work and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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.2022.1013501/full#supplementary-material

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Effect of Danhong injection on prognosis and inflammatory factor expression in patients with acute coronary syndrome during the perioperative period of percutaneous coronary intervention: A systematic review and meta-analysis

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Objectives: In China, Danhong injection (DHI) is recommended by expert consensus and is widely used in the perioperative management of patients with acute coronary syndrome (ACS). This study investigates the effect of perioperative DHI administration and the timing of DHI administration on patients with ACS undergoing percutaneous coronary intervention (PCI) by analyzing the prognosis and anti-inflammatory effects. This article summarizes the most up-to-date clinical evidence on DHI, and in this study, we assesses treatment efficacy of DHI in patients with ACS.

Methods: A total of seven databases (PubMed, Embase, Cochrane Library, SINOMED, CNKI, Wanfang, and VIP) were searched from the time of their inception to 1 July 2022. Clinical randomized controlled trials (RCTs) of DHI combined with PCI for the treatment of ACS were included. RCT quality was assessed using the Cochrane Handbook risk-of-bias tool, and STATA 17.0 was used for meta-analysis.

Results: In total, 33 studies including 3,458 patients with ACS undergoing PCI were included in the meta-analysis. Compared with conventional therapy alone, the combination of DHI and conventional therapy significantly decreased the incidence of major adverse cardiovascular events (MACEs; $P<0.001$) and improved the reperfusion rate ($P < 0.001$). Serum high-sensitivity C-reactive protein (hs-CRP) and interleukin (IL)-6 levels were substantially reduced in the test group ($P<0.001$). In addition, the plasma levels of myocardial injury markers and cardiac troponin T (cTnT) declined significantly ($P < 0.01$). Compared with the control group, DHI improved the left ventricular ejection fraction (LVEF; $P < 0.001$) and reduced B-type natriuretic peptide (BNP; $P < 0.001$) levels. Subgroups were established based on different timings

of DHI administration: preoperative, intraoperative, and postoperative groups. The results showed that the incidence of MACEs and the reperfusion rate did not differ between the groups. Among the subgroups, the postoperative group exhibited significantly lower levels of BNP, hs-CRP, and IL-6 serum and a significantly higher level of LVEF ($P < 0.05$).

Conclusion: The combination of DHI and conventional therapy results in a better therapeutic effect than that observed with conventional therapy alone in patients with ACS. To improve treatment efficacy, postoperative initiation of DHI is recommended as a standard treatment. Further research is needed to confirm these results.

Systematic review registration: Identifier: CRD42022344830.

KEYWORDS

Danhong injection (DHI), perioperative period, acute coronary syndrome (ACS), prognosis, inflammatory factor expression, percutaneous coronary intervention (PCI)

Introduction

Acute coronary syndrome (ACS) is caused by the rupture of atherosclerotic plaque and subsequent thrombosis, resulting in unstable angina, non-ST segment elevation myocardial infarction, and ST segment elevation myocardial infarction (1, 2). In China, the incidence of coronary heart disease (CHD) and associated mortality rates is increasing annually (3). ACS is the most extreme type of CHD. Percutaneous coronary intervention (PCI) has an immediate effect on the revascularization of the infarct-related artery, and it may be more effective in restoring myocardial perfusion, reducing the incidence of myocardial ischemia or infarction, and improving clinical outcomes. PCI is widely used for the treatment of ACS (4). However, PCI may be complicated by no reflow, slow coronary flow, diverse arrhythmias, myocardial ischemia–reperfusion injuries (MIRIs), and in-stent restenosis (ISR). MIRI seriously affects patients' heart function and prognosis. Therefore, these complications of PCI cannot be ignored.

As a complementary or adjuvant therapy, DHI is a standardized traditional Chinese medicine (TCM) product. The main active ingredients are protocatechuic aldehyde, tanshinone, salvianolic acid, and catechin (5). Based on the TCM theory, the pathogenesis of CHD is closely related to stagnant blood, while DHI promotes blood flow and resolves the blood stasis. Modern pharmacological studies have reported that DHI promotes multiple pharmacological activities that have anti-thrombotic, anti-platelet aggregate, anti-inflammatory, hypolipidemic, anti-oxidative damage, and pro-human microcirculation effects (6). In clinical practice, DHI has been used to treat cardiovascular diseases and to reduce the incidence of major adverse cardiovascular events (MACEs), myocardial necrosis marker levels, and inflammatory factor levels (7–10).

A previous meta-analysis reported that DHI combined with conventional therapy for the treatment of patients with ACS improved the total efficacy rate and decreased the incidence of MACEs after PCI (11). However, it did not measure indicators such as myocardial injury or analyze the effect of the timing of DHI.

Therefore, this systemic review and meta-analysis summarizes the results of more recent RCTs regarding DHI. The efficacy of DHI in patients with ACS undergoing PCI and the effect of the timing of DHI on the incidence of MACEs and myocardial injury and inflammatory biomarker levels are assessed to provide clinical evidence regarding DHI.

Materials and methods

This analysis followed the PRISMA guidelines (12), and the review protocol was registered with PROSPERO (CRD42022344830).

Search strategy

For this study, seven databases (PubMed, Embase, Cochrane Library, SINOMED, CNKI, Wanfang, and VIP) were searched from their inception to 1 July 2022, using the following subject terms: "percutaneous coronary intervention," "Danhong injection," "acute coronary syndrome," "myocardial infarction," "unstable anginas," "percutaneous coronary intervention," and "randomized controlled trial." The search terms were changed according to databases and languages. Language restrictions were not applied for included studies. The different databases used a corresponding combination of subject words, free words, and keywords. In total, two researchers (YXL and YL)

independently evaluated the eligibility of the retrieved studies. A third researcher (DL) was consulted in case of disagreement. The bibliography of each article was manually searched for additional studies.

Inclusion and exclusion criteria

The prespecified eligibility criteria were as follows: (a) RCTs including patients with ST elevation myocardial infarction or unstable angina/non-ST elevation myocardial infarction, as defined by the European Society of Cardiology guidelines, and undergoing PCI were included in the meta-analysis; (b) all studies including a control group undergoing conventional therapy and a test group undergoing DHI combined with conventional treatment; and (c) studies reporting at least one of the following findings or outcomes: MACEs, thrombolysis in myocardial infarction (TIMI) flow grade, ST segment resolution (STR), high-sensitivity C-reactive protein (hs-CRP), interleukin (IL)-6, creatine kinase (CK), CK–myocardial band (MB), cardiac troponin T (cTnT), left ventricular ejection fraction (LVEF), or brain natriuretic peptide (BNP).

The exclusion criteria were as follows: (a) duplicate studies, (b) studies in which other TCMs were used in control or experimental groups, and (c) case reports, narrative reviews, meta-analyses, systematic literature reviews, observational studies, animal studies, or *in vitro* studies.

Data extraction

In this study, two researchers (YXL and YL) independently extracted data from each study, including the first author, year of publication, participant characteristics, sample size, intervention, duration of intervention, and outcome assessment, and any differences were resolved *via* discussion.

Quality assessment

The quality of the included studies was assessed following the Cochrane Handbook of Systematic Review. Random sequence generation, assignment confounding, blinding of participants and hospital staff, blind outcome assessment, incomplete outcome data, selective reporting, and other sources of bias were considered in the quality assessment. The results were cross-checked by the same two researchers (YXL and YL), and any disagreements were resolved *via* discussion.

Data synthesis and statistical analysis

STATA 17.0 was used for the meta-analysis (13). Data are presented as risk ratios (RRs) and standardized mean differences (SMDs) with 95% confidence intervals (CIs). Potential heterogeneity was assessed using Cochran Q and I^2 statistical tests. A fixed-effects model was used to compare data from studies with low heterogeneity (14), whereas a random-effects model was used to compare data from studies with high heterogeneity ($P < 0.05$, $I^2 > 50\%$). Subgroup, sensitivity, and meta-regression analyses were used to examine heterogeneity between the outcomes. The potential of a publication bias was assessed using Egger's and Begg's tests.

Results

Literature search and screening

First, the database search identified 448 articles for evaluation (Wanfang: 120 articles; CNKI: 102 articles; VIP: 93 articles; SINOMED: 76 articles; Embase: 22 articles; Cochrane Library: 20 articles; and PubMed: 12 articles). Subsequently, 316 duplicate records were identified and removed. After screening titles and abstracts, 82 articles met the exclusion criteria. Finally, 33 articles (7–10, 15–43) were included in the meta-analysis (Figure 1).

Study characteristics and quality assessment

The 33 studies included in this meta-analysis were published between 2007 and 2022 (Table 1) and were conducted in China. Among them, two studies were published in English (35, 42) and 31 studies were in Chinese. A total of 3,458 patients with ACS who underwent PCI were enrolled, among whom 1,722—control group—patients received a PCI-based conventional treatment, including medicines for anti-platelet aggregation, anti-coagulation, lipid lowering and plaque stabilization, and inhibition of ventricular remodeling, and the remaining 1,736—test group—patients received DHI and the conventional treatment. A DHI dose of 20–40 mL was used, administered by intravenous drip or injection. DHI was administered before (preoperatively), during (intraoperatively), or after (postoperatively) PCI. The treatment duration was 7–14 days. The outcome indicators of response prognosis observed in the included studies were MACEs, STR, and ISR, and the inflammatory factors were hs-CRP, tumor necrosis factor (TNF)-α, IL-6, IL-10, and matrix metalloproteinase (MMP)-9. Markers of myocardial injury (CK, CK-MB, and cTnT) and cardiac function (LVEF and BNP) were also reported.

The Cochrane Handbook tool was used for assessing the risks of bias in this study. In all the studies, subjects were randomly assigned. A total of 15 studies that provided the detailed information about that random assignment method had a low risk of bias; two studies that randomized subjects according to the time of admission and treatment protocol had a

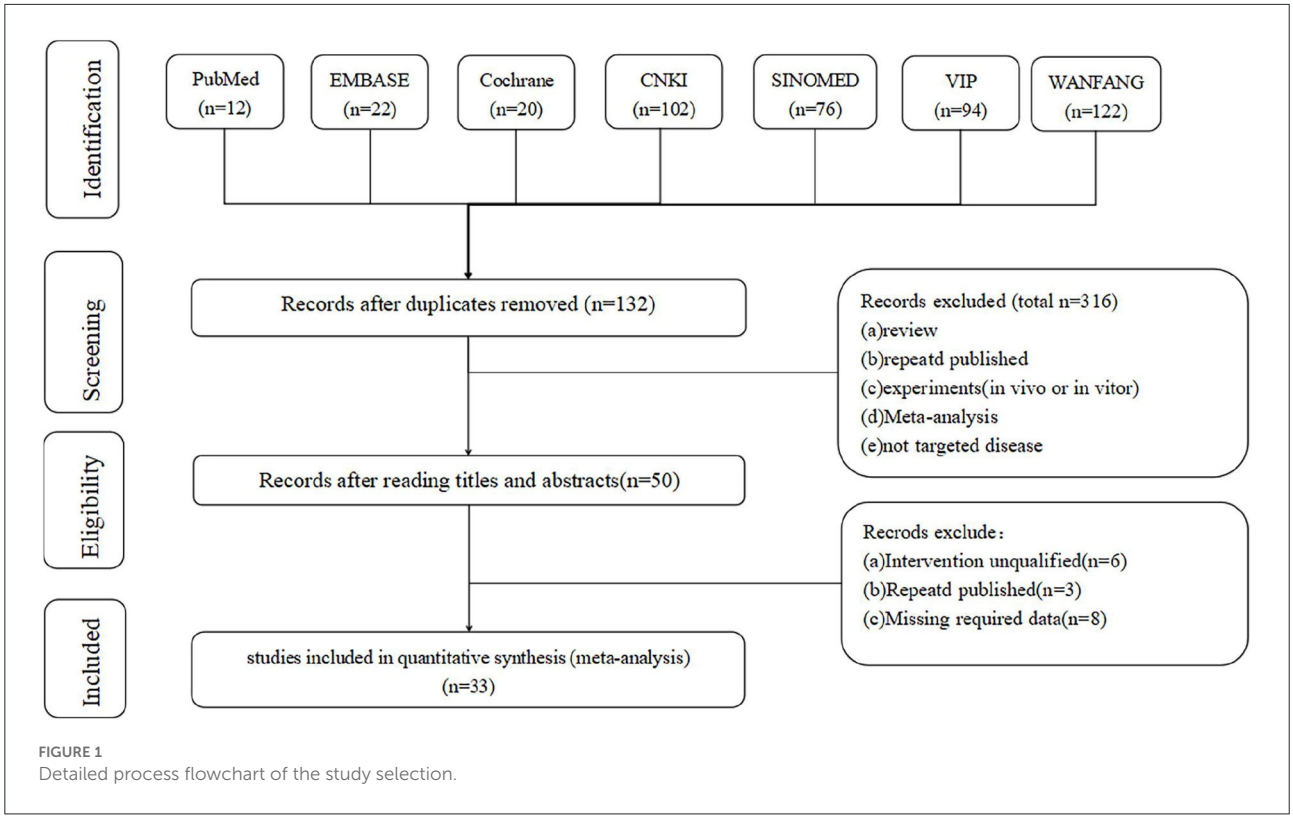

FIGURE 1
Detailed process flowchart of the study selection.

high risk of bias; eight studies that did not report information about the randomization method had an unclear risk of bias. Regarding allocation concealment, two studies that provided descriptions of allocation methods had a low risk of bias. The remaining 29 studies that did not describe the allocation process had an unclear risk of bias. Regarding blinding methods, two studies that blinded the patients but not the investigators had a low risk of bias. The remaining studies that did not report patient or investigator blinding had an unclear risk of bias. In addition, two studies that did not report the results of the pre-specified indicators had a high risk of bias due to selective outcome reporting. The remaining 31 studies that included complete data results had a low risk of bias. All included studies stated that the baseline characteristics of the two groups were not significantly different (Figure 2).

Outcome measures and subgroup analyses

MACE

A total of 15 (three preoperative, three intraoperative, and nine postoperative) studies reported MACEs, including malign arrhythmias, angina pectoris, recurrent myocardial infarction, recurrent hemodialysis, heart failure, and cardiac death that occurred during follow-up. No significant heterogeneity was determined between the studies. Overall, the incidence of MACEs was significantly lower in the test group than in the control group (RR = 0.45, 95% CI [0.37, 0.56], $P < 0.05$, $I^2 = 0\%$), and no differences between preoperative, intraoperative, and postoperative groups were identified (Figure 3).

Reperfusion rate

The reperfusion rate was assessed using the postprocedural TIMI flow grade and STR. A TIMI ≥ grade 3 and an STR rate ≥ 50% indicated successful reperfusion.

A total of three studies reported TIMI flow grades, and all studies applied DHI intraoperatively. Low heterogeneity was detected between the studies. The TIMI flow grade was significantly better in the test group than in the control group (RR = 0.22, 95% CI [0.10, 0.50], $P < 0.05$, $I^2 = 0\%$), suggesting that DHI could improve the TIMI flow grade of patients (Figure 4A).

Overall nine (three intraoperative and six postoperative) studies with high heterogeneity reported STR. STR was significantly better in the test group than in the control group (RR = 1.33, 95% CI [1.13, 1.58], $P < 0.05$, $I^2 = 66\%$). After excluding one study (32), the heterogeneity significantly reduced, although the findings did not significantly change (RR = 1.24, 95% CI [1.12, 1.37], $P < 0.05$, $I^2 = 29\%$).

TABLE 1 Study characteristics.

References	Disease	Sample size (T/C)	Participants (Male/Female)	Age (years)		Intervention		DHI (dosage and method)	Duration	Intervention time	OutCOME
				T	C	T	C				
Feng et al. (15)	ACS	91(46/45)	66/ 25	67.2 ± 16.2	65.6 ± 17.3	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	28 days	Preoperative	MACEs+hs-CRP+TC+TG+LDL+ET-1+Fg
Gao et al. (16)	STEMI	61(31/30)	38/ 23	60.1 ± 10.6	59.8 ± 7.6	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	MACEs+hs-CRP
Chen et al. (17)	ACS	100(50/50)	62/ 38	63.1 ± 9.7	67.5 ± 8.8	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	hs-CRP+CD62p+GP+FIB-C
Chen et al. (18)	STEMI	58(29/29)	43/ 16	61.9 ± 5.2	65.2 ± 4.5	DHI+PCI+CT	PCI+CT	20 ml qd ivgtt	14 days	Postoperative	STR+hsCRP+ET-1+LVEF
Zhao et al. (19)	ACS	70(36/34)	37/ 33	54.00 ± 9.00	54.00 ± 9.00	DHI+PCI+DAAT +PS+UFH	PCI+DAAT+PS +UFH	40 ml qd ivgtt	14 days	Postoperative	hs-CRP+ET-1+sP-sel
Zhang and Zhang (20)	ACS	68(34/34)	37/ 31	55.7 ± 7.4	54.5 ± 8.2	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	STR+hs-CRP+CD62p+ET-1
Wang et al. (21)	STEMI	60(30/30)	44/ 16	65.22 ± 7.54	63.61 ± 8.21	DHI+PCI+DAAT	PCI+DAAT	20 ml iv+20 ml qd ivgtt	st	intraoperative	STR+IL-6
Cui and Wang (22)	AMI	180(90/90)	106/ 74	72.1 ± 5.8	72.3 ± 5.8	DHI+PCI+CT	PCI+CT	30 ml qd ivgtt	10 days	Postoperative	Clinical efficiency+hs-CRP+SOD
Dong (10)	UA	120(60/60)	90/ 30	58.3 ± 10.2	56.8 ± 8.6	DHI+PCI+DAAT +PS+a-gent	PCI+DAAT +PS+a-gent	40 ml qd ivgtt	7 days	intraoperative	MACEs+TIMI+hsCRP+IL-6+CK-MB+cTnT+SOD+Vwf+sICAM-1+LVWM
Qin et al. (9)	AMI	112(56/56)	61/ 51	52.31 ± 11.24	55.12 ± 10.52	DHI+PCI+DAAT +PS+ARB/ACEI+β blockers +UFH	PCI+DAAT+PS +ARB/ACEI+β blockers +UFH	40 ml qd ivgtt	7 days	Postoperative	hs-CRP+CK-MB+cTnT+SOD+BNP+LVEF
Chen et al. (8)	ACS	120(60/60)	65/ 55	61.38 ± 8.63	61.47 ± 9.38	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	MACEs+ISR+LDL-C+TC+TG+CD6P+PAGT+PADT+LVEF
Guo et al. (7)	ACS	125(62/63)	69/ 56	62.1 ± 10.6	61.5 ± 10.3	DHI+PCI+CT+CE	PCI+CT+CE	40 ml qd ivgtt	14 days	Postoperative	MACEs+CRP+IL-1+TNF-α+vWF+FMD+ET-1+NO

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TABLE 1 (Continued)

References	Disease	Sample size (T/C)	Participants (Male/Female)	Age (years)		Intervention		DHI (dosage and method)	Duration	Intervention time	OutCOME
				T	C	T	C				
Guo et al. (23)	ACS	78(38/40)	45/ 33	60.1 ± 10.6	61.6 ± 11.2	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	hs CRP+ICAM-1+VCAM-1
Jia et al. (24)	AMI	120(60/60)	75/ 45	62. 23 ± 11. 26	64. 56 ± 12. 85	DHI+PCI+CE	PCI+CE	20mg st ivgtt	st	intraoperative	TIMI+CRP
Xu et al. (25)	AMI	71(36/35)	49/ 22	65 ± 13	63 ± 11	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	MACEs+STR+CK+CK-MB+cTnT+ET-1+BNP+LVEF
Yang et al. (26)	STEMI	57(28/29)	30/ 27	64 ± 12. 3	65 ± 11. 7	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	10 days	Postoperative	MACEs+STR+TIMI+CK+CK-MB+cTnT+ET-1+IRA +BNP+LVEF
Zheng et al. (27)	STEMI	300(150/150)	186/ 114	61.7 ± 7.4		DHI+PCI+DAAT+UFH	PCI+DAAT+UFH	30 ml qd ivgtt	10 days	Postoperative	STR+IL-17+IL-6+MIS+LVEF
Zhou et al. (28)	UA	100(50/50)	70/ 30	58.0 ± 9.2		DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	7 days	Preoperative	Clinical efficiency+hs-CRP+IL-6+cTnT
Liu et al. (29)	ACS	104(52/52)	55/ 49	58.73 ± 8.45	59.21 ± 8.57	DHI+PCI+DAAT+UFH	PCI+DAAT+UFH	40 ml qd ivgtt	14 days	Postoperative	Vwf+ET-1+NTG+NO+FMD+pentraxin-3+IL-18+IL-18/IL-10+LpPLA2+IL-10+BNP+LVEF
Zhang et al. (30)	ACS	100(50/50)	67/ 33	71.26 ± 4.82	68.28 ± 4.88	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	hs-CRP+ET-1+IL-6+Vwf+NO+FMD
Zeng et al. (33)	STEMI	120(60/60)	64/ 56	65.13 ± 2.38	64.38 ± 2.12	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	MACEs+Clinical efficiency+IL-6+IL-17+LVEF+MIS
Liu et al. (31)	NSTEMI	180(90/90)	NR	NR	NR	DHI+PCI+DAAT+UFH	PCI+DAAT+UFH	20 ml qd ivgtt	14 days	Postoperative	hs-CRP+Clinical efficiency+ET+LVEF
Wu et al. (32)	STEMI	80(44/36)	NR	NR	NR	DHI+PCI+CT	PCI+CT	4 ml iv+20 ml st ivgtt	st	intraoperative	STR+MMP-9+CRP+IL-6
Qin et al. (34)	AMI	126(63/63)	62/ 64	63.98 ± 1.25	63.41 ± 1.16	DHI+PCI+CT	PCI+CT	4 ml iv+20 ml ivgtt st	st	intraoperative	MACEs+TIMI+IL-6+Cys-C+Hcy+LVEF
You et al. (35)	STEMI	110(57/53)	95/ 15	56.8 ± 8.9	55.4 ± 9.5	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	4-6 days	Preoperative	MACEs+CK-MB+cTnT+MIS+LVEF

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TABLE 1 (Continued)

References	Disease	Sample size (T/C)	Participants (Male/Female)	Age (years)		Intervention		DHI (dosage and method)	Duration	Intervention time	OutCOME
				T	C	T	C				
Hu (36)	AMI	86(43/43)	65/ 21	50.28 ± 0.43	50.62 ± 0.53	DHI+PCI+CT	PCI+CT	30 ml qd ivgtt	14 days	Postoperative	Clinical efficiency+CRP+IL-6+FIB+D-Dimer+CD63+CD62P+SOD+MDA
Lv (37)	AMI	100(50/50)	62/ 38	60 ± 5.8	59 ± 6	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	14 days	Postoperative	MACEs+STR+hs-CRP+cTnT+CK-MB+NT-proBNP
Wen-long (42)	UA	78(39/39)	58/ 20	61.03 ± 9.03	60.74 ± 10.82	DHI+PCI+CT	PCI+CT	40 ml qd ivgtt	7 days	intraoperative	MACEs+CK+CK-MB+cTnT+FFR+IMR
Chen et al. (38)	STEMI	93	56/ 37	62.9 ± 9.5	63.2 ± 8.5	DHI+PCI+CT	PCI+CT	40 ml st ivgtt	st	intraoperative	MACEs+TIMI+STR+Arrhythmia+hs-CRP+CK+CK-MB+CTNI+FIB+LDH+NT-proBNP
Cui et al. (39)	AMI	90	46/ 44	57.53 ± 3.35	56.35 ± 3.23	DHI+PCI+CT	PCI+CT	30 ml qd ivgtt	60 days	Postoperative	MACEs+STR+ISR+Clinical efficiency+hsCRP+MMP-9+TNF-α+ET-1
Feng (40)	STEMI	157	82/ 76	60.19 ± 1.38	60.25 ± 1.21	DHI+PCI+CT	PCI+CT	20 ml qd ivgtt	7 days	Postoperative	ANGPTL4+Sst2+LVEF
Niu et al. (41)	UA	61	NR	NR	NR	DHI+PCI+CT	PCI+CT	40 ml bid ivgtt	7 days	Preoperative	MACEs+CK-MB+Metabolome
Li et al. (43)	AMI	82	49/ 33	62.5 ± 4.6	62.3 ± 4.5	DHI+PCI+CT	PCI+CT	20 ml qd/20 ml bid	7 days	Postoperative	MACEs+Clinical efficiency+hs-CRP+TNF-α+IL-6+LEVF

T, trial group; C, control group; NR, no report; ACS, acute coronary syndrome; UA, unstable angina; AMI, acute myocardial infarction; STEMI, ST segment elevation myocardial infarction; CT, conventional therapy, no details were given; DHI, Danhong injection; PCI, percutaneous transluminal coronary intervention; DAAT, double anti-platelet aggregation; PS, plaque stabilization (atorvastatin calcium tablets, etc.); a-gent, antihypertensive; UFH, unfractionated heparin; CE, coronary enlargement; MACEs, major adverse cardiovascular events; STR, ST segment resolution; TIMI, thrombolysis in myocardial infarction; CRP, C-reactive protein; hs-CRP, hypersensitive C-reactive protein; LVEF, left ventricular ejection fraction; CD62P, P-selectin CD62P; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; ET-1, endothelin-1; Fg, fibrinogen; GP, glucose protein; FIB-C, fibrinogen C; sP-sel, soluble P-selectin; IL, interleukin; SOD, superoxide dismutase; Vwf, von Willebrand factor; sICAM-1, intercellular adhesion molecule-1; BNP, brain natriuretic peptide; MDA, malonaldehyde; ISR: in-stent restenosis; FMD, flow-mediated dilation; NO, nitric oxide; VCAM-1, vascular cell adhesion molecule-1; MIS, myocardial infarction size; NTG, endothelial non-dependent vascular dilation reactions; LpPLA2, lipoprotein-associated phospholipase A2; MMP, matrix metalloproteinase; Cys-C, cystatin-C; Hcy, homocysteine; IMR, index of microcirculation resistance; TNF-α, tumor necrosis factor-α; ANGPTL4, angiopoietin-like4; Sst-2, soluble suppression of tumorigenicity 2.

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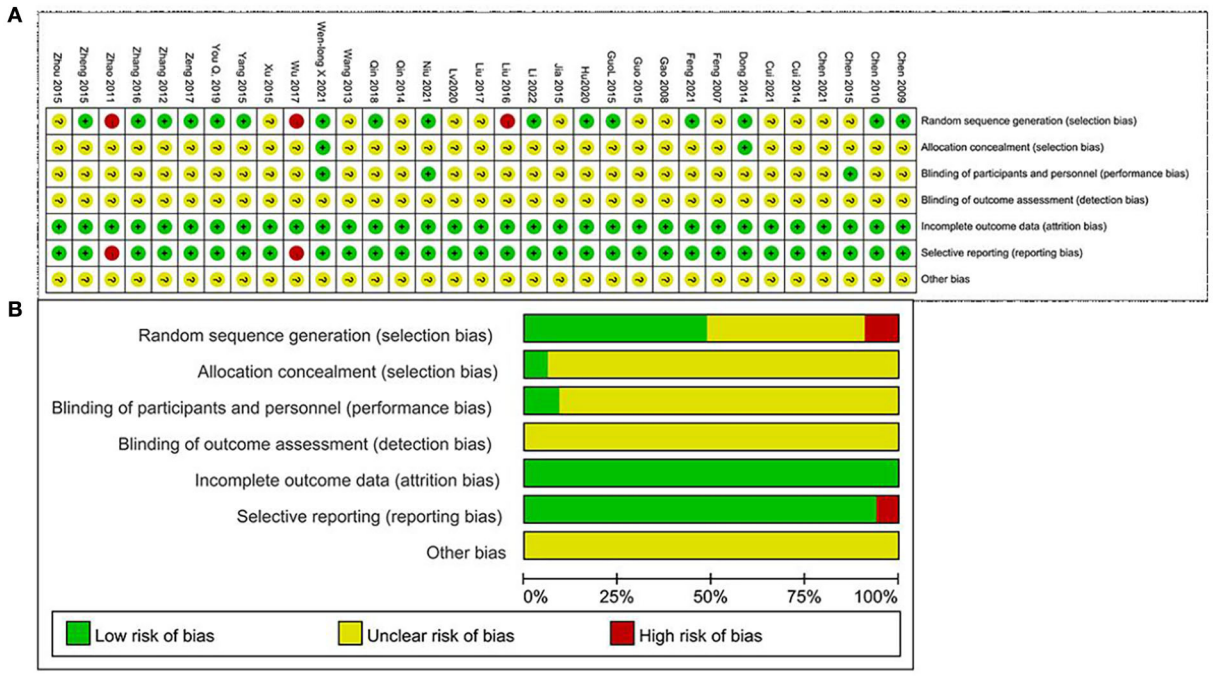


FIGURE 2
(A) Risk-of-bias summary. **(B)** Risk-of-bias graph.

The comparison of intraoperative and postoperative indicators between the two groups showed no differences. The reperfusion rate was more favorable in the test group than in the control group. The timing of DHI (intraoperatively or postoperatively) did not significantly affect the reperfusion rate (Figure 4B).

Inflammatory factors

A total of 18 studies reported serum hs-CRP levels. Random-effects models were applied owing to the high heterogenicity. The serum hs-CRP levels more significantly decreased in the test group than in the control group (SMD $= -1.14$, 95% CI $[-1.58, -0.7]$, $P < 0.05$, $I^2 = 94.2\%$). The hs-CRP level more significantly decreased when DHI was administered postoperatively than pre- or intraoperatively (Figure 5A). A meta-regression was conducted to identify the possible sources of the high heterogeneity, and the hs-CRP test method was identified as a source of heterogeneity ($P < 0.05$; Figure 5B). According to the test method, a subgroup analysis was undertaken based on the explicit test method. We excluded one study (22) to reduce the heterogeneity of the explicit test method groups. Subsequently, there was no heterogeneity in the explicit test method groups. The use of the unspecified assay method to measure the hs-CRP level was identified as a specific potential source of heterogeneity (Figure 5C).

A total of 10 studies reported serum IL-6 levels, and high heterogeneity was detected among these studies. Serum IL-6 levels more significantly decreased in the test group than in the control group (SMD $= -1.35$, 95% CI $[-1.84, -086]$, $P < 0.05$, $I^2 = 92.7\%$; Figure 6A). A meta-regression analysis determined that the heterogeneity was independent of disease type, detection time, and drug dose and was correlated with the timing of DHI (Figure 6B).

Heterogeneity within the data of the intraoperative group originated from one study (34). After excluding that study (34), heterogeneity of the intraoperative group decreased from $I^2 = 79.2\%$ to $I^2 = 54\%$. But the source of heterogeneity in the postoperative group was not identified after the separate exclusion of each study; therefore, the postoperative group was identified as the main source of heterogeneity.

Myocardial injury index

In all, seven studies reported serum cTnT levels, five of which included patients with acute myocardial infarction in whom DHI was administered postoperatively, and the remaining two studies involved patients with unstable angina for whom DHI was administered preoperatively or intraoperatively. Random-effects models were applied because of high heterogeneity. The

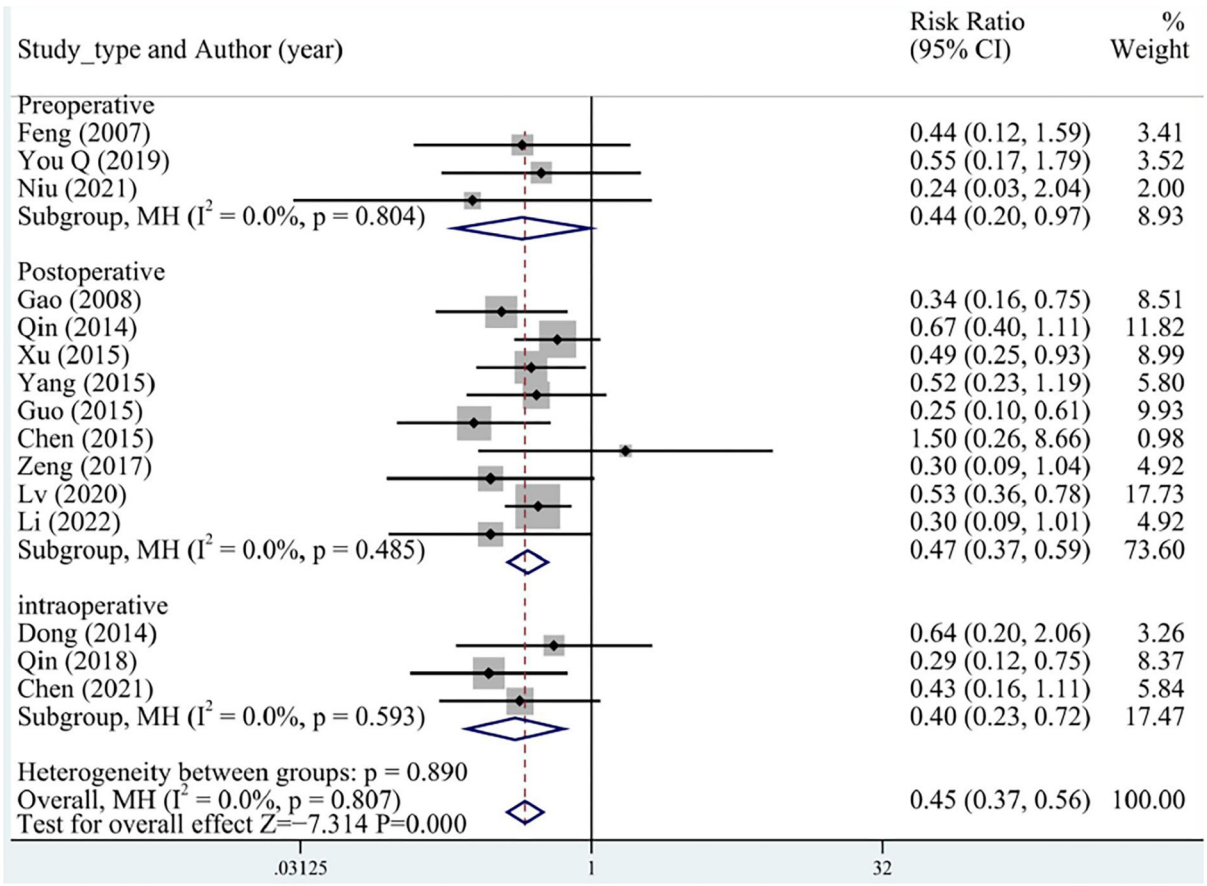


FIGURE 3
MACE subgroup analysis. MACE, major adverse cardiovascular event.

results indicated that the peak cTnT level more significantly decreased in the test group than in the control group (SMD = −1.59, 95% CI [−2.48, −0.69], $P < 0.05$, $I^2 = 96\%$). We removed one study (9) after sensitivity analysis with no significant heterogeneity within the subgroups. The results did not change significantly (SMD = −1.56, 95% CI [−2.60, −0.52], $P<0.05$, $I^2 = 96.5\%$; Figure 7A). Among the three subgroups, the intraoperative group had a significantly higher cTnT peak level.

A total of four studies reported CK levels. Fixed-effects models were applied because of low heterogeneity. The peak CK level more significantly decreased in the test group than in the control group (SMD = −0.86, 95% CI [−1.10, −0.62], $P < 0.05$, $I^2 = 0\%$; Figure 7B).

A total of nine studies reported CK-MB levels. Random-effects models were applied because of high heterogeneity. The peak CK-MB level more significantly decreased in the test group than in the control group (SMD = −1.05, 95% CI [−1.55, −0.55], $P < 0.05$, $I^2 = 90.6\%$). We searched for the source of heterogeneity by conducting a sensitivity analysis. After excluding two studies (9, 37), the heterogeneity decreased significantly. The peak CK-MB level more significantly decreased in the test group than in the control group (SMD = −0.66, 95% CI [−0.86, −0.46], $P < 0.05$, $I^2 = 29.6\%$). No differences between the intraoperative and postoperative groups were observed (Figure 7C).

Cardiac function

LVEF and BNP levels were analyzed to assess cardiac function. A total of 12 studies reported LVEF levels. LVEF levels more significantly increased in the test group than in the control group (SMD = 0.96, 95% CI [0.68, 1.25], $P < 0.05$, $I^2 = 85.1\%$). The source of heterogeneity was determined by conducting a sensitivity analysis, and one study was identified as the main source of heterogeneity (27). After excluding that study (27), each subgroup had significantly lower heterogeneity. LVEF levels more significantly increased in the test group than in the control group (SMD = 0.84, 95% CI [0.73, 0.96], $P < 0.05$,

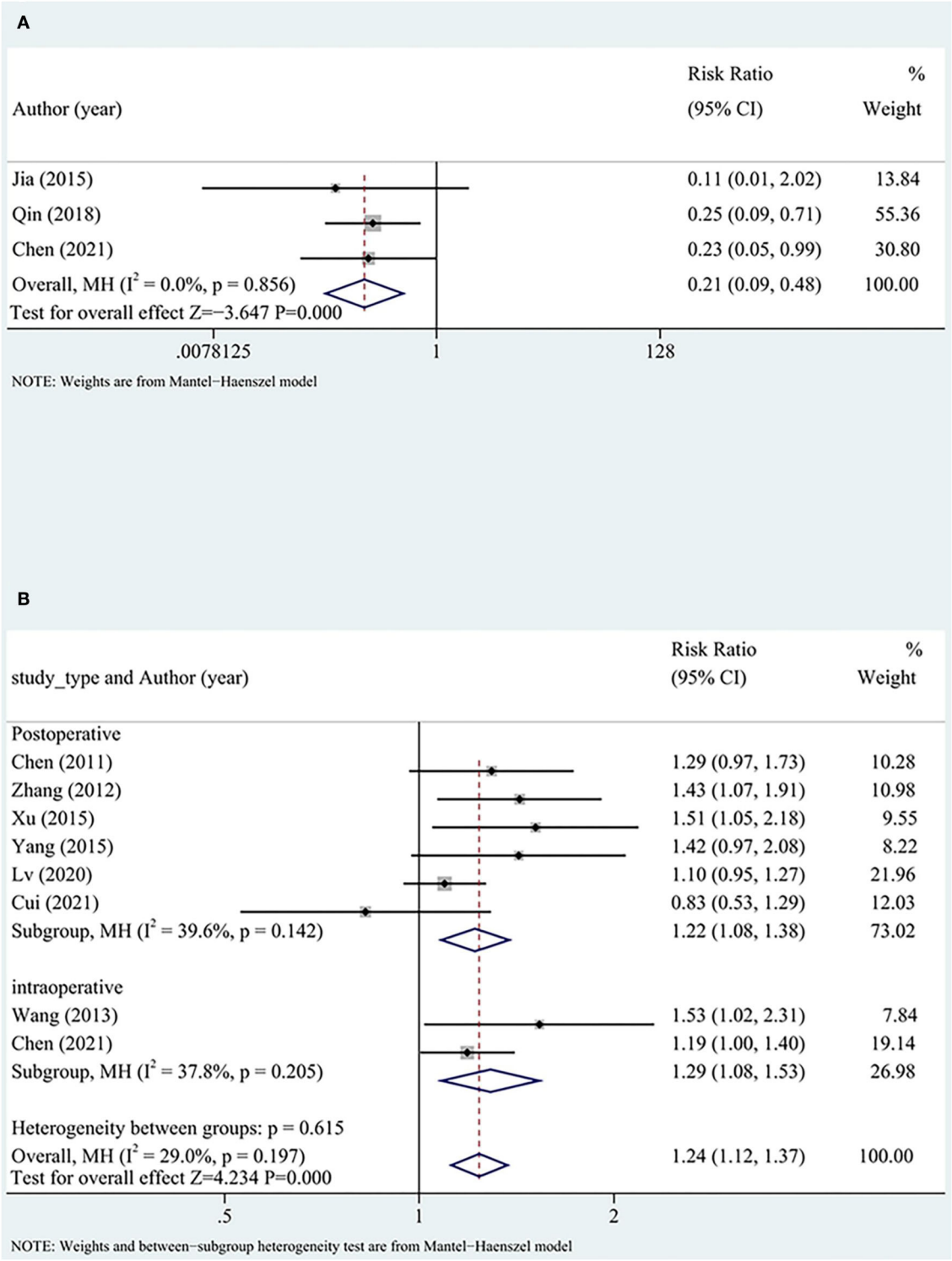
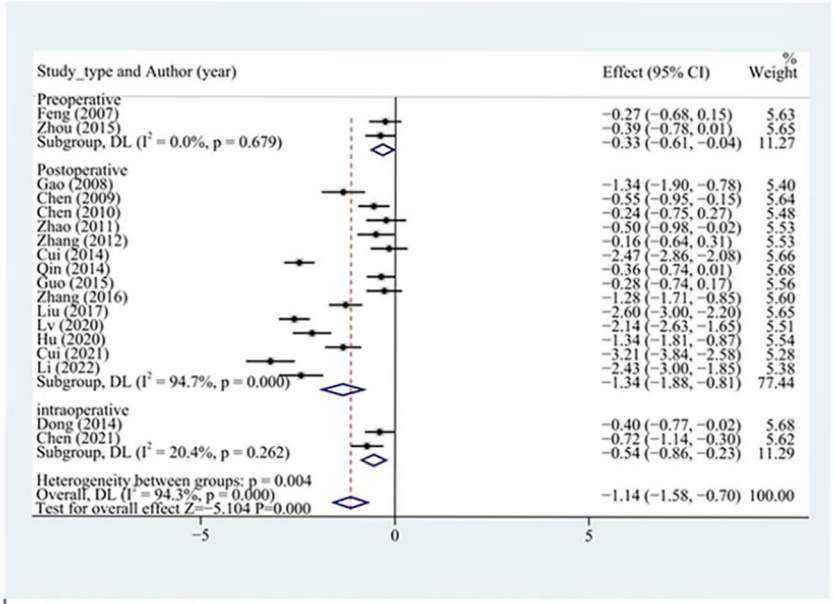


FIGURE 4
Reperfusion subgroup analysis including **(A)** TIMI flow grade and **(B)** STR. TIMI, thrombolysis in myocardial infarction; STR, ST segment resolution.

A



B

```
. metareg _ES testmethod  dosage detection, wsse(_seES) bsest(reml)

Meta-regression                                 Number of obs  =      18
REML estimate of between-study variance         tau2           =   .4472
% residual variation due to heterogeneity       I-squared_res  =  89.79%
Proportion of between-study variance explained  Adj R-squared  =  51.30%
Joint test for all covariates                   Model F(3,14)  =   6.33
With Knapp-Hartung modification                 Prob > F       =  0.0062
```

_ES	Coefficient	Std. err.	t	P>|t|	[95% conf.	interval]
testmethod	-1.124853	.3987028	-2.82	0.014	-1.979985	-.2697208
dosage	.0714698	.0382337	1.87	0.083	-.0105334	.1534729
detection	.0128443	.0140122	0.92	0.375	-.0172088	.0428974
_cons	13.51084	6.774601	1.99	0.066	-1.019234	28.04092

C

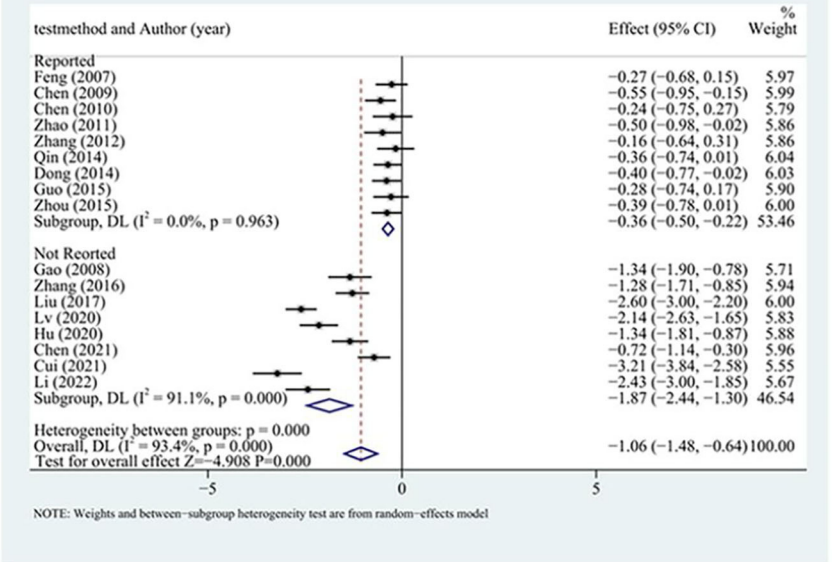
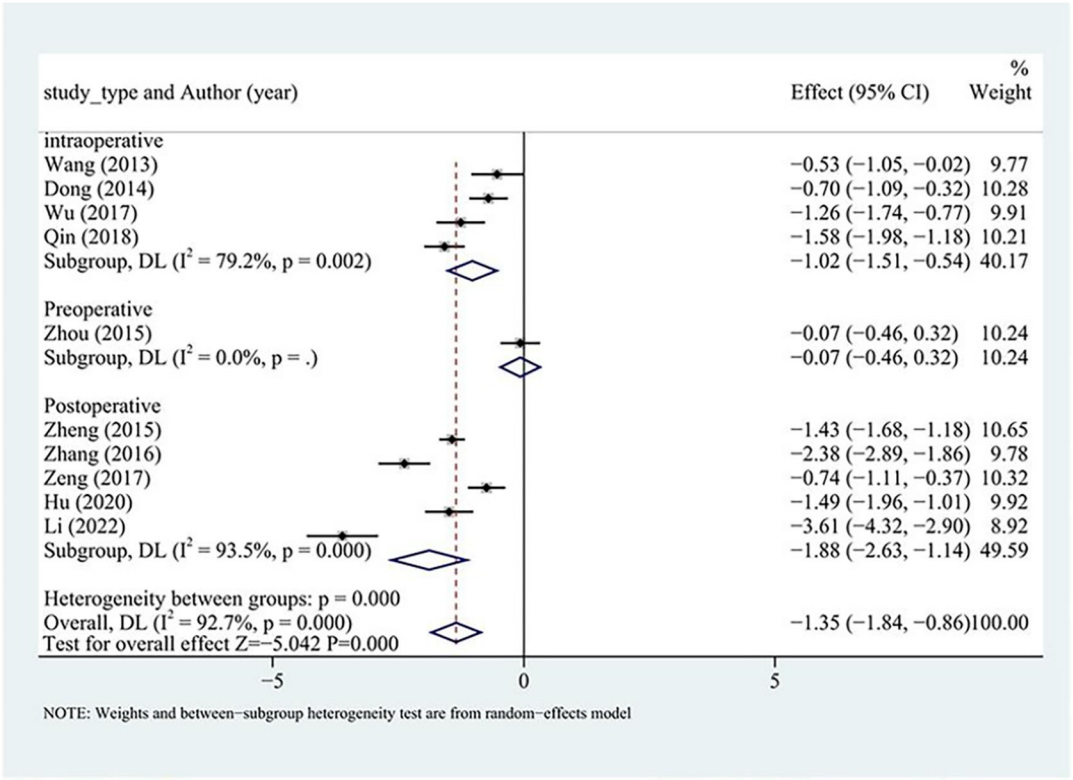


FIGURE 5
(A) hs-CRP subgroup analysis. **(B)** Results of meta-regression. **(C)** Subgroup analysis of test methods. Hs-CRP; high sensitivity C-reactive protein.

A



B

Meta-regression — Number of obs = 10
REML estimate of between-study variance — tau2 = .4545
% residual variation due to heterogeneity — I-squared_res = 85.64%
Proportion of between-study variance explained — Adj R-squared = 50.02%
Joint test for all covariates — Model F(4,5) = 2.78
With Knapp-Hartung modification — Prob > F = 0.1458

_ES	Coefficient	Std. err.	t	P>|t|	[95% conf.	interval]
detectiontime	-.2375528	.1204584	-1.97	0.106	-.547201	.0720954
interventiontimepoints	-.6692331	.2996711	-2.23	0.076	-1.439562	.1010959
disease	.3117122	.4836164	0.64	0.548	-.9314634	1.554888
dosage	-.049324	.0732921	-0.67	0.531	-.2377274	.1390793
_cons	2.466637	2.193295	1.12	0.312	-3.171408	8.104681

FIGURE 6
(A) IL-6 subgroup analysis. **(B)** Results of meta-regression. IL, interleukin.

$I^2 = 44.5\%$). The postoperative group had the most significantly increased LVEF levels ($P < 0.05$; Figure 8A).

In all, four studies reported BNP levels. The BNP levels more significantly decreased in the test group than in the control group (SMD = −1.96, 95% CI [−3.70, −0.21], $P < 0.05$, $I^2 = 97.7\%$). We searched for the source of heterogeneity by conducting a sensitivity analysis. After the exclusion of the study (29), the heterogeneity was significantly lower in each subgroup. The BNP levels more significantly decreased in the test group than in the control group (SMD = −0.58, 95% CI [−0.84, −0.32], $P<0.05$, $I^2 = 37.4\%$). The postoperative group had the most significantly decreased BNP levels (Figure 8B).

Publication bias

Since more than 10 studies reported MACEs, hs-CRP, IL-6, and LVEF, we performed Egger's and Begg's tests to identify publication bias for these studies. The results showed that there was no possibility of publication bias (Figure 9).

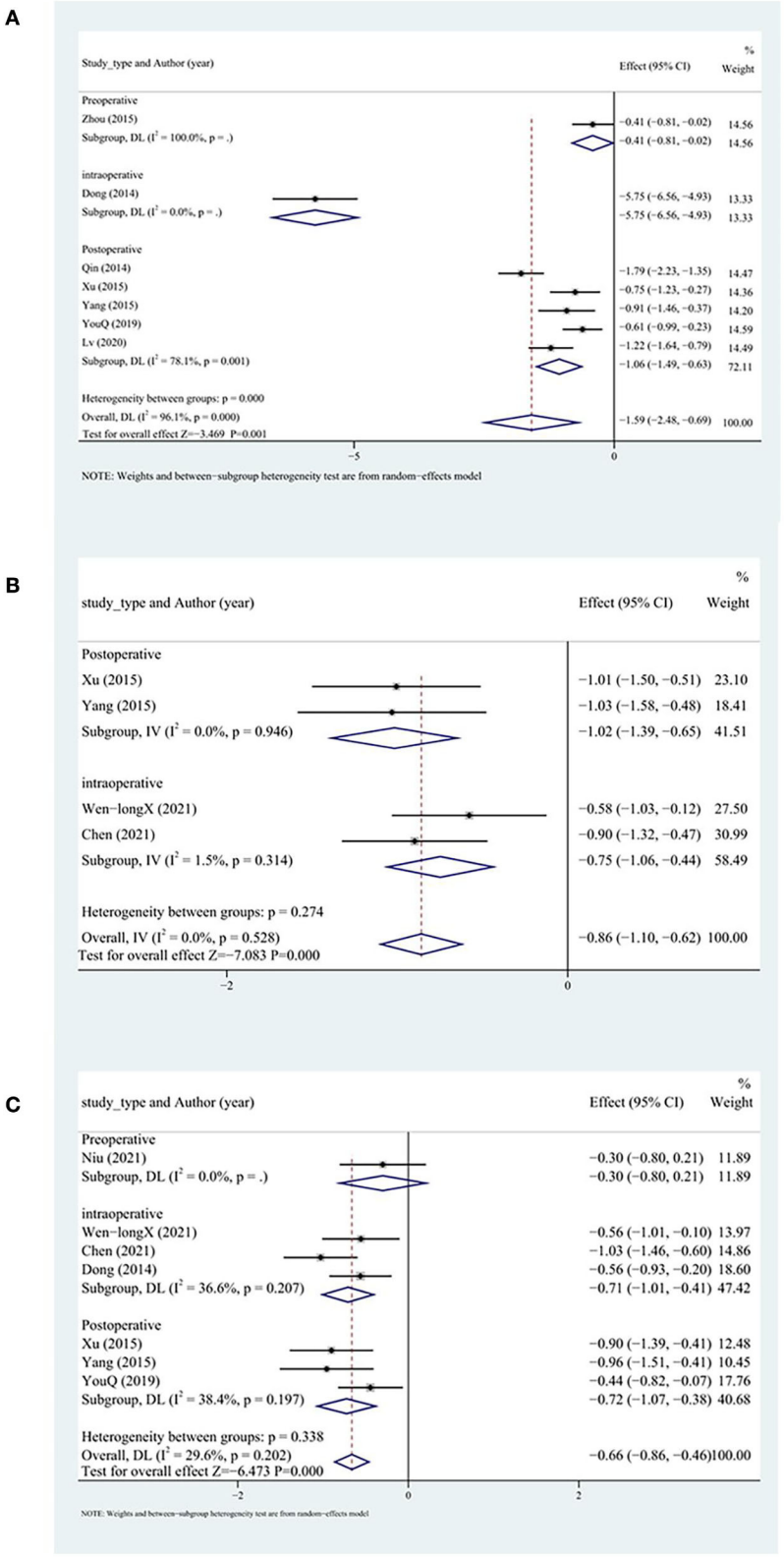


FIGURE 7
Myocardial injury index subgroup analysis including **(A)** cTnT, **(B)** CK, and **(C)** CK-MB. cTnT, cardiac troponin T; CK, creatine kinase.

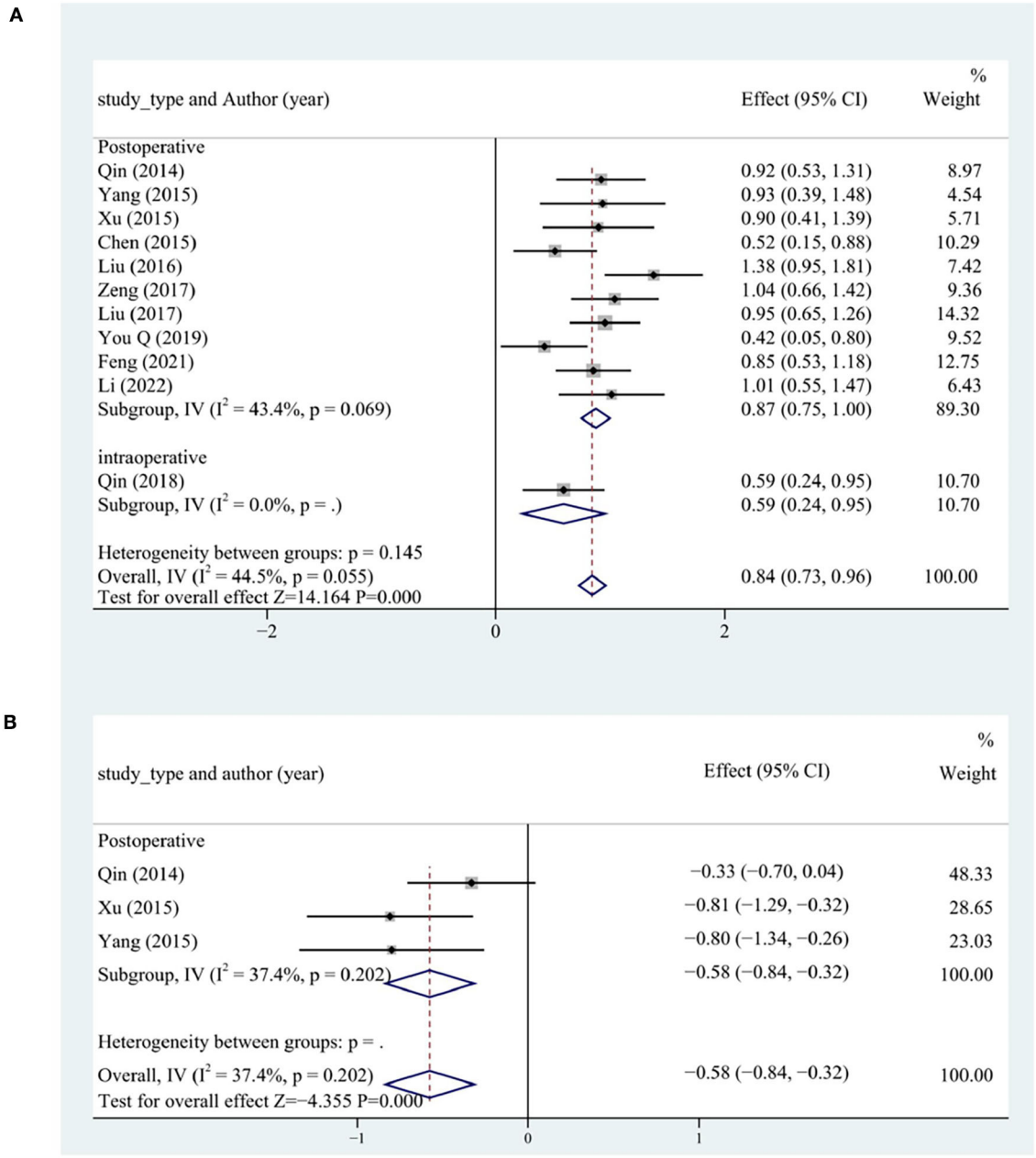


FIGURE 8
Cardiac function subgroup analysis including **(A)** LVEF and **(B)** BNP. LVEF, left ventricular ejection fraction; BNP, B-type natriuretic peptide.

Discussion

Overview of evidence

A total of 33 studies, including four in which DHI was administered preoperatively, seven in which DHI was administered intraoperatively, and 22 in which DHI was administered postoperatively. In our study, 3,458 patients were included in meta-analysis. Data regarding the use of DHI in patients with ACS during the perioperative period of PCI were summarized. The combination of DHI and conventional treatment effectively decreased the number of inflammatory factors, the incidence of no reflow, myocardial injury, and the incidence of MACEs, and increased cardiac function. Postoperative DHI may result in more favorable suppression of the inflammatory response and improvement in cardiac function and patients' quality of life.

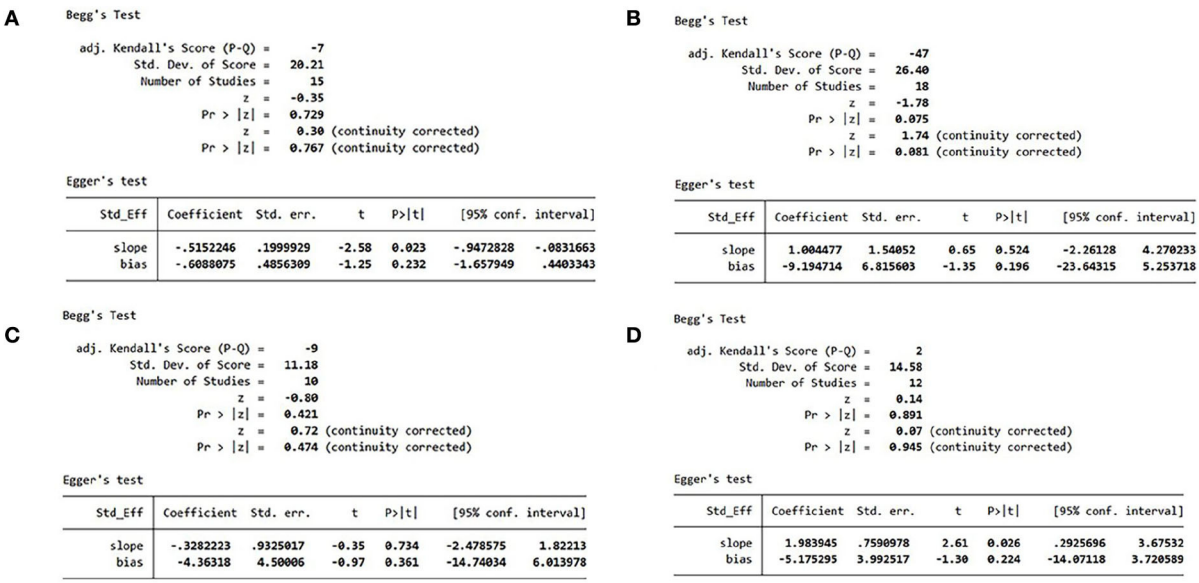


FIGURE 9
(A) MACE publication bias analysis. **(B)** hs-CRP publication bias analysis. **(C)** IL-6 publication bias analysis. **(D)** LVEF publication bias analysis. MACE, major adverse cardiovascular event; hs-CRP, high-sensitivity C-reactive protein; IL, interleukin; LVEF, left ventricular ejection fraction.

Clinical application of DHI to ACS

The annual CHD incidence in China is increasing. ACS is the most serious type of CHD, and PCI is an effective treatment for ACS (3). PCI may be complicated by no reflow, arrhythmias, and MIRI. MIRI impairs cardiac function and negatively affects prognosis in patients undergoing cardiac surgery (44); there is a high risk of MIRI even when patients are administered conventional drug therapy, and MIRI contributes to up to 50% of the final infarct size (45). Therefore, the development of a method for attenuating myocardial injury to increase PCI efficacy is necessary.

In China, DHI is recommended for patients with ACS and patients undergoing PCI (46, 47). DHI is widely used in clinical practice and includes phenolic acid, C-glycosyl quinone chalcones, flavonoid glycosides, cyclic enol ether terpene glycosides, organic acids, amino acids, and nucleosides (46).

Several clinical studies had reported that the combination of DHI and conventional therapy may result in more favorable clinical outcomes than standard therapy alone. This meta-analysis also clarifies the clinical efficacy of DHI. Moreover, subgroup analyses are conducted to determine the optimal timing of DHI. Angina pectoris, heart failure, severe arrhythmia, recurrent myocardial infarction, re-bleeding, and cardiogenic death during follow-up are some of the MACEs. MACE is a reflection of the prognosis of patients; therefore, MACE is a primary endpoint in this study.

DHI improves patients' prognosis. When combined with conventional therapy, DHI decreases the incidence of MACEs. MIRI leads to adverse ventricular remodeling, resulting in progressive heart failure and poor outcomes. The results of the current meta-analysis indicate that DHI ameliorates cardiac function. After treatment with DHI, the LVEF level significantly improved ($P < 0.05$), and the BNP level significantly reduced ($P < 0.05$).

As a TCM standardized product, DHI has several targets and multiple effects. Hence, the mechanism of action of DHI remains unclear. DHI improves the reperfusion rate ($P<0.05$) and decreases the CK, CK-MB, and cTnT levels in patients with ACS during the perioperative period of PCI ($P < 0.05$), indicating that DHI decreases the incidence of myocardial injury by improving the reperfusion rate. This may be the mechanism of action of DHI to increase cardiac function and improve patients' prognosis.

Myocardial ischemic injury and MIRI are associated with inflammation. Previous clinical studies have examined whether DHI exerts a protective effect by suppressing the inflammatory response. In the current meta-analysis, IL-6 and hs-CRP are used as indicators of pro-inflammatory responses. IL-6 induces inflammatory cell adhesion and injures vascular endothelium, and hs-CRP is a predictor of cardiovascular events. DHI more significantly decreases hs-CRP and IL-6 levels than standard therapy ($P < 0.05$). The mechanism of action of DHI in inhibiting inflammation may be multi-faceted. It is reported that

DHI reduces inflammatory cytokines, such as IL-1, IL-18, MMP-9, and TNF-α, resulting in a broad anti-inflammatory effect (7, 27, 30, 32, 39, 43). Inhibition of inflammation by DHI is an important mechanism for its cardioprotective effect.

Although DHI is recommended for patients with ACS or patients undergoing PCI, the timing of DHI has not optimized yet. Patients included in this meta-analysis were divided into preoperative, intraoperative, and postoperative subgroups. The incidence of MACEs, TIMI flow grade, and STR was not significantly different between the subgroups. However, hs-CRP and IL-6 levels reduced more significantly in patients in whom DHI was administered postoperatively. The peak cTnT level was significantly decreased in the intraoperative group than in the other subgroups. In the postoperative group, the LVEF level was significantly improved ($P < 0.05$), and the BNP level was significantly reduced.

The results of this study suggest that DHI is effective in patients with ACS. While the incidence of MACEs is not affected by the timing of DHI, postoperative DHI may suppress the inflammatory response and improve cardiac function more significantly. Therefore, postoperative DHI is recommended to optimizing the efficacy of conventional treatment. However, the potential adverse effects of DHI must be considered. The incidence of adverse reactions of DHI is 3.50 per 1,000, and common adverse reactions include pruritus, rash, sweating, dizziness, and headache. Severe adverse effects such as anaphylactic shock are very rare (48). Current clinical studies do not report adequate data regarding the adverse effects of DHI. We suggest all clinical studies in progress to report adverse effects.

Anti-inflammatory effects of DHI

DHI enhances cardiac function by inhibiting the inflammatory response, increasing the reperfusion rate, alleviating myocardial injuries, and ultimately reducing the incidence of MACEs. Inflammatory response plays an important role in MACEs and is closely related to ischemia–reperfusion injury. DHI alleviates myocardial injuries *via* anti-inflammatory effects exerted by multi-target pathways.

Shortly after myocardial ischemic injury, necrotic cardiomyocytes release alarmins to activate the immune system and trigger neutrophil infiltration in the ischemic necrosis area (49). Neutrophils generate pro-inflammatory responses that trigger the infiltration of monocytes (50). When reperfusion is performed at this time, fibroblasts release granulocyte–macrophage colony-stimulating factor to promote neutrophil and monocyte infiltration in the ischemic necrotic area (51). Activation or degranulation of mast cells results in the release of pro-inflammatory mediators, and the derived angiotensin II (AngII) induces reperfusion arrhythmias by activating the renin–angiotensin system (52, 53). Few hours to days after myocardial ischemic injury, the composition of immune cells changes, and the spleen becomes a major source of monocytes. Monocyte migration to sites of myocardial injury is regulated by IL-1β, AngII, and the binding of chemokine ligand 2 and chemokine receptor 2. The first monocytes to migrate to the site of myocardial injury are pro-inflammatory Ly6C^{high} monocytes, which differentiate into activated pro-inflammatory macrophages; express IL-1β, IL-6, TNF-α, and protein hydrolases; and secrete MMPs, which degrade the extracellular matrix (54–57). At a later stage, both Ly-6C^{low} monocytes and the M2 phenotype are involved in angiogenesis and collagen deposition, forming scar tissue to replace lost cardiomyocytes in areas of ischemic necrosis and promoting the healing response of the ischemic myocardium (Figure 10).

The sustained and severe pro-inflammatory response during this process leads to adverse ventricular remodeling. Inflammation is an important novel target to ameliorate the prognosis of patients after PCI. Postoperative DHI better inhibits the inflammatory response and increases cardiac function, which may be related to adverse ventricular remodeling caused by the inhibition of the inflammatory response by DHI.

The results of this study indicate that DHI significantly reduces the hs-CRP and IL-6 levels ($P < 0.05$). IL-6 induces inflammatory cell adhesion and injures vascular endothelium, and hs-CRP is a predictor of cardiovascular events. In addition, DHI reduces the TNF level in patients with ACS (39, 43), leading to reduced expression of chemoattractant protein-1 in monocytes to inhibit inflammatory responses (58). Indicators of myocardial injury are further reduced after decreased inflammation in patients with ACS, and cardiac function is significantly improved. Cardiac function is closely related to the prognosis of patients, and the improvement of cardiac function reduces the incidence of MACEs.

Strengths and limitations

A previous meta-analysis reported that DHI improved the total efficacy rate, reduced the inflammatory response, and inhibited oxidative stress in patients with ACS (59). Liao et al. reported that DHI reduced mortality and the incidence of MACEs, which were considered to be associated with improved cardiac function and reperfusion, in patients with acute myocardial infarction (60). Zou et al. conducted a meta-analysis of the effects of DHI in patients with ACS undergoing interventional procedures and showed that DHI improved the overall response rate of treatment and reduced the incidence of MACEs (11). However, no previous study evaluated the effects of the timing of DHI.

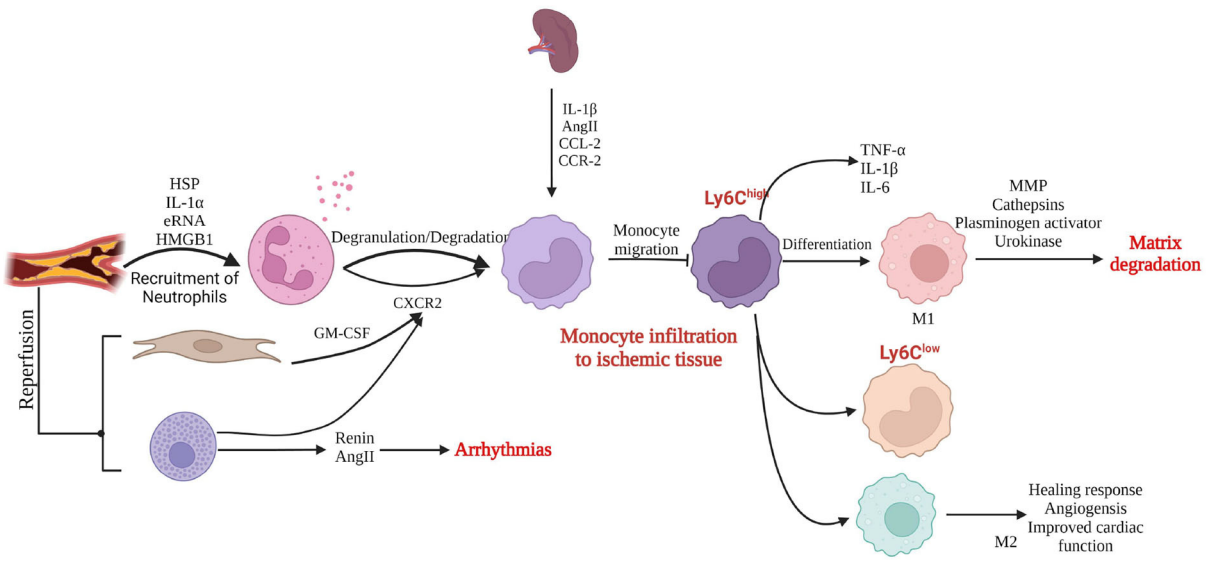


FIGURE 10
Inflammatory response to myocardial injury (created with BioRender.com). HSP, heat shock protein; IL, interleukin, RNA, ribonucleic acid; HMGB1, high mobility group box 1; GM-CSF, granulocyte–macrophage colony-stimulating factor; AngII, angiotensin II; CXCR2, CXC chemokine receptor 2; CCL-2, C-C chemokine ligand 2; CCR-2, C-C chemokine receptor type 2; TNF, tumor necrosis factor; MMP, matrix metalloproteinase.

This study is a state-of-the-art study involving 3,458 patients and evaluates the clinical effects of the combination of DHI and conventional therapy in patients with ACS. Also, this study clarifies the effectiveness of DHI during the perioperatively period of PCI in patients with ACS, proposes a possible mechanism of action, and assesses the timing of DHI.

This study also has several limitations. First, the included studies were all conducted in China. To generalize these results to other populations, multi-national investigations should be conducted in future. Second, some included studies achieved low scores on quality assessment. Thus, future trials should be designed to meet the CONSORT criteria. Third, the number of studies regarding preoperative/intraoperative DHI was low, limiting the strength of the conclusions, which should be interpreted carefully.

Conclusion

The combination of DHI and conventional therapy results showed a better therapeutic effect than conventional therapy alone in patients with ACS undergoing PCI. DHI decreases the incidence of MACEs and improves the reperfusion rate. DHI has multiple effects, including reducing inflammation, reducing myocardial injury, and adjusting cardiac function to play a cardioprotective role. DHI could be a useful supplement to perioperative PCI for patients with ACS. Therefore, postoperative DHI is recommended as a standard treatment for patients with ACS.

Data availability statement

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

Author contributions

YuL and DL carried out the protocol and drafted the article. DL and WW assisted in data collection, quality control, and project administration. WW and XL assisted with analysis methods. XL, PL, and YZ were involved in data management and analysis. YaL and QL designed and managed this protocol. All authors contributed to the final version of the article.

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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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J-shaped association between serum albumin levels and long-term mortality of cardiovascular disease: Experience in National Health and Nutrition Examination Survey (2011–2014)

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Background: Cardiovascular disease (CVD) is a constellation of heart, brain, and peripheral vascular diseases with common soil hypothesis of etiology, and its subtypes have been well-established in terms of the albumin-mortality association. However, the association between albumin and the mortality of CVD as a whole remains poorly understood, especially the non-linear association. We aimed to investigate the association of albumin levels with long-term mortality of CVD as a whole.

Materials and methods: This study included all CVD patients who participated in the National Health and Nutrition Examination Survey (NHANES 2011–2014). CVD was defined as coronary heart disease, stroke, heart failure, or any combination of these two or three diseases. Serum albumin was tertile partitioned: tertile 1, <4.1; tertile 2, 4.1–4.3; and tertile 3, >4.3 g/dl. COX proportional hazards model was used to assess the association between the serum albumin levels and CVD mortality. Restricted cubic spline (RCS) curves were used to explore the non-linear relationship.

Results: A total of 1,070 patients with CVD were included in the analysis, of which 156 deaths occurred during a median 34 months of follow-up. On a continuous scale, per 1 g/dl albumin decrease was associated with an adjusted HR (95% CI) of 3.85 (2.38–6.25). On a categorical scale, as compared with tertile 3, the multivariable adjusted hazard ratio (95% CI) was 1.42 (0.74–2.71) for the tertile 2, and 2.24 (1.20–4.16) for the tertile 1, respectively, with respect

to mortality. RCS curve analysis revealed a J-shaped association between albumin and CVD mortality.

Conclusion: A J-shaped association between low serum albumin levels and increased long-term mortality of CVD has been revealed. This J-shaped association's implications for CVD prevention and treatment are deserving of being further studied.

KEYWORDS

albumin levels, cardiovascular disease, mortality, J-shaped association, NHANES

Introduction

Cardiovascular disease (CVD) mainly includes coronary heart disease (CHD), stroke, heart failure (HF), and also covers other cardiovascular conditions (1). Accumulating evidence has shown that low albumin level is a well-established risk factor for its subtypes (2), such as stable CHD (3), acute coronary syndrome (4–9), acute and chronic HF (10–18), and ischemic stroke (19–21). In patients with acute coronary syndrome, as compared with albumin ≥3.5 g/dl, those with albumin <3.5 g/dl were associated with a 2.8-fold greater risk of adverse outcomes (death, acute heart failure, cardiogenic shock, and reinfarction) (4); A 3-year follow-up study of patients with first-onset acute myocardial infarction found per 1 g/dl decrease was associated with an unadjusted hazard ratio (95% CI) of 4.11 (3.17–5.33) (6); Hypoalbuminemia (≤3.4 g/dl) predicted 1-year mortality in acute decompensated heart failure [HR (95% CI), 2.05 (1.10–3.81)], and also predicted one and a half year- mortality in patients with chronic heart failure [HR (95% CI), 5.74 (4.08–8.07)], when compared with non-hypoalbuminemia (11, 14). In addition, a prospective community-based cohort study has demonstrated that low albumin level is an independent predictor of cerebro-cardiovascular death [HR (95% CI), 5.26 (1.59–16.67)] (19). However, cardiovascular disease is a constellation of heart, brain, and peripheral vascular diseases with common soil hypothesis of etiology, and the association between albumin and mortality in CVD as a whole remains poorly understood, especially the non-linear association. We aimed to investigate the association between albumin levels and long-term mortality of CVD as a whole.

Abbreviations: NHANES, National Health and Nutrition Examination Survey; CVD, cardiovascular disease; CHD, coronary heart disease; HF, heart failure; BM, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; ADA, American Diabetes Association; A1C, glycated hemoglobin; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; SD, Standard deviation; HR, Hazard ratio; CI, confidence interval.

Materials and methods

Study population

We obtained publicly available data from the National Health and Nutrition Examination Survey (NHANES). NHANES, with a probability sampling design to represent the civilian non-institutionalized population of the United States, was designed to assess the health and nutritional status of the general population. The NHANES survey included not only demographic, socioeconomic, dietary, and health-related interview data, but also examination data for medical, dental, and physiological measurements. The survey protocol was approved by the National Institute of Health Research Ethics Review Board, and all participants signed and provided informed consent. For this study, we included patients who were diagnosed with CVD in the continuous two cycles of NHANES survey of 2011–2014. The study population was divided into three groups according to the tertiles of albumin levels (<4.1, 4.1–4.3, and >4.3 g/dl).

Outcomes, exposures, covariates, and their definitions or measurements

The primary outcome was CVD mortality. CVD diagnosis includes CHD, stroke, heart failure, or any combination of these two or three diseases. The exposure was the serum albumin levels. We adjusted for covariates with respect to demographics, lifestyle, chronic diseases, and blood indicators, including age, sex, ethnicity, BMI, pulse, drinking, smoking, hypertension, diabetes mellitus, albumin, triglycerides, LDL-C, and HDL-C. The definition of smoking referred to New Zealand standards (22). Hypertension was measured as SBP ≥ 140 and or DBP ≥ 90 mmHg or as self-reported one by asking the question, "Has a doctor or other health professional ever told you that you have hypertension?". The diagnosis of diabetes referred to the most recent ADA criteria (A1C ≥ 6.5% or

FPG ≥ 7.0 mmol/L or 2-h OGTT ≥ 11.1 mmol/L or a random plasma glucose ≥ 11.1 mmol/L) (23). BMI was evaluated by body mass (kilograms) and body height (m^2). Albumin levels were measured by DcX800 method. Triglycerides and HDL-C were measured by the Roche/Hitachi Modular P Chemistry Analyzer (Mod P) in Mobile Examination Centers (MECs). LDL-C was calculated from measured values of total cholesterol, triglycerides, and HDL-C according to the Friedewald calculation. Information on all variables and their measurement methods are publicly available on the NHANES website (24).

Statistical analysis

Frequency distribution diagram was used to investigate the distribution of albumin. Independent groups were compared using the Wilcoxon rank test or likelihood ratio test as appropriate. Kaplan-Meier curves were used to compare survival differences between different albumin tertiles. COX proportional hazards model was used to assess the association between albumin levels and CVD mortality. Restricted cubic spline (RCS) curves were used to explore the non-linear relationship between albumin levels and CVD mortality. Subgroup analyses were performed to test whether the association between albumin levels and CVD mortality was consistent in different groups. Interactions between albumin and other covariates were examined. Adjusted covariates included age, sex, ethnicity, BMI, pulse, drinking, smoking, hypertension, diabetes mellitus, triglycerides, LDL-C, and HDL-C. Multiple imputation (Chained equations, 25 times) was used to fill in the missing values. All analyses were conducted using Stata 15.1 (Stata Corp, TX, USA). All tests were two-sided. Statistical significance was considered when a $P < 0.05$.

TABLE 1 Baseline characteristics of the study population stratified by albumin tertiles.

Factor	Tertile 1 (<4.1 g/dl)	Tertile 2 (4.1–4.3 g/dl)	Tertile 3 (>4.3 g/dl)	*P* for trend
n	404	398	268	
Age, year	67 ± 13	66 ± 13	64 ± 14	0.002
Female, sex, *n* (%)	234 (58)	168 (42)	95 (35)	<0.001
Ethnicity, *n* (%)				0.068
Mexican American	29 (7)	32 (8)	27 (10)	
Non-Hispanic White	192 (48)	218 (54)	147 (55)	
Non-Hispanic Black	128 (32)	87 (22)	40 (15)	
Non-Hispanic Asian	17 (4)	22 (6)	21 (8)	
Others	38 (9)	39 (10)	33 (12)	
BMI, kg/m^2	32 ± 9	30 ± 7	28 ± 5	<0.001
Pulse, beats/min	71 ± 13	69 ± 12	71 ± 12	0.633
Drinking, *n* (%)				<0.001
Never	71 (19)	50 (14)	28 (11)	
Former	151 (40)	117 (32)	75 (31)	
Current	154 (41)	200 (54)	144 (58)	
Smoking, *n* (%)				<0.001
Never	177 (44)	156 (39)	114 (43)	
Former	133 (33)	152 (38)	99 (37)	
Current	94 (23)	90 (23)	55 (20)	
Hypertension, *n* (%)	131 (34)	111 (29)	67 (26)	0.016
Diabetes mellitus, *n* (%)	200 (50)	163 (41)	103 (38)	0.003
Triglyceride, mmol/L	1.6 ± 1.5	1.5 ± 1.1	1.6 ± 1.0	0.920
LDL-C, mmol/L	2.6 ± 0.9	2.6 ± 0.8	2.6 ± 1.0	0.793
HDL-C, mmol/L	1.3 ± 0.4	1.3 ± 0.4	1.4 ± 0.5	0.005
Albumin, g/L	38 ± 2	42 ± 0.8	45 ± 1.6	<0.001
Death cause, *n* (%)				
Cardiac deaths	28 (6.9)	15 (3.8)	5 (1.8)	<0.001
Hypertensive death	26 (6.4)	5 (1.3)	1 (0.3)	<0.001
Diabetic death	11 (2.7)	4 (1.0)	2 (0.7)	<0.001
Cancerous deaths,	7 (1.7)	9 (2.3)	5 (1.8)	0.008
Other caused deaths	9 (2.3)	17 (4.3)	12 (4.4)	<0.001
Deaths, *n* (%)	81 (20)	50 (13)	25 (9)	<0.001

Continuous and categorical variables were presented as mean ± SD or percentages *n* (%), respectively.
BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

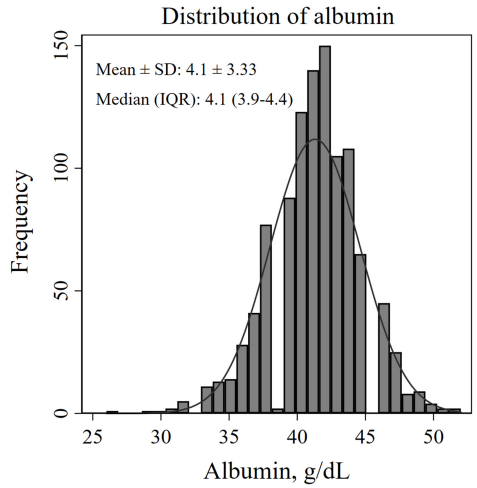

FIGURE 1
Histogram for estimating the distribution of albumin levels.

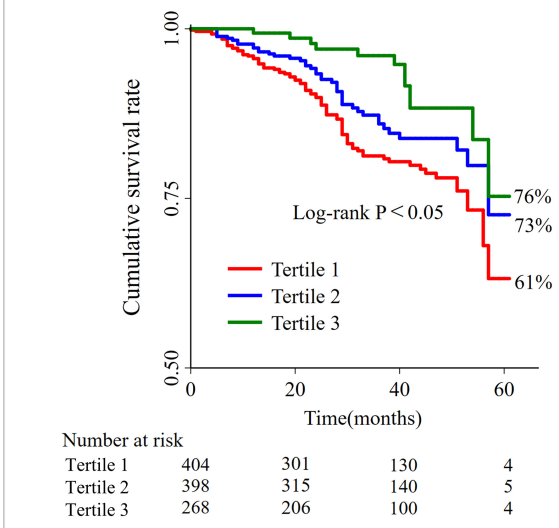

FIGURE 2
Kaplan-Meier survival curves for mortalities between different albumin tertiles.

Results

Baseline characteristics and albumin distribution

A total of 1,070 patients with CVD were included in the analysis, of which 156 deaths occurred during a median 34 months of follow-up. Patients in the lower tertiles were older, and more frequently observed in females. They also had a higher prevalence of diabetes and hypertension, and a higher mortality. Frequency distribution diagram showed an approximately normal distribution of albumin with an interval of 2.6–5.2 g/dl, with a median (IQR) of 4.1 (5) g/dl, and with a mean ± SD of 4.1 ± 3.33 g/dl. Details in Table 1 and Figure 1.

The predictive role of albumin on long-term mortality in cardiovascular disease

The 5-year cumulative Kaplan–Meier survival were 61% (95% CI: 48–72%) for tertile 1, 73% (95% CI: 57–84%) for tertile 2, and 76% (95% CI: 51–89%) for tertile 3 (Figure 2). On a continuous scale, per 1 g/dl decrease was associated with an adjusted HR (95% CI) of 3.85 (2.38–6.25). On a categorical scale, as compared with tertile 3, the multivariable adjusted hazard ratio (95% CI) was 1.42 (0.74–2.71) for the tertile 2, and 2.24 (1.20–4.16) for the tertile 1, respectively, with respect to mortality. The *P*-values for trend test were <0.001. Details in Table 2.

A J-shaped association of albumin levels with long-term mortality in cardiovascular disease

Non-linear analysis showed that the association between albumin levels and CVD mortality took on a J-shaped association, when the lowest albumin level was used as a reference (2.6 g/dl), as shown in Figure 3. The turning point for this J-shaped association was around 4.3 g/dl, beyond which, this association started to accelerate.

Subgroup and sensitivity analysis

Subgroup analysis largely confirmed the associations of albumin levels with CVD mortality revealed in the current study across a broad spectrum of risk factors as shown in Figure 4. To test the sensitivity of models, we selected participants from the 2011–2012 cycle for the same analysis and found a similar relationship between albumin levels and CVD mortality (data not shown).

Discussion

The key findings revealed in the current study included that (i) to the best of our knowledge, the J-shaped albumin-CVD mortality association was first revealed; (ii) on a continuous scale or on a categorical scale, low albumin levels were significantly associated with higher CVD mortality, and this association remained substantially unchanged after adjusting for confounding factors; and (iii) the threshold effects were observed in terms of the albumin-CVD mortality association.

TABLE 2 Association of albumin levels and cardiovascular disease (CVD) mortality in the COX model.

		Crude		Adjusted	
CVD mortality		**HR (95% CI)**	***p* for trend**	**HR (95% CI)**	***p* for trend**
On a continuous scale		4.55 (2.86–7.14)	<0.001*	3.85 (2.38–6.25)	<0.001*
Ona categorical scale	Tertile 1	2.98 (1.64–5.43)		2.24 (1.20–4.16)	
	Tertile 2	1.90 (1.00–3.59)	<0.001†	1.42 (0.74–2.71)	<0.001†
	Tertile 3	Reference		Reference	

Adjusted covariates: Age, sex, ethnicity, BMI, pulse, drinking, smoking, hypertension, diabetes mellitus, triglycerides, LDL-C, and HDL-C.
*per 1 g/dl decrease.
†per one tertile decrease.

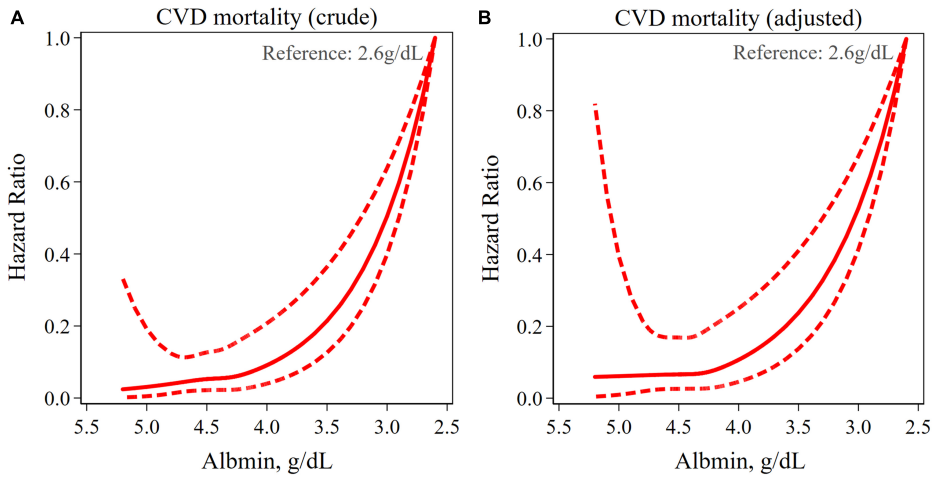

FIGURE 3
Restricted cubic spline (RCS) curves for analyzing the non-linear association between albumin levels and cardiovascular disease (CVD) mortality. Same variables adjusted as **Table 2**.

On a continuous scale, the current study showed an adjusted 3.85-fold increase in the risk of CVD death for every 1 g/dl decrease in albumin. A 3-year follow-up study demonstrated a 4.11-fold increase in the risk of death in patients with acute myocardial infarction for each 1 g/dl decrease in albumin in the univariate model, consistent with our findings (4). Another study described findings that for each 1/dl decrease in albumin, the risk of cerebro-cardiovascular death increased by 4.26-fold in the general population at 15 years of follow-up (19). Slightly higher than our findings. The reasonable explanations include 15-year long-term follow-up with more events recorded. On a categorical scale, previous studies also revealed an association between albumin tertiles and CVD mortality. Patients with acute coronary syndrome with albumin <3.5 g/dl had a 2.80-fold higher risk of death compared to those with albumin ≥3.5 g/dl [OR (95% CI), 2.80 (1.11 1–7.00)] (4). A 5-year follow-up of patients with systolic heart failure found a 2.2-fold higher risk of death from hypoalbuminemia (albumin ≤3.4 g/dl) compared to non-hypoalbuminemia [HR (95% CI), 2.2 (1.4–3.3)] (10). A large meta-analysis showed that during the 3-month follow- up period, compared with 4 to 4.49 g/dl group, patients with acute ischemic stroke in <3.5 g/dl group had a 2.13-fold higher risk of death [HR (95% CI), 2.13 (1.41–3.23)] (21). We reported adjusted hazard ratios (95% CI) of 1.42 (0.74–2.71) and 2.24 (1.20–4.16) for the tertile 2 (4.1∼4.3 g/dl) and for the tertile 1 (<4.1 g/dl), respectively, when compared to the tertile 3 (>4.3 g/dl), which are very similar to those aforementioned studies.

To the best of our knowledge, a J-shaped association between albumin and CVD mortality was first revealed in the current study. This J-shaped albumin-CVD association and the presence of a threshold effect may have potential implications for CVD prevention and treatment: in the steep limb of the J-shaped curve, a slight change in the serum albumin levels will dramatically increase or decrease the risk of CVD death; by contrast, in the almost horizontal limb of the J-shaped curve, infusion of albumin to achieve a higher level of serum albumin (beyond the threshold) will not further result in the reduction of CVD death. The J-shaped predictive effect of serum albumin levels in CVD mortality may be mainly related to its irreplaceable physiological functions, including its effects on antitumor, anti-inflammatory, antioxidant and antithrombotic activity. Decreased albumin levels weaken the antioxidant capacity of the vascular endothelium, initiate and

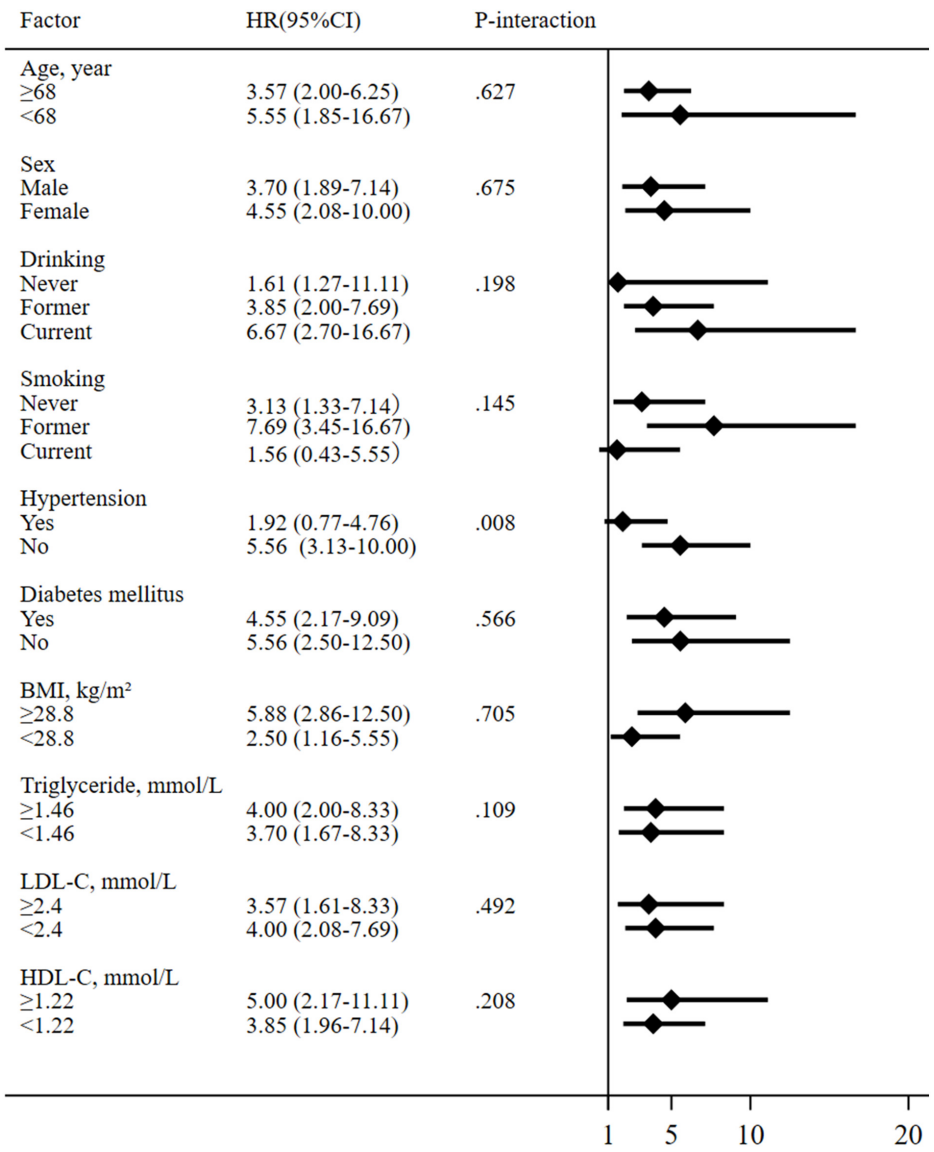

FIGURE 4
Subgroup analysis for the association between albumin levels and cardiovascular disease (CVD) mortality. Continuous variables were dichotomized at their corresponding medians. All models are adjusted as in **Table 2**.

exacerbate the inflammatory process, increase blood viscosity, and increase the risk of thrombosis, all of which, in combination or separately, contribute to the poor prognosis of CVD (25–28).

Limitations

Several limitations of the current study should be discussed. First, the design of the study was limited in that not all patients obtained serum albumin values at the same time interval and the etiology of low albumin levels was not distinguished; Second, some of the covariates selected for this study such as smoking, drinking, hypertension, and diabetes were obtained in combination with subjective questionnaires, which may bias the results; Third, our study was conducted on patients in the general U.S. population, so it is not clear whether the J-shaped predictive effect of albumin is also applicable to other populations or ethnic groups.

Conclusion

A J-shaped association between low serum albumin levels and increased long-term mortality of CVD has been revealed. This J-shaped association's implications for CVD prevention and treatment are deserving of being further studied.

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.

Ethics statement

This survey protocol was approved by the Research Ethics Review Board of the NCHS and all participants have signed and provided informed consent.

Author contributions

XL and YZ wrote the main manuscript. W-XC and Y-MH provided statistical guidance and were responsible for the final revisions. YH, K-XL, R-NX, HW, and T-BJ provided graphic art direction and supported the successful completion of the work. 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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Edgeworthia gardneri (Wall.) Meisn. extract protects against myocardial infarction by inhibiting NF-κB-and MAPK-mediated endothelial inflammation

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Background: Experimental and clinical evidence has demonstrated a pivotal role of inflammation in the pathogenesis of ischemic heart disease, and targeting inflammation has been shown to provide clinical benefits for patients with coronary disease. Endothelial cells constitute the majority of non-cardiomyocytes in the heart. Endothelial pro-inflammatory activation is recognized as a critical component in the pathophysiology of cardiovascular disease. The dried flowers of *Edgeworthia gardneri* (Wall.) Meisn. (EG) have been widely used as Tibetan folk medicine to ameliorate a range of metabolic disorders, such as diabetes mellitus, hyperlipidemia, hypertension, and obesity. However, its role in modulating endothelial inflammation and ischemic heart disease has not been evaluated.

Methods and results: Herein, using a preclinical rat model of coronary artery ligation-induced myocardial infarction (MI), we demonstrated that systemic administration of EG extract (EEEG) attenuated ischemic cardiac injury. EEEG reduced myocardial infarct size, improved cardiac function, and ameliorated adverse cardiac remodeling. Moreover, the cardioprotective effects of EEEG were associated with decreased MI-induced myocardial inflammation. Consistent with the anti-inflammatory role of EEEG *in vivo*, EEEG attenuated TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) activation and monocyte-endothelial cell firm adhesion *in vitro*. Mechanistically, our data showed that EEEG's mode of action suppresses the activation of NF-κB, ERK, and p38 MAPK signaling pathways in ECs. Importantly, we demonstrated that EEEG inhibits endothelial inflammation in an NF-κB- and p38 MAPK-dependent manner using pharmacological inhibitors.

Conclusion: Collectively, this study identified EG as a potential therapeutic agent in attenuating endothelial inflammation and managing ischemic cardiovascular disease.

KEYWORDS

Edgeworthia gardneri (Wall.) Meisn., myocardial infarction, inflammation, endothelial cells, NF-κB, MAPK

Introduction

Myocardial infarction (MI) refers to an event of a heart attack characterized by inhibiting blood flow to the heart that irreversibly causes cardiac injury and impairs cardiac function, eventually leading to the pathogenesis and progression of heart failure (1, 2). It has been reported that there are currently about 126.5 million cases of ischemic heart disease (IHD), with a concomitant of over 9 million deaths per year in the world, making IHD a leading cause of morbidity and mortality worldwide (3, 4). Over the past years, despite significant advances in the clinical practice for preventing and treating IHD, it has caused a substantial burden on healthcare resources worldwide (5, 6). Therefore, much work remains to be done to seek new effective strategies for managing IHD.

Inflammation is implicated in MI and plays a critical role in the onset and progression of IHD (7). Upon cardiac injury following MI, the release of danger signals activates the innate immune signaling and triggers an overwhelming inflammatory response to clear the necrotic cardiomyocytes from the injured heart (7). This pro-inflammatory state is a finely orchestrated process followed by an anti-inflammatory state to promote cardiac repair (7, 8). Notably, a proper physiologic equilibrium between these pro-inflammatory and anti-inflammatory phases contributes to optimal post-infarct healing and cardiac remodeling (8). Excessive and prolonged inflammation may impair wound healing and cause cell loss and cardiac dysfunction, leading to sustained cardiac injury and adverse remodeling events (8). Anti-inflammatory therapies among patients with recent MI or chronic coronary disease have resulted in a lower risk of ischemic cardiovascular events (9, 10), indicating that controlled inflammation may be a fundamental determinant for favorable cardiovascular outcomes. In the heart, endothelial cells (ECs) are recognized to be the most abundant non-cardiomyocytes. The endothelial pro-inflammatory activation plays a critical role in the recruitment of leukocytes into the infarcted area and the subsequent inflammatory cascades after MI (8, 11). Thus, manipulating endothelial inflammation is a potential therapeutic strategy for attenuating cardiac inflammatory damage during MI.

Nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs), including p38 MAPK, extracellular signal-regulated protein kinases1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) are pivotal intracellular signaling molecules. They jointly regulate many physiological and pathological processes, including inflammation (8, 12, 13). In the context of MI, intracellular signaling converges on the activation of NF-κB and MAPK pathways to induce cardiac inflammatory responses (8). Remarkably, NF-κB and MAPK inhibition have been demonstrated to be beneficial for MI-induced injury with the manifestation of attenuated infarct size, reduced inflammatory responses, and improved cardiac function (14, 15). Therefore, targeted intervention in NF-κB and MAPK signaling pathways provides an attractive strategy for protecting against MI injury via modulating immune responses.

The dried flowers of *Edgeworthia gardneri* (Wall.) Meisn. (EG), also known as "Lv Luo Hua" in China, have long been widely used as traditional Tibetan medicine to prepare a herbal beverage to treat and prevent a range of diseases such as diabetes mellitus, hyperlipidemia, hypertension, and obesity (16–19). In addition, the active components of EG, including quercetin (20), tiliroside (21), umbelliferone (22), and pentadecanoic acid (22), have been identified and shown to possess excellent pharmacological properties such as islet protection, α-glucosidase inhibition and PPARγ/β activation. However, the precise role of EG in IHD remains elusive. Thus, in the present study, we aim to investigate whether EG affects the pathogenesis of MI and, if so, to reveal the potential mechanisms using a rat model of acute ischemic myocardial injury.

Materials and methods

Chemicals and reagents

Ethanol (64-17-5) and petroleum ether (8032-32-4) were purchased from Xilong Science (Guangzhou, China). AB-8 Macroporous Adsorptive Resin was provided by Baoen Chemical (BE1003, Hebei, China). Sodium carboxymethyl cellulose (CMC-Na) was obtained from Zhanyun Chemical (9004-32-4, Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Solarbio (D8370, Beijing, China). 2,3,5-triphenyl tetrazolium chloride (TTC) was obtained from Solarbio (T8170, Beijing, China). Xylene was provided by

Damao Chemical (3833, Tianjin, China). 10x Zinc Fixative solution was purchased from BD Biosciences (552658, Franklin Lakes, NJ, USA). Tumor necrosis factor-α (TNF-α) was purchased from PeproTech (300-01A, New Jersey, USA). Vascular endothelial growth factor-A (VEGF-A) was obtained from Lonza (CC-4114A, Basel, Switzerland). p65 (6956), p-p65 (3033), p38 MAPK (8690), p-p38 MAPK (9216), ERK (4696), and p-ERK (9101) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). CD45 (ab10558), CD68 (ab125212), and CD31 (ab64543) antibodies were purchased from Abcam (Cambridge, UK). Goat anti-rabbit IgG (I31203) and goat anti-mouse IgG (I31107) were purchased from TransGen Biotech (Beijing, China). Biotin-conjugated goat anti-rabbit IgG (H + L) (ABM120002-100) and goat anti-mouse IgG (H + L) (ABM120001-100) was purchased from Embime Biology (Beijing, China). Streptavidin-Horseradish Peroxidase was purchased from Lianke Biotech (SH001, Hangzhou, China). 3,3′-diaminobenzidine was purchased from Invitrogen (750118, Carlsbad, CA, USA). Masson's trichrome Staining Kit was purchased from Sigma-Aldrich (1004850001, St. Louis, MO, USA). Hematoxylin (51275) and eosin (E4009) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizol reagent was purchased from Invitrogen (15596-018, Carlsbad, CA, USA). High-Capacity cDNA Reverse Transcription Kit (RR047A) and SYBR Green PCR Master Mix (RR820A) were purchased from Takara (Shiga, Japan). EGM-2 endothelial growth medium was purchased from Lonza (CC-3156 & CC-4176, Basel, Switzerland). MTT was purchased from Sigma-Aldrich (M5655, St. Louis, MO, USA). RIPA buffer was purchased from KeyGen (KGP703-100, Jiangsu, China). Protease and phosphatase inhibitors were purchased from Vazyme (E312-01, Jiangsu, China). NF-κB inhibitor BAY11-7082 (B5556), ERK inhibitor SD98059 (P215), and p38 MAPK inhibitor SB203580 (S8307) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material

The medicinal plant material was purchased from Zangxi Tang Company (Tibet, China), and its sample was authenticated as dried flowers of *Edgeworthia gardneri* (Wall.) Meisn. by Dr. Guoyue Zhong (Jiangxi University of Chinese Medicine). A voucher sample (GH827) was deposited at Jiangxi University of Chinese Medicine.

Preparation of EG extract

EG (2.7 kg) was initially soaked in 70% ethanol (Xilong Science) and then decocted to an extract solution, followed by rotary evaporation of the extract solution under reduced pressure. Subsequently, the concentrated extracts were further extracted with petroleum ether (Xilong Science), and then the petroleum ether extract and the residue fraction were obtained, respectively. The residue fraction was concentrated under reduced pressure and then separated by macroporous resin column chromatography (Baoen Chemical) and eluted with a gradient system of water-ethanol (100:0 and 70:30). The resultant 30% ethanol fraction of EG extract (EEEG) was harvested and evaporated with a rotary evaporator under reduced pressure followed by vacuum freeze drying. Before *in vitro* studies, EEEG was dissolved in DMSO (Solarbio) to get a stock solution followed by further dilution to a final concentration with the cell culture medium. For animal experiments, EEEG was dissolved in 0.1% CMC-Na (Zhanyun Chemical).

Ultra performance liquid chromatography (UPLC)-mass spectrometry (MS) analysis

Identification and characterization of chemical constituents of EEEG were performed using UPLC-Q-TOF-MS. 50 mg EEEG was dissolved in 10 ml water with ultrasound followed by centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was filtered and injected for further UHPLC-Q-TOF-MS analysis. The reference compound tiliroside (20316-62-5, Desite Biotechnology, Sichuan, China) with purity >98% was also used for this analysis. The separation was performed on a Nexera X2LC-30A system (Shimazdu Corp., Japan) using an Acquity UPLC HSS T3 column (100 × 2.1 mm, i.d., 1.8 μm, Waters, USA). Chromatographic separation conditions were as follows: the sample injection volume was 5 μl, the column temperature was 40°C, and the flow rate was 0.3 ml/min. The mobile phase was composed of 0.1% formic acid in water (A) and acetonitrile (B), and the gradient elution procedure was as follows: 0–3 min, 2–10% B; 3–12 min, 10–17.5% B; 12–20 min, 17.5–18% B; 20–27 min, 18–30% B; 27–34 min, 30–60% B; 34–39 min, 60–95% B; 39–42 min, 95% B; 42–42.01 min, 95–2% B; 42.01–45 min, 2% B. The Q-TOF-MS analysis was performed using TripleTOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA) in positive electrospray ionization mode. The scan range was m/z 50–1,250, with a spray voltage of 4,500 V, a nebulizing gas temperature of 500°C, a curtain gas pressure of 25 psi, a nebulizing gas pressure of 50 psi, an auxiliary gas pressure of 50 psi, a declustering potential of 100 V, and a collision energy of 30 eV. Data analysis was performed using Analyst TF 1.6 software and PeakView software (AB SCIEX, Framingham, MA, USA). Chemical constituents of EEEG were determined according to the retention time and MS fragmentations. The total ion chromatograms (TICs) of EEEG and the reference compound by UPLC-Q-TOF-MS, data analysis of UPLC-Q-TOF-MS and the chemical structures of identified constituents

in EEEG were shown in **Supplementary Figures 1–3** and **Supplementary Table 1**.

Animals

Adult male Sprague-Dawley rats (200 ± 20 g) were obtained from Silaike (SCXK-2013-0004, Hunan, China) and housed in the Experimental Animal Center of Jiangxi University of Chinese Medicine (license number: SYXK-2017-0004). All studies were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of Jiangxi University of Chinese Medicine. All rats were maintained in controlled conditions with free access to food and water (temperature: 23 ± 2°C, humidity: 60 ± 5%, and 12 h/12 h light/dark cycle). The rats used in this study were randomly assigned to three groups (n = 8 per group): sham operation group, MI surgery group, and MI + EEEG group. EEEG (10 g/kg) was orally administered by gastric gavage every other day for rats in the MI + EEEG group after MI surgery. The gavage dose in the present study was determined according to the equivalent patient dose. The rats in the sham group and the MI group received 0.1% CMC-Na (Zhanyun Chemical), and all rats in this study were treated for 4 weeks.

Induction of MI

Myocardial infarction was induced by permanent ligation of the left anterior descending (LAD) coronary artery. Briefly, rats were initially anesthetized with sodium pentobarbital (60 mg/kg by intraperitoneal injection). Subsequently, the left thoracotomy was performed, the pericardium was excised, and the LAD artery was exposed. A 6/0 silk suture was then placed around the proximal LAD coronary artery 2–3 mm from its origin and tightly tied to cause occlusion of the LAD coronary artery. Next, the heart was immediately put back into the chest cavity, followed by manual air evacuation and closure of muscle and the skin. After fully recovering from anesthesia, the rats were placed back into the housing facility. The same procedure was performed for sham-operated rats without ligating the LAD coronary artery. At 4 weeks after surgery, rats were sacrificed, and tissues were collected for subsequent experiments.

Echocardiography

Rats were mildly anesthetized using sodium pentobarbital. After removing the hairs on the chest with depilatory paste, the rat was placed on an experimental animal plate with a heating function. Then the electrocardiogram metal electrode on the animal plate was coated with a conductive paste. Next, the chest of each rat was covered with an ultrasonic coupling agent. M-mode echocardiography was performed to measure cardiac function using a Vevo3100 instrument with a 38 MHz transducer (Visual Sonics). The cardiac parameters including left ventricular end-systolic diameter (LVIDs), left ventricular end-diastolic diameter (LVIDd), anterior wall thickness (AWT), the ratio of mitral peak velocity during early diastole to atrial contraction (E/A ratio), ejection fraction (EF), and fractional shortening (FS) were measured and calculated.

Measurement of myocardial infarct size

Briefly, rat hearts were harvested and cleared of blood with chilled PBS. Next, the rat hearts were frozen at −80°C for 10 min and then sliced transversally from apex to base at 3–5 mm thick, followed by the incubation of 1% TTC solution (Solarbio) for 30 min at 37°C in the dark. Subsequently, the slices were treated with 1x Zinc Fixative solution (BD Biosciences) for 24 h. Mark the infarct size and calculate using ImageJ (National Institutes of Health, Bethesda, MD, USA). Express the infarct size as a ratio of the infarct area (the unstained necrotic area) vs. the total left ventricle (LV) area.

Histology and immunohistochemistry

Heart specimens were rinsed with cold PBS, fixed in 1x Zinc Fixative solution (BD Biosciences), embedded in paraffin, and cut into 5 μm thick sections. These sections were then incubated with a graded ethanol series for deparaffinization and hydration. Heart sections were stained with Masson's trichrome (Sigma-Aldrich) to visualize myocardial fibrosis, hematoxylin, and eosin (H&E) (Sigma-Aldrich) to show the heart structure, as previously described (23, 24). For immunohistochemistry, sections were incubated with anti-CD45 (Abcam), anti-CD68 (Abcam), and anti-CD31 (Abcam) primary antibodies, followed by biotin-conjugated secondary antibodies (Embime). Next, sections were treated using Streptavidin-Horseradish Peroxidase (Lianke) and 3,3′-diaminobenzidine (Invitrogen), followed by counter-staining with hematoxylin (Sigma-Aldrich).

Quantitative real-time PCR (qRT-PCR)

Total RNA from rat hearts and cultured cells was isolated using Trizol reagent (Invitrogen), and reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Takara). The resulting cDNA was amplified for 35 cycles using SYBR Green PCR Master Mix (Takara) by the ABI 7500 Real-Time PCR System. Data were normalized by *Gapdh* software. The sequences of primers used in this study are shown in **Supplementary Table 2**.

Culture of human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained from the Vascular Biology and Therapeutics Program of Yale University and cultured in an EGM-2 endothelial growth medium (Lonza). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Passage 4–10 was used for experiments.

Cell viability and proliferation assay

Cell viability was assessed by MTT assay. Briefly, HUVECs (1.2×10^4 cells/well) were cultured in 96-well plates. Upon growth to sub-confluence, the cells were treated with or without different concentrations of EEEG for 24 h. After washing with PBS, MTT (5 mg/ml) (Sigma-Aldrich) was used to treat cells at 37°C for 3 h. Subsequently, the medium was discarded, and 100 μl of DMSO (Solarbio) was added into each well to dissolve formazan blue generated within the cells. The optical density was measured at 490 nm using a microplate reader (Bio-Rad). Cell viability in each well was expressed as a percentage of the vehicle control. Cell proliferation assay was performed by direct cell counting. HUVECs were seeded at 1×10^5 cells/well in 24-well plates and treated with EEEG (500 μg/mL) for indicated time points. After incubation, cells were harvested and counted using a hemocytometer.

Cell adhesion assay

HUVECs (1×10^5 cells/well) were seeded in 24-well plates and pretreated with EEEG (500 μg/mL) for 24 h. When the cells reached confluence, cells were stimulated with TNF-α (10 ng/mL) (PeproTech) for 4 h. Subsequently, the medium was removed, and Dil-labeled human acute monocytic leukemia THP-1 cells (5×10^4 cell/well) were added to HUVECs. Cells were co-cultured for another 2 h in a 5% CO_2 incubator at 37°C. Next, wash with PBS to remove the non-adherent cells. THP-1 monocyte adhesion was quantified in five random fields per well by a fluorescence microscope (Nikon).

Western blot

Cells were homogenized in cold RIPA buffer (KeyGen) supplemented with protease and phosphatase inhibitors (Vazyme). Total protein extracts (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore). Five percentage BSA in Tris-buffered saline/Tween-20 (TBST) was used to block membranes for 1 h. The primary antibody incubation was performed overnight at 4°C followed by secondary antibody treatment for 1 h at room temperature. Signals were visualized using ODYSSEY Infrared Imaging System (LI-COR). β-actin and Hsp90 were used as loading controls. Signal intensities were quantified using Image Studio software (LI-COR).

Statistical analysis

All data are presented as mean ± standard errors of the means (SEM). Statistical analysis was performed using Prism 8 software (GraphPad, CA, USA). The statistical significance of differences was evaluated using a 2-tailed Student's *t*-test for comparisons between two groups and one-way ANOVA followed by the Tamhane T2 test for multiple groups comparisons. Differences with a p-value < 0.05 were considered to denote statistical significance.

Results

EEEG attenuates MI-induced infarction and improves cardiac function *in vivo*

To determine whether EEEG affects ischemic myocardial injury, we performed permanent LAD ligation to induce MI in rats, followed by oral administration of EEEG or vehicle. First, we measured infarct size using TTC staining to examine MI-induced myocardial necrosis. As expected, the infarct size was markedly increased in vehicle-treated rats that underwent 4 weeks of MI compared with sham-operated controls (**Figures 1A, B**). The hearts of EEEG-treated rats showed significantly decreased infarct size compared to vehicle controls 4 weeks after MI (**Figures 1A, B**). Next, we performed M-mode echocardiography to investigate the influence of EEEG on cardiac function upon MI injury. As demonstrated by representative echocardiographic images (**Figure 1C**) and analyses (**Figures 1D–F**), EEEG exhibited protective effects on ventricular functions as shown by reduced LVIDs, higher E/A ratio, improved EF and FS compared to vehicle controls. Furthermore, EEEG treatment decreased heart weight-to-femur length and heart-to-body weight ratios, indicating their improved cardiac hypertrophy and function at 4 weeks post-MI compared to vehicle-treated rats (**Supplementary Figure 4**). These data suggest that EEEG treatment protects against myocardial damage and cardiac dysfunction resulting from MI.

EEEG alleviates MI-induced adverse cardiac remodeling *in vivo*

To evaluate the effects of EEEG on myocardial fibrosis remodeling after MI, Masson's trichrome staining was used to examine collagen deposition in cardiac tissues. As expected,

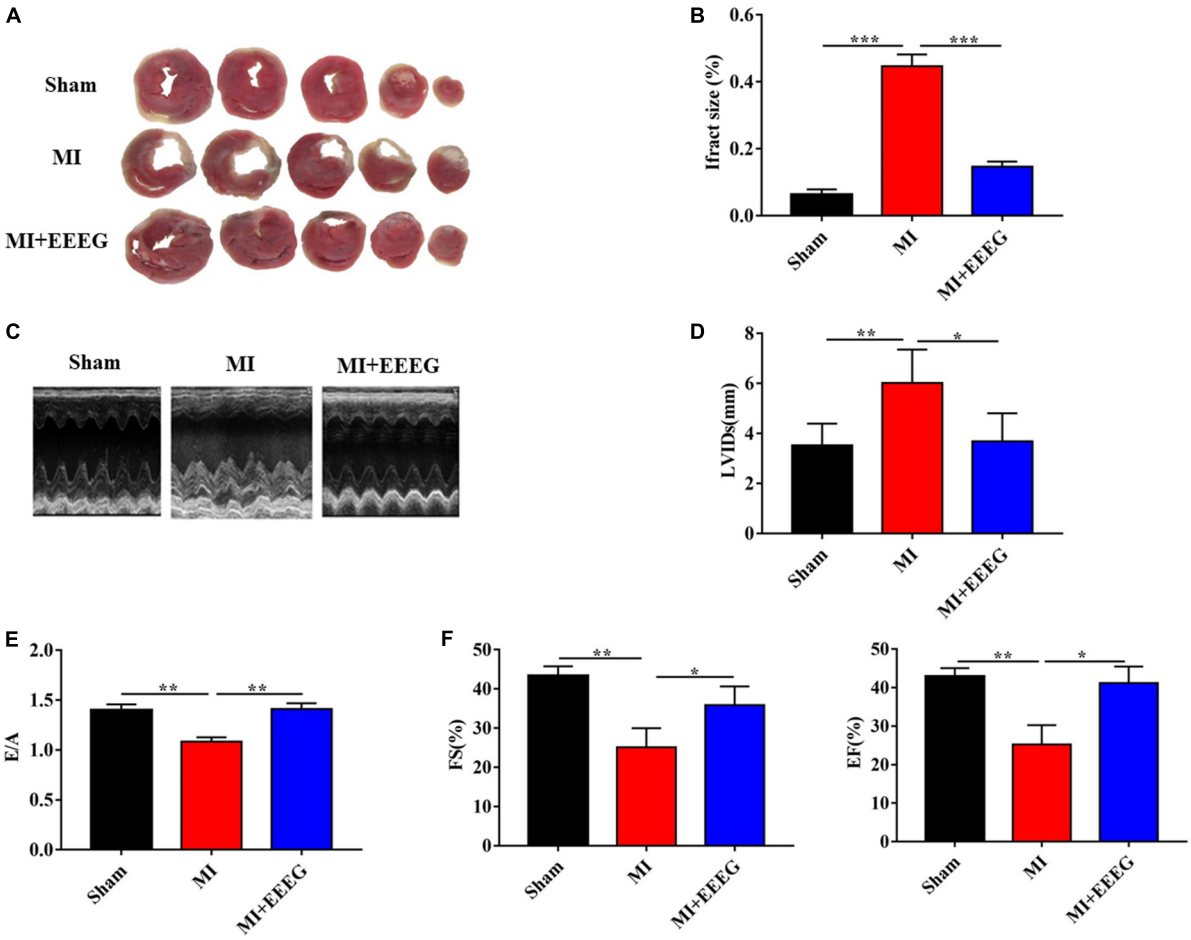

FIGURE 1
EG extract (EEEG) reduces infarct area and protects against cardiac dysfunction after MI. **(A)** Representative TTC staining of cardiac tissue obtained from vehicle and EEEG (10 g/kg) treated rats at 4 weeks after MI or sham operation. **(B)** Quantitative analysis of TTC-stained infarct area 4 weeks after MI or sham operation in vehicle and EEEG (10 g/kg) treated rats (n = 8). **(C)** Representative M-mode tracing for vehicle and EEEG (10 g/kg) treated rats at 4 weeks after MI or sham operation are shown. **(D–F)** Echocardiographic analysis of left ventricular end-systolic diameter **(D)**, the ratio of mitral peak velocity during early diastole to atrial contraction **(E)**, ejection fraction (**F**, left panel), and fractional shortening (**F**, right panel) at 4 weeks after MI or sham operation in vehicle and EEEG (10 g/kg) treated rats (n = 8). Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

MI surgery resulted in a considerable increase in collagen deposition and the extent of cardiac fibrosis as compared with sham-operated rats (Figures 2A, B). EEEG treatment decreased collagen deposition in the infarcted hearts compared to vehicle controls at 4 weeks post-MI (Figures 2A, B). In addition, H&E staining was performed to examine the pathological morphologies of heart tissue. As shown in Figure 2C, Myocardial cells are arranged orderly without inflammatory cell infiltration observed in sham-operated hearts. In contrast, disorderly arranged myocardial fibers with large amounts of fibrous tissue hyperplasia in the intercellular space and massive inflammatory cell infiltration were noticed in hearts that underwent 4 weeks of MI. These cardiac pathological changes were notably counteracted by EEEG treatment (Figure 2C). Collectively, these results demonstrate that EEEG administration attenuates adverse cardiac remodeling in response to MI injury.

EEEG reduces inflammation in the heart after MI

MI injury leads to the activation of innate immune signaling and the induction of a heightened inflammatory response. The impaired suppression of post-infarction inflammation has been reported to result in adverse cardiac remodeling associated with major adverse clinical events (7, 8). To evaluate the effects of EEEG on cardiac inflammatory response following MI, rats subjected to either MI or sham surgery were treated with EEEG or vehicle, and immunohistochemical

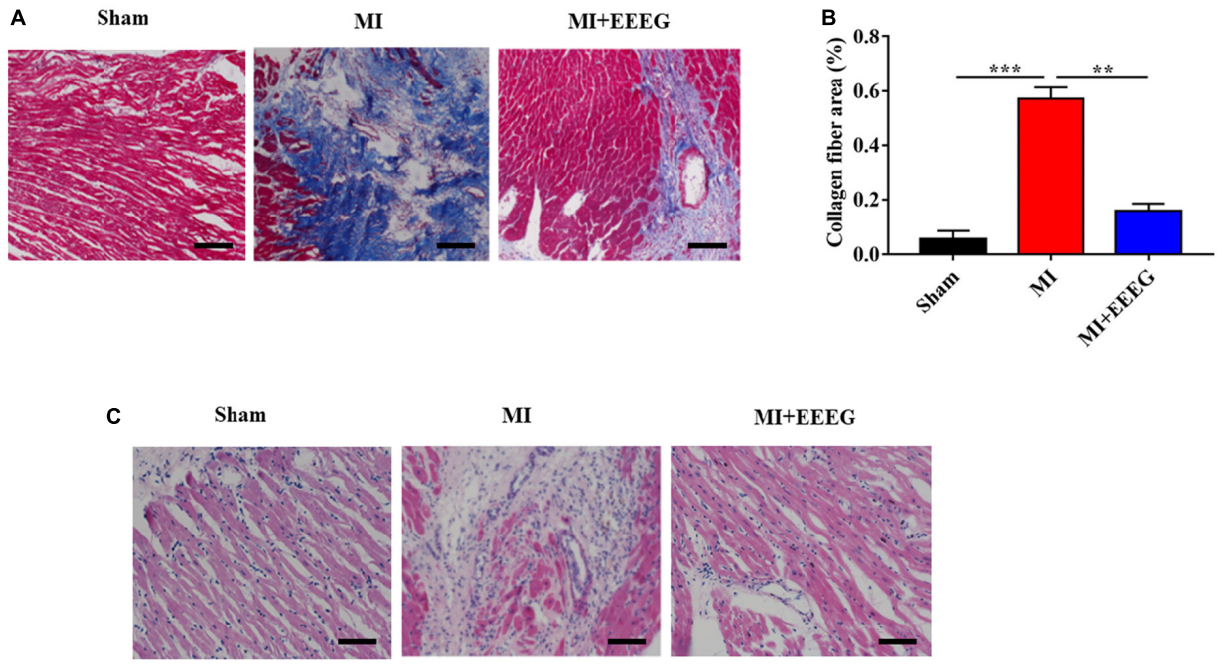

FIGURE 2
EG extract (EEEG) alleviates fibrotic remodeling and histopathologic changes of cardiac tissue in response to MI. **(A)** Representative Masson's trichrome-stained sections of vehicle and EEEG (10 g/kg) treated rat hearts 4 weeks after MI or sham operation (scale bar, 50 μm). **(B)** Masson's trichrome staining quantified the total cardiac fibrotic area using sections of vehicle and EEEG (10 g/kg) treated rat hearts at 4 weeks after MI or sham operation (n = 8). **(C)** Representative H&E staining of heart tissue 4 weeks after MI or sham operation from rats treated with vehicle or EEEG (10 g/kg) (scale bar, 50 μm). Data are mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

staining was used to examine the infiltration of $CD45^+$ leukocytes and $CD68^+$ macrophages in the myocardium. LAD ligation markedly upregulated the number of infiltrated $CD45^+$ leukocytes and $CD68^+$ macrophages in hearts obtained from vehicle-treated MI rats compared to those from sham-operated rats (**Figures 3A, B**). However, the infiltration of these immune cells was significantly reduced by EEEG administration in comparison with the vehicle controls at 4 weeks after MI (**Figures 3A, B**). The extent of cardiac inflammation was also investigated by examining the expression of pro-inflammatory mediators. Quantitative RT-PCR analysis showed that MI surgery significantly increased the expression of cell adhesion molecule *Icam-1* and pro-inflammatory cytokines *Il-6*, *Tnf*-α, and *Il-1*β. These effects were primarily reversed by EEEG treatment (**Figure 3C**). Taken together, these findings demonstrate that EEEG exerts an anti-inflammatory property which may serve as the underlying mechanism to protect against MI injury. In addition, we also observed that EEEG treatment induced a higher percentage of ECs in the infarcted hearts, as indicated by immunohistochemical analysis of CD31 positivity 4 weeks after MI. This exciting finding suggested that EEEG promoted neovascularization and wound healing (**Figures 3D, E**), which is worth further study.

EEEG attenuates endothelial pro-inflammatory activation through modulation of NF-κB and MAPK signaling

EC activation and inflammation are critical elements in the pathogenesis of ischemic heart failure (25). Upon stimulation by pathogenic mediators, endothelial pro-inflammatory cascades are triggered, including intensified adhesive interaction with circulating leukocytes and upregulated production of pro-inflammatory cytokines, which may contribute to ischemic myocardial injury when dysregulated (8). We then tested whether the anti-inflammatory effect of EEEG *in vivo* could be attributed to the attenuation of endothelial pro-inflammatory activation. Thus, TNF-α-stimulated HUVECs were used as a model of endothelial inflammation. First, we examined HUVEC viability by using an MTT assay. Our result showed that EEEG treatment alone did not affect the viability of HUVECs (**Figure 4A**). Interestingly, a time-dependent and dose-dependent increase in cell proliferation was observed in EEEG-treated HUVECs (**Figure 4B** and **Supplementary Figure 5**). Monocyte adhesion mediated by increased expression of endothelial adhesion molecules is a hallmark of EC pro-inflammatory activation (8). We, therefore,

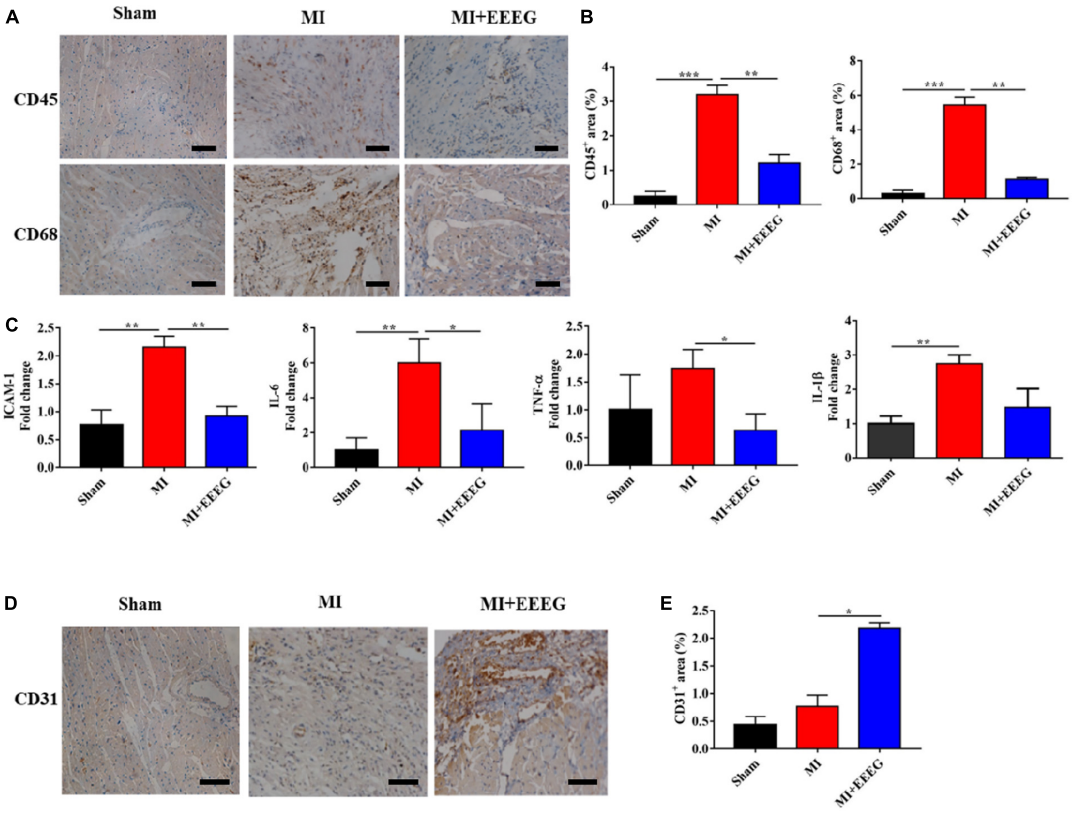

FIGURE 3
EG extract (EEEG) attenuates myocardial inflammation after MI. **(A)** Representative immunohistochemical staining of CD45 and CD68 in the heart tissue from vehicle and EEEG (10 g/kg) treated rats at 4 weeks after MI or sham operation (scale bar, 50 μm). **(B)** According to immunohistochemical staining, $CD45^+$ and $CD68^+$ positive areas were quantified using hearts 4 weeks after MI or sham operation from vehicle and EEEG (10 g/kg) treated rats (n = 8). **(C)** qRT-PCR analysis of *Icam-1*, *Il-6*, *Tnf-α*, and *Il-1β* was performed using mRNA isolated from vehicle and EEEG (10 g/kg) treated rat hearts at 4 weeks after MI or sham operation (n = 8). **(D)** Immunohistochemical analyses of CD31 in the cardiac tissue at 4 weeks after MI or sham operation (scale bar, 50 μm). **(E)** Quantification of $CD31^+$ positive area in vehicle and EEEG (10 g/kg) treated rat hearts 4 weeks after MI or sham operation (n = 8). Data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

investigated whether EEEG could suppress THP-1 cell adhesion to TNF-α-stimulated HUVECs. As shown in **Figures 4C, D**, after TNF-α stimulation, monocytes' firm adhesion was significantly increased as compared with negative controls. However, pretreatment of HUVECs with EEEG marked a decrease in monocyte adhesion to TNF-α-activated HUVECs (**Figures 4C, D**). Since EEEG could suppress TNF-α-induced cell adhesion, we analyzed whether EEEG could inhibit the expression level of adhesion molecules and pro-inflammatory cytokines in HUVECs in response to TNF-α. As expected, TNF-α stimulation significantly upregulated the gene expression of adhesion molecule *Vcam-1* as well as pro-inflammatory cytokines *Tnf-α*, *Il-1β*, and *Il-6* (**Figure 4E**). EEEG markedly attenuated the effect of TNF-α-induced expression of *Vcam-1*, *Tnf-α*, *Il-6*, and to a lesser extent *Il-1β* in HUVECs (**Figure 4E**).

NF-κB and MAPKs are the major signaling molecules that regulate endothelial inflammatory cascades (8, 12). To further investigate the mechanisms by which EEEG attenuates endothelial pro-inflammatory activation, we examined the effects of EEEG on activating these signaling pathways in HUVECs. In response to TNF-α stimulation, NF-κB p65 showed increased phosphorylation within 10 min. At the same time, EEEG-treated HUVECs exhibited a pronounced decrease in the phosphorylation of NF-κB p65 compared with vehicle controls (**Figures 5A, B**). Besides, VEGF-A is implicated in the induction of inflammatory response in addition to its well-known pro-angiogenic role (26, 27). We thus also used VEGF-A-stimulated HUVECs as a second model of endothelial inflammation besides TNF-α stimulation. Upon treatment of EEEG, the phosphorylation of ERK and p38 MAPK induced by VEGF-A was significantly reduced (**Figures 5C, D**). These data indicate that EEEG may attenuate endothelial pro-inflammatory activation by suppressing the activation of NF-κB and MAPK signaling pathways. To further validate our hypothesis, we investigated the effect of inhibiting NF-κB, ERK, and p38 MAPK signaling in TNF-α-induced HUVECs with their respective specific pharmacological inhibitors BAY11-7082, SD98059, and SB203580. We compared the expression of pro-inflammatory

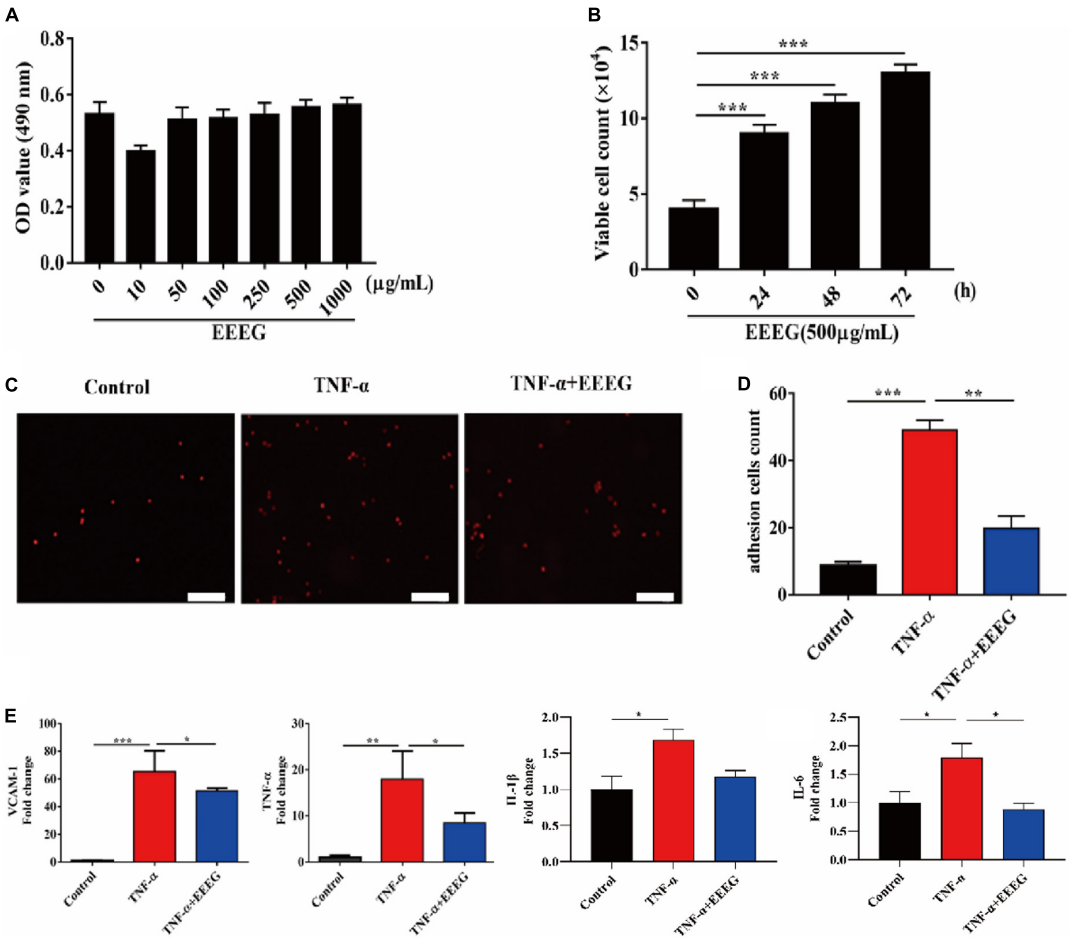

FIGURE 4
EG extract (EEEG) suppresses pro-inflammatory activation of HUVECs. **(A)** HUVECs were treated with different doses of EEEG (0, 10, 50, 100, 250, 500, and 1,000 μg/ml) for 24 h, and cell viability was determined by MTT assay (n = 5). **(B)** HUVECs were incubated with EEEG (500 μg/mL) for 0, 24, 48, and 72 h, and then cell proliferation was examined by direct cell counting (n = 3). **(C,D)** Representative images of adhesion of fluorescence-labeled THP-1 cells to HUVECs pretreated with or without EEEG (500 μg/mL) for 24 h followed by stimulation with or without TNF-α (10 ng/ml) for 4 h **(C)** (scale bar, 50 μm), and quantification of the adherent THP-1 cells (n = 3) **(D)**. **(E)** Gene expression levels of *Vcam-1*, *Tnf-α*, *Il-1β*, and *Il-6* in HUVECs pretreated with or without EEEG (500 μg/mL) for 24 h followed by stimulation with or without TNF-α (10 ng/ml) for 4 h (n = 3). Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

mediators *Tnf*-α, *Il-1*β, and *Il-6* in HUVECs at baseline, with TNF-α stimulation in the presence of EEEG, treatment with pharmacological inhibitors, or both. The expression of pro-inflammatory mediators *Tnf*-α, *Il-6* and to a lesser extent *Il-1*β, was markedly decreased in the presence of EEEG, BAY11-7082 or SB203580 compared to TNF-α-treated cells (**Figures 5E, F**). Notably, co-treatment of EEEG with BAY11-7082 or SB203580 showed that EEEG exerted no effect in further attenuating the expression of *Tnf*-α, *Il-1*β, and *Il-6* as compared with BAY11-7082 or SB203580 treatment alone, indicating no synergistic or additive effect on the inhibition of endothelial pro-inflammatory activation (**Figures 5E, F**). In contrast, co-treatment of EEEG with SD98059 exhibited a synergistic effect on the reduction of *Il-1*β expression level (**Supplementary Figure 6**). These data suggest that EEEG suppresses endothelial pro-inflammatory activation, at least in part, through NF-κB and p38 MAPK, but not ERK signaling pathway. Together with our *in vivo* findings, it is conceivable that inhibition of NF-κB- and p38 MAPK-mediated endothelial activation by EEEG contributes to the attenuated cardiac inflammatory response following MI and, thus, protects against MI-induced myocardial injury.

Discussion

This study used a preclinical rat model of permanent LAD ligation-induced MI to explore whether EG has potential pharmacological effects on an ischemic cardiac injury. EEEG, the 30% ethanol fraction of EG extract, significantly attenuated myocardial infarct size, improved cardiac function,

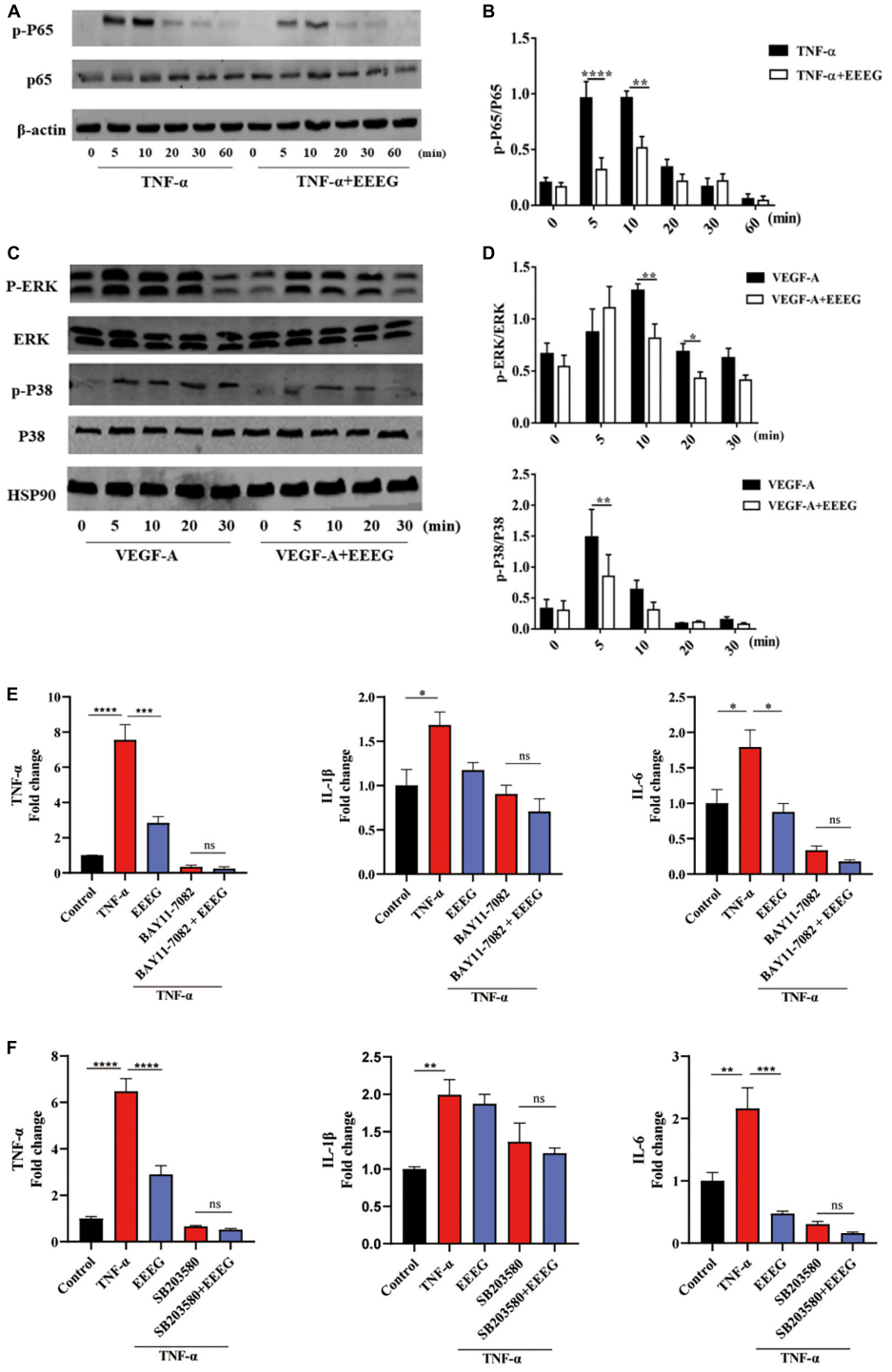

FIGURE 5
EG extract (EEEG) inhibits endothelial pro-inflammatory activation by targeting NF-κB and p38 MAPK signaling pathways. **(A,B)** Western blot analysis of the phosphorylation of p65 in HUVECs pretreated with or without EEEG (500 μg/mL) for 24 h followed by TNF-α (10 ng/ml) stimulation for 0, 5 10, 20, 30, and 60 min **(A)** and quantification of phosphorylated p65 normalized to total p65 (n = 3) **(B)**. **(C,D)** Western blot analysis of the phosphorylation of ERK and p38 MAPK in HUVECs pretreated with or without EEEG (500 μg/mL) for 24 h followed by stimulation with VEGF-A (150 ng/ml) for 0, 5 10, 20, and 30 min **(C)**; quantification of phosphorylated ERK and p38 MAPK normalized to total ERK and p38 MAPK (n = 3) **(D)**. **(E)** Gene expression levels of *Tnf-α*, *Il-1β*, and *Il-6* in HUVECs pretreated with or without EEEG (500 μg/mL) while in the presence or absence of NF-κB inhibitor BAY11-7082 (5 μM) for 24 h, followed by stimulation with or without TNF-α (10 ng/ml) for 4 h (n = 3). **(F)** Gene expression levels of *Tnf-α*, *Il-1β*, and *Il-6* in HUVECs pretreated with or without EEEG (500 μg/mL) while in the presence or absence of p38 MAPK inhibitor SB203580 (20 μM) for 24 h, followed by stimulation with or without TNF-α (10 ng/ml) for 4 h (n = 3). Data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

prevented adverse cardiac remodeling, and decreased cardiac inflammation after MI in rats. Mechanistically, we demonstrated that EEEG suppressed endothelial pro-inflammatory activation by inhibiting NF-κB, ERK, and p38 MAPK signaling pathways, which may subsequently counteract inflammatory damage to the ischemic heart. Our data provided the cellular and molecular basis through which EG exerts a cardioprotective effect in response to MI.

Experimental and clinical evidence has indicated a crucial role of inflammation in the pathogenesis of IHD (28). The recent immuno-suppressive Colchicine Cardiovascular Outcomes Trial (COLCOT) has shown encouraging results among patients with MI and strongly supported the inflammatory hypothesis of the pathogenesis of coronary artery disease (9, 10). Therefore, inflammation is now recognized as a treatment target for acute ischemic cardiovascular events (29). EG is a traditional Tibetan folk medicine that has long been widely used for health care. However, the direct pharmacological action of EG on IHD has yet to be investigated. The current study indicated that EEEG protects against MI injury induced by permanent LAD ligation (**Figures 1**, **2**). The therapeutic benefit of EEEG lies in its ability to suppress cardiac inflammatory responses, as demonstrated by decreased infiltration of $CD45^+$ leukocytes and $CD68^+$ macrophages and reduced expression of pro-inflammatory mediators in the infarcted myocardium (**Figures 3A–C**). Significantly, attenuated myocardial inflammation mediated by EEEG facilitated the cardiac reparative process, characterized by increased $CD31^+$ ECs after MI (**Figures 3D, E**). These findings suggest that EEEG may promote neovascularization and post-infarct healing, which is worth further investigation.

Ischemia leads to cardiac tissue damage and necrosis following MI, which initiates an acute pro-inflammatory response. Among cellular effectors of the inflammatory reaction in the ischemic heart, ECs play a fundamental role (8, 11). Danger signals released by dying cardiomyocytes induce rapid endothelial activation, manifesting upregulated expression of endothelial adhesion molecules as well as pro-inflammatory cytokines and chemokines, thereby promoting immune cell infiltration and complicating inflammation in the infarcted heart (30). In this study, using the TNF-α-stimulated HUVECs model of endothelial inflammation, we showed that EEEG significantly decreased the expression of adhesion molecule *Vcam-1* and pro-inflammatory cytokines *Tnf*-α, *Il-6*, and to a lesser extent, *Il-1*β, and inhibited monocyte-EC adhesion (**Figures 4C–E**). These results strongly indicate the inhibitory role of EEEG in endothelial pro-inflammatory activation. Since EEEG attenuated the infiltration of CD45+ leukocytes and CD68+ macrophages and reduced the production of inflammatory mediators in the ischemic myocardium *in vivo* (**Figures 3A–C**), it is reasoned that EEEG protects against MI-induced cardiac inflammation through a mechanism involving attenuation of endothelial pro-inflammatory activation.

NF-κB is a central intracellular signaling molecule involved in the induction of inflammatory responses (8). It has been known that the production of endothelial adhesion molecules and inflammatory cytokines is under the tight control of NF-κB (31). Upon inflammatory stimulation, NF-κB p65 becomes activated and binds to the promoter region of pro-inflammatory genes, triggering a series of inflammatory responses (31). Additionally, ERK and p38 MAPK also participate in response to inflammatory stimulation (32). Blockade of MAPK signaling activity suppresses endothelial pro-inflammatory activation, leading to the improvement of cellular damage (33). In the current study, we showed that the levels of phosphorylation of NF-κB p65, ERK, and p38 MAPK were markedly decreased in TNF-α- or VEGF-A-induced HUVECs as a result of EEEG pretreatment (**Figures 5A–D**).

Further mechanistic studies using pharmacological inhibitors demonstrated that EEEG specifically targets NF-κB and p38 MAPK signaling to suppress endothelial pro-inflammatory activation (**Figures 5E, F**). Moreover, previous studies have evidenced that both ERK and p38 MAPK positively regulates NF-κB activity in activated HUVECs, and inhibition of these MAPKs decreases NF-κB activation and immune disorders (34, 35). In this regard, it is reasoned that the reduced ERK and p38 MAPK activation after EEEG pretreatment in this study may also be involved in preventing NF-κB activation, eventually resulting in attenuated adhesion molecules and pro-inflammatory cytokine gene expression.

Although our data suggest that EEEG attenuates endothelial pro-inflammatory activation, we cannot exclude its pharmacological action on other cell types may also contribute to the reduced inflammatory response post-MI. Besides ECs, it has been shown that cardiomyocytes, neutrophils, monocytes/macrophages, mast cells, lymphocytes, and fibroblasts are critical cellular effectors of the inflammatory response after MI (8, 36). Thus, the role of EEEG in these cells in the context of MI-induced inflammation is worthy of further investigation. Furthermore, it has been reported that myocardial angiogenesis plays an essential role in cardiac repair and tissue remodeling. After MI, an adequately coordinated angiogenic response can be boosted to reduce scarring and adverse LV remodeling (37, 38). In this study, we observed that $CD31^+$ cells were significantly increased in the hearts of MI rats treated with EEEG (**Figures 3D, E**). Consistently, HUVEC proliferation was also markedly accelerated by EEEG treatment *in vitro* (**Figure 4B** and **Supplementary Figure 5**). These observations imply that EEEG may promote myocardial angiogenesis and the post-infarct healing process, which potentially contributes to the protective effects of EEEG on MI injury. The primary regulators of angiogenesis include a series of signaling pathways such as PI3K/Akt, MAPK, Notch, JAK/STAT, Wnt/β-catenin, Hippo, and Sonic hedgehog (39). Previous studies have demonstrated that MAPK activation promotes angiogenesis (40, 41). In contrast, our data showed that EEEG attenuated

ERK and p38 MAPK signaling in VEGF-A-induced HUVECs (**Figures 5C, D**), suggesting that the altered MAPK activity may not be responsible for EEEG-induced endothelial proliferation and angiogenesis. Thus, further studies are warranted to substantiate the role of EEEG in angiogenesis and explore the underlying molecular mechanism.

In summary, for the first time, the present study provides evidence that EEEG protects against ischemic cardiac injury and attenuates myocardial inflammation in response to MI *in vivo*. We propose a model in which EEEG inhibits NF-κB, ERK, and p38 MAPK signaling pathways, thereby suppressing endothelial pro-inflammatory activation and cardiac inflammatory response in the hearts following MI-induced injury. Our study provides an experimental basis for understanding the protective role of EG in MI and sheds light on the clinical use of EG in managing ischemic cardiovascular disease.

Data availability statement

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

Ethics statement

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Jiangxi University of Chinese Medicine.

Author contributions

CZ and JY designed the study and coordinated all experimental work. DW, LT, XL, YZ, LS, SZ, and CZ conducted the experiments and collected the data. DW, LT, XL, YZ, LS, SZ, AT, MQ, LQ, CZ, and JY analyzed and interpreted the data. DW, LT, CZ, and JY wrote the manuscript with valuable input from all other authors. All authors approved the submitted 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1013013/full#supplementary-material

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Bifurcation strategies using second-generation drug-eluting stents on clinical outcomes in diabetic patients

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Background: Diabetes mellitus (DM) is a critical risk factor for the pathogenesis and progression of coronary artery disease, with a higher prevalence of complex coronary artery disease, including bifurcation lesions. This study aimed to elucidate the optimal stenting strategy for coronary bifurcation lesions in patients with DM.

Methods: A total of 905 patients with DM and bifurcation lesions treated with second-generation drug-eluting stents (DES) from a multicenter retrospective

patient cohort were analyzed. The primary outcome was the 5-year incidence of target lesion failure (TLF), which was defined as a composite of cardiac death, target vessel myocardial infarction, and target lesion revascularization.

Results: Among all patients with DM with significant bifurcation lesions, 729 (80.6%) and 176 (19.4%) were treated with one- and two-stent strategies, respectively. TLF incidence differed according to the stenting strategy during the mean follow-up of 42 ± 20 months. Among the stent strategies, T- and V-stents were associated with a higher TLF incidence than one-stent strategy (24.0 vs. 7.3%, $p < 0.001$), whereas no difference was observed in TLF between the one-stent strategy and crush or culotte technique (7.3 vs. 5.9%, $p = 0.645$). The T- or V-stent technique was an independent predictor of TLF in multivariate analysis (hazard ratio, 3.592; 95% confidence interval, 2.117–6.095; $p < 0.001$). Chronic kidney disease, reduced left ventricular ejection fraction, and left main bifurcation were independent predictors of TLF in patients with DM.

Conclusion: T- or V-stenting in patients with DM resulted in increased cardiovascular events after second-generation DES implantation.

Clinical trial registration: https://clinicaltrials.gov/ct2/show/NCT03068494?term=03068494&draw=2&rank=1, identifier: NCT03068494.

KEYWORDS

coronary bifurcation angioplasty, diabetes mellitus, stent strategy, second-generation drug-eluting stent, clinical outcome, percutaneous coronary intervention (complex PCI)

1. Introduction

Diabetes mellitus (DM) is an independent predictor of long-term death, myocardial infarction, and revascularization in patients undergoing percutaneous coronary intervention (PCI) (1–3). This may be due to impaired endothelial function caused by DM, which promotes a pro-inflammatory vasoconstrictive state and prompts arterial atherothrombosis (4, 5). Thus, newer-generation drug-eluting stents (DES) are recommended for patients with DM undergoing PCI rather than bare-metal stent or early-generation DESs (6). However, despite stent technology and strategy improvements, patients with DM after PCI presented poorer clinical outcomes than patients without DM (7, 8).

A new stent technology achieving high therapeutic drug concentrations in the arterial tissue using a reservoir recently presented better clinical outcomes in patients with DM after PCI (9). These results may lead to identifying DM-specific treatments. However, little is known concerning the optimal stent strategy for complex PCI cases, such as coronary bifurcation diseases associated with atherosclerosis progression and thrombosis due to higher endothelial shear stress, especially in patients with DM (10, 11). In addition, clinical outcomes of stent strategies for coronary bifurcation lesions in patients with DM using second-generation DES have not been fully elucidated.

Thus, this study aimed to investigate the impact of stenting strategies on clinical outcomes in patients with DM and coronary bifurcation lesions using second-generation DES.

2. Methods

2.1. Study population

This retrospective study cohort was based on the coronary bifurcation stent III registry (NCT03068494) and consisted of 2,648 patients treated between January 2010 and December 2014 in 21 Korean tertiary hospitals. The design and detailed description of the registry have been previously reported (12). The coronary bifurcation stent III registry is a real-world registry of second-generation DES use, and from the registry, patients with DM ($N = 905$) were included in this study. The inclusion criteria were age >19 years and main vessel (MV) diameter ≥2.5 mm and side branch (SB) diameter ≥2.3 mm, confirmed using core laboratory quantitative coronary angiography analysis. Patients who experienced cardiogenic shock or cardiopulmonary resuscitation during hospitalization, had protected left main disease, or had severe left ventricular

systolic dysfunction (ejection fraction <30%) were excluded from the registry. The institutional review board of each hospital approved the study protocol, which was conducted in accordance with the principles of the Declaration of Helsinki. Each institutional review board waived the requirement for informed consent due to the retrospective nature of the study.

2.2. Percutaneous coronary bifurcation intervention

Index PCI was performed according to the relevant standard guidelines during each procedure. Before PCI, all patients received loading doses of antiplatelet medications (aspirin 300 mg and P2Y12 inhibitors [clopidogrel 300–600 mg, prasugrel 60 mg, or ticagrelor 180 mg]) unless they had previously received antiplatelet therapy. An activated clotting time of 250–300 s was maintained during PCI using low-molecular-weight or unfractionated heparin. The PCI strategy, including stent strategy, proximal optimization technique (POT) or re-POT, access site, DES type, glycoprotein IIb/IIIa inhibitor use, and intravascular imaging or invasive physiological assessments, was based on the operator's discretion. In addition, the duration of dual antiplatelet therapy and DM medication was at the operator's discretion.

2.3. Data collection and quantitative coronary angiography analysis

Patient information, including demographics; medication; and laboratory, angiographic, and procedural data, was collected for analysis through a web-based reporting system. Follow-up clinical outcomes were obtained from electronic medical records of the outpatient clinic. For the quantitative coronary angiography (QCA) analysis, an angiographic core laboratory (Heart Vascular Stroke Institute, Samsung Medical Center, Seoul, South Korea) with a validated automated edge-detection system (Centricity CA 1000; GE, Waukesha, WI, USA) reviewed and analyzed all baseline and procedural coronary angiograms. QCA analysis was performed pre- and post-procedure, bifurcation angle (the angle between the distal MV and the SB at its origin, measured using the angiographic projection with the widest separation of both branches), minimum lumen diameter, reference vessel diameter, and lesion length for each vessel were measured. In addition, percent diameter stenosis (100 × [reference vessel diameter/minimum lumen diameter]/reference vessel diameter) for each vessel was determined.

2.4. Primary and secondary outcomes

The primary outcome was the 5-year incidence of target lesion failure (TLF), defined as the composite of cardiac death, target vessel myocardial infarction (TVMI), and target lesion revascularization (TLR). The secondary outcomes were the individual components of the primary outcome. An independent clinical event adjudication committee composed of independent interventional cardiology experts who had not participated in patient enrollment verified all the clinical events. Deaths were of cardiac cause unless an undisputed non-cardiac cause could be established. TVMI was myocardial infarction with evidence of an elevated creatine kinase-myocardial band or a troponin level higher than the standard upper limit with concomitant ischemic symptoms or electrocardiography findings indicative of ischemia in the vascular territory of the previously treated target vessels. TLR was repeat PCI of the lesion within 5 mm of the stent deployment.

2.5. Statistical analysis

Continuous variables are presented as mean ± standard deviation and were compared using the Student's *t*-test for parametric data and the Mann–Whitney test for non-parametric data. Categorical variables are presented as numbers (percentages) and were compared using the Chi-squared test or Fisher's exact test. The cumulative incidences of clinical events are presented as Kaplan–Meier estimates and compared using a log-rank test. Patients were censored at 5 years (1,825 days) or when events occurred. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using the Cox proportional hazards models. In multivariable models, variables with p-values <0.10 in the univariate analysis were included in the multivariate analysis using backward elimination and multivariable Cox regression to determine the independent predictors of clinical events. After univariate analysis, adjusted HR was obtained from Cox regression based on taking insulin for DM, chronic kidney disease, preserved left ventricular ejection fraction (LVEF; ≥50%), left main (LM) bifurcation, stent strategy, post-procedural distal minimal lumen diameter of the SB, and final kissing balloon (FKB) inflation. All probability values were two-sided, and p-values <0.05 were significant. Propensity scores were estimated using a non-parsimonious multiple logistic regression model for stent strategy. Age, sex, initial presentation, hypertension, taking insulin for DM, dyslipidemia, current smoking status, chronic kidney disease, previous myocardial infarction, previous percutaneous coronary intervention, left ventricular ejection fraction <50%, transradial approach, use of intravascular ultrasound, left main bifurcation, and true bifurcation were selected to estimate the propensity score. A local optimal algorithm using the caliper method was used to develop propensity score-matched pairs without

replacement (2:1 matching). To ensure that poorly fitting matches were excluded, a matching caliper of 0.2 SDs from the estimated propensity score logit was enforced using the MatchIt package in R Core Team (2015). R: Language and environment for statistical computing (version 3.6.0, R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/). SPSS version 25.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the results.

3. Results

3.1. Patients' baseline characteristics

A total of 905 patients with DM and significant bifurcation lesions were enrolled in the study: 729 (80.6%) were treated with a one-stent strategy, and 176 (19.4%) were treated with a two-stent strategy. The baseline clinical and procedural characteristics of all patients with DM according to the stenting strategy are presented in Table 1. There was no significant difference between the two-stent and one-stent groups except in the prevalence of insulin use (14.2 vs. 8.2%, $p = 0.022$). The two-stent group had a higher prevalence of intravascular ultrasound use (54.5 vs. 35.8%, $p < 0.001$), LM bifurcation (53.4 vs. 35.9%, $p < 0.001$), true bifurcation lesions (78.4 vs. 38.8%, $p < 0.001$), and FKB inflation (87.5 vs. 17.3%, $p < 0.001$) than the one-stent group. The transradial approach was used less often in the two-stent group than in the one-stent group (38.1 vs. 61.2%, $p < 0.001$). The QCA results are presented in Supplementary Table 1.

3.2. Outcomes and TLF predictors

The cumulative TLF incidence was higher in patients treated with two- than one-stent strategy (13.6 vs. 7.3%, $p = 0.005$; Figure 1). Notably, at a mean follow-up of 42 ± 20 months, there was a significant difference in TLF incidence based on the stenting strategy (Supplementary Figure 1; p for trend <0.001), and the T- or V-stent strategy demonstrated a higher TLF incidence than the one-stent strategy (24.0 vs. 7.3%, $p < 0.001$). However, the crush or culotte technique and the one-stent strategy presented similar outcomes (5.9 vs. 7.3%, $p = 0.645$; Figure 2). In the Cox multivariate analysis, the T- or V-stent technique remained significantly associated with TLF (HR, 3.349; 95% CI, 1.960–5.721; $p < 0.001$), mainly driven by TLR (HR, 4.688; 95% CI, 2.478–8.869; $p < 0.001$), with no significant differences in cardiac death or TVMI. Meanwhile, the crush and culotte techniques did not have significantly different clinical outcomes (Figure 3). In addition, chronic kidney disease (CKD; HR, 3.071; 95% CI, 1.728–5.456; $p < 0.001$), reduced LVEF (HR, 2.436; 95% CI, 1.478–4.017; $p < 0.001$), and LM bifurcation (HR, 2.030; 95% CI, 1.290–3.195; $p = 0.002$) during follow-up were independent predictors of TLF in patients with DM after multivariate adjustment (Table 2). After propensity score matching (Supplementary Table 2), although there was no TLF difference between the one- and two-stent groups (11.8 vs. 8.9%, $p = 0.334$; Supplementary Figure 2A), the T- or V-stent technique had a higher TLF incidence than the others (Supplementary Figure 2B; p for trend = 0.025). In addition, the T- or V-stent technique remained significantly associated with TLF (HR, 2.269; 95% CI, 1.123–4.584; $p = 0.022$) in the propensity score matching.

4. Discussion

This study investigated the impact of stenting strategies on the clinical outcomes of patients with DM who were treated for coronary bifurcation lesions. This study revealed that the one-stent strategy for coronary bifurcation lesions presented a lower TLF incidence than the two-stent strategy, in patients with DM. The T- or V-stent technique also revealed a higher TLF incidence, mainly driven by TLR, than the one-stent strategy and crush or culotte technique. Furthermore, except for the T- or V-stent techniques, there was no difference in TLF incidence between the one- and two-stent strategies. Additionally, using the T- or V-stent technique in patients with DM undergoing PCI for coronary bifurcation lesions was an independent predictor of TLF in the multivariate analysis. CKD, reduced LVEF, and left main bifurcation lesions were independent TLF predictors.

Patients with DM are at high risk of progressive atherosclerosis regarding coronary plaque rupture and neointimal proliferation, which leads to an increased incidence of adverse clinical outcomes (4, 13). Moreover, PCI with coronary bifurcation lesions presented a higher incidence of MI, thrombosis, and revascularization than PCI with simple coronary lesions (14). A plausible explanation for the adverse prognosis after coronary bifurcation PCI is the unique local flow pattern that increases the endothelial shear stress and affects plaque development. According to our previous study, the 5-year TLF incidence was 7.8% in patients with and without DM who underwent PCI for coronary bifurcation lesions (15). The 5-year incidence rates of TLF for the one- and two-stent strategies were 7.6 and 12.1%, respectively. In this study, the 5-year incidence rate of TLF in patients with DM was 8.5%. This difference in TLF rate between the total and DM populations was primarily due to the different events in the two-stent strategy (12.1 vs. 13.6%, respectively).

To date, no randomized controlled trial or large-scale observational study has reported the clinical outcomes of coronary bifurcation lesions in patients with DM regarding stent strategy in the second-generation drug-eluting stent era. In this study, the T- or T and small protrusion (TAP) technique and V- or simultaneous kissing stenting revealed a higher TLF incidence than the other techniques and were independent predictors of TLF in multivariable analysis. To our knowledge, this is the

TABLE 1 Patients' baseline clinical and procedural characteristics.

	Total ($N = 905$)	One-stent ($n = 729$)	Two-stent ($n = 176$)	*P*-value
Age, years	65.3 ± 10.0	65.0 ± 10.2	66.2 ± 9.4	0.147
Male sex	653 (72.2%)	533 (73.1%)	120 (68.2%)	0.224
Initial presentation				0.861
Stable angina	370 (40.9%)	295 (40.5%)	75 (42.6%)	
NSTE-ACS	458 (50.6%)	371 (50.9%)	87 (49.4%)	
STEMI	77 (8.5%)	63 (8.6%)	14 (8.0%)	
Hypertension	592 (65.4%)	481 (66.0%)	111 (63.1%)	0.522
DM taking insulin	85 (9.4%)	60 (8.2%)	25 (14.2%)	0.022
Dyslipidemia	362 (40.0%)	297 (40.7%)	65 (36.9%)	0.401
Current smoking status	241 (26.6%)	199 (27.3%)	42 (23.9%)	0.407
CKD	72 (8.0%)	56 (7.7%)	16 (9.1%)	0.642
Previous MI	43 (4.8%)	34 (4.7%)	9 (5.1%)	0.957
Previous PCI	145 (16.0%)	113 (15.5%)	32 (18.2%)	0.450
LVEF <50%	138 (15.2%)	105 (14.4%)	33 (18.8%)	0.186
Transradial approach	513 (56.7%)	446 (61.2%)	67 (38.1%)	< 0.001
IVUS use	357 (39.4%)	261 (35.8%)	96 (54.5%)	< 0.001
LM bifurcation	356 (39.3%)	262 (35.9%)	94 (53.4%)	< 0.001
True bifurcation	421 (46.5%)	283 (38.8%)	138 (78.4%)	< 0.001
Stent strategy				
Simple crossover	562 (62.1%)	562 (77.1%)	-	
One-stent with SB balloon	167 (18.5%)	167 (22.9%)	-	
Crush	81 (9.0%)	-	81 (46.0%)	
T (or TAP)	55 (6.1%)	-	55 (31.2%)	
Culotte	16 (1.8%)	-	16 (9.1%)	
V (or kissing)	20 (2.2%)	-	20 (11.4%)	
Other	4 (0.4%)	-	4 (2.3%)	
DES type				0.287
Everolimus eluting stent	446 (49.3%)	348 (47.7%)	98 (55.7%)	
Zotarolimus eluting stent	249 (27.5%)	207 (28.4%)	42 (23.9%)	
Biolimus eluting stent	163 (18.0%)	134 (18.4%)	29 (16.5%)	
Others	47 (5.2%)	40 (5.5%)	7 (4.0%)	
Kissing balloon inflation	280 (30.9%)	126 (17.3%)	154 (87.5%)	< 0.001
Proximal optimization technique	283 (31.3%)	228 (31.3%)	55 (31.2%)	>0.999
MV success	899 (99.3%)	723 (99.2%)	176 (100.0%)	0.490
SB success	649 (71.7%)	476 (65.3%)	173 (98.3%)	< 0.001

CKD, chronic kidney disease; DM, diabetes mellitus; IVUS, intravascular ultrasound; LM, left main; LVEF, left ventricular ejection fraction; MI, myocardial infarction; MV, main vessel; NSTE-ACS, non-ST elevation–acute coronary syndrome; PCI, percutaneous intervention; SB, side branch; STEMI, ST-segment elevation myocardial infarction; TAP, T, and small protrusions.

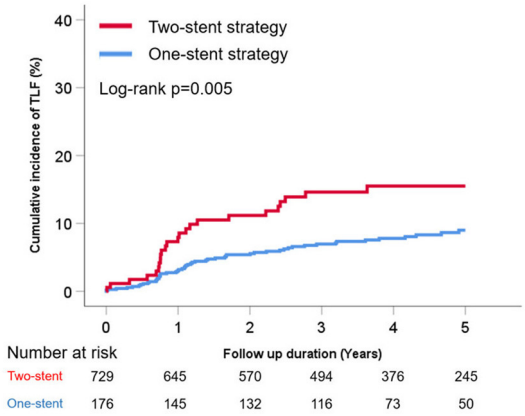

FIGURE 1
Cumulative incidence of target lesion failure (TLF) of one- vs. two-stent strategy for treating coronary bifurcation lesions in patients with diabetes mellitus.

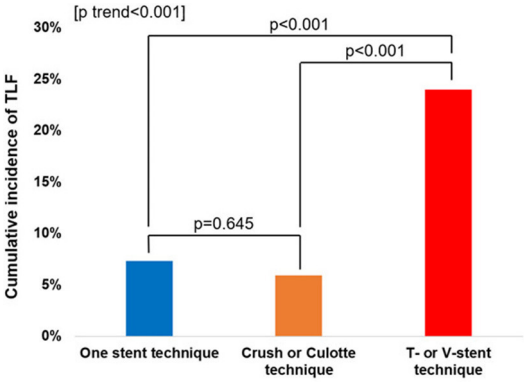

FIGURE 2
Incidence of target lesion failure (TLF) for one-stent, T-stent, V-stent, and crush or culotte techniques.

first study to reveal that different stent strategies in the two-stent technique may be associated with clinical outcomes in patients with coronary bifurcation lesions. Consistent with this study, a meta-analysis of various randomized controlled trials on coronary bifurcation stent strategies reported that the TLR rate of the T-stent or TAP technique was higher than that of other techniques (16). In the DEFINITION II trial, which included 35% of patients with DM, the double kissing crush technique had a 1-year TLR rate, superior to that of the provisional stent group (14.5 %) using the T-stent or TAP technique for bailout stenting (17).

A possible explanation for these results is the association between DM and shear stress at the bifurcation level (18). The T-stent technique is commonly used when the SB is compromised during provisional stenting; however, it has the inherent risk of suboptimal SB ostium coverage, which may lead to restenosis (19). The V-stent technique has a similar limitation regarding the suboptimal coverage of the SB ostium. The TAP stent technique has a lower risk of missing the SB ostium (19); however, it creates a metallic neocarina, where a significant portion of the unappositioned stent remains in the vessel. In addition, the post-procedural minimal lumen diameter of the main branch ostium was an independent TLF predictor. Theoretically, metallic neocarina may risk narrowing the main branch ostium. A recent study suggested obtaining the maximal diameter of the main vessel stent in PCI for a non-left main bifurcation lesion (12). In our previous study, DM was an independent TLF predictor in non-LM lesions but not in LM bifurcation lesions (12). DM is a potential factor in atherosclerosis progression and neointimal proliferation (4) and a vasoconstrictive endothelial response along with an inflammatory and prothrombotic milieu in DM. A synergistic relationship with shear stress at the bifurcation level may lead to a worse prognosis (13, 18, 20). In this study's subgroup analysis, except for the T-stent, TAP stent, and V-stent techniques, there was no significant difference in the TLF between the one- and two-stent strategies. These observations suggest that accurate stent deployment and optimization of the bifurcation carina site could reduce TLF incidence, especially in the very high-risk population with DM and bifurcation CAD (19).

In addition, CKD, reduced LVEF, and LM bifurcation were independent TLF predictors in the diabetic population. CKD is associated with a poor prognosis due to its strong correlation with various risk factors, such as hypertension, DM, and dyslipidemia, which could be a cause or consequence (21). In addition, DM is an extremely high-risk factor for patients referred for treatment of LM bifurcation. Our study also emphasizes that PCI for LM bifurcation is an independent TLF predictor in patients with DM, even in the second-generation DES era, consistent with that reported in previous studies (12, 15, 22, 23).

This study had several limitations. First, its inherent limitation is the nature of the observational registry. The multivariate adjustment was performed; nonetheless, potential bias due to unmeasured variables or confounding factors, such as body mass index, serum glucose level, and mean blood pressure, could not be excluded. Second, the range of baseline and follow-up glycemic levels of these patients could have influenced our results, and insulin use revealed a borderline significant risk of TLF (adjusted HR, 1.811; $p = 0.061$), which suggests that poor DM control may affect clinical outcomes, highlighting the need for further research. Finally, treatment strategy, intravascular imaging, stent type, and concomitant medication use were based on the physician's preferences. In this context, FKB inflation was relatively poorly performed in patients treated with the two-stent strategy, and the rate of POT was relatively low. However, our study has analyzed the largest real-world PCI dataset for bifurcation lesions in patients with DM.

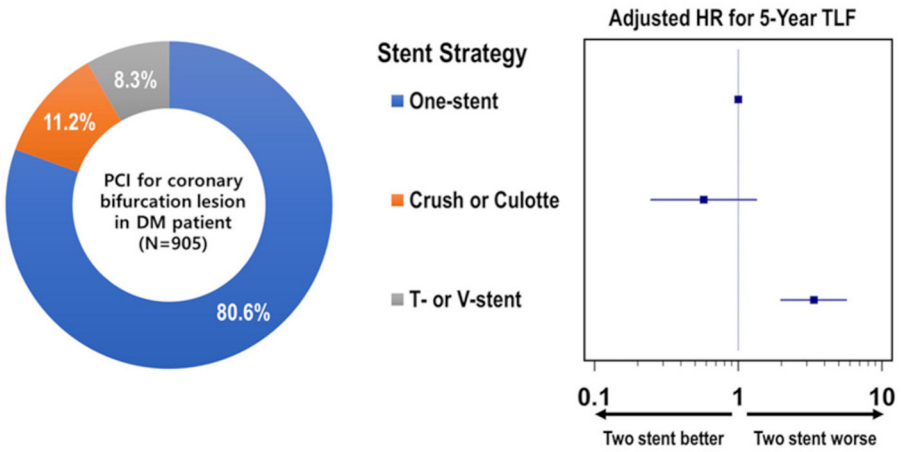

FIGURE 3
Distribution and individual risk of stent strategy for coronary bifurcation lesions in patients with diabetes mellitus.

TABLE 2 Independent predictors of 5-year target lesion failure in DM patients treated for coronary bifurcation lesion with stent implantation.

All patients with DM ($N = 905$)	Crude HR	Final model, stepwise backward elimination (HR and 95% CI)	*P*-value
Use of insulin	2.143	1.772 (0.951–3.301)	0.074
Chronic kidney disease	3.793	3.034 (1.710–5.385)	<0.001
Preserved LVEF (≥50%)	0.405	0.419 (0.254–0.690)	0.001
Radial artery approach	0.633	-	-
Left main bifurcation	1.975	1.932 (1.233–3.028)	0.004
T- or V-stent technique	1.330	3.592 (2.117–6.095)	<0.001
Kissing balloon inflation	0.470	-	-
Post- procedural distal MLD of SB	0.410	-	-

CI, confidence interval; DM, diabetes mellitus; HR, hazard ratio; LVEF, left ventricular ejection fraction; MLD, minimal lumen diameter; SB, side branch.

5. Conclusion

T- or V-stenting in patients with DM showed increased cardiovascular events after second-generation DES implantation compared with one- or other two-stent strategies.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board of each hospital including Korea University Anam Hospital and conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1018802/full#supplementary-material

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Frontiers and hotspots evolution in anti-inflammatory studies for coronary heart disease: A bibliometric analysis of 1990–2022

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Background: Coronary heart disease (CHD) is characterized by forming of arterial plaques composed mainly of lipids, calcium, and inflammatory cells. These plaques narrow the lumen of the coronary artery, leading to episodic or persistent angina. Atherosclerosis is not just a lipid deposition disease but an inflammatory process with a high-specificity cellular and molecular response. Anti-inflammatory treatment for CHD is a promising therapy; several recent clinical studies (CANTOS, COCOLT, and LoDoCo2) provide therapeutic directions. However, bibliometric analysis data on anti-inflammatory conditions in CHD are lacking. This study aims to provide a comprehensive visual perspective on the anti-inflammatory research in CHD and will contribute to further research.

Materials and methods: All the data were collected from the Web of Science Core Collection (WoSCC) database. We used the Web of Science's systematic tool to analyze the year of countries/regions, organizations, publications, authors, and citations. CiteSpace and VOSviewer were used to construct visual bibliometric networks to reveal the current status and emerging hotspot trends for anti-inflammatory intervention in CHD.

Results: 5,818 papers published from 1990 to 2022 were included. The number of publications has been on the rise since 2003. Libby Peter is the most prolific author in the field. "Circulation" was ranked first in the number of journals. The United States has contributed the most to the number of publications. The Harvard University System is the most published organization. The top 5 clusters of keywords co-occurrence are inflammation, C-reactive protein, coronary heart disease, nonsteroidal anti-inflammatory, and myocardial infarction. The top 5 literature citation topics are chronic inflammatory diseases, cardiovascular risk; systematic review, statin therapy; high-density lipoprotein. In the past 2years, the strongest keyword reference burst is "Nlrp3 inflammasome," and the strongest citation burst is "Ridker PM, 2017 (95.12)."

Conclusion: This study analyzes the research hotspots, frontiers, and development trends of anti-inflammatory applications in CHD, which is of great significance for future studies.

KEYWORDS

anti-inflammatory, coronary heart disease, atherosclerosis, C-reactive protein, bibliometric analysis, inflammation

Introduction

In recent decades, inflammation has been a growing concern in atherosclerotic coronary artery disease. Meanwhile, the basic and clinical anti-inflammation studies in coronary artery disease have been widely explored (1). In 1986, Professor Russell Ross (2) explicitly stated that atherosclerosis is an inflammatory disease and excessive defensive response to injury. In 1996, Michael A Mendall (3) investigated the relationship between chronic low-grade systemic inflammation (c-reactive protein, CRP) and coronary artery disease through a cross-sectional study. The results suggest that the body's response to inflammation may affect the development of atherosclerosis in the middle-aged population. In 1999, John Danesh (4) explored the relevance of low-grade inflammatory processes to cardiovascular disease and vascular risk factors. It was concluded that hypersensitive C-reactive protein (hs-CRP) is a strong predictor of future cardiovascular events. CANTOS confirms the relationship between inflammation and coronary heart disease, and reducing inflammation reduces the risks of heart disease (5). This hypothesis has been continuously proven scientifically, from pathological studies of the blood vessel wall to epidemiological studies of circulating inflammatory factors in preliminary intervention studies. More studies are focusing on the mechanisms of anti-inflammatory action in coronary heart disease and exploring new therapeutic approaches for anti-inflammatory drugs (6–9).

Bibliometry is the cross-science of quantitatively analyzing all knowledge carriers using mathematical and statistical methods (10). Bibliometric analysis can capture literature groups' characteristics and hot trends within a topic domain (11). Therefore, a comprehensive understanding can be gained using bibliometric analysis methods, which greatly help scientific research. In recent years, bibliometric analysis has played a role in medicine with the surge of medical papers (12). However, the bibliometric analysis of inflammation in CHD is still lacking. In this paper, we conducted bibliometric research on anti-inflammation use in CHD to explore its development trends.

Materials and methods

The literature data was collected from the Web of Science Core Collection (WoSCC) database through the Science Citation Index Expanded (SCI-E) on August 9, 2022. Our search strategy was: TS = (antiinflammatory or anti-inflammatory or anti-inflammation) AND TS = (coronary heart disease or unstable angina pectoris or angina pectoris or Acute coronary syndrome or heart failure or myocardial infarct) AND PY = (1990–2022) AND LA = (English). Only articles and reviews met the requirement and were included. The literature search was conducted by two authors independently (JL Z and CY J). After data normalization, all documents, including the complete records and cited references, were exported in pure text format. All valid data were imported to VOSviewer and CiteSpace for visual analysis. We analyze the essential characteristics of the literature. Microsoft Excel 2019 was used to predict the growth trend of publications in 2022. Figure 1 shows the literature's prediction graph and the literature's screening graph.

VOSviewer is a procedure for building and viewing bibliometric maps (13). It can be used to build author, journal, or keyword maps based on co-occurrence data (14). CiteSpace focuses on analyzing the potential knowledge contained in the scientific literature (15). It can be used to visualize the comprehensive research situation over a certain period and to predict the development trend of the related field (16). CiteSpace has certain advantages in revealing the dynamic development law of the discipline and the research frontier (17). VOSviewer can be selected to draw the knowledge map in presenting the relationship between the subject themes (18).

Results

Distribution of literature

A total of 5,818 articles focus on anti-inflammatory studies in CHD. Among the most prolific authors, Libby Peter (USA) was ranked first with 28 articles, followed by Aukrust, Pal (Norway), and Ridker, Paul M (USA), with 27 and 26 articles, respectively. Anker, Stefan D (Germany) and Tousoulis, Dimitris (Greece) were ranked fourth and fifth with 22 and 20 articles, respectively. For the details of all literature, see Supplementary material 1.

In terms of publications, they have now been published in over 1,458 journals. Circulation has published 98 articles on the application of anti-inflammatory in CHD. This was followed by PloS One (84 papers), Atherosclerosis (79 papers), International Journal of Cardiology (68 papers), and Current Pharmaceutical Design (65 papers). Impact Factor (IF) is a quantitative index representing a journal's impact and general evaluation of international journals' academic level and publication quality (19). According to the latest impact factor published in 2022, Circulation had the highest impact factor of 39.918, followed by Atherosclerosis with an IF of 6.847.

The literature on anti-inflammatory intervention in CHD has been published in 103 countries and regions. The United States leads the way, with 1,708 publications, followed by China (929 publications), Italy (496 publications), Germany (397 publications), And England (394 publications).

Among the research institutions, 4,959 institutions were involved in the research field. According to the statistical analysis, Harvard University ranked first with 213 articles, followed by the University of California System (149 publications), Brigham and Women's Hospital (144 publications), University of London (124 publications), and Institut National De La Sante Et De La Recherche Medicale Inserm (111 publications). In conclusion, the top three institutions are all research institutions in the US, where Brigham and Women's Hospital are affiliated hospitals under Harvard Medical School. The US remains a leader in anti-inflammatory intervention for coronary heart disease, and Harvard University's system ranks first in various organizations' publications.

The top 20 most cited references besides the clinical guidelines are shown in Table 1. The top 3 papers were all from the relevant Harvard University team. The first paper comes from Peter Libby's review in Circulation (Inflammation and atherosclerosis) (20), published in 2002. His review of Inflammation in atherosclerosis was published in Nature in the same year (21). The total citation frequency of the two articles reached 12,576 times. It is clearly stated that inflammation is a therapeutic target in atherosclerosis. In 1997, Paul M. Ridker published in The New England Journal of Medicine (NEJM) in "Inflammation, Aspirin, and the Risk of Cardiovascular Disease in Apparently Healthy Men" (22), suggesting that anti-inflammatory drugs may have a clinical benefit in preventing cardiovascular disease. In 2017, Paul M. Ridker published "Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease" (23) in NEJM. This randomized, double-blind,

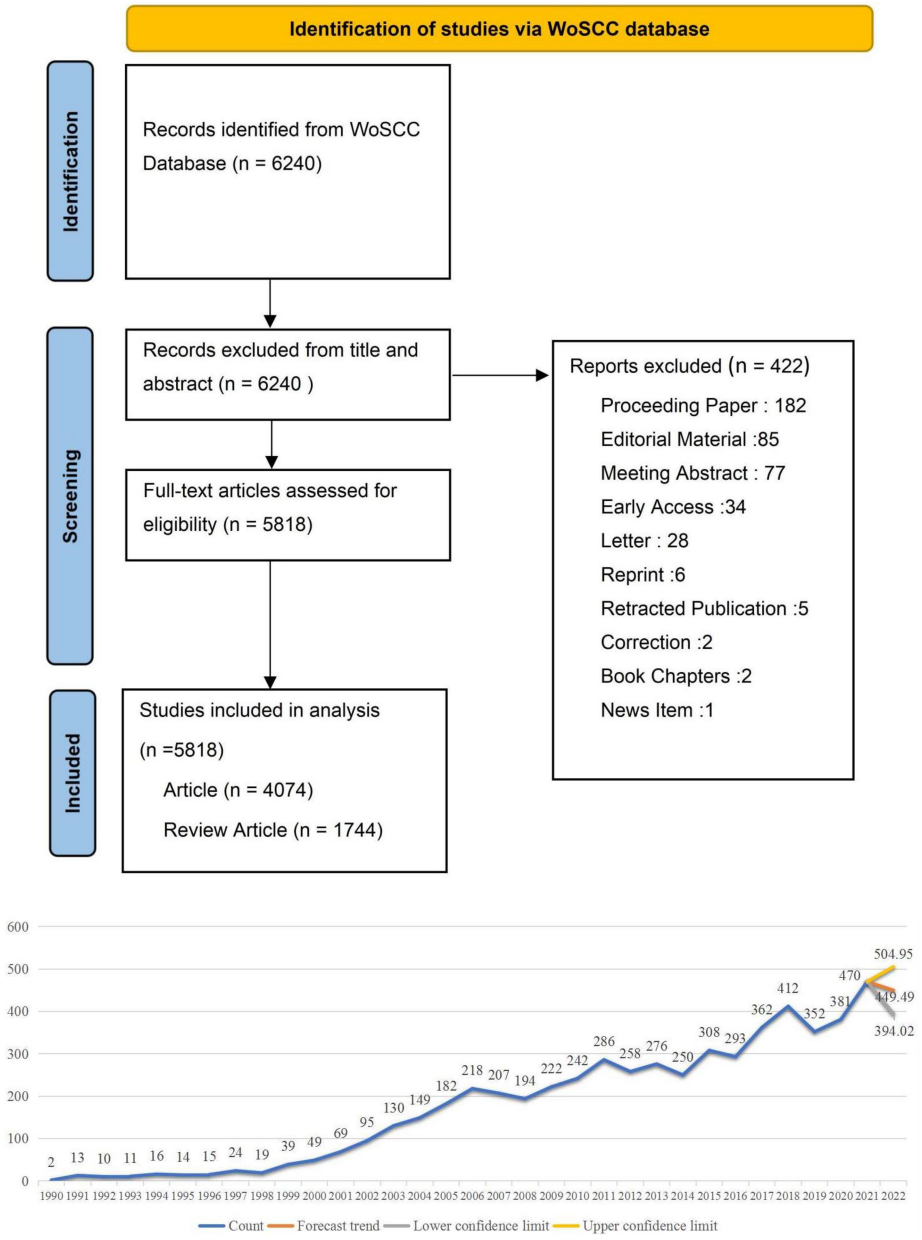

FIGURE 1
The literature's prediction graph and the literature's screening flow chart. This is the trend chart of the distribution and growth of the literature, and we predict that publications in 2022 were between 394 and 504.

placebo-controlled international multi-center clinical trial created the first new era of anti-inflammatory treatment of atherosclerotic diseases and was cited more than 4,000 times.

Cooperative analysis

Big data reveals that cooperation between high-level academics or research institutions can produce more effective results (24). The analysis of literature authors and their cooperative network is conducive to grasping the cooperation between high-yielding authors and academic groups in this research field (25, 26).

In the national cooperation analysis, there are three main trends. First, the United States and China as the central core cluster. Second, it has three obvious geographical advantages, the American continent research cluster, the research cluster of European countries, and the East Asian research cluster, and third, there are spatio-temporal change trends. Before 2014, mainly in Europe and America and other developed countries, after 2014, China and developing countries in Asia began to emerge (Figure 2A).

In the analysis of institutional cooperation, from the perspective of the frequency of institutional collaboration, the Harvard University system was dominated in the early stage. Harvard Medical School, Brigham & Women's Hosp participated; after 2010, the University of

TABLE 1 The top 20 most cited references.

Title	Authors (top five)	Journal	Year	DOI	Total Citation	Impact Factor
Inflammation and atherosclerosis	Libby, P; Ridker, PM; Maseri, A	CIRCULATION	2002	10.1161/hc0902.104353	5,729	39.9175
Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men	Ridker, PM; Cushman, M; Stampfer, MJ; Tracy, RP; Hennekens, CH	NEW ENGLAND JOURNAL OF MEDICINE	1997	10.1056/NEJM199704033361401	4,513	176.0774
Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease	Ridker, PM; Everett, BM; Thuren, T; MacFadyen, JG; Chang, WH et al.	NEW ENGLAND JOURNAL OF MEDICINE	2017	10.1056/NEJMoa1707914	4,415	176.0774
Chemistry and Biological Activities of Flavonoids: An Overview	Kumar, Shashank; Pandey, Abhay K	SCIENTIFIC WORLD JOURNAL	2013	10.1155/2013/162750	2,163	NA
Chronic subclinical inflammation as part of the insulin resistance syndrome - The Insulin Resistance Atherosclerosis Study (IRAS)	Festa, A; D'Agostino, R; Howard, G; Mykkanen, L; Tracy, RP et al.	CIRCULATION	2000	10.1161/01.CIR.102.1.42	1925	39.9175
Adipose tissue, inflammation, and cardiovascular disease	Berg, AH; Scherer, PE	CIRCULATION RESEARCH	2005	10.1161/01.RES.0000163635.62927.34	1,599	23.213
Recent advances in the relationship between obesity, inflammation, and insulin resistance	Bastard, JP; Maachi, M; Lagathu, C; Kim, MJ; Caron, M et al.	EUROPEAN CYTOKINE NETWORK	2006	PMID: 16613757	1,463	3.45
Interleukin-1 in the pathogenesis and treatment of inflammatory diseases	Dinarello, Charles A.	BLOOD	2011	10.1182/blood-2010-07-273,417	1,432	25.476
Review of the biology of quercetin and related bioflavonoids	Formica, JV; Regelson, W	FOOD AND CHEMICAL TOXICOLOGY	1995	10.1016/0278-6,915(95)00077-1	1,404	5.572
Risk of cardiovascular events associated with selective COX-2 inhibitors	Mukherjee, D; Nissen, SE; Topol, EJ	JAMA-JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION	2001	10.1001/jama.286.8.954	1,375	157.335
Omega-3 fatty acids in inflammation and autoimmune diseases	Simopoulos, AP	JOURNAL OF THE AMERICAN COLLEGE OF NUTRITION	2002	10.1080/07315724.2002.10719248	1,375	3.571
Effect of statin therapy on C-reactive protein levels - The Pravastatin Inflammation/CRP Evaluation (PRINCE): A randomized trial and cohort study	Albert, MA; Danielson, E; Rifai, N; Ridker, PM	JAMA-JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION	2001	10.1001/jama.286.1.64	1,374	157.335

(Continued)

TABLE 1 (Continued)

Title	Authors (top five)	Journal	Year	DOI	Total Citation	Impact Factor
Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly	Harris, TB; Ferrucci, L; Tracy, RP; Corti, MC; Wacholder, S et al.	AMERICAN JOURNAL OF MEDICINE	1999	10.1016/S0002-9343(99)00066-2	1,215	5.928
Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer	Kris-Etherton, PM; Hecker, KD; Bonanome, A; Coval, SM; Binkoski, AE et al.	AMERICAN JOURNAL OF MEDICINE	2002	10.1016/S0002-9343(01)00995-0	1,210	5.928
Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials	Bhala, N; Emberson, J; Merhi, A; Abramson, S; Arber, N et al.	LANCET	2013	10.1016/S0140-6736(13)60900-9	1,103	202.7275
Antiinflammatory properties of HDL	Barter, PJ; Nicholls, S; Rye, KA; Anantharamaiah, GM; Navab, M et al.	CIRCULATION RESEARCH	2004	10.1161/01.RES.0000146094.59640.13	1,024	23.213
Flavonoids: Old and new aspects of a class of natural therapeutic drugs	Di Carlo, G; Mascolo, N; Izzo, AA; Capasso, F	LIFE SCIENCES	1999	10.1016/S0024-3205(99)00120-4	1,010	6.78
The Biological Basis for Cardiac Repair After Myocardial Infarction From Inflammation to Fibrosis	Prabhu, Sumanth D; Frangogiannis, Nikolaos G	CIRCULATION RESEARCH	2016	10.1161/CIRCRESAHA.116.303577	958	23.213
Update on uses and properties of Citrus flavonolds: New findings in anticancer, cardiovascular, and anti-inflammatory activity	Benavente-Garcia, O; Castillo, J	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY	2008	10.1021/jf8006568	808	5.895

The Scientific World Journal was deselected from SCIE on September 15th, 2014. All the impact factors come from the latest Journal Citation Report published by Clarivate in 2021.

Amsterdam, Wuhan University, and Nanjing Medical University participated in the research. China followed closely in the study, with a small-scale but systematic cluster of research institutions (Figure 2B).

In the author's cooperation analysis, there are two apparent characteristics; Developed countries such as Europe and the United States have presented a research pattern of the cooperative cluster formed by a group of Harvard University experts led by Peter Libby and Paul M. Ridker. Developing countries, represented by Chinese experts' concentrated research, including Qizhu Tang's (27–33) and Wei Wang's (34–39) team, have characteristics that emphasize the role of traditional medicine and natural products in the anti-inflammatory treatment of CHD (Figure 2C).

Journal analysis

Academic journals serve as a vehicle for disseminating disciplinary knowledge, and the relationship between the journals cited in each discipline can reflect the flow of knowledge between journals. Journal coupling analysis refers to the reference situation where two pieces of literature are cited together (40), i.e., if two articles cite the same literature simultaneously, there is a coupling relationship between the two articles. Coupling analysis connects journals from the perspective of knowledge absorption, exploring the academic classification, determining the core or peripheral status of periodicals, the disciplinary nature of journals, and the degree of correlation among various domains. Journal co-citation

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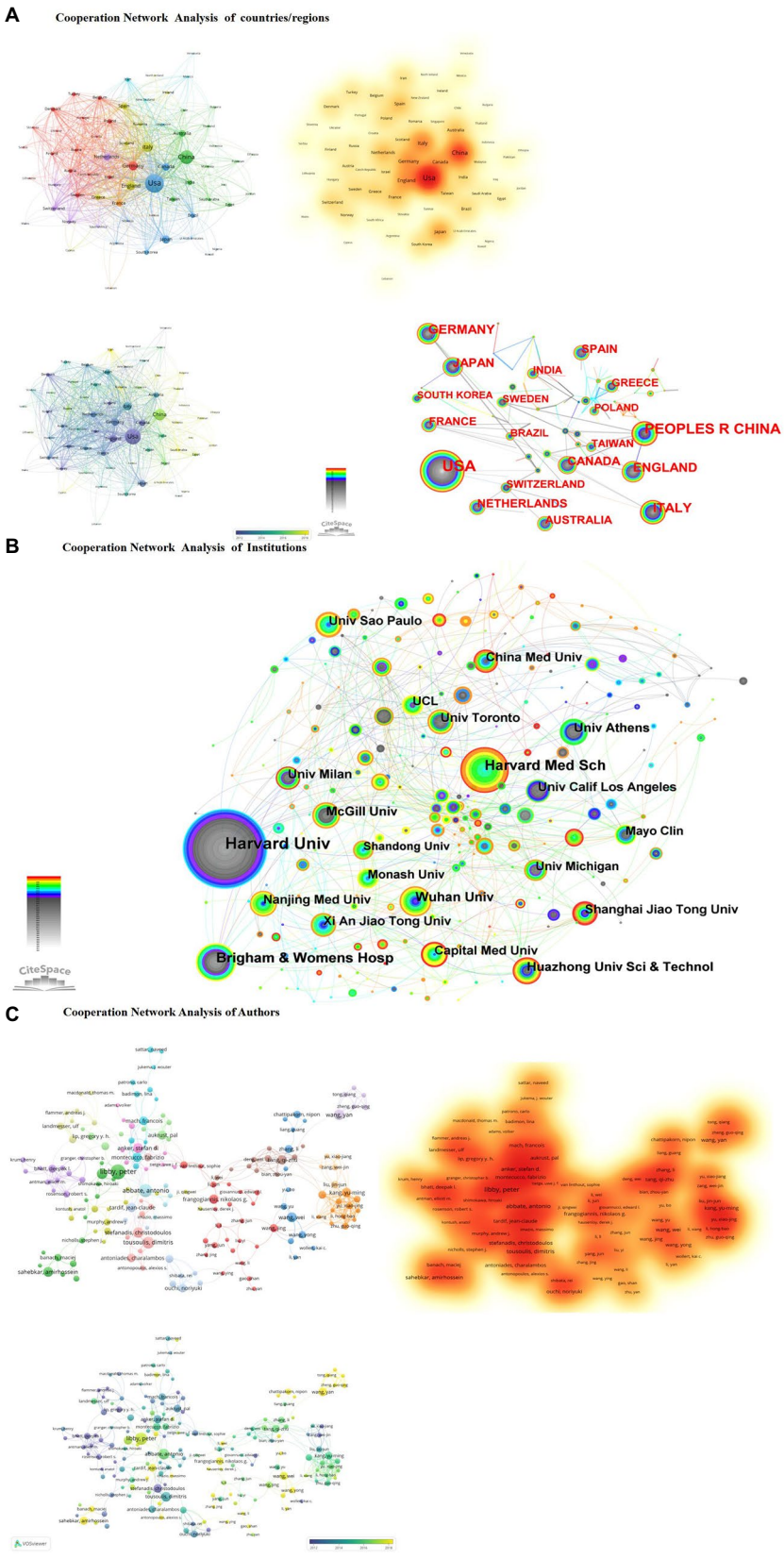

FIGURE 2
(A) Cooperation network analysis of countries/regions. **(B)** Cooperation network analysis of institutions. **(C)** Cooperation network analysis of authors.

refers to the co-citation of two articles that appear in the reference list of a third cited article (41). Journal co-citation analysis examines associations between journals from the perspective of knowledge output, and looking at co-citation relationships between journals provides a glimpse into the scholarly communication patterns of disciplinary research.

Journal co-citation analysis

All journals had a total of 346,140 citations. From the total citations of the journals, Circulation ranked first with 21,817 citations, followed by NEJM. NEJM published only nine publications, but these nine references' total citations reached 14,256 times. Lancet published 12 related articles with a total frequency of 6,285 citations. Among the latest journal impact factor, the Circulation score was 39.918, ranking second in Cardiology and Cardiovascular Medicine. The lancet score was 202.731, and the NEJM impact factor was indexed third in the Medicine disciplines, at 176.079. Although the number of publications is low, internationally renowned academic journals greatly influence co-citation (Figure 3A).

Journal coupling analysis

The journal coupling method can be used to analyze the relationship between journals, help journal classification and explore the internal knowledge structure of the disciplines. The larger node also shows the more significant influence of the journals. We screened journals with a minimum citation count of five times, yielding 294 publications. In the journal coupling analysis, we can find that; Early years focus on the

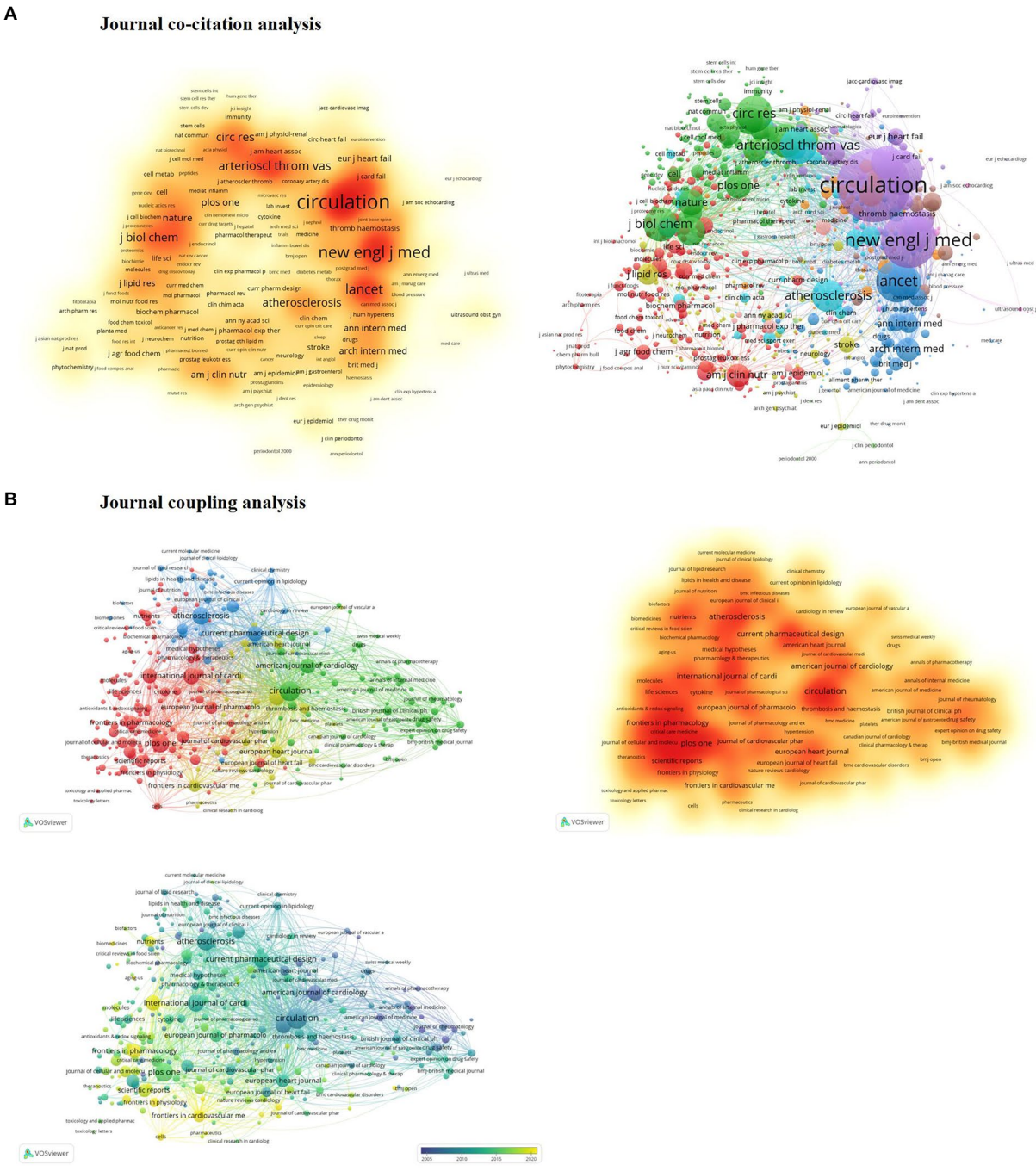

FIGURE 3
(A) Journal co-citation analysis. **(B)** Journal coupling analysis.

association of traditional publishing journals. Moving on to open access journals after 2015. With an apparent trend, early years as Circulation, American Journal of Cardiology, Atherosclerosis, International Journal of Cardiology, Current Pharmaceutical Design's multiple non-open access journals, as the leading core group. Journal coupling density maps formed at later stages, including coupled clusters with open access journals (Figure 3B).

Cluster analysis of keywords

Keywords are the values used to identify specific data items in the literature, mainly to briefly and accurately describe the article's topic, and are primarily used for indexing or cataloging (42). Therefore, we can understand publications' characteristics and evolution trends by analyzing the changes in keywords.

A keyword merged coexistence network with 459 nodes and 5,024 links were constructed using CiteSpace. The parameters of the software are set as follows. Time slicing: from 1990 to 2022, 1 year per slice. Node types: reference. Selection criteria: select the top 50 levels of most occurred items from each slice. Cluster analysis was performed based on these keywords

and the results are shown in (Figure 4A). The smaller the cluster tag number

the larger the cluster size. According to the cluster results analysis

the critical clusters are coronary heart disease

c-reactive protein

cardiovascular disease

myocardial infarction

rheumatoid arthritis

and gastrointestinal toxicity. According to the VOSviewer analysis of the keyword cooperative network and keyword density

the top five core keywords are inflammation

C-reactive protein

coronary heart disease

nonsteroidal antiinflammatory

and myocardial infarction (Figure 4B)

Keywords and references with the strongest citation bursts

CiteSpace provides burst detection that can perceive significant changes in references and keywords over a certain period (43). We screened the top 20 keywords and the 25 references according to burst intensity (Figure 5). The strongest keywords were "coronary heart disease (20.73)," and most recently "heart failure (18.28)," nonsteroidal anti-inflammatory drug (17.51), oxidative stress (16.62), c reactive protein (12.18), tumor necrosis factor (10.36). In addition, the Nlrp3 inflammasome in the last 2 years strongly references the sudden hot spot, reflecting the current hot spot trend. Moreover, the most cited citation burst is "Ridker PM, 2017 (95.12)," ranked by citation time. The top five are Tardif JC, 2019 (52.59), Ridker PM, 2019 (37.9), Ridker PM, 2018 (22.82), Prabhu SD, 2016 (23.51), and Ridker PM, 2017 (95.12).

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Cluster analysis of reference co-citation

According to the analysis of study topics, reference co-citation clustering is a superior function of CiteSpace, enabling us to study issues and hotspot trends comprehensively. Each cluster is considered to represent a hot frontier of research. Therefore, we used CiteSpace to describe the cluster view (Figure 6A) and the timeline view (Figure 6B) of the reference co-citations to analyze the trend of anti-inflammatory applications in CHD. The parameters of the software are set as follows. The top 50 papers (TOP = 50) references of each time section were also extracted to construct a literature co-citation network. Contains 4,146 lines and 964 nodes. The analysis yielded a cluster modularity value of 0.799 (Q value), reflecting that the clustering is apparent.

The clustering results jointly cited by references give us some hints: early attention focus on the anti-inflammatory effects of statins (44–46), recent hot topics pay more attention to the study of combined diseases and comorbidities, to explore the common mechanism of anti-inflammation in coronary heart disease and other significant chronic non-communicable complex diseases such as diabetes and obesity (47–49). In addition, the application of new anti-inflammatory drugs is also one of the hot concerns (50–53).

Discussion

Coronary heart disease has become the highest mortality disease in the world. The number of cardiovascular diseases in China is 290 million, containing 11 million coronary heart diseases, and morbidity trends are increasing yearly (54, 55). With the development of research, it found that inflammation plays an important role in the development of CHD. The inflammatory response is an essential mechanism of CHD and significantly impacts the progression of coronary atherosclerotic plaque and adverse cardiovascular events (56, 57). The inflammatory response accelerates the formation of atherosclerotic plaques. Some inflammatory factors reduce the tensile strength of the plaque fiber cap and increase the necrotic lipid core, leading to damaged endothelium and plaque rupture (58–60). Meanwhile, anti-inflammatory treatment has been proven effective in the secondary prevention of coronary heart disease, reducing acute coronary events and improving the prognosis (61, 62).

The number of anti-inflammatory interventions in CHD-related studies has generally increased over the past 30 years. It shows that inflammation, as a critical pathological change during atherosclerosis progression, is attracting increasing attention from researchers (63). Early studies confirmed the correlation between CRP and inflammatory mechanisms in CHD. Acute phase CRP has been shown to reflect systemic and possible vascular inflammation and to predict future cardiovascular events in asymptomatic individuals. In addition, CRP promotes the release of TNF-α, IL-1β, and IL-6 from macrophages and foam cells in the neointima (64). The release of these pro-inflammatory factors promotes atherosclerosis and recruits early monocytes and

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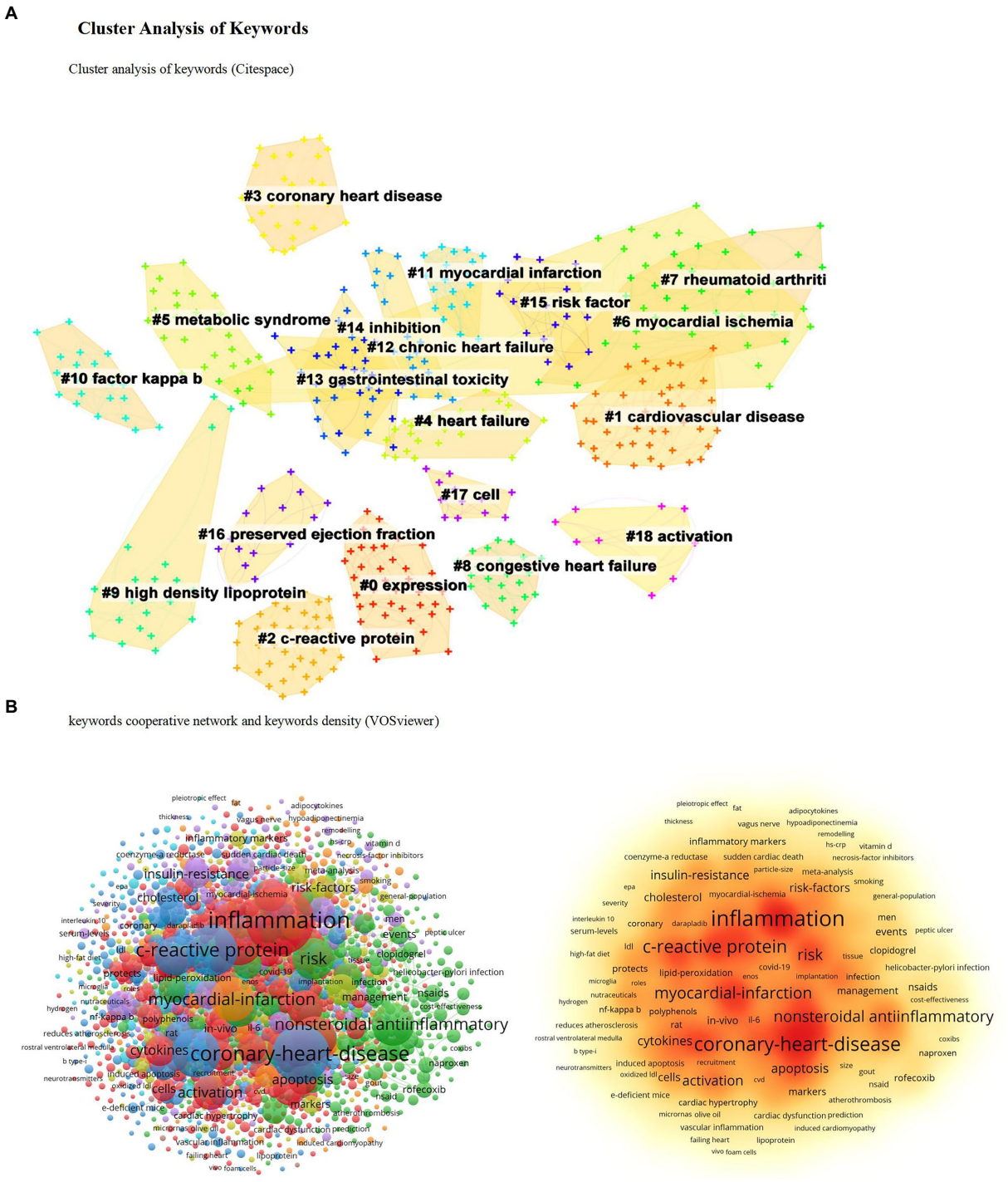

FIGURE 4
Cluster analysis of keywords. **(A)** Cluster analysis of keywords (Citespace). **(B)** Keywords cooperative network and keywords density (VOSviewer).

lymphocytes in the intima (65). One study (66) showed that serum hs-CRP levels were significantly higher in patients with moderate and severe arterial stenosis than in patients with mild stenosis. A panel of experts from the Centers for Disease Control and Prevention and the American Heart Association (67) has recommended hs-CRP as the clinical test with the highest reliability for assessing and predicting the risk of cardiovascular disease. Hs-CRP has become an independent predictor of atherosclerosis and cardiovascular disease, classifying individuals into three risk categories based on hs-CRP levels. Low <1 mg/l; medium 1–3 mg/l; and high >3 mg/l. Each standard deviation increase in hs-CRP concentration increases the risk by 45%, so the level of hs-CRP can be applied to distinguish low-risk from high-risk for atherosclerosis and cardiovascular disease.

In addition, there is a class of clusters focused on rheumatoid arthritis, which is not exploring the comorbid mechanisms of RA and CHD, but instead uses anti-inflammatory drugs to treat rheumatoid arthritis. Recent studies have focused on mechanistic intervention in cardiovascular disease with the anti-inflammatory drugs allopurinol

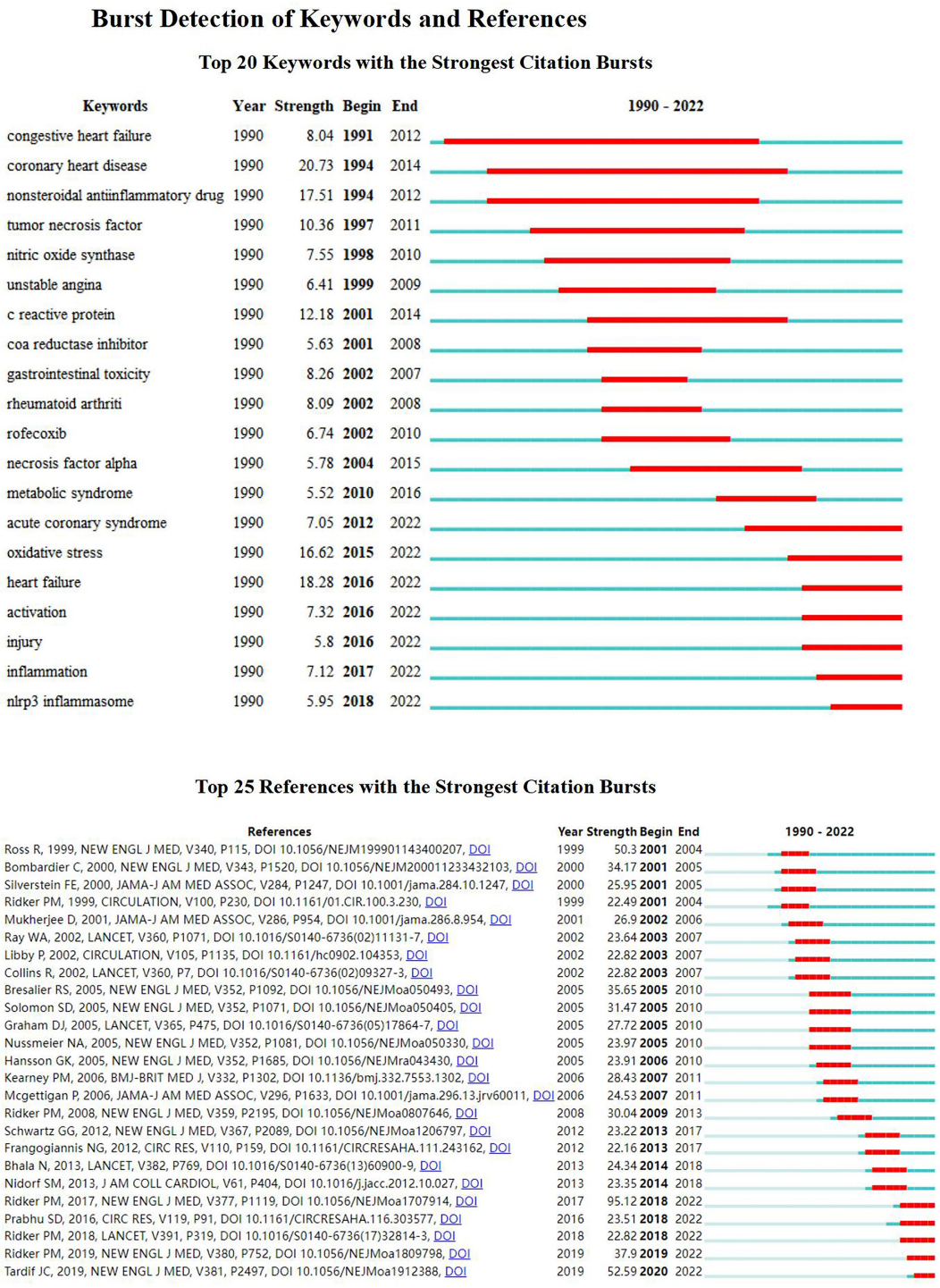

FIGURE 5
Burst detection of keywords and references. Top 20 keywords with the strongest citation bursts. Top 25 references with the strongest citation bursts.

(68, 69) and colchicine (70–74), promising agents for intervention in cardiovascular disease (75). Moreover, there have been concentrated studies on gastrointestinal toxicity of NSAIDs (76–78), such as targeting gastrointestinal side effects of anti-inflammatory medications COX-2 inhibitors (79, 80), focusing on the drug's safety, and preventing adverse events (81, 82).

According to the time trend analysis of the keyword strongest burst, from the late 1990s, the anti-inflammatory application of traditional indicators (tumor necrosis factor, CRP) was concerned. Around 2010, research focused on NSAIDs' use in CHD, including the gastrointestinal toxicity of NSAIDs (77, 83). In the past 5 years, more attention has been paid to the mechanism of the inflammasome in coronary heart disease (84), and the NLRP3 inflammasome-driven IL-1 release has led to atherosclerotic progression and accelerated vascular inflammatory response (85, 86). The research on inflammasome may become one of the research hotspots for future anti-inflammatory interventions in

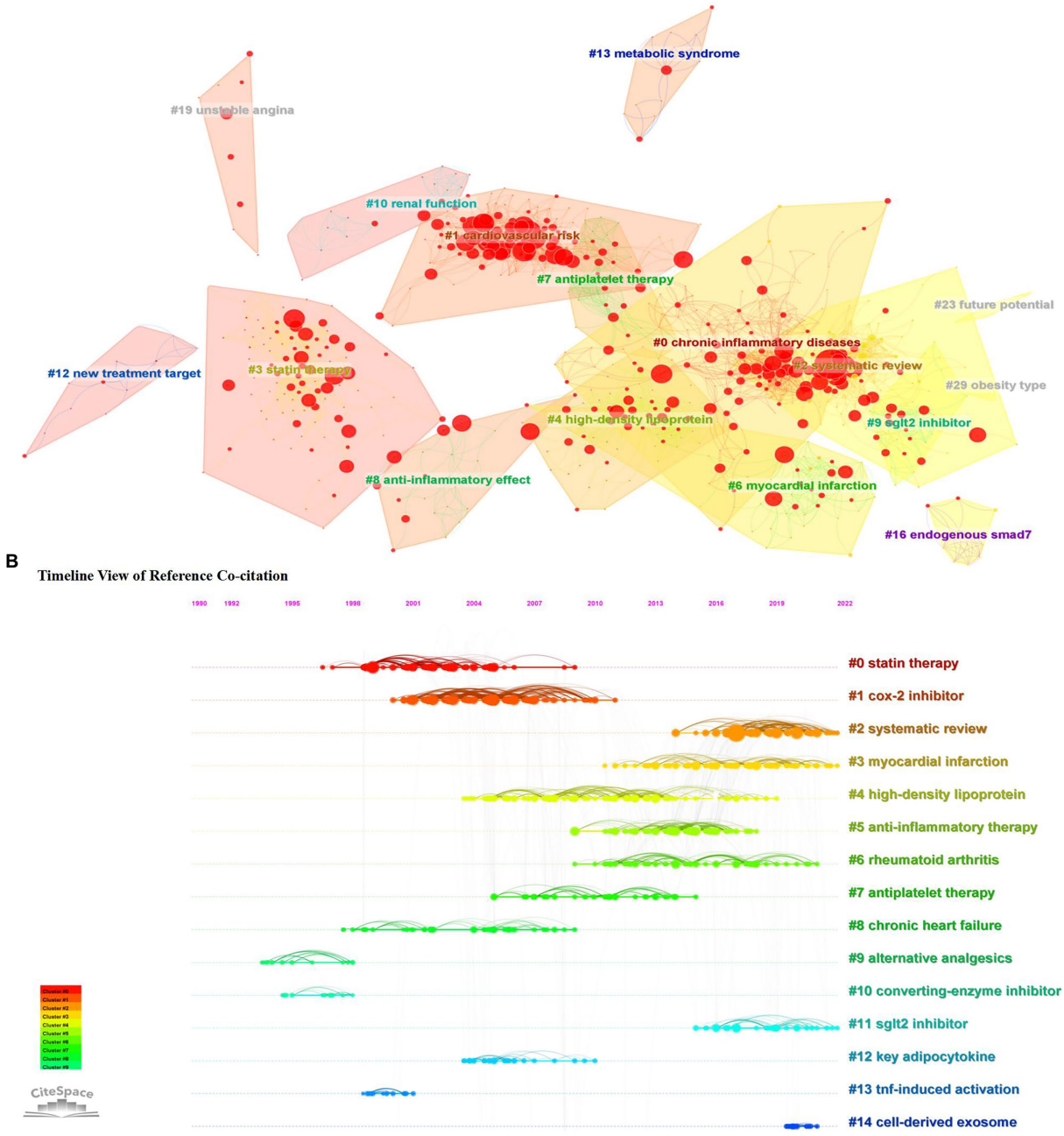

FIGURE 6
Cluster analysis of reference co-citation. **(A)** Cluster view of reference co-citation. **(B)** Timeline view of reference co-citation.

CHD. Although hs-CRP can be used clinically as a biomarker for risk prediction, and high levels of hs-CRP are associated with adverse cardiovascular outcomes after acute coronary syndrome (ACS) (87), most mechanistic studies suggest that CRP itself is unlikely to be an ideal target for specific anti-inflammatory therapies (88). Upstream movement of the inflammatory cascade from CRP to IL-6 to IL-1 offers new therapeutic opportunities for atherosclerosis protection (89). IL-1β, a critical pro-inflammatory cytokine, is involved in various autoimmune inflammatory responses (90). The level of IL-1β is synergistically regulated by Toll-like receptors (TLRs) and Nod-like receptors (NLRs). Activation of TLRs induces the synthesis of precursor IL-1β and precursor interleukin-18, and the activation of NLRs induces assembly in the host cell cytoplasm to form a multimeric protein complex, the inflammasome. The inflammasome is central to the production of IL-1β and IL-18.

The NLRP3 inflammasome/IL-1β signaling pathway plays an important role in the development of AS. Caspase-1, IL-1β, and IL-18, related components of the NLRP3 inflammasome signaling pathway, were highly expressed in atherosclerotic plaques, and the expression levels were higher in vulnerable plaques than in stable plaques (91),

indicating that the NLRP3 inflammasome pathway plays an essential role in the development of AS and affects plaque stability. Cytokine release inhibitory drug 3 (MCC 950), a selective inhibitor of the NLRP3 inflammasome (92), was shown to reduce the maximum stenosis significantly, mean plaque size and volume, minimize macrophage aggregation and inflammatory factor levels, and increase plaque stability in the aorta of apoE−/− mice (93). MCC 950 blocks the NLRP3 inflammasome/IL-1β signaling pathway from upstream, reducing the production of not only IL-1β but also inflammatory factors such as IL-1α and IL-18 simultaneously (94). Theoretically, it is a safer and more effective therapeutic measure than the anti-IL-1β monoclonal antibody canakinumab and has good research prospects. Targeted modulation of the NLRP3 inflammasome/IL-1β is expected to be one of the hot studies for future anti-inflammatory interventions in the prevention and treatment of CHD (95, 96).

Similar results were shown according to the timeline of the research topic. The early 90s focused on traditional mechanisms such as tumor necrosis factor (97, 98) and the mechanism of a nuclear transcription factor (99–101) in atherosclerosis. Several cytokines, including TNF-α, TGF-β, and different interleukins, are involved in developing various inflammatory cardiac pathologies (102, 103). It was found that the combined action of the NF-κB signaling pathway and IL-23/IL-17 inflammatory axis allows IL-1β and TNF-α to accumulate in macrophage foam cells and inflammatory responses, both of which are involved in the pathological development of CHD and related diseases (104).

Around 2000, attention was paid to the anti-inflammatory effects of statins in patients by regulating CRP in CHD (105–108). Despite aggressive statin therapy, publications (109, 110) show that inflammation may be an important driver of residual cardiovascular risk in coronary artery disease. Due to the inability of lipid-lowering to slow the progression of atherosclerosis completely, the identification of inflammatory biomarkers as independent risk factors for cardiovascular disease events has facilitated trials using anti-inflammatory strategies to treat atherosclerosis (111, 112). Since 2010, the focus has been on the mechanisms of NSAIDs in CHD, especially pilot studies using anti-rheumatoid arthritis drugs such as colchicine (62, 113) and methotrexate (114) to intervene in CHD and modulate the level of inflammation. Different from the keyword burst, in the last 5 years, on the one hand, attention has been paid to clinical studies, and the integration of evidence has been carried out to evaluate the evidence systematically (115, 116). On the other hand, studies on the potential association between macrophages and anti-inflammatory have been carried out, which will help drive the formation of new therapies (117–119). In addition, the anti-inflammatory efficacy of fish oil in cardiovascular diseases is included as an area of exploration in nutrition (120–122).

The references with the strongest citation bursts revealed that recent attention has focused on the anti-inflammatory clinical studies of Canakinumab and colchicine in cardiovascular diseases (61, 123–126). The CANTOS study (23) enrolled 10,061 patients from 39 countries with myocardial infarction combined with elevated hs-CRP (>2 mg/l). Canakinumab is a selective, high-affinity, fully humanized monoclonal antibody that targets the inhibition of interleukin-1β (IL-1β). The study showed that canakinumab could further reduce adverse cardiovascular events with myocardial infarction on top of lipid-lowering drug therapy (57, 127, 128). The CANTOS confirmed the clinical importance of the pro-atherosclerotic of IL-1β and identified the IL-1 to IL-6 to CRP inflammatory pathway as a central target for atherosclerotic protection. These data support further drug discovery of atherosclerotic thrombosis therapies targeting IL-18 or IL-6. Due to the role of IL-1β in promoting various pro-inflammatory factors previously, the search for signaling pathways upstream of IL-1β (e.g., NLRP3 inflammasome inhibitors) and possible inflammation targets for intervention has become a hot topic of current research (129). In a follow-up study (130) of 4,833 CANTOS participants, inhibition of the IL-6 signaling pathway was associated with reducing cardiovascular events and all-cause mortality. IL-6 is involved in the pathogenesis of multiple inflammatory diseases, and plasma IL-6 levels strongly predict future vascular events independent of traditional risk factors (131). The results of this study also suggest that lower IL-6 ratios may lead to a lower proportion of cardiovascular events.

Allopurinol is commonly used as a first-line agent to lower serum uric acid and prevent acute attacks in patients with gout, and cardiovascular benefits have also been reported (68, 69). A population-based case–control study (132) found that allopurinol was associated with a lower risk of non-fatal acute myocardial infarction and that the longer patients took the drug, the greater the reduction in infarction risk, suggesting additional cardiovascular protection. In a recent ALL-HEART trial conducted in the United Kingdom (133), allopurinol combined with conventional therapy did not improve cardiovascular outcomes (non-fatal myocardial infarction, non-fatal stroke, or cardiovascular death) in patients with ischemic heart disease. Therefore, the trial shows that allopurinol may not be recommended for the secondary prevention of cardiovascular events in patients with ischemic heart disease. New evidence for the cardiovascular benefit of allopurinol remains to be further investigated.

Colchicine is widely used in clinical practice for gout (113, 134, 135). The LoDoCo (136) study suggested that colchicine reduced the relative risk of the primary endpoint event (acute coronary syndrome, out-of-hospital cardiac arrest, or non-cardiogenic embolic ischemic stroke) by 67% in 532 patients with stable coronary artery disease treated with low-dose colchicine (0.5 mg/day) (HR, 0.33; 95% CI, 0.18 to 0.59; $p < 0.001$). The COLCOT (137) published in 2019 is a large randomized controlled trial (RCT) evaluating the effect of the colchicine group (0.5 mg/day) on recurrent cardiovascular events in patients who had a myocardial infarction within 30 days. The results showed a significant 23% (HR, 0.77; 95% CI, 0.61 to 0.96; $p = 0.02$) reduction in the risk of the primary endpoint event (including cardiovascular death, cardiac arrest, non-fatal myocardial infarction, non-fatal stroke, and urgent revascularization due to angina). The results of a CT coronary angiography study (138) of colchicine intervention in ACS showed that low-dose colchicine treatment was effective in modifying coronary plaques with ACS and that the anti-inflammatory properties of colchicine may drive the improvement in plaque morphology. Colchicine may be beneficial as an additional secondary prevention drug in patients post-ACS. Recent meta-analyses (139–142) have shown that colchicine positively reduces the incidence of MACE, MI, stroke, and revascularization and decreases cardiovascular events, inflammatory markers, hs-CRP, and IL-6 in patients with coronary artery disease. But with a higher incidence of gastrointestinal distress and no effect on all-cause mortality. In a recent Australian study (143), patients with ACS were treated with colchicine 0.5 mg/d twice daily for the first month, then 0.5 mg daily for 11 months, and after 1 year of follow-up, there was no significant difference in the primary adverse event composite endpoint in patients taking colchicine compared to the placebo. After 2 years follow-up (144), the primary adverse cardiovascular event endpoint incidence was significantly lower. This sustained effect may be attributed to colchicine's anti-inflammatory and plaque-modulating properties, reducing the potential development of high-risk plaque volume and ischemic complications. Since the drug was only used for

12 months, the results sustained over 2 years suggest that colchicine may have a legacy effect. Several studies with different trial designs, including colchicine dose, the timing of administration, and the different endpoint events, may have influenced the trial results. More comprehensive and in-depth studies are needed to provide definitive evidence for the clinical use of colchicine. In summary, these findings initially suggest an opportunity to reduce the burden of coronary heart disease in patients using either drug targeting IL-1β or other inflammatory inhibitory pathways (145). Future trials of other new anti-inflammatory agents may help to understand the role of anti-inflammation in the prevention of severe cardiovascular disease events in high-risk patients. We list recent ongoing clinical studies of anti-inflammatory interventions for coronary artery disease being recruited by ClinicalTrials.gov in Supplementary material 2.

In conclusion, integrating the burst of the keywords and the thematic timeline, the current research is focused on the mechanism of anti-inflammation and anti-inflammatory drugs in CHD, and the association between inflammatory vesicles NLRP3 levels and coronary heart disease risk is one of the hot topics (84). In addition, supplementing dietary nutrients and trace elements (146), including omega-3 fatty acids (147–149), provides a nutritional perspective for anti-inflammatory intervention in cardiovascular disease. A recent study has also found (150) that ferroptosis plays a crucial role in the development of CHD and that antioxidants may be the most promising inhibitors of ferroptosis in widespread use. Ferroptosis inhibition is a good option for treating CHD. Moreover, smartphone-based applications (151) for health management in the anti-inflammatory treatment of coronary heart disease can help bridge the digital divide and may be one of the next hot spots in the post-COVID-19 era.

To the best of our knowledge, this is the first study summarizing the research progress on anti-inflammatory in CHD studies by bibliometric analysis, intuitively presenting contributors, collaboration networks, research hotspots, and development prospects through visualization. This paper analyzes the research trends and hot spots of anti-inflammation in CHD. Researchers can refer to the research trends and grasp the current research hotspots. Meanwhile, researchers can adjust the study design according to the research hotspots to make the study more innovative and feasible. The future can focus on three main points to concentrate on exploration.

1. Inflammatory mechanisms in cardiovascular diseases. Recent studies demonstrating that anti-inflammatory interventions can prevent atherosclerotic complications have only scratched the surface of the potential for developing new therapies. Targeting IL-1β highlights the inflammatory vesicle pathway as a promising avenue for further therapeutic interventions (152). NLRP3 inflammasome and the downstream cytokines IL-1β (153), IL-18 (154, 155), and IL-6 (156) are attractive candidates for intervention.
2. Clinical studies of targeted anti-inflammation. The inflammatory process affects all stages of the atherosclerotic plaque life cycle and is a well-established target for intervention in the disease. CANTOS confirms that IL-1β is a tempting target for anti-inflammatory therapy in CHD and suggests that patients with residual inflammation risk (RIR) are the main population for anti-inflammatory therapy (157). It shows that future anti-inflammatory treatment should move from macro to precision anti-inflammatory therapeutics (158–162). Macrophages are involved in the entire process of atherosclerosis formation, progression, and regression (163). They are the primary inflammatory cells involved in atherosclerosis, and their retention within the arterial tubes is necessary for atherosclerosis (164). The accumulation and functional activation of macrophages in the subintima and the secretion of various pro-inflammatory factors lead to the progression of plaques into chronic complex lesions. Therapeutic strategies that promote the conversion of the macrophage phenotype to an anti-inflammatory phenotype may benefit the prognosis of atherosclerotic cardiovascular disease. Several new anti-inflammatory and anti-cytokine agents, including but not limited to direct upstream inhibitors of the NLRP3 inflammasome, and natural inhibitors of IL-6, can be expected to be used in atherosclerosis by targeting the NLRP3, IL-1, IL-6 to CRP pathway. The way to incorporate these anti-inflammatory agents in practice is long and challenging. Still, discovering potential inflammatory targets demonstrates the importance of addressing this factor for CHD risk prevention.
3. Natural products as anti-inflammatory supplements. Recently, anti-inflammatory nutritional supplements, including fish oil, have attracted widespread attention. Inflammation-induced by dietary components is usually chronic and often caused by alterations in the intestinal flora (165). Therefore, microbial-targeted therapies, such as probiotics, prebiotics, and synbiotics, have great potential in systemic inflammatory diseases. Besides, there is a class of studies (166–168) focusing on the mechanisms of action of natural products in anti-inflammatory intervention for CHD. Evidence suggests that medical plants' phenolics, and saponins could reduce inflammatory reactions. In addition, various nutritional components within plant flavonoids (169–172), antioxidant vitamins (173–176), and fruit polyphenols (177–179) have the potential to modulate susceptibility to chronic inflammation.

However, it is important to consider that our study still has these limitations. First, this study only contains literature written in English and no literature in other languages, which may bias the study results. Second, we only retrieved data from the WoSCC database, which may lead to incomplete literature collection. Still, it is noteworthy that the academic community recognizes WoSCC as one of the most authoritative literature data platforms covering most studies. Third, the parameter setting and analysis methods of CiteSpace software lack systematic standards, which may lead to discrepancies in the results, so we used CiteSpace combined VOSviewer to achieve a complete visual presentation. Furthermore, articles published in the past 2 years with high impact factor publications were less cited. Therefore, some recently published papers with high quality should have been included in the analysis of highly cited articles.

Conclusion

This study systematically analyzes the global research results of anti-inflammatory intervention for CHD over the past 30 years. It explores the already published papers' hotspots, frontiers, and trends. Overall, the literature on anti-inflammatory intervention in coronary heart disease has increased yearly. It is expected that nearly 500 articles will be published in the whole year 2022. Further investigation into the interaction between inflammatory mechanisms may be a future research direction, and the treatment's screening and efficacy evaluation of

anti-inflammatory drugs is the focus. Our results summarize the current status of the study and are of great significance for future clinicians and researchers to condense their research directions.

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

JH and HT designed this study. JZ and CJ collected all the articles and wrote the manuscript. CJ carried out software operation and figure drawing. XZ contributed to the final version. 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1038738/full#supplementary-material

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Transcriptome analysis reveals ADAMTS15 is a potential inflammation-related gene in remote ischemic postconditioning

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Background: Remote ischemic postconditioning (RIPostC) induced by brief episodes of the limb ischemia is a potential therapeutic strategy for myocardial ischemia/reperfusion injury, achieved by reducing cardiomyocyte death, inflammation and so on. The actual mechanisms underlying cardioprotection conferred by RIPostC remain unclear. Exploring gene expression profiles in myocardium at transcriptional level is helpful to deepen the understanding on the cardioprotective mechanisms of RIPostC. This study aims to investigate the effect of RIPostC on gene expressions in rat myocardium using transcriptome sequencing.

Methods: Rat myocardium samples from the RIPostC group, the control group (myocardial ischemia/reperfusion group) and the sham group were performed transcriptome analysis using RNA sequencing. The levels of cardiac IL-1β, IL-6, IL-10 and TNFα were analyzed by Elisa. The expression levels of candidate genes were verified by qRT-PCR technique. Infarct size was measured by Evans blue and TTC staining. Apoptosis was assessed by TUNEL assays and caspase-3 levels were detected using western blotting.

Results: RIPostC can markedly decrease infarct size and reduce the levels of cardiac IL-1β, IL-6 and increase the level of cardiac IL-10. This transcriptome analysis showed that 2 genes were up-regulated (Prodh1 and ADAMTS15) and 5 genes (Caspase-6, Claudin-5, Sccpdh, Robo4 and AABR07011951.1) were down-regulated in the RIPostC group. Go annotation analysis showed that Go terms mainly included cellular process, metabolic process, cell part, organelle, catalytic activity and binding. The KEGG annotation analysis of DEGs found only one pathway, amino acid metabolism, was up-regulated. The relative mRNA expression levels of ADAMTS15, Caspase-6, Claudin-5 and Prodh1 were verified by qRT-PCR, which were consistent with the RNA-seq results. In addition, the relative expression of ADAMTS15 was negatively correlated with the level of cardiac IL-1β ($r = -0.748$, $P = 0.005$) and positively correlated with the level of cardiac IL-10 ($r = 0.698$, $P = 0.012$). A negative correlation statistical trend was found between the relative expression of ADAMTS15 and the level of cardiac IL-6 ($r = -0.545$, $P = 0.067$).

Conclusions: ADAMTS15 may be a potential inflammation-related gene in regulation of cardioprotection conferred by remote ischemic postconditioning and a possible therapeutic target for myocardial ischemia reperfusion injury in the future.

KEYWORDS

acute myocardial infarction, ischemia reperfusion injury, inflammation, remote ischemic postconditioning (RIPostC), ADAMTS15, transcriptome analysis

Introduction

In recent years, early reperfusion therapy has become the most effective treatment for patients with acute myocardial infarction (AMI). However, as a side effect of early reperfusion treatment, ischemia reperfusion (IR) injury almost inevitably causes cardiomyocyte death or no reflows, seriously affecting the prognosis of patients with AMI (1, 2). Therefore, it is necessary to seek effective methods to alleviate IR injury at present. Remote ischemic conditioning (RIC), referring to repeated and transient ischemia/reperfusion episodes of distant organs such as limbs, could effectively reduce myocardial IR injury. In 1993, Przyklenk et al. found that remote ischemic preconditioning (RIPC), performed before prolonged myocardial ischemia, could protect the heart against myocardial IR injury (3). As the time of acute coronary occlusion is unpredictable, the application of RIPC has been greatly limited. Therefore, RIC implemented immediately following myocardial reperfusion, called remote ischemic postconditioning (RIPostC), attracts the attentions of researchers. In 2013, Crimi et al. divided 100 patients with AMI receiving primary percutaneous coronary intervention (PCI) into RIPostC group together with PCI control group, and found that the area under the CK-MB curve decreased significantly in RIPostC group (4). Furthermore, a series of clinical and basic studies confirmed the cardioprotective effect of RIPostC, which made it a feasible strategy in treatment of AMI.

At present, the mechanism of cardioprotection conferred by RIPostC is not clear (5, 6). Previous studies have shown that RIC can induce cardioprotective protein factors, such as hypoxia-inducible factor 1α (HIF-1α), stroma cell-derived factor-1 (SDF-1), nitrite, etc. These factors are usually up-regulated in heart tissue and can activate signal transduction pathways in myocardium, finally lead to significant cardioprotection (7–10). In view of current research highlights, the inflammation hypothesis of cardioprotection becomes an attractive target. RIC has been proved to play a significant anti-inflammatory role in myocardial IR injury and relate to decreased levels of the pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-6 (IL-6), after myocardial reperfusion (11). Cai et al. found RIPC can induce the increase of interleukin-10 (IL-10) which was mostly considered as a protective anti-inflammatory cytokine in plasma and heart, and then activate the protective signal pathway in the heart to induce the protection of myocardial IR injury (12–14).

In addition, previous studies on the mechanism of cardioprotection conferred by RIC are mainly focusing on the levels of protein expressions (15–17). The proteomics technology played a prominent role in revealing the mechanism of cardioprotection conferred by RIC. In 2012, Michele hepponstall et al. carried out proteomic analysis on the plasma of 5 healthy volunteers treated with limb RIPC. They found that the expression of 6 proteins changed by using 2-dimensional difference in gel electrophoresis and the expressions of 48 proteins changed by using liquid chromatography-mass spectrometry (LC-MS). The expression of apolipoprotein A-I, fibrin and fibrinogen was changed in the above two technologies (18). Further studies showed that apolipoprotein A-I up-regulated in plasma and heart tissue and played a cardioprotective role in RIPC (19). Transcriptome research is also an important method of functional genome research, which can explore gene expressions and functions more efficiently but is insufficient in RIC research (20, 21). To clarify the effects of RIPostC on gene expressions at transcriptional level in myocardium using transcriptome analysis is of great significance to figure out the mechanism of RIPostC. Therefore, this study aims to investigate the effect of RIPostC on gene expressions in rat myocardium using RNA sequencing.

Materials and methods

Animals

The animal experiment was approved by the Animal Care Committee of Peking University. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Eighteen male Sprague-Dawley (SD) rats (22) with body weights of 250–300 g were used in this study and were divided into three groups randomly ($n = 6$):

RIPostC: 30 min of myocardial ischemia followed by 3 h of reperfusion. Four cycles of 5 min ischemia and 5 min reperfusion on lower limbs were applied immediately after 30 min myocardial ischemia.
Con (Control): the same treatment as RIPC, except that no ischemia on lower limbs was applied.
Sham: sham operation was implemented on rats.

Myocardial IR model

SD rats were anesthetized and then subjected to 30 min myocardial ischemia by ligation of left anterior descending coronary artery, followed with 3 h reperfusion by releasing the ligation to simulate myocardial IR.

The implementation of RIC

Tourniquets were used to bind both lower limbs of rats (22), causing bilateral femoral artery occlusion. After the lower limbs ischemia lasting for 5 min, the tourniquets were relaxed and the lower limbs were reperfusion for 5 min, which was repeated for 4 times. The signs of successful femoral artery occlusion were pale skin, decreased body temperature and loss of pulse. Reperfusion is marked by rapid hyperemia and redness of the distal limb, with return of body temperature and presence of pulse.

Assessment of myocardial infarct size and inflammatory level

After myocardial reperfusion in rats, the anterior descending coronary artery was ligated at the original ligation position again. 2 ml of 1% Evans blue solution was injected to dye the non-ischemic myocardium into blue. The heart was frozen and 4–5 slices of frozen heart tissue were quickly cut. The slices were placed into 1% TTC-PBS buffer and incubated in a constant temperature water bath at 37°C for 20 min in dark. Then heart slices were transferred into 10% formalin solution. The infarction area was gray-white, the non-ischemic area was blue, and the ischemic risk area was red and white. Image J software was used for analysis and myocardial infarction area (%) was valued by infarction area/risk area. Rat troponin I (TnI) in plasma and cardiac IL-1β, IL-6, IL-10 and TNFα were analyzed by enzyme linked immunosorbent assay (Elisa) using commercial kits according to the manufacturer's protocol (Abcam, USA).

Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay

For the detection of TUNEL-positive cells, the In Situ Cell Death Detection Kit (Roche, CH) was used according to the manufacturer's instructions.

Western blotting

Western blotting was carried out according to the previous literature description (12). Bands were normalized to eukaryotic translation initiation factor 5 (eIF5) expression.

Transcriptome sequencing

Rat myocardium samples from the RIPostC group ($n = 3$) and the control group ($n = 3$) were used for transcriptome analysis by RNA sequencing. Trizol (Invitrogen, USA) was used to extract total RNA from tissue samples. The concentration and purity of RNA were detected using Nanodrop2000. The integrity of RNA was measured by agarose gel electrophoresis, and the value of RNA integrity was acquired using Agilent2100. The cDNA library was established according to the instructions of Illumina TruseqTM RNA sample prep kit. The cDNA library was then sequenced using Illumina novaseq 6000.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from tissues using Trizol (Invitrogen, USA). A total of 2μg RNA were transcribed into cDNA using PrimeScript^{TM} RT Reagent Kit (TAKARA, Japan) according to the manufacturer's instructions. Amplification was performed using SYBR Green Real Time PCR Master Mix (TOYOBO, Japan). The sequences of primers are shown in **Table 1**. The relative amount of mRNA was calculated by $2^{-\Delta\Delta CT}$ method. We used α-tubulin as a housekeeping gene, and all reactions were executed in triplicate for the 12 samples ($n = 6$ for RIPostC group and Con group).

Bioinformatic analysis

Raw reads were processed with fastp software (version 0.19.5). Clean reads were obtained after removal of reads containing poly-N and low-quality reads. Mapped reads were acquired by comparison of clean reads with reference genome Rattus_Norvegicus (Rnor_6.0) using hisat software (version 2.1.0). Then mapped reads were assembled using StringTie (version 2.1.2) software.

The genes/transcripts were compared with NR (version 2020.06), GO (version 2020.0628) and KEGG (version 2020.07) databases, and the annotation of each database was statistically analyzed. TPM (transcripts per million reads) was obtained by RSEM (version 1.3.3) software to detect the expression levels of genes and transcripts. Venn analysis was performed to evaluate the common and unique expressed genes/transcripts. Principal Component Analysis (PCA) was carried out to access the relationship and variation between samples using R (version 3.3.1) software package. DESeq2 (Version 1.24.0) software was used to identify differentially expressed genes (DEGs). An

TABLE 1 Primer sequences used in qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Caspase-6	AGCATGACGTGCCATTGGT	ACGTTGTCGTCCAGCTTGTCT
Prodh1	ACTGTGGACCTACTGGACTGGAAC	CATCCTCTTCATCTGCTGCTCTTCC
Adamts15	AAATGTGGCGTGTGTGGTGGAG	CTGGCGGATGTCAATGCTGGAG
Claudin-5	TACTCAGCACCAAGGCGAACCAC	GCGGCTTCCCACATCGGTC
α-tubulin	TTGAGCGCCCAACCTACACT	TCAGGGCCCCATCAAATCT

adjusted *P*-value <0.05 and fold change >2 or fold change <0.5 were considered as thresholds for significantly differential expressions (23). The volcano map and heatmap for DEGs was conducted by R (version 3.3.1). GO and KEGG annotation for DEGs was completed using R (version 3.3.1) software package.

Statistical analysis

The data are expressed in mean ± standard error (SE). R (version 3.3.1) software and Graphpad Prism 8.0 (La Jolla, CA, USA) were used for statistical analysis. Variables were tested for normal distribution using the Kolmogorov–Smirnov test and Q–Q plots. Student's *t* test was used to compare variables with normal distribution and the Mann–Whitney *U* test was used to compare variables without a normal distribution. $P < 0.05$ was considered to be statistically significant. Pearson's correlation was used to investigate the association between the relative expression of ADAMTS15 ($2^{-\Delta CT}$ method) and cardiac inflammatory indicators.

Results

RIPostC induce protection against myocardial IR injury in rat model

Rats were subjected to RIPostC immediately after prolonged myocardial ischemia (Figure 1A). We assessed the cardioprotective effect of RIPostC by detecting myocardial infarct area in myocardial IR model using Evans blue-TTC staining method. RIPostC markedly reduced infarct size compared with the Con group (Figures 1B,C). Besides, the level of plasma TnI significantly decreased in the RIPostC group (Figure 1D). In addition, compared to the Con group, the number of TUNEL-positive cells significantly decreased in the RIPostC group, and the level of cleaved-caspase 3 significantly downregulated in the RIPostC group, showing that RIPostC could markedly suppress cardiac apoptosis during myocardial IR (Figure 2 and Supplementary Figure S1). These data illustrated that RIPostC can confer powerful protection against myocardial IR injury.

RIPostC significantly reduce cardiac inflammation in myocardial IR injury

Compared to the Con group, the levels of cardiac IL-1β and IL-6 significantly decreased in the RIPostC group and the level of cardiac IL-10 significantly elevated (Figure 3 and Supplementary Figure S2). These data showed that RIPostC can play a powerful anti-inflammatory role in myocardial IR injury.

Expression differences between the Con and the RIPostC group

The transcriptome analysis of 6 samples was completed, the clean reads of each sample was more than 7.26 GB, and the percentage of Q30 base was >94.38%. The clean reads of each sample were mapped with the specified reference genome, and the alignment rate was >95.29%. 11,493 known genes were expressed in the Con group as well as the RIPostC group, while 287 and 290 genes were only expressed in the Con group or the

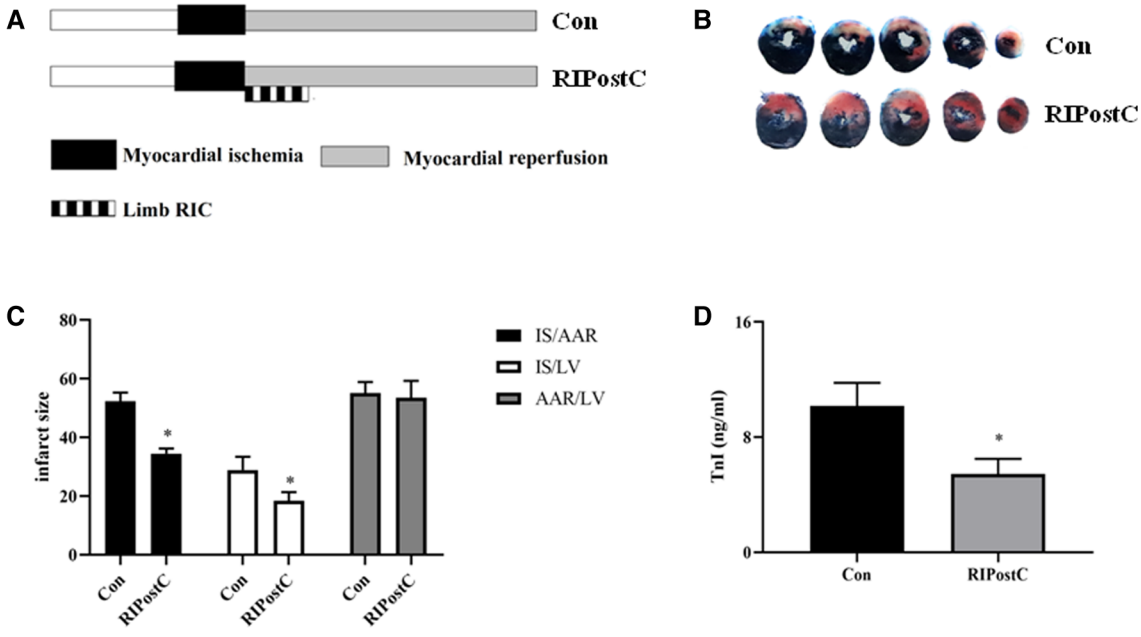

FIGURE 1
RIPostC significantly reduced cardiac injury in rat myocardial IR model. (A) The protocol of experimental design. (B,C) Evans blue and TTC staining. Representative five heart slices stained by Evans blue and TTC in two groups. IS, infarct size; AAR, the ischemic area at risk; LV, left ventricle. (D) The level of plasma TnI. *Represents $P < 0.05$, $n = 6$ for each group.

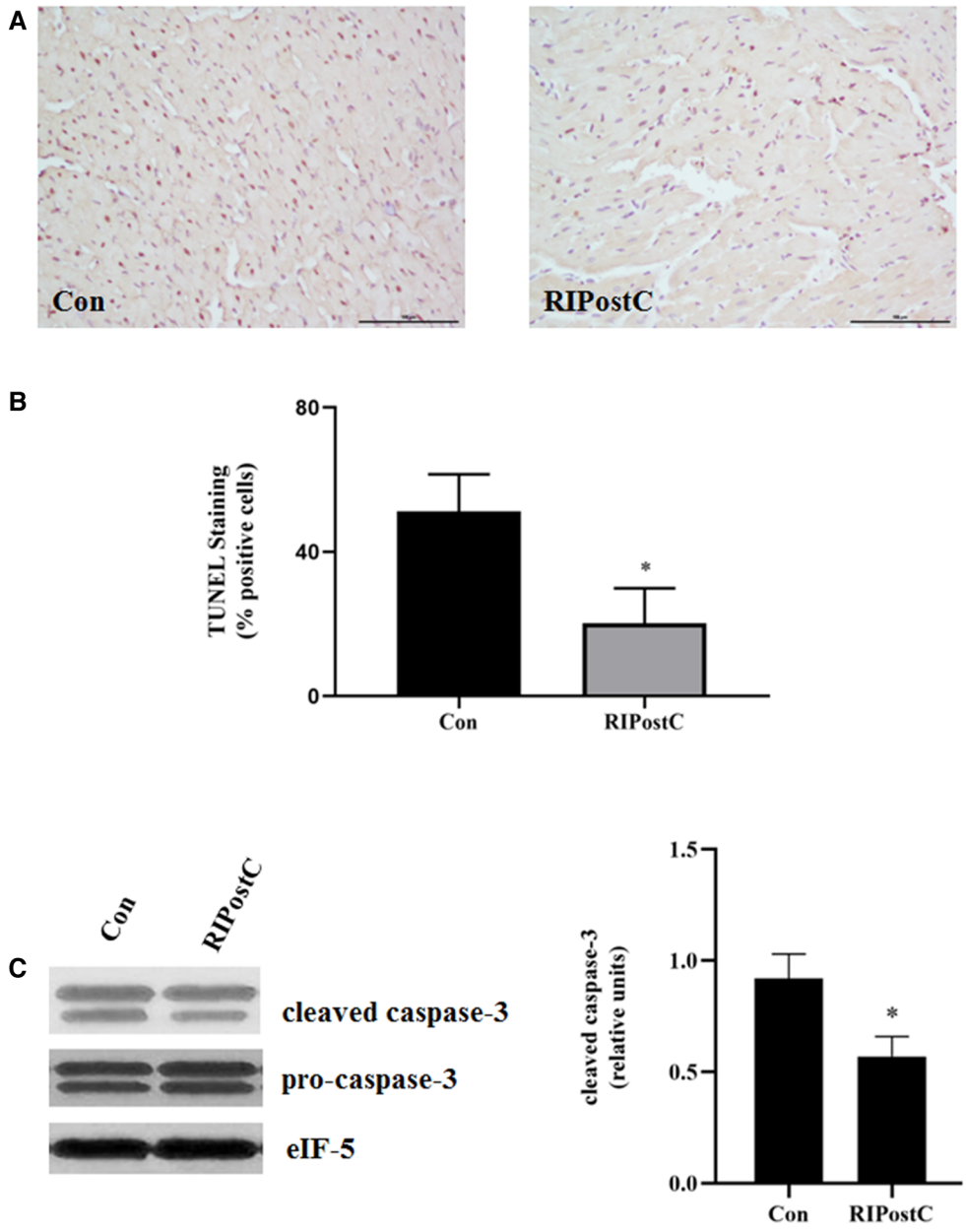

FIGURE 2
RIPostC significantly reduced the apoptosis of heart. (A) Representative myocardial apoptosis detected by TUNEL assay. (B) The percentage of TUNEL-positive cells in the total cells. Scale bar: 100 μm. *Represents $P < 0.05$, $n = 6$ for each group. (C) Detection of caspase-3 levels in heart after RIPostC using western blotting.

RIPostC group respectively (Figure 4A). PCA results showed that differences existed in a certain extent on gene expression patterns between the two groups (Figure 4B).

DEGs identification and analysis

Volcano map showed that the number of DEGs in the RIPostC group vs. to the Con group was 7. The heatmap displayed the expression levels of DEGs in every sample between two groups. The 2 up-regulated genes are: ADAMTS15 (ADAM metallopeptidase with thrombospondin type 1 motif, 15) and Prodh1 (proline dehydrogenase 1). The 5 down-regulated genes are: Cldn5 (Claudin-5), Sccpdh (saccharopine dehydrogenase), Robo4 (roundabout guidance receptor 4), LOC103689977 (Caspase-6) and AABR07011951.1 (Heat shock cognate 71 kDa protein) (Figure 5). Expression differences between the Con and the Sham group were shown in Supplementary Figure S3 and Table S1.

GO and KEGG annotation analysis of DEGs

To identify the functions of DEGs induced by RIPostC, GO terms were annotated. In biological process, cellular process,

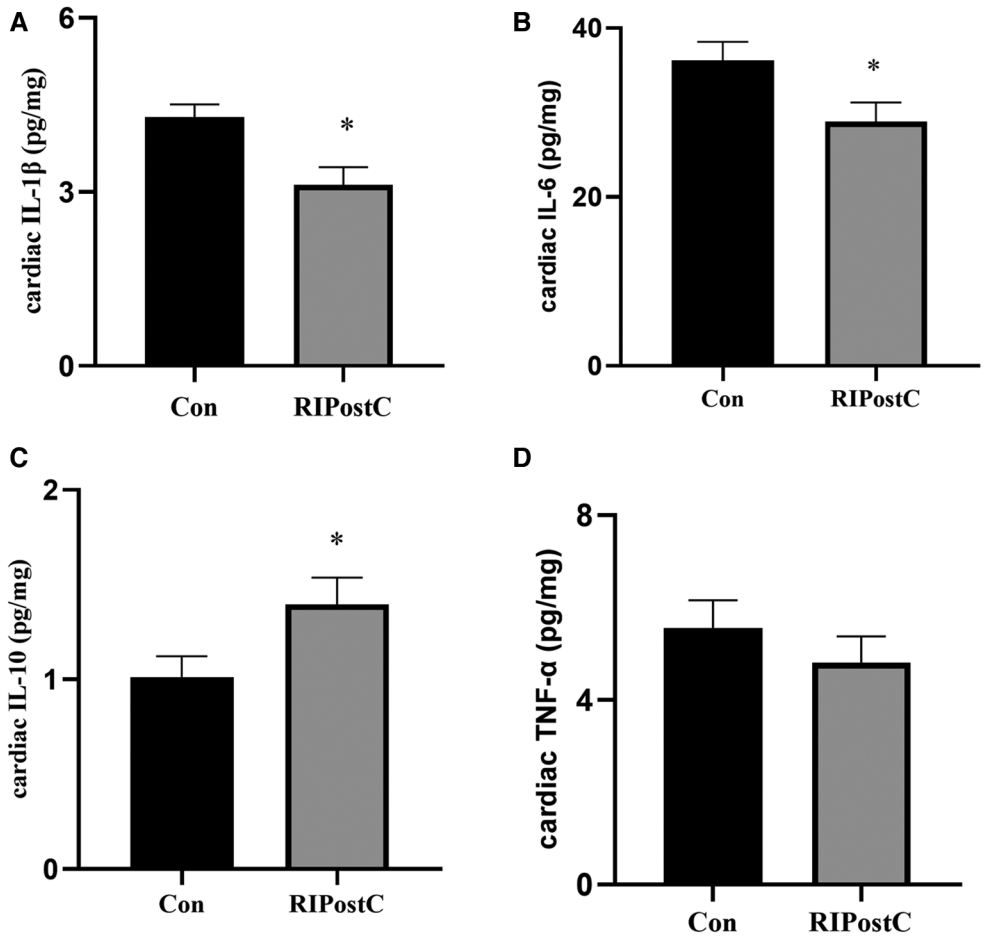

FIGURE 3
The comparison of the levels of cardiac inflammatory factors between the Con and the RIPostC group. (A–D) The comparison of cardiac IL-1β, IL-6, IL-10 and TNFα levels respectively in the RIPostC and the Con group. *Represents $P < 0.05$, $n = 6$ for each group.

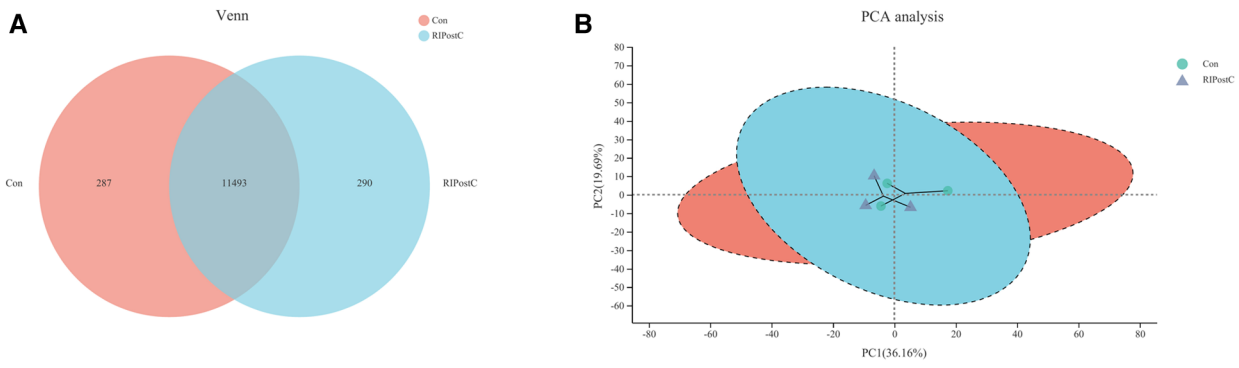

FIGURE 4
Expression differences between the Con and the RIPostC group. (A) Shared and unique expressed genes displayed by Venn analysis. (B) Differences of expression pattern exhibited by PCA analysis.

metabolic process and response to stimulus correspond to 5, 3 and 2 DEGs respectively. In cellular component, cell part, organelle, and membrane correspond to 5, 3 and 2 DEGs respectively. In molecular function, catalytic activity and binding correspond to 5 different genes respectively. Therefore, this GO annotation analysis showed that GO terms mainly included cellular process, cell part, binding, catalytic activity, organelle and metabolic process (Figure 6A). Meanwhile, the KEGG annotation analysis of DEGs between the two groups found that only one pathway, amino acid metabolism, was up-regulated (Figure 6B).

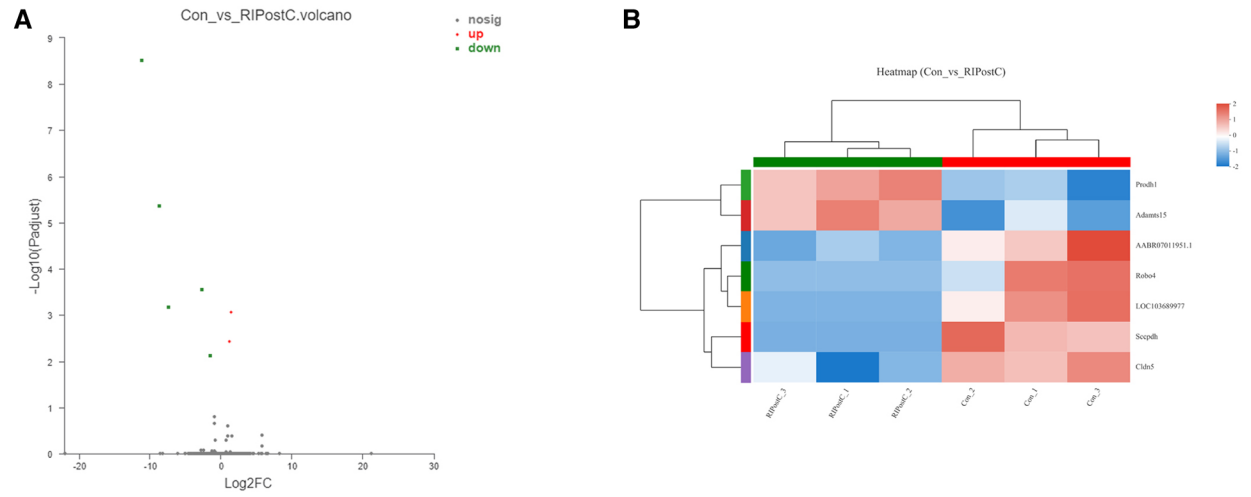

FIGURE 5
DEGs identification and analysis. (**A**) Red and green dots represented up and down regulated genes by Volcano map. (**B**) The expression levels of DEGs and similarity between samples as well as DEGs showed by heatmap.

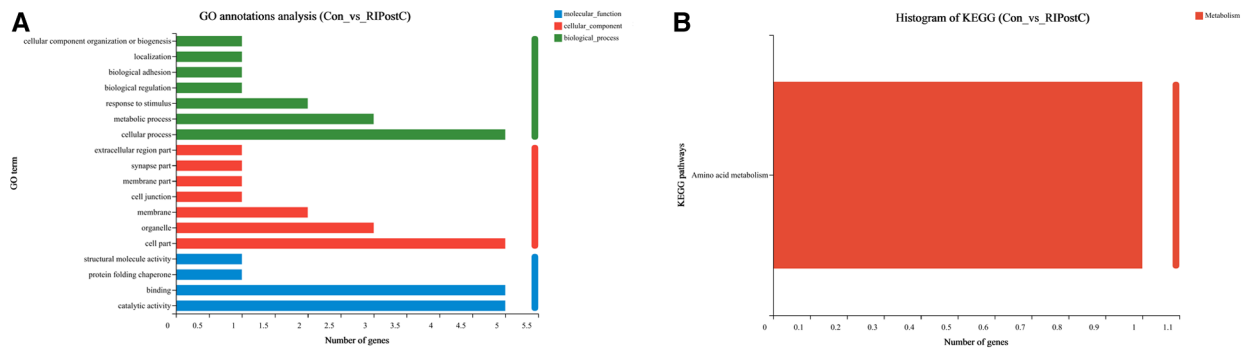

FIGURE 6
Go and KEGG annotation analysis of DEGs. (**A**) Annotated GO terms of DEGs. (**B**) Annotated KEGG pathways of DEGs.

Verification of RNA sequencing data by qRT-PCR

The expression levels of candidate genes were verified by qRT-PCR. As shown in **Figure 7**, compared to Con group, the relative mRNA expression levels of Caspase-6 and Claudin-5 were decreased, Prodh1 and ADAMTS15 were increased in the RIPostC group, which were consistent with the RNA-seq results.

The correlation between the relative expression of ADAMTS15 and cardiac inflammatory indicators

The scatter diagrams and correlation analysis demonstrated that the relative expression of ADAMTS15 were corelated to the levels of cardiac inflammatory indicators (**Figure 8**). The relative expression of ADAMTS15 was negatively correlated with the level of cardiac IL-1β ($r = -0.748$, $P = 0.005$) and positively correlated with the level of cardiac IL-10 ($r = 0.698$, $P = 0.012$). In addition, a negative correlation trend was found between the relative expression of ADAMTS15 and the level of cardiac IL-6 ($r = -0.545$, $P = 0.067$). There was no statistical correlation between the relative expression of ADAMTS15 and the level of cardiac TNF-α ($r = -0.406$, $P = 0.190$).

Discussion

Inflammation is a key factor of myocardial IR injury. Excessive inflammation mediates a series of interactions between neutrophils and vascular endothelial cells, which plays a fundamental role in the inflammatory process of myocardial IR injury. Inflammatory signal cascades triggered during myocardial IR injury can lead to the release of proinflammatory cytokines, such as IL-1β, IL-6 and IL-18, promote leukocyte adhesion to endothelial cells, and trigger leukocyte infiltration into inflammatory damage. Therefore, anti-inflammatory strategy is a potential effective therapeutic method

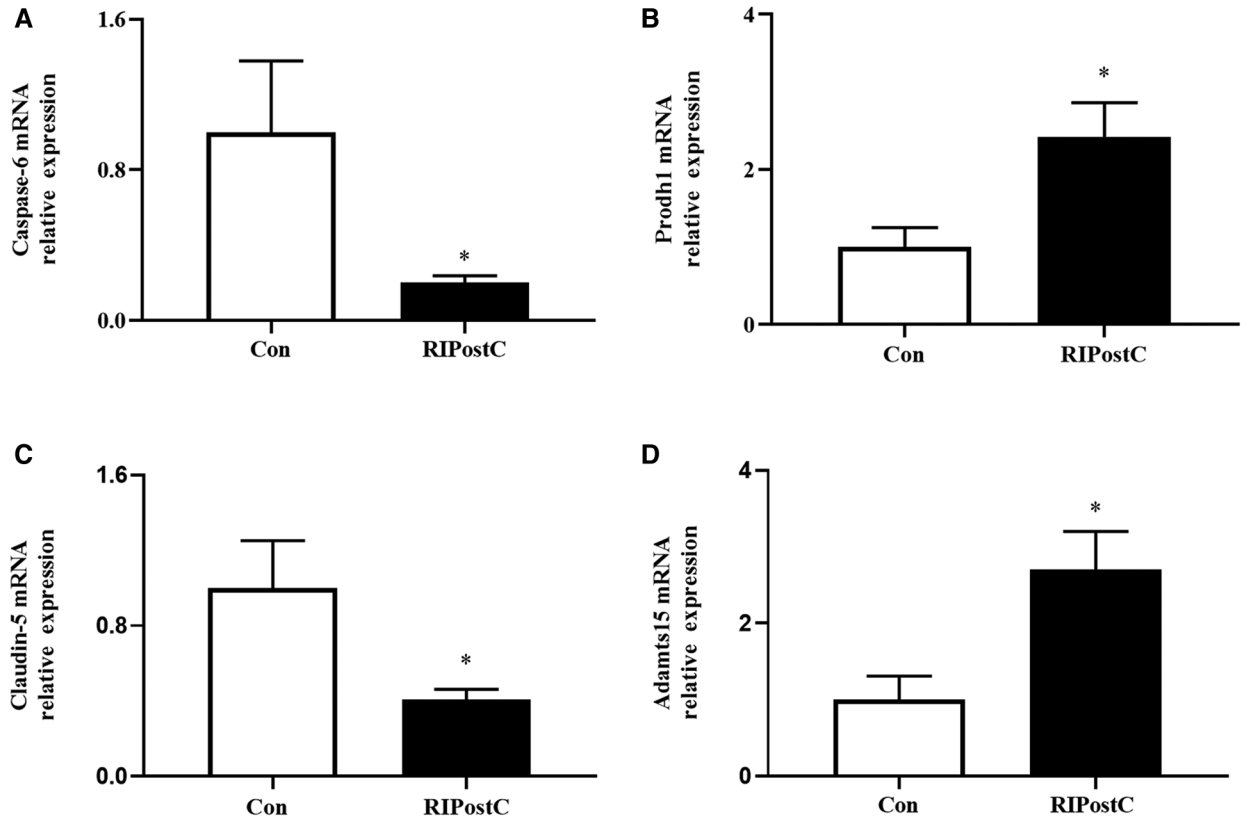

FIGURE 7
Expression levels of DEGs validated by qRT-PCR. (A–D) The relative mRNA expression comparisons of Caspase-6, Prodh1, Claudin-5 and ADAMTS15 respectively in the RIPostC and the Con group. *Represents $P < 0.05$, $n = 6$ for each group.

to alleviate myocardial IR injury (11, 24). In several studies, RIC was associated with the reduction of proinflammatory cytokines, such as IL-1β, IL-6 (11). Besides, RIC was proved to increase the level of protective cytokine IL-10. The up-expression of cardiac IL-10 can alleviate the cytokine response and lead to significant cardioprotection in RIC (12). Consistent with the results in previous studies, we found that RIPostC can reduce the levels of cardiac IL-1β, IL-6 and increase the level of cardiac IL-10, showing powerful anti-inflammatory role of RIPostC.

In this study, we investigated the effect of RIPostC on gene expressions in myocardium by using transcriptome sequencing in rat models. Our results showed that 7 genes were differently expressed between the RIPostC group and the Con group. Go analysis showed that DEGs associated GO terms mainly included cellular process, cell part, binding, catalytic activity, organelle and metabolic process. Further KEGG annotation analysis showed that these DEGs are associated with amino acid metabolism pathway. Compared to the Con group, the relative mRNA expression levels of Caspase-6 and Claudin-5 decreased, Prodh1 and ADAMTS15 increased in the RIPostC group by qRT-PCR, which were consistent with the RNA-seq data. In addition, the relative expression of ADAMTS15 was negatively correlated with the level of cardiac IL-1β and positively correlated with the level of cardiac IL-10. To our knowledge, this is the first study to investigate the transcriptome changes in the myocardium using RNA sequencing technology and demonstrate the overall mechanism changes induced by RIPostC. The potential role of ADAMTS15 in myocardial IR injury and RIPostC was a novel discovery.

ADAMTS are members of proteolytic enzyme family, which are secreted by fibroblasts, smooth muscle cells, macrophages and other cells (25). ADAMTS is a kind of secreted zinc endopeptidase, which contains a signal peptide, a variable length anterior domain, a metalloproteinase domain, an integrin like domain, a central platelet reactive protein type 1 sequence repeat (TSR) motif, a cysteine rich spacer domain and auxiliary domain. The auxiliary domain determines the differences among members of the ADAMTS protein family. ADAMTS are associated with a variety of diseases, including cardiovascular disease (25). Clinical studies have shown that low ADAMTS13 level was associated with an increased risk of AMI (26). ADAMTS5 is considered to have a protective effect on atherosclerosis by regulating the catabolism of vascular proteoglycan and improving the deposition of lipoprotein (27). The expression level of ADAMTS7 was up-regulated in damaged plaques and may promote vascular smooth muscle cells migration and proliferation and aggravate vascular remodeling (28). Therefore, ADAMTS can play key roles in the occurrence and development of coronary heart disease.

Interestingly, ADAMTS is a family of enzymes closely related to acute and chronic inflammation. It is increasingly recognized that inflammation stimulates thrombosis, which in turn promotes inflammation. Up to now, ADAMTS were proved to be key

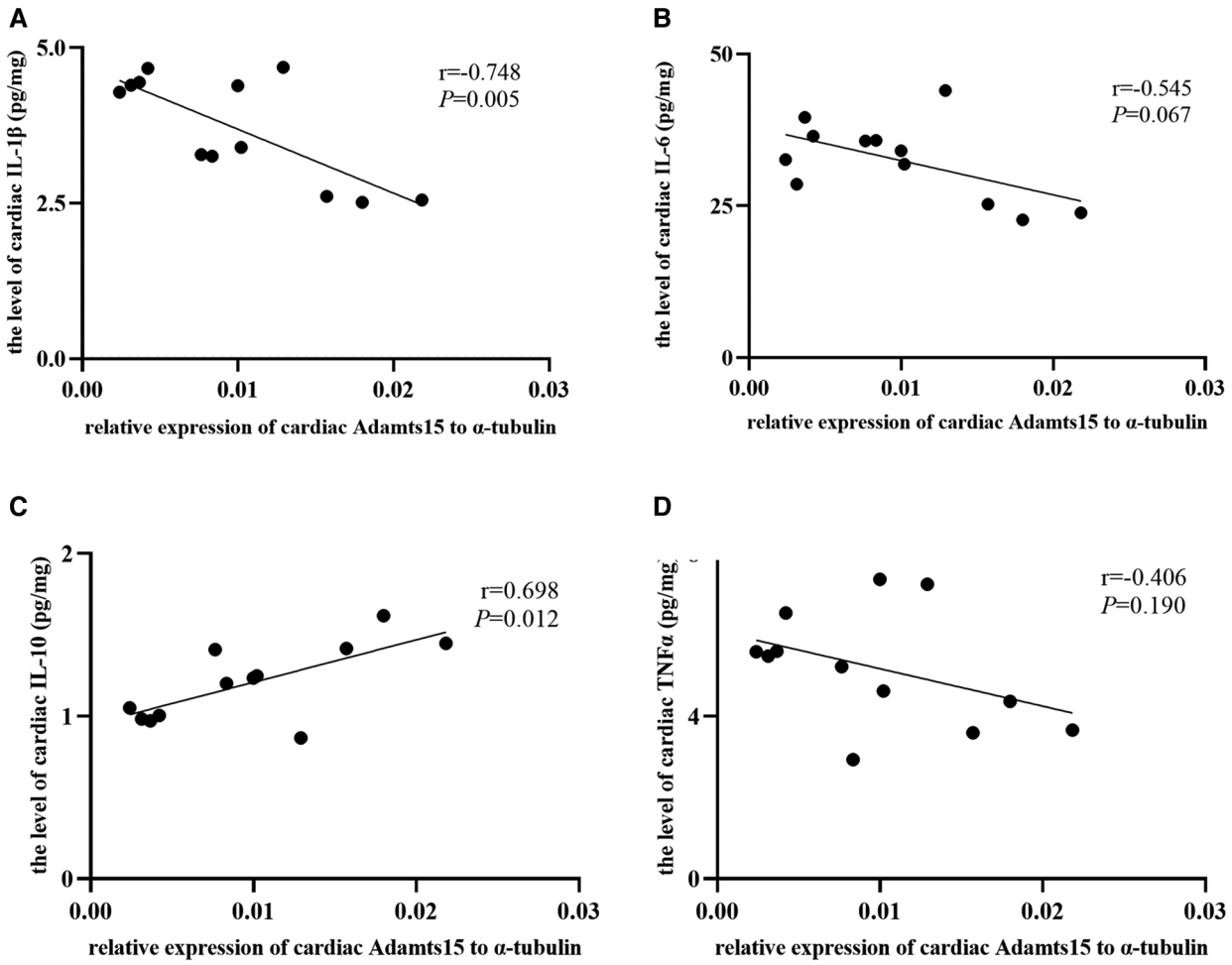

FIGURE 8
The correlation between the relative expression of ADAMTS15 and the levels of cardiac inflammatory factors. (A–D) The correlation between the relative mRNA expression of ADAMTS15 and the levels of cardiac IL-1β, IL-6, IL-10 and TNFα respectively in the RIPostC and the Con group. *Represents $P < 0.05$, $n = 6$ for each group.

contributors to thrombo-inflammation. For example, literatures have shown that ADAMTS13 gene deficiency mice have proinflammatory and thrombogenic phenotypes. In addition, the administration of recombinant ADAMTS13 can alleviate inflammation in myocardial IR injury. ADAMTS13 can prevent excessive platelet and/or leukocyte recruitment in ischemic myocardium by cleaving von Willebrand factor (vWF). The treatment of wild type mice with recombinant human ADAMTS13 resulted in a smaller infarct area and decreased number of neutrophils infiltrating ischemic myocardium, indicating that ADAMTS13 had an effective anti-inflammatory effect during myocardial IR injury (29, 30).

Similar with other ADAMTS members, ADAMTS15 is also a metalloproteinase with thrombospondin domain. It was reported that ADAMTS15 played a tumor suppressor role in prostate cancer and colorectal cancer (31). However, to our knowledge, the role of ADAMTS15 in myocardial IR injury and RIPostC has not been investigated. In this study, we found that the relative expression of ADAMTS15 was negatively correlated with the level of cardiac IL-1β and positively correlated with the level of cardiac IL-10, indicating that ADAMTS15 may be a potential inflammation-related gene in cardioprotection conferred by RIPostC and a possible effective target for myocardial IR injury. The inflammation-related role and mechanism of ADAMTS15 in RIC need to be further studied in future.

In this study, we found that the expression of Caspase-6 was down-regulated after the implementation of RIPostC. Caspases is a family of enzymes that regulate programmed cell death, inflammation and other biological functions. Caspase-6 is generally identified as an executioner of apoptosis, a kind of non-inflammatory cell death (32, 33). In contrast to apoptosis, other common programmed cell death forms, pyroptosis and necroptosis, for instance, may result in the increase of interleukins release, such as IL-1β and IL-18, to enhance cardiac inflammation in myocardial IR injury, mostly considered to be associated with activation of NLRP3 inflammasome and NF-κβ pathway. Recent studies showed that Caspase-6 may facilitate ZBP1-mediated inflammasome activation and promote the activation of programmed cell death pathways, including pyroptosis and necroptosis (34). Whether Caspase-6, with or not

with ADAMTS15, involves in cardioprotection conferred by RIC via regulating inflammation related pyroptosis and necroptosis is an interesting hypothesis and worth exploring in the future.

Claudin-5 is a transmembrane protein, which is widely expressed in epithelium and endothelial cells of brain, heart and so on. Claudin-5 was found to mediate the occurrence and development of cerebral ischemia-reperfusion injury (35, 36). In the ischemic penumbra, the level of Claudin-5 decreased in the early stage of vascular remodeling and increased in the later stage (37). However, the change of Claudin-5 expression over time in myocardial IR was still unknown. The role and mechanism of Claudin-5 in RIPostC need to be further investigated in future. In this study, the expression of Prodh1 is up-regulated after RIPostC. Prodh1 is a kind of enzyme that catalyzes the first step of proline catabolism (38). Wang et al. found that the enhancement of proline metabolism induced by over-expression of prodh decreased the level of reactive oxidative stress and apoptosis, while prodh gene knockout had the opposite effect in rat cardiomyocytes under hypoxia, indicating a cardioprotective role by the enhancement of proline metabolism (39).

Our KEGG annotation analysis showed that amino acid metabolism pathway may involve in regulation of cardioprotection conferred by RIPostC. In 2016, a metabolomic study showed that RIPC was associated with a decrease in ornithine and an increase in kynurenine (KYN) together with glycine concentrations in both rat and human plasma samples (40). Further study showed that inhibition of KYN synthesis may eliminate the cardioprotective effect induced by RIPC (41). At present, there are few studies on the role of amino acid metabolism in cardioprotection induced by RIC, which needs further exploration in our future research work.

Meanwhile, there are some limitations in this study. First, our results of transcriptome sequencing are based on a small sample size, which may not provide enough information to support our hypothesis. Second, the subjects of this study were rats, and species heterogeneity may lead to differences in results (42). Third, RIC is a physiological process involving multiple targets, such as limb, heart and blood. This study only focused on the expressions of DEGs in myocardial tissue and could not fully reflect the mechanism of RIC. Finally, as an association study, this research lacks evidence for causality between the transcriptomics changes and the observed cardioprotection. Thus, further study should be performed to explore how these DEGs involve in cardioprotection induced by RIC, especially ADAMTS15. In addition, the mechanism of the cardioloprotective role of RIPostC should be explored by ex-vivo experiments in the future.

Conclusion

In conclusion, 7 differently expressed genes were found after RIPostC by using transcriptome sequencing in rat models. ADAMTS15 may be a potential inflammation-related gene in regulation of cardioprotection conferred by RIPostC and a possible therapeutic target for myocardial IR injury in future. Caspase-6 mediated cell death may possibly involve in cardioprotection induced by RIPostC. Amino acid metabolism pathway, may involve in regulation of cardioprotection. Further studies are needed to explore the complex mechanisms of cardioprotection induced by RIPostC.

Data availability statement

The data presented in the study were deposited in the NCBI repository which can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA847371/.

Ethics statement

The animal study was reviewed and approved by The Animal Care Committee of Peking University and the approval number was LA2016063.

Author contributions

BZ and ZL were devoted to the analysis and interpretation of the data. BZ and SZ were devoted to writing for this article. GW and ZL designed this research. 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.2023.1089151/full#supplementary-material.

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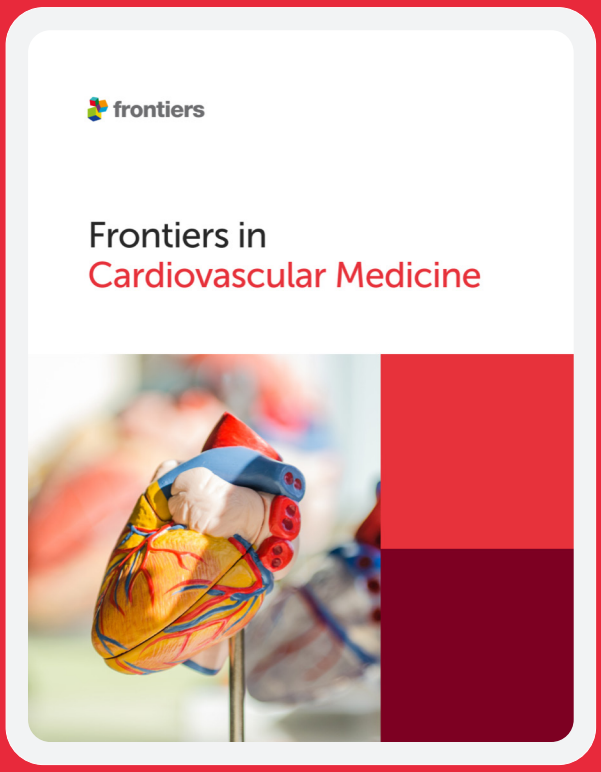

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