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IDENTIFICATION AND VALIDATION OF CARDIOPROTECTIVE MICRORNAS IN CELLULAR MODELS OF ISCHEMIA/REPERFUSION INJURY

PhD thesis

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List of Abbreviations

ANGII – angiotensin II

ATRA – all-trans retinoic acid

CABG – coronary artery bypass grafting

CAD – coronary artery disease

CVD – cardiovascular disease

ECG – electrocardiogram

ESC – European Society of Cardiology

FBS – fetal bovine serum

GO – gene ontology

H/R – hypoxia/reoxygenation

hiPSC-CM – human induced pluripotent stem cell-derived cardiomyocytes

I/R – ischemia/reperfusion

IHD – ischemic heart disease

IPostC – ischemic postconditioning

IPreC – ischemic preconditioning

ISOP – isoprenaline

KEGG – Kyoto Encyclopedia of Genes and Genomes

LAD – left anterior descending

LDL-C – low-density lipoprotein cholesterol

MI – myocardial infarction

miRNA – microRNA

MRI – magnetic resonance imaging

ncRNA – non-coding RNA

NMCM – neonatal mouse cardiomyocytes

NRCM – neonatal rat cardiomyocytes

NSTEMI – non-ST-elevation myocardial infarction

PCA – principal component analysis

PPCI – primary percutaneous coronary intervention

pre-miRNA – precursor miRNA

pri-miRNA – primary miRNA

RIC – remote ischemic conditioning

RIPerC – remote ischemic perconditioning

RISC – RNA-induced silencing complex

ROS – reactive oxygen species

RT-PCR – real-time polymerase chain reaction

sI/R – simulated ischemia/reperfusion injury

STEMI – ST-elevation myocardial infarction

1. Introduction

1.1. Ischemic heart disease

1.1.1. Epidemiology

Ischemic heart disease (IHD) or coronary artery disease (CAD) is the leading cause of death worldwide. In 2021, around 9 million people died from IHD, according to the Global Burden of Disease Study that analyzed the mortality data in 204 countries (1). Metabolic risk factors, like high systolic blood pressure, high low-density lipoprotein-cholesterol (LDL-C) levels, high fasting glucose, or high body mass index, are the leading drivers of IHD (2, 3). Lifestyle choices, including smoking, alcohol consumption, a high-sugar diet, and a lack of physical activity, contribute to the onset of these metabolic risk factors and, consequently, the manifestation of IHD (4). Older people account for the majority of IHD-related deaths; however, premature deaths also increased in the past 30 years (2, 3).

1.1.2. Pathophysiology of myocardial infarction

Atherosclerotic plaque erosion, which leads to thrombosis and subsequently to coronary artery occlusion, is the major cause of acute myocardial infarction (MI). Plaque buildup gradually narrows arteries, reducing blood flow. When myocardial demand increases, this causes insufficient oxygen supply, resulting in ischemia and angina pectoris (5). Besides atherosclerosis, coronary embolism due to endocarditis, spontaneous coronary artery dissection, or arteritis due to autoimmune or infectious causes may also lead to coronary artery occlusion or narrowing and, consequently, MI (5). During an ischemic event, cardiomyocytes lose their contractile ability within 5 minutes due to the rapid inhibition of contractile proteins by inorganic phosphate accumulation and reduced calcium binding caused by intracellular acidosis. 10-20 minutes of ischemia leads to a sustained contractile dysfunction caused by the generation of reactive oxygen species (ROS) that oxidize contractile proteins and calcium overload-induced damage in cardiomyocytes. Early cardiac dysfunction is reversible if blood flow is restored within 5-15 minutes; however, the prolonged dysfunction can last for 24 hours even after the complete restoration of the blood flow (6). MI also impairs oxidative phosphorylation and subsequently ATP production in cardiomyocytes. Consequently, anaerobic glycolysis using glycogen stores becomes the primary energy source. However, anaerobic glycolysis is less efficient, leading to lactate accumulation and intracellular acidosis, which inhibits glycolytic

enzymes. After 15-20 minutes of ischemia, the glycolysis is completely impaired, and the severe ATP depletion causes irreversible damage to the cardiomyocytes (5). The indicators of irreversibly damaged cardiomyocytes are sarcolemmal disruption and alterations in the mitochondrial structure, such as mitochondrial swelling or disorganized cristae (7).

Both apoptosis and necrosis play a role in cardiomyocyte cell death following MI. Apoptosis is a regulated process involving cellular shrinkage and dead cell removal by phagocytic cells (7). DNA damage, elevated ROS, and increased cytosolic calcium trigger apoptosis, resulting in the release of cytochrome c from mitochondria, caspase activation, and DNA fragmentation (6). In contrast, necrosis is an uncontrolled event marked by cell swelling, membrane damage and inflammation due to the failure of ion pumps, acidosis, and calcium overload (6, 7). Recent findings indicate that apoptosis and necrosis may occur simultaneously; however, their relative contribution to cardiomyocyte death is poorly understood. It is proposed that ischemia primarily causes necrosis, while reperfusion triggers apoptosis (7).

Several factors determine the infarction onset, including the duration of the coronary occlusion, the size of the myocardial region affected by the coronary occlusion, known as the ischemic area at risk, and the scope of coronary microvascular dysfunction. The infarction occurs in a characteristic pattern, beginning in the subendocardium and moving outward into the subepicardial layers and the border zones of the area at risk as the coronary occlusion continues (5, 7). The acute loss of myocardium results in a remodeling process in the border zone of the infarct and the noninfarcted myocardium. Cardiomyocyte necrosis and the subsequent increase in cardiac load activate biochemical pathways that initiate reparative changes and cardiac remodeling. These include ventricular dilatation, cardiomyocyte hypertrophy compensating increased workload, and collagen scar formation (7, 8). The ventricular remodeling process may persist for weeks or months (8). Necrotic cardiomyocytes also release danger signals that trigger inflammation, which activate the immune system (7). Inflammatory cells infiltrate the infarcted region to help the removal of necrotic cells and to initiate a healing response, which allows scar tissue development (9). These alterations result in the loss of contractile myocardium, leading to impaired systolic function, elevated mortality rates, a higher

incidence of arrhythmias, and a poorer overall prognosis (7). Figure 1 summarizes the key mechanisms in the pathophysiology of MI.

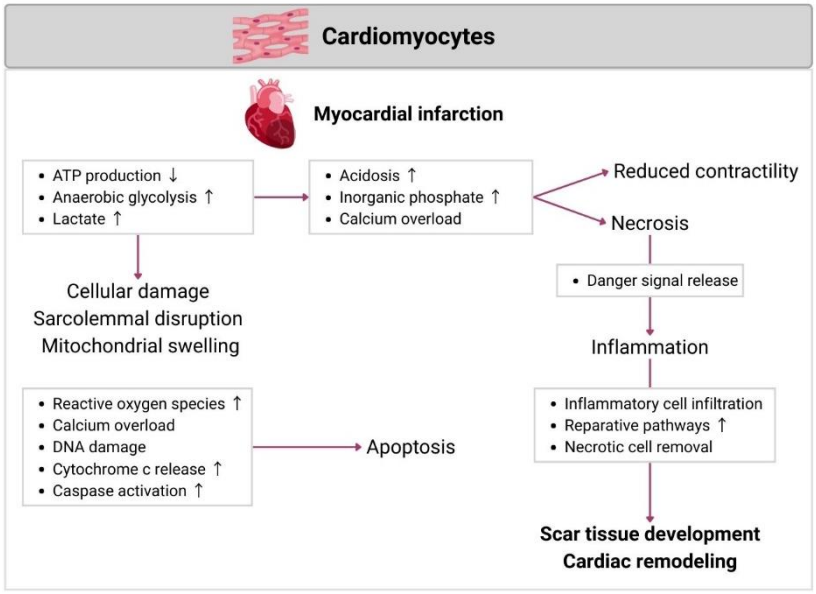


Figure 1. Overview of the key pathophysiological processes occurring in cardiomyocytes after myocardial infarction (5-9). This figure was created with Canva.com.

1.1.3. Diagnosis of myocardial infarction

Acute chest discomfort is the most common symptom that raises suspicion for acute coronary syndrome. Immediate medical help should be requested if the chest pain lasts longer than 15 minutes and/or recurring pain is present within one hour. The resting 12-lead electrocardiogram (ECG) is the primary diagnostic tool for evaluating patients with suspected acute coronary syndrome. Based on the initial ECG, patients can be categorized into two primary diagnostic groups: (i) patients with acute chest pain and persistent ST-segment elevation (STEMI), and (ii) patients with acute chest pain but without persistent ST-segment elevation (NSTEMI). Biomarkers, like cardiac troponins, have a complementary role in diagnostics. In MI patients, cardiac troponin levels usually rise quickly after symptoms begin, and stay elevated for several days (10).

1.1.4. Current clinical practice in ischemic heart disease treatment

For patients experiencing acute coronary syndromes, immediate invasive management is crucial. An emergency angiography is required as quickly as possible for all STEMI patients or very high-risk NSTEMI patients with cardiogenic shock, life-threatening arrhythmias, or cardiac arrest (10, 11). For STEMI patients, primary percutaneous coronary intervention (PPCI) is the preferred reperfusion strategy, optimally within 120

minutes of the ECG-based diagnosis (10). PPCI is a minimally invasive procedure that mechanically opens narrowed coronary arteries with stents (12). In situations where immediate PPCI is not possible, fibrinolysis should be initiated within 12 hours of symptom onset that involves the intravenous administration of streptokinase, alteplase, reteplase, or tenecteplase to dissolve the thrombus in the coronary artery (10). In Western nations, approximately 80% of STEMI patients are treated with PPCI, about 10% receive fibrinolysis as their initial reperfusion therapy, resulting in approximately 10% of patients not undergoing any reperfusion therapy (13).

Emergency coronary artery bypass grafting (CABG) should be considered in patients with an open infarct-related artery but anatomy unsuitable for PPCI, particularly if a large area of myocardium is at risk or cardiogenic shock is present (10). In contrast to PPCI, CABG is an open-heart surgery, during which healthy vessels from another part of the body are grafted to the coronary artery, bypassing the blocked sections. CABG has a higher procedural risk, but improved survival can be observed compared to PPCI by reducing the occurrence of new MIs (12).

1.1.5. Pharmacotherapy for ischemic heart disease

Anticoagulation plays a key role in the initial management of acute coronary syndrome during PPCI and is typically stopped immediately after PCI unless specific conditions, such as left ventricular aneurysm with thrombus or atrial fibrillation, require continued anticoagulation (10).

Antithrombotic therapy is crucial for CAD patients undergoing myocardial revascularization, with treatment choice guided by patient factors, comorbidities, clinical context, and revascularization type (PPCI or CABG). Both ischemic and bleeding risks critically impact patient outcomes and mortality, so treatment decisions must carefully balance these risks (11). Standard treatment involves 12 months of dual antiplatelet therapy, followed by a single antiplatelet therapy(14).

Secondary prevention after acute MI is recommended to be started as early as possible to improve quality of life and reduce morbidity and mortality. Dyslipidemia is usually managed by combining lifestyle changes and pharmacotherapy. Lowering LDL-C is strongly linked to reduced cardiovascular event rates. High-intensity statin therapy is recommended ideally before PPCI, with lipid levels monitored 4-6 weeks after starting treatment to ensure targets are met. If LDL-C goals are not achieved with maximal statins,

additional lipid-lowering agents are recommended (10). In some instances, other therapeutic agents might be recommended post-MI to prevent recurring MI and to reduce mortality (10). Healthy lifestyle changes such as quitting smoking, limiting alcohol, a balanced diet, and regular aerobic and resistance exercise are essential for all CAD patients (10, 15). Although there are various pharmacological treatments available to improve the quality of life for patients, none are able to cure the tissue damage caused by MI, highlighting the necessity for continued research and the development of novel causative therapeutic agents.

1.1.6. Stem cell and exosome therapies

In the last decade, regenerative medicine has also attracted significant attention to revolutionize the treatment of MI. Several types of stem cells have been investigated for MI treatment in several preclinical studies, which can contribute to cardiac regeneration by differentiating into cardiomyocytes and other cardiac lineages, releasing paracrine factors that stimulate angiogenesis, reduce inflammation, prevent fibrosis, and regulate immune responses (16). However, due to the complex structural and functional characteristics of the myocardium, the simple transplantation of stem cells into the infarcted region is unlikely to result in effective cardiac repair unless the engrafted cells successfully integrate with the native myocardium, which is a crucial factor for restoring functional cardiac tissue (17). Stem cell-derived exosomes loaded with microRNAs (miRNAs) and paracrine factors present a promising, cell-free approach that might contribute to cell survival, angiogenesis, and immune modulation. However, advancing knowledge in this field is still required to develop novel therapeutic strategies that utilize microvesicles to treat MI (16, 17).

1.1.7. Reperfusion injury

It is well established that urgent coronary intervention in STEMI patients provides significant clinical benefits. This intervention helps to preserve viable myocardium, reduce the infarct size, and attenuate adverse left ventricular remodeling. However, paradoxically, restoring myocardial blood flow through reperfusion can exacerbate tissue damage by increasing infarct size, a phenomenon known as ischemia/reperfusion (I/R) injury (9).

Reperfusion is characterized by a greater ROS generation than ischemia, which degrades cytoskeletal and sarcolemmal proteins (6). Ischemia also leads to succinate accumulation,

which undergoes rapid oxidation upon reperfusion, triggering the generation of ROS. The sustained elevation of ROS contributes to a vicious cycle of cardiac hypertrophy, myocyte death, and ventricular remodeling through matrix metalloproteinase activation (9). During the early phase of reperfusion, calcium overload and the subsequent increased calcium cycling between the sarcoplasmatic reticulum and cytosol lead to uncoordinated myofibrillar contractions (6). Besides the elevated levels of ROS and intracellular calcium, reperfusion also exacerbates proteinase activity, collectively contributing to the degradation of proteins, lipids, and DNA (18). These changes result in chronic mitochondrial remodeling, impaired energy production, and ultimately promote the development of heart failure (9). To date, there are no clinically approved cardioprotective therapeutic approaches targeting I/R injury; therefore, there is an unmet need to develop new therapeutic agents.

1.1.8. Ischemic conditioning

Ischemic conditioning, during which short cycles of ischemia and reperfusion are applied to the heart or distant organs, was shown to be a promising strategy in preclinical settings to reduce infarct size. Ischemic conditioning can be applied in experimental models before, during, or after a prolonged ischemic event, known as preconditioning, perconditioning, or postconditioning, respectively (19).

The first study on ischemic conditioning, conducted by Murry et al., demonstrated that ischemic preconditioning (IPreC), consisting of four cycles of 5-minute coronary artery occlusion each followed by 5 minutes of reperfusion prior to a sustained 40-minute coronary occlusion, significantly reduced infarct size in dogs, as measured after 4 days of reperfusion (20). Despite the successful preclinical studies, the effective translation of IPreC to clinical treatment remains limited. In a clinical setting, IPreC would require predicting an upcoming ischemic event and a direct intervention to the heart during surgery, both of which are not feasible scenarios (19).

Ischemic postconditioning (IPostC) is applied during early reperfusion in a similar approach to IPreC by performing short reocclusion-reperfusion on the coronary artery following a longer ischemic period (6). Zhao et al. concluded that the infarct size-reduction and preservation of endothelial function achieved by IPostC in dogs are comparable to those observed with IPreC (21). Although IPostC could be a more applicable approach in clinical settings, results from clinical trials investigating its

efficacy have been inconsistent (22). The first randomized clinical trial demonstrated that repeated inflation and deflation of the angioplasty balloon reduces the infarct size by 36% (23). Shortly thereafter, the same research group reported improvements in myocardial function in patients one year after undergoing IPostC (24). Controversially, other research groups failed to prove cardioprotection and even suggested a potentially harmful effect of IPostC in STEMI patients (25-27).

Remote ischemic conditioning (RIC), which involves brief cycles of occlusion and reperfusion applied to distant organs such as the limbs or abdominal organs, has also been shown to significantly reduce myocardial infarct size in preclinical models. RIC can be performed before, during, or after the prolonged ischemic event, which makes this procedure more versatile compared to IPreC or IPostC (19, 28). The first demonstration of the infarct size-reducing effect of RIC was conducted in a canine model, where four cycles of 5-minute circumflex branch occlusion and 5-minute reperfusion were applied before a 1-hour left anterior descending (LAD) coronary artery occlusion (29). RIC is non-invasive, easy to perform, and can be applied in a clinical setting during a coronary occlusion before PPCI, which makes it an advantageous intervention compared to IPreC or IPostC (6). However, the clinical trials investigating the effect of RIC in STEMI patients are controversial. RIC has demonstrated promising results in numerous clinical trials, showing a reduction in infarct size and myocardial edema (30-33), an increase in myocardial salvage index (31, 34), and a decrease in biomarkers of myocardial injury (32, 33). In contrast, the large-scale, multicenter CONDI-2/ERIC-PPCI trial, including 5401 STEMI patients undergoing PPCI, did not find any significant long-term benefit of RIC on cardiac death or heart failure hospitalization (35).

1.1.9. Pharmacological conditioning

Several cardioprotective pharmacological agents were also identified in preclinical experimental studies by discovering signaling cascades involved in endogenous cardioprotection (22). Many substances have been shown to reduce infarct size when administered exogenously in preclinical models; however, these results have often lacked confirmation by independent research groups (6). Although nitrates have been shown to protect ischemic myocardium through pharmacological preconditioning in rat models (36), their use has not reduced infarct size or ventricular remodeling in acute MI patients (37). Similarly, a randomized controlled trial found that administering sodium nitrite

intravenously before PPCI did not decrease infarct size, improve ejection fraction, or lower troponin I levels (38). Another clinical trial did not find evidence of cardioprotective effects from inhaled nitric oxide following PPCI in STEMI patients (39). Another promising area of research involves targeting the mitochondrial permeability transition pore. The opening of these pores, which occurs at the beginning of myocardial reperfusion, plays a key role in mediating reperfusion injury in the heart (19). Cyclosporine A, a mitochondrial permeability transition pore inhibitor, initially showed promising results in reducing infarct size in small and large animal models (40, 41). Moreover, early phase 2 clinical trials indicated that Cyclosporine A could reduce perioperative myocardial injury in CABG patients (42) and decrease infarct size in STEMI patients (43). However, two large multicenter clinical trials did not confirm any improvement in clinical outcomes when Cyclosporine A was administered before reperfusion in STEMI patients (44, 45). Besides these approaches, several other agents have been tested for cardioprotection in various preclinical and clinical studies, all of which have not yet been proved to be clinically effective drugs (6, 17, 19, 22, 46).

1.1.10. Clinical translation of cardioprotective therapeutics

Although multiple promising proof-of-concept clinical studies have demonstrated cardioprotective effects of mechanical and pharmacological interventions in STEMI patients, an increasing number of recent phase 2 trials have failed to show reductions in IS, and several large phase 3 trials have not confirmed improvements in clinical outcomes despite encouraging earlier phase 2 results (22). The failure of clinical translation of the therapeutic approaches for I/R injury might be related to several factors, including the lack of conclusive evidence of cardioprotection in preclinical studies before clinical trials; the limitations of current animal models of I/R injury, which do not adequately reflect the comorbidities found in patients with cardiovascular disease (CVD) or other influencing factors such as age and other medical treatments; and the design flaws in clinical studies, which have not properly incorporated findings from experimental research (6, 19, 22). To ensure that only robust and reproducible cardioprotective therapies advance to clinical trials, it would be beneficial to establish standardized guidelines for preclinical evaluation (22). The advancement of cardioprotective treatments depends on a deeper understanding of the pathophysiological mechanisms underlying I/R injury (6). Additionally, identifying molecular signaling pathways involved in ischemic conditioning, which provide potent

cardioprotection in preclinical models, is essential for understanding the mechanisms that preserve myocardial integrity. Improving clinical translation may also benefit from a multitarget strategy, either using single agents with multiple targets or combining therapies addressing different mechanisms. Such a multitargeted approach is likely more effective than single-target therapies, given the complex and multifaceted nature of acute myocardial I/R injury (22).

1.2. Preclinical models of ischemia/reperfusion injury

Preclinical models are essential for understanding the mechanisms of myocardial I/R injury and developing cardioprotective therapies. Since the underlying mechanisms of diseases often cannot be directly studied in patients, experimental models are crucial for gaining deeper mechanistic insights. To achieve this, experimental studies usually take a reductionist approach that may have limited relevance to clinical conditions, for example, by temporarily increasing or decreasing the expression of a specific gene in certain cells. Another important objective of preclinical models is to generate mechanistic knowledge that can be effectively translated to clinical practice. For this purpose, experimental models need to closely mimic the clinical situation to ensure their findings are relevant and translatable. This requirement can contradict the reductionist approach, highlighting a fundamental challenge in designing preclinical studies (47).

1.2.1. In vivo small and large animal models

In vivo, small and large animal models are used to investigate the progression of MI induced by coronary artery occlusion, with or without subsequent reperfusion (48).

Small animal models such as mice, rats, and rabbits are commonly used in cardioprotection research due to their size, cost-effectiveness, and ease of handling (49). The first mouse model of myocardial I/R was developed to study the pathophysiology of acute and chronic coronary occlusion by examining cardiac histological changes and infarct size (50). Several factors must be considered when selecting a mouse model for MI. The C57BL6 strain is recommended due to its consistent infarct size compared to other strains (51). Young mice aged 4-8 weeks are typically used, as older animals may have a reduced response to cardioprotective interventions. Female mice exhibit inherent cardioprotection, likely linked to sex-specific β1-adrenergic signaling responses and higher estrogen levels. These variables limit the translational relevance of mouse models; however, they are widely used in preclinical I/R studies, primarily because mice are more

cost-effective than larger animals and their genome can be easily manipulated to produce knockout and transgenic lines (49).

The rat model of MI was first developed in 1946 and has since been refined by various research groups (52). This model has become popular because rats are easy to handle and maintain, have short gestation periods, allow for genetic manipulation, and have lower maintenance costs than larger animals. These advantages make both rats and mice suitable for high-throughput experimental studies (49). Lewis and Wistar strains are preferred for I/R experiments due to their intermediate infarct size compared to other strains (53). Similar to mice, estrogen has a cardioprotective effect in female rats (54). Therefore, for initial I/R experiments, male rats aged 3-4 months and weighing between 200 and 300 grams are recommended (49).

New Zealand white rabbits are also commonly used in MI studies due to several advantages. As a medium-sized animal, it is easy to handle thanks to its calm and non-aggressive nature, making it cost-effective for research purposes. Crucially, the rabbit heart lacks collateral circulation and has fewer fatal arrhythmias and deaths following coronary occlusion than other animal models (49).

Large animal models, such as pigs and historically dogs, possess significant translational relevance and are crucial to be involved in the preclinical stage of drug development before initiating clinical trials (49).

Pigs are a commonly used large animal model for IHD research because of their anatomical, physiological, and pathophysiological similarities to humans (55). The heart size and the coronary anatomy of pigs are similar to those of humans, as both species have left and right coronary arteries arising from the aortic root and branching into the atrial and ventricular regions. Additionally, the porcine heart has minimal coronary collateral circulation, which allows for consistent and reproducible induction of ischemic injury, making pigs especially suitable for MI models. The close anatomical and physiological similarities between pig and human hearts increase the translational relevance of porcine models. These models allow accurate simulation of human cardiac conditions and offer a reliable platform for evaluating the effectiveness and safety of different therapeutic approaches (55). Unlike in small animal models, strain and gender have not been shown to affect MI outcomes or cardioprotection in pigs significantly. However, younger animals (6-7 months old) are still preferred over older ones for

experimental consistency (49). Pigs are also well-suited for a closed-chest coronary occlusion using a balloon catheter, a procedure that closely resembles clinical PCI (56). Unlike genetically uniform small rodents, large animals exhibit greater genetic diversity, resulting in increased variability in infarct size development. While this variability enhances translational relevance by better reflecting patient heterogeneity, it also complicates experimental consistency. Additionally, developing transgenic large animal models is expensive, time-consuming, and technically challenging (49).

In animal models, myocardial infarct size and the area at risk are key endpoints for assessing I/R injury and cardioprotection. Infarct size depends on the area at risk, ischemia duration, severity, and systemic hemodynamics. In small animals, infarct size is measured postmortem using 2,3,5-triphenyltetrazolium chloride staining, while area at risk is identified by Evans blue dye. In large animals, infarct size is assessed via delayed gadolinium-enhanced cardiac magnetic resonance imaging (MRI), similar to clinical practice (6). Echocardiography is commonly used to evaluate cardiac performance after I/R injury, and ECG can detect arrhythmias and ST-segment elevation. Additional endpoints to determine the cardioprotection include quantifying inflammation, neovascularization, and evaluating microvascular damage (57). These parameters collectively provide insight into the extent of myocardial injury and the efficacy of therapeutic agents.

1.2.2. Ex vivo models

Ex vivo models of myocardial I/R injury primarily involve isolated perfused hearts or myocardial tissue slices (48). Retrograde perfusion is most commonly performed on adult rat or mouse hearts using a physiological saline solution (57). The advantages of isolated perfused heart models is that cardiac cells remain in the native environment; however, the nervous system and other systemic effects are excluded from the model (49). Myocardial tissue slices allow the studying of the cardiomyocyte function, as well as other cell populations, in their physiological state (48). Both models provide valuable insights into I/R injury mechanisms that closely resembles the *in vivo* physiology, while offering controlled experimental conditions (57).

1.2.3. In vitro cardiomyocyte models

For ethical reasons, minimizing the use of laboratory animals in preclinical research is highly desirable. Therefore, there is a need to develop reliable *in vitro* cell-based

platforms to model cardiac cells and CVDs. *In vitro* models, including cultured myocardial cells, are valuable tools for investigating the cellular and molecular mechanisms underlying ischemic injury (48). To simulate I/R injury in isolated fresh or cultured cardiomyocytes, hypoxia and reperfusion by reoxygenation (H/R) are performed in controlled conditions (57). Ideally, freshly isolated adult cardiomyocytes are considered the gold standard for H/R experiments; however, alternatives such as neonatal cardiomyocytes, cell lines like AC16, H9C2, and HL-1, and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are also commonly utilized (57).

Fresh isolation of cardiomyocytes eliminates potential phenotypic changes associated with cell culture (57). Primary adult cardiomyocytes are most commonly isolated from mice, rats, or rabbits, though they can also be sourced from guinea pigs, dogs, pigs, and humans (58). However, isolating adult myocytes is technically challenging, requires a high number of animals, and their limited survival in culture restricts longer H/R protocols or procedures requiring transfection before H/R (57, 59). Therefore, neonatal cardiomyocytes are more frequently used due to their easier isolation, sustained viability in culture for several days, and ability to be genetically modified. However, they exhibit greater resistance to hypoxia compared to adult cardiomyocytes, which may limit the translational relevance of findings (57).

In place of primary cardiomyocytes, several immortalized cell lines generated from primary cells are also used as experimental models of I/R. Kimes and Brandt first described the rat cardiomyoblast cell line H9C2 in 1976 (60). The mouse atrial myocyte-derived HL-1 line was introduced by Claycomb et al. in 1998 (61), which was followed by the establishment of the human AC16 cell line by Davidson et al. in 2005 (62). These cell lines are characterized by rapid growth in culture and consistent responses to stimuli, making them suitable for high-throughput drug screening and modeling cardiomyopathies, hypertrophic responses, adverse cardiac remodeling, metabolic changes, and I/R injury. Furthermore, their maintenance is easier and less costly than that of primary isolated cells. On the other hand, cardiac cell lines have several limitations, including the loss of specific cardiac characteristics and increased resistance to ischemic injury (63). These factors can significantly affect the translation of *in vitro* findings to *in vivo* cardiac mechanisms. To address these issues, differentiation protocols have been

developed to induce the cells to adopt a more mature cardiac phenotype; however, thorough characterization of these differentiated cells remains limited (62, 64, 65).

In addition to primary and immortalized cardiomyocytes, stem cell-derived cardiomyocytes, including hiPSC-CMs and embryonic-stem cell derived cardiomyocytes, have recently gained considerable attention as experimental models (66). Cardiomyocytes derived from rodent or human embryonic stem cells are promising tools for drug testing and regenerative medicine. However, ethical concerns, risk of tumor formation and immune rejection limit their therapeutic application (67). Differentiation protocols of hiPSCs yield cultures with cardiomyocyte purity exceeding 80%, yet these cultures are heterogeneous, comprising a mixture of atrial, ventricular, and nodal-like cells with diverse phenotypes (68). Due to their human origin, functional properties, and degree of differentiation, hiPSC-CMs are more relevant for translational studies than other cardiomyocyte models. However, they typically retain fetal-like characteristics and have limited commercial availability, which may influence their applicability (69). While hiPSC-CMs overcome some of the challenges associated with primary cells, their response to H/R injury is not fully understood and appears to be influenced by their maturation status (70). Despite these limitations, hiPSC-CMs are particularly valuable for investigating complex cellular interactions, making them a promising tool for enhancing the physiological relevance of *in vitro* cardiovascular research (49).

In vitro models can be used to assess the direct effects of H/R on cardiomyocytes without influence from other cell types or circulating factors. The *in vitro* conditions simulation of *in vivo* ischemia include a low oxygen level (1% O_2, 5% CO_2, 94% N_2) and can be combined with serum- and glucose-deprivation (57). The duration of hypoxia and reoxygenation influence cardiomyocyte viability and must be carefully considered to optimize the experimental model (71). After H/R, cardiomyocytes undergo apoptosis or necrosis; thus, cell viability assays are usually performed to determine the cardioprotective effect of genetic or pharmacological interventions. Cell viability can be measured by lactate dehydrogenase release, various fluorescent dyes, or ATP assay, while apoptosis is commonly measured by TUNEL or annexin V staining. Mitochondrial membrane potential and ROS production is also frequently measured due to their role in H/R injury. Additionally, cardiomyocyte morphology, contractility, calcium handling,

and electrical activity can also be assessed to gain more insight of the functional properties of cells (57).

1.3. MicroRNAs

Non-coding RNAs (ncRNAs) are RNA molecules that do not code for proteins. They regulate processes linked to cellular functions and various diseases, including IHD. They act as both pathogenic factors and mediators of protective or reparative signals. ncRNAs are classified based on structure, function, mechanism, and length. Small ncRNAs include miRNAs, small nuclear RNAs involved in splicing, small nucleolar RNAs that modify ribosomal RNA, transfer RNA-derived small RNAs, and piwi-interacting RNAs. Long ncRNAs consist of linear long ncRNAs and circular RNAs (72).

miRNAs are double-stranded, 19-23 nucleotide-long RNA molecules. They regulate gene expression by binding to complementary sequences on target mRNAs, leading to mRNA degradation or inhibition of translation. Since binding relies on a short complementary region, the 7-nucleotide seed sequence located between positions 2 and 8, each miRNA is capable of targeting hundreds of different mRNA transcripts due to sequence similarities (73). It is estimated that over 2588 mature miRNAs are expressed in human cells, with unique expression patterns that vary by tissue type and developmental stage. These miRNAs are believed to regulate more than 60% of human genes, highlighting their role in controlling a wide range of physiological functions (74). The first miRNA was identified in *Caenorhabditis elegans* in 1993, a discovery for which Victor Ambros and Gary Ruvkun were awarded the Nobel Prize in Physiology or Medicine in 2024 (75, 76). Since then, it has become clear that miRNAs are expressed across all animal and plant species, with some exhibiting conserved sequences across different species (77).

1.3.1. MicroRNA biogenesis and gene regulation mechanisms

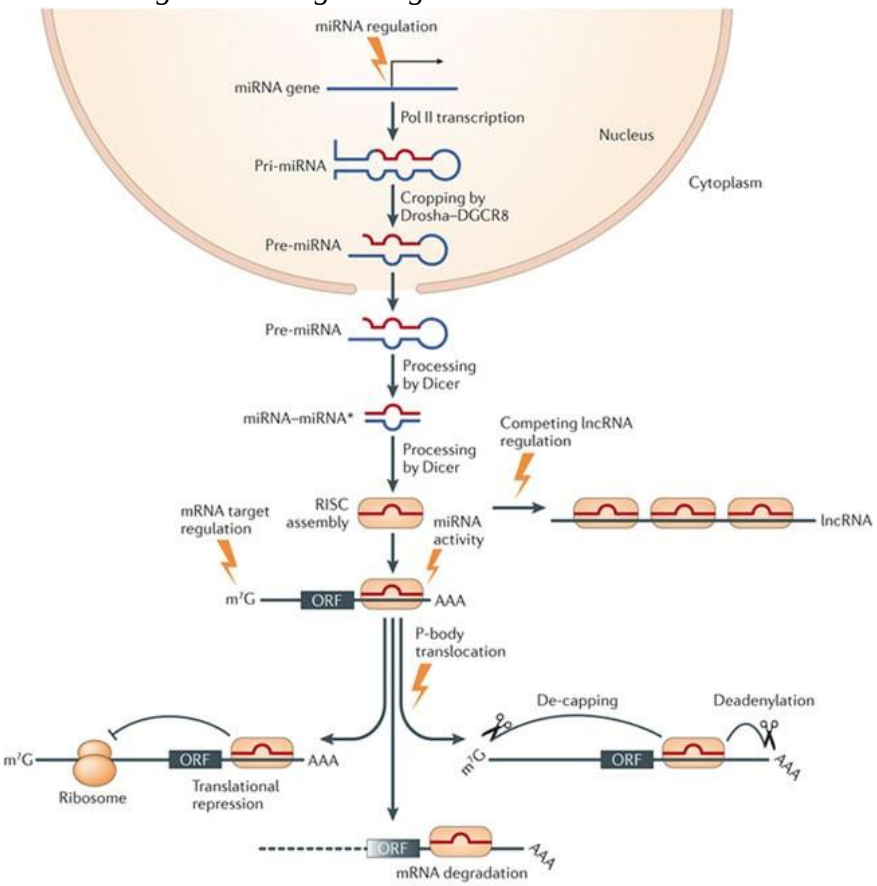


Figure 2. Biogenesis and mechanism of miRNAs (78). miRNAs are transcribed from specific gene regions as primary miRNAs (pri-miRNA), which are processed by the Drosha-DGCR8 complex into precursor miRNAs (pre-miRNA) in the nucleus. Pre-miRNAs are then transported to the cytoplasm, where Dicer processes them into their final duplex form. The active strand of the miRNA duplex is loaded into the RISC complex, which facilitates binding to the 3' untranslated region of their target mRNA. This binding leads to target mRNA de-capping, deadenylation, degradation or repression of its translation into protein. Figure is obtained from van Rooij et al, with permission from Springer Nature (License number: 6107640524318). DGCR8: DiGeorge Syndrome Critical Region 8, lncRNA: long non-coding RNA, mRNA: messenger RNA, miRNA: microRNA, ORF: open reading frame, Pol II: RNA polymerase II, RISC: RNA-induced silencing complex.

The canonical miRNA biogenesis begins with the transcription of miRNA genes or intergenic regions, producing primary miRNAs (pri-miRNAs) that contain hairpin structures (Figure 2). These pri-miRNAs undergo a cleavage by the Drosha-DiGeorge Syndrome Critical Region 8 (DGCR8) complex in the nucleus to precursor miRNAs (pre-miRNAs), followed by export to the cytoplasm. Then, they are further processed by the

Dicer-transactivation response element RNA binding protein (TRBP) endoribonuclease complex into two short mature miRNA strands from the 5' and 3' arms of the pre-miRNA, miR-5p and miR-3p, both of which can be loaded separately into the Argonaute 2 (AGO2) protein to form the RNA-induced silencing complex (RISC). The RISC then silences target gene expression through mRNA degradation, deadenylation, or translational repression (72, 78).

1.3.2. The role of microRNAs in myocardial infarction

miRNAs regulate diverse processes relevant to IHD, including cardiomyocyte proliferation, cell death, angiogenesis, hypertrophy, fibrosis, and inflammation (72). The miR-15 and miR-34 families were identified to be upregulated by ischemia, and promote cardiomyocyte apoptosis. Cardiac-enriched miRNAs like miR-1 and miR-499 enhance cardiac differentiation and lineage commitment in pluripotent stem cells, highlighting their therapeutic potential for reprogramming fibroblasts into cardiomyocytes (79). The miR-212/132 family, activated during heart failure, influences cardiomyocyte hypertrophy and autophagy by modulating the transcription factor FOXO3 (80). In preclinical studies, targeting these miRNAs seemed to be promising (79, 80); however, additional evidence is needed to confirm their therapeutic benefits.

1.3.3. MicroRNAs as therapeutic agents

Since their discovery, there has been growing interest in the therapeutic potential of miRNAs due to several reasons. First, miRNAs are natural molecules with precise regulatory functions. In addition, their ability to target multiple mRNA targets simultaneously allows the modulation of complex phenotypes, which is particularly important in certain diseases (73). Although the results in preclinical research are promising, miRNA-based diagnostics and therapeutics remain in early development with only a few miRNA therapies entering clinical trials and none reaching phase III or drug approval. Current efforts focus on two main types of miRNA drugs: mimics and inhibitors (antagomiRs), with an increasing number of these therapeutics being tested for various diseases (74). miRNA mimics replicate a desired endogenous miRNA strand, while their complementary passenger strand, which is usually a perfect complement, is usually chemically modified to prevent its incorporation into the RISC complex (73). AntagomiRs bind to the endogenous miRNAs through complementary base pairing, and

are chemically modified to improve binding strength, resistance to nucleases, and cellular uptake (78).

1.3.4. Development and challenges of microRNA therapeutics

The development of miRNA therapeutics begins with identifying targets and discovering miRNAs through patient samples or preclinical disease models, combining bioinformatics and biological validation. The relevance and effective modulation of miRNAs in specific diseases are then confirmed using cell culture and animal models. The stability of therapeutic miRNAs is enhanced by various chemical modifications such as 2′-O-methyl, 2′-fluoro, locked nucleic acid, phosphorothioate, and peptide nucleic acid modifications. Precise delivery to target tissues and organs is achieved by conjugating miRNAs with biomolecules and optimizing delivery systems like lipid nanoparticles. Pharmacokinetic and pharmacodynamic studies follow, along with efficacy and toxicity testing *in vivo*. Subsequently, manufacturing, regulatory submissions, and comprehensive Phase I-III clinical trials assessing dosage, safety, and efficacy are conducted. This complex and costly process ultimately aims for a medical approval based on rigorous evaluation of safety and effectiveness (74).

Despite the potential of miRNA-based therapeutics, numerous challenges remain. Each miRNA regulates multiple genes, including unrelated ones, which can cause side effects across different tissues. This complex regulation means systemic overexpression or inhibition may yield both beneficial and harmful effects, complicating therapeutic use. In addition, most *in vivo* studies focus only on target tissues, often overlooking off-target effects. Preclinical doses may also be clinically unfeasible, highlighting the need for further research to determine safe and effective dosing (78). Another challenge is the delivery of miRNA therapeutics specifically to the desired cell types, such as vascular cells, cardiomyocytes, fibroblasts, and macrophages in IHD. Effective delivery relies on appropriate administration methods and vectors that ensure specific cellular uptake and reduce immune responses. Addressing these challenges is essential for advancing miRNA therapies, especially for IHD (72).

1.3.5. Clinical trials of microRNA therapies for cardiovascular diseases

Despite the challenges of miRNA therapeutics development, some miRNA targeting agents are currently being tested in clinical trials for CVDs. The inhibitor of miR-132, CDR132L, which is a synthetic oligonucleotide developed by Cardior Pharmaceuticals

for the treatment of heart failure, was the first to reach clinical trials in cardiology (72). Overexpression of miR-132-3p in cardiac cells contributes to cardiac remodeling and heart failure by suppressing important molecules like the anti-hypertrophic transcription factor FoxO3 and calcium-handling genes such as SERCA2A (81). The efficacy of CDR132L was validated in various models, including a porcine model of heart failure (82). The first phase Ib clinical trial confirmed CDR132L to be well-tolerated and safe in heart failure patients, producing a dose-dependent reduction of the miR-132 levels in plasma (83). CDR132L is currently being evaluated in the multicenter, phase II HF-REVERT trial. This randomized, placebo-controlled study involves 280 patients with cardiac dysfunction after MI, with an ejection fraction ≤45%, and tests two doses of CDR132L against placebo (81).

MRG-110, an locked nucleic acid-based inhibitor of miR-92a-3p by miRagen Therapeutics, showed promise in treating ischemia in animal models, including pigs with MI. A phase I trial with 48 healthy volunteers with small skin wounds and a single intradermal injection of MRG-110 had positive results, but the therapy has not yet advanced to trials in patients with IHD or other conditions (72).

1.3.6. ProtectomiR concept

Nevertheless, several promising candidates for cardioprotective miRNA therapeutics have emerged from preclinical studies. Our research group previously demonstrated the differential expression of miRNAs in an *ex vivo* rat model of ischemic pre- and postconditioning compared to I/R alone, and validated the cytoprotective effects of miR-139-5p, miR-125b*, and let-7b mimics, and the miR-487b antagomiR *in vitro*. We have termed the miRNAs that are potential therapeutic targets for cardioprotection as “protectomiRs” (84). In addition, several other miRNAs, such as miR-1, miR-21, miR-107, miR-133, miR-144, and miR-210, have been identified as regulators of various ischemic conditioning protocols, underscoring their crucial role in modulating cardioprotection (85-88). This suggests that miRNAs play a key regulatory role in cardioprotection, highlighting the importance of identifying and understanding additional miRNAs involved in protective mechanisms.

2. Objectives

In vitro cardiomyocyte models, including primary isolated cardiomyocytes, cell lines, and induced pluripotent stem cell-derived cardiomyocytes, have been previously characterized individually; however, a systematic comparison of these models has not yet been performed. These cardiomyocyte models are also well-suited for testing novel cardioprotective agents against ischemia/reperfusion injury, which are currently lacking in clinical practice. We hypothesize that novel therapeutic molecules or targets can be identified by investigating the molecular mechanisms underlying cardioprotective ischemic conditioning within a relevant and translational model of acute myocardial infarction. Cardioprotective miRNAs, termed protectomiRs, are promising therapeutic candidates; thus, their identification in clinically relevant models could provide new treatments for ischemia/reperfusion injury.

To deepen our understanding of *in vitro* cardiomyocyte models and to identify protectomiRs, the following objectives have been defined:

I. Systematic comparison of commonly used cardiomyocyte models from rats, mice, and humans based on transcriptomic analysis, their response to hypertrophic and adrenergic stimuli, and their tolerance to simulated ischemia/reperfusion injury, in order to determine their similarities or cell-specific characteristics.

II. ProtectomiR identification and validation:

IIa. Analysis of the miRNA expression profile of a clinically relevant closed-chest porcine model of acute myocardial infarction in combination with ischemic preconditioning, postconditioning, or remote perconditioning, to identify miRNAs in association with cardioprotection (protectomiRs).

IIb. Validation of the cardiocytoprotective effect of identified protectomiR candidates in cardiomyocyte models subjected to simulated ischemia/reperfusion injury.

IIc. Investigation of the mechanism of action of the identified protectomiRs through bioinformatics target prediction and subsequent functional analysis.

Together, these objectives aim to provide comprehensive insights into the properties of different cardiomyocyte models, and to propose novel therapeutic agents or targets for myocardial ischemia/reperfusion injury.

3. Methods

3.1. Ethical statements

All experimental procedures were conducted in compliance with the ethical standards set by the institutional and national committees on human experimentation, following the Helsinki Declaration (1975). Written informed consent was obtained from every patient participating in the study, by the protocol approved by the Local Ethics Committees of the Institute of Cardiology, Warszawa, Poland (IK-NP-0021–24/1426/14).

The study adhered to the Guide for the Care and Use of Laboratory Animals, 8th edition issued by the National Research Council (US) Committee (National Academies Press (US), 2011) and the EU Directive (2010/63/EU), with approval granted by the Institutional Animal Care and Use Committee of Semmelweis University, Budapest, Hungary (PE/EA/1784–7/2017).

3.2. H9C2 cell line maintenance and differentiation

The rat myoblast cell line, H9C2, was obtained from the European Collection of Authenticated Cell Culture (#CRL-1446, Salisbury, UK). H9C2 cells were maintained in DMEM (#10-014-CVR, Corning Inc., Corning, NY, USA), supplemented with 10% fetal bovine serum (FBS, #35-015-CV, Corning Inc., Corning, NY, USA), 2 mM L-glutamine (#25-005-CI, Corning Inc., Corning, NY, USA) and 1% Antibiotic-Antimycotic solution (#30-004-CI, Corning Inc., Corning, NY, USA) at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged every 2-3 days at 70% confluence to maintain morphological characteristics. All experiments were performed within 10 passages after thawing. The differentiation of H9C2 cells to cardiomyocytes was conducted as described previously (89). To test the differentiation protocol, cells were seeded onto 6-well or 96-well plates. Upon reaching full confluence (day 0), the growth medium was replaced with 1% FBS medium for 24 hours. On day 1, 10 nM all-trans retinoic acid (ATRA, #R2625, Merck, Brighton, MA, USA) was added to the medium, which was changed every two to three days. ATRA was kept and used in the dark to prevent degradation. Cells in 6-well plates were collected either during the proliferation stage (one day before confluence; day -1) or after differentiation (days 8–10) using Qiazol Lysis buffer (#79306, Qiagen, Hilden, Germany) for RNA isolation. Cells in 96-well plates were used for sI/R experiments (Figure 3).

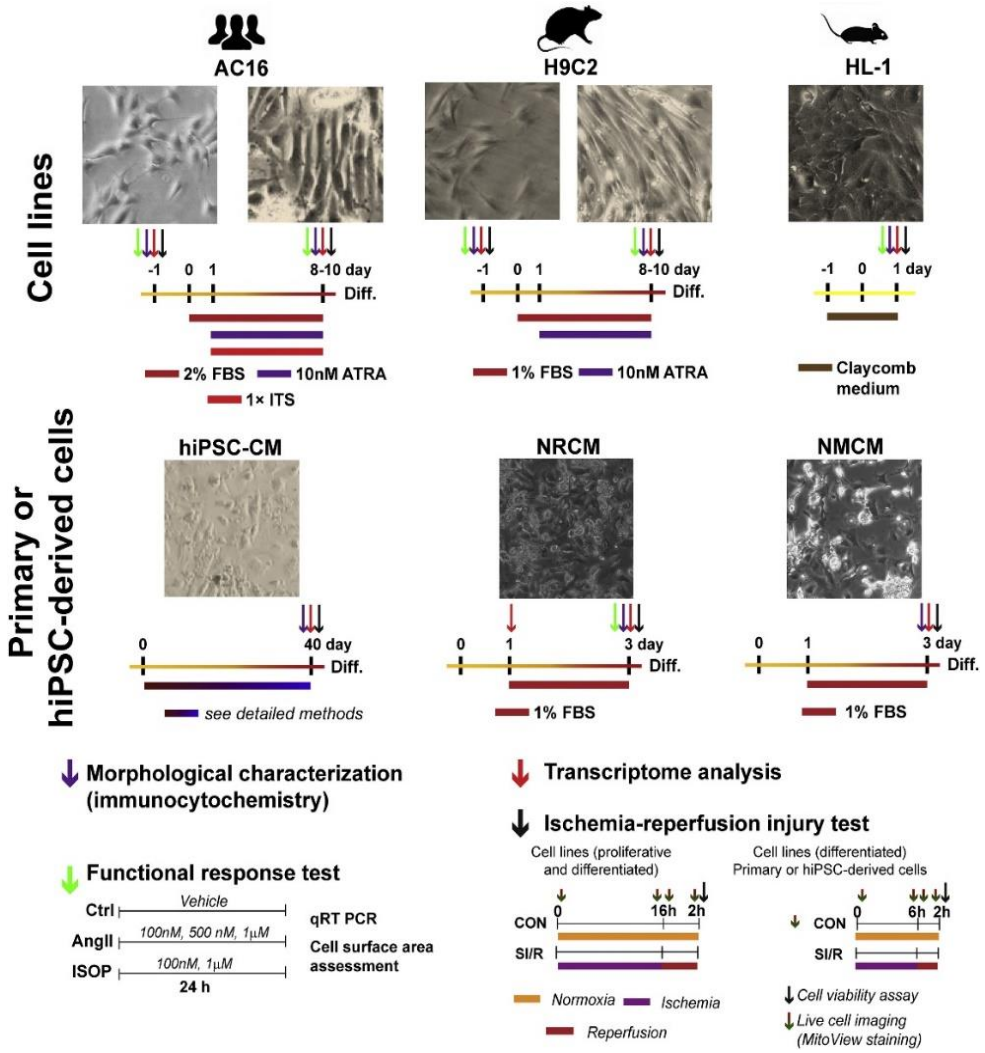


Figure 3. Overview of the experimental design for the systematic comparison of cardiomyocyte models. Obtained from Onódi et al. (89) FBS: fetal bovine serum, ATRA: all-trans retinoic acid, ITS: insulin-transferrin-selenium supplement, Diff: differentiated, hiPSC-CM: human induced pluripotent stem cell derived cardiomyocytes, NRCM: neonatal rat cardiomyocytes, NMCM: neonatal mouse cardiomyocytes, Ctrl/CON: control, AngII: angiotensin II, ISOP: isoprenaline, SI/R: simulated ischemia-reperfusion.

3.3. AC16 cell line maintenance and differentiation

The human cardiomyocyte cell line, AC16, was obtained from Merck (LOT: RD1606008, #SCC109, Brighton, MA, USA). AC16 cells were maintained in DMEM-F12 (#04-687F/U1, Lonza, Basel, Switzerland), supplemented with 12.5% FBS (#35-015-CV, Corning Inc., Corning, NY, USA), 2 mM L-glutamine (#25-005-CI, Corning Inc., Corning, NY, USA), 10 mM HEPES (#15630080, Thermo Fisher Scientific, Waltham, MA, USA) and 1% Antibiotic-Antimycotic solution (#30-004-CI, Corning Inc., Corning, NY, USA) at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged every

2-3 days at 90% confluence to maintain morphological characteristics. All experiments were performed within 10 passages after thawing. To test differentiation protocols, cells were seeded onto 6-well or 96-well plates. Upon reaching 80% confluence (day 0), the growth medium was replaced with differentiation medium containing 2% FBS, 1x insulin-transferrin-selenite supplement (ITS, #I3146, Merck, Brighton, MA, USA), and 10 nM ATRA (#R2625, Merck, Brighton, MA, USA). Differentiation was carried out with or without cell passaging (referred to as AC16 Diff + Pass and AC16 Diff, respectively) according to the observed confluency during differentiation. Cells were harvested either during the proliferation stage (day -1, before full confluence) or at the differentiated stage (days 8–10) using Qiazol Lysis buffer (#79306, Qiagen, Hilden, Germany) for RNA isolation. Cells seeded in 96-well plates were used for sI/R experiments (Figure 3).

3.4. HL-1 cell line maintenance

The mouse cardiomyocyte cell line, HL-1 (LOT: RD1601001, #SCC065), was obtained from Merck. HL-1 cells were maintained in Claycomb medium (#51800C, Merck, Brighton, MA, USA), supplemented with 10% HL-1 Qualified FBS (#TMS-016-B, Merck, Brighton, MA, USA), 2 mM L-glutamine (#25-005-CI, Corning Inc., Corning, NY, USA), 100 nM norepinephrine (#A0937, Merck, Brighton, MA, USA, 100x stock in 30 mM L-Ascorbic Acid (#A7506, Merck, Brighton, MA, USA)) and 1% Antibiotic-Antimycotic solution (#30-004-CI, Corning Inc., Corning, NY, USA) at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged every 2-3 days at 100% confluence to maintain morphological characteristics. All experiments were performed within five passages after thawing. To compare with primary cell cultures and heart tissue, cells were seeded onto 6-well or 96-well plates. Once cells reached full confluence (day 0) and began beating, typically by day 1, they were harvested in Qiazol Lysis buffer (#79306, Qiagen, Hilden, Germany) for RNA isolation. Cells seeded in 96-well plates were used for sI/R experiments (Figure 3).

3.5. Human induced pluripotent stem cell (hiPSC) culture and differentiation

Human induced pluripotent stem cells-derived cardiomyocytes were differentiated from the XCL-1 stem cell line, purchased from XCellScience (#CAXIP-001-1V, Novato, CA, USA). The hiPSCs were cultured under standard conditions in a 5% CO_2 incubator at 37°C on Matrigel-coated (#356237, Corning Inc., Corning, NY, USA) 6-well or 96-well

plates using mTeSR medium (#100-0276, Stemcell Technologies, Vancouver, Canada). Differentiation into dense cardiomyocyte monolayers (75–90% confluence) was initiated at passage 26 by switching to RPMI 1640 medium (#21875034, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with insulin-free B27 (#17504044, Thermo Fisher Scientific) and 10 μM CHIR99021 (#S1263, Selleckchem, Houston, TX, USA). On day 2, the medium was replaced with RPMI plus insulin-free B27, followed on day 3 by RPMI with insulin-free B27 containing 2.5 μM C59 Wnt inhibitor (#S7037, Selleckchem). From day 5 onward, the medium was changed every other day with RPMI supplemented with B27 containing insulin (#A1895601, Thermo Fisher Scientific). Beating areas typically appeared around day 7 post-induction. Between days 10 and 14, metabolic selection was performed by culturing cells in glucose-free RPMI (#11879020, Thermo Fisher Scientific) supplemented with B27 for four days. All experiments were conducted between days 35 and 40 (Figure 3).

3.6. Primary neonatal rat and murine cardiomyocyte isolation

Neonatal rat and murine cardiomyocytes (NRCM and NMCM, respectively) were isolated from newborn (1-3 day-old) Wistar rats or C57BL/6 mice obtained from Toxi-Coop Zrt. with the Pierce™ Primary Cardiomyocyte Isolation Kit purchased from Thermo Fisher Scientific (#88281, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, after cervical dislocation of the animals, the chest was opened, and the heart was dissected using forceps. The hearts were immediately placed in ice-cold Hanks' Balanced Salt Solution (#88284, Thermo Fisher Scientific, Waltham, MA, USA). Then, the hearts were minced into 1-3 mm pieces using sterile scissors under a laminar hood. The tissue pieces were washed twice with Hanks' Balanced Salt Solution, then Cardiomyocyte Isolation Enzyme 1 (#88288, Thermo Fisher Scientific, Waltham, MA, USA) and Enzyme 2 (#88289, Thermo Fisher Scientific, Waltham, MA, USA) were added to each sample. Following a 30-minute digestion period at 37 °C, the samples were washed twice with Hanks' Balanced Salt Solution. Then, the digested tissue was resuspended in DMEM (#88287, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (#35-015-CV, Corning Inc., Corning, NY, USA) and 1% Antibiotic-Antimycotic (#30-004-CI, Corning Inc., Corning, NY, USA), quantified, and seeded onto 6-, 24- or 96-well plates according to the experimental setup.

The cells were kept at 37 °C in a humidified incubator with 5% CO_2. Cell cultures were used at day 1 or day 3 after isolation. (Figure 3).

3.7. Tissue collection from human, rat, and mouse hearts

Whole heart tissue samples were collected from humans, rats, and mice for RNA sequencing, as previously described (89):

Human heart tissues were collected at the Department of Heart Failure and Transplantology, Cardinal Stefan Wyszyński Institute of Cardiology, Warsaw, Poland, as previously described (90). Healthy hearts were obtained from organ donors (n=3) whose hearts were not used for transplantation due to technical reasons, such as donor-recipient incompatibility. These donors had no significant cardiovascular history and showed no abnormalities in ECG or echocardiography. Left ventricular tissue samples were taken from the free wall at the time of heart explantation, avoiding scarred, fibrotic, or adipose tissue, as well as the endocardium, epicardium, and coronary vessels. Patient characteristics are summarized in Table 1.

Table 1. Patient characteristics. Obtained from Onódi et al. (89).

	Patient #1	**Patient #2**	**Patient #3**
Sex, age	Female, 41	Male, 27	Male, 30
Body mass index (kg/m2)	20.3	31.7	27.7
Cause of death	subarachnoid hemorrhage	head injury	head injury
Medical history	ø relevant conditions		
Echocardiography	ejection fraction > 60%, normal left ventricle dimensions		
Medications administered before explantation	noradrenaline, mannitol, furosemide	noradrenaline, levofloxacin, metoprolol, enoxaparin	noradrenaline, metronidazole, piperacillin + tazobactam

Left ventricular tissue was collected from healthy male Wistar rats (7-8 weeks old, 280-330 g, n=3) and male C57BL/6 mice (11-12 weeks old, 25-30 g, n=3). Following anesthesia with an intraperitoneal injection of 60 mg/kg pentobarbital (#17F015, Produlab Pharma, Raamsdonksweer, The Netherlands), hearts were excised. Samples were immediately rinsed in saline, blotted dry, flash-frozen in liquid nitrogen, and stored at -80 °C until further analysis.

3.8. RNA isolation, library preparation, and RNA sequencing

Cells and tissue samples were lysed in 1 mL of QIAzol Lysis Reagent (#79306, Qiagen, Hilden, Germany). Total RNA was extracted from 350 µL of lysate using the Direct-zol RNA MiniPrep System with on-column DNase I treatment (#R2053, Zymo Research,

Irvine, CA, USA) following the manufacturer's instructions. RNA integrity and concentration were assessed using the RNA ScreenTape system with the 2200 TapeStation (#5067-5579, Agilent Technologies, Santa Clara, CA, USA) and the RNA HS Assay Kit with Qubit 3.0 Fluorometer (#Q32855, Thermo Fisher Scientific, Waltham, MA, USA), respectively. For gene expression profiling, libraries were prepared using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (#015.96, Lexogen GmbH, Vienna, Austria) following the manufacturer's protocol. Library quality and quantity were evaluated with the High Sensitivity DNA1000 ScreenTape system on the 2200 TapeStation (#5067-5585, Agilent Technologies) and the dsDNA HS Assay Kit with Qubit 3.0 (#Q32854, Thermo Fisher Scientific). Pooled libraries were diluted to 1.8 pM and sequenced with 1x86 bp single-end reads using the 75-cycle High Output v2.5 Kit on the NextSeq 500 Sequencing System (#20024906, Illumina, San Diego, CA, USA) according to the manufacturer's guidelines.

3.9. Bioinformatic analysis of RNA sequencing

The analysis of RNA sequencing data was described previously (89). Briefly, raw RNA-sequencing reads were preprocessed using Cutadapt (v1.15) to trim adapters, poly(A) tails, and bases below Phred score 30, filtering out reads shorter than 19 nt. Quality control was done with FastQC (v0.11.8) and MultiQC (v1.7). HISAT2 (v2.0.4), featureCounts (Subread 2.0.0), and DESeq2 (v1.10.1) were used for alignment, annotation, normalization, and differential expression analysis. Reference genomes from *Homo sapiens* (GRCh37), *Rattus norvegicus* (Rnor_6.0), and *Mus musculus* (GRCm38) guided species-specific analysis. Principal component analysis (PCA) via DESeq2 was performed to visually compare whole transcriptome similarities among groups. Functional analysis of transcriptomic profiles involved manually selecting Gene Ontology (GO) biological process terms related to cardiovascular functions. Mean DESeq2-normalized gene abundances were calculated per group, and functional expression vectors were created by filtering genes annotated to each GO term or its descendants via "*is a*" relationships. Construction used Gene Ontology OBO v1.2 and taxon-specific GAF v2.1 files. To compare biological functions, a signed cosine distance was computed between the groups' functional expression vectors and reference tissue, indicating the magnitude and direction of their differences. The signed cosine distance was calculated using the following formula:

$$Signed\ cosine\ distane = \left(1 - \frac{\sum_{i=1}^{n} A_i B_i}{\sqrt{\sum_{i=1}^{n} A_i^2}\sqrt{\sum_{i=1}^{n} B_i^2}}\right) sgn\left(\frac{\sum_{i=1}^{n} A_i (B_i - A_i)}{\sqrt{\sum_{i=1}^{n} A_i^2}\sqrt{\sum_{i=1}^{n} (B_i - A_i)_i^2}}\right)$$

where Ai and Bi represent the components of the reference and studied vectors, respectively. The distance metrics were computed using scripts developed in R (version 3.2.3).

3.10. Functional response to hypertrophic and adrenergic signals

AC16 cells, differentiated AC16 cells, H9C2 cells, differentiated H9C2 cells, and NRCMs were seeded into 6-well plates or 13 mm cover slips in 24-well plates for real-time polymerase chain reaction (RT-PCR) or cell surface area measurements, respectively. The cells were treated with 100 nM, 500 nM or 1 μM angiotensin II (ANGII, #A9525, Sigma, St. Louis, MO, USA) for 24 hours, or 100 nM or 1 μM isoprenaline (ISOP; #I2760, Sigma, St. Louis, MO, USA) for 24 hours to test their functional response to hypertrophic or adrenergic stimuli. Following treatment, the cells were harvested for RNA isolation and RT-PCR as described in Section 3.11, or their cell surface area was measured as described in Section 3.12.

3.11. Real-time polymerase chain reaction

Total RNA isolation, reverse transcription, and RT-PCR were performed as previously described (89). Briefly, the cells were homogenized in Qiazol (#79306, Qiagen, Hilden, Germany), and total RNA was isolated using a chloroform/isopropanol precipitation method. The isolated RNA samples were digested by DNase I (#AM2224, Thermo Fisher Scientific, Waltham, MA, USA), and then RNA concentrations were determined with NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). The reverse transcription using 2 μg RNA was conducted by High-Capacity cDNA Reverse Transcription Kit (#4374966, Applied Biosystems, Foster City, CA, USA). The target genes were amplified using the SYBR® Select Master Mix (#4472920, Thermo Fisher Scientific, Waltham, MA, USA) using LightCycler® 480 (Roche Molecular Systems, Pleasanton, CA, USA) with the following protocol: 95 °C for 1 minute, 60 cycles of 95 °C for 10 seconds, and 60 °C for 10 seconds, followed by final cooling to 40 °C for 10 seconds. *Rplp0* was used as a reference gene. The forward and reverse primer sequences are provided in Table 2. Gene expression differences were calculated by the ΔΔCt method.

Table 2. List of primers used for real-time polymerase chain reaction analysis. Obtained from Onódi et al (89). Tm: melting temperature; Rplp0: ribosomal protein lateral stalk subunit P0; Ctgf: connective tissue growth factor; Col1a1: collagen type I alpha 1 chain; Nppb: natriuretic peptide B; Col3a1: collagen type III alpha chain.

Gene	Species	Forward	Reverse	Product length	Tm (°C)
Rplp0	Rat	TTGAAATCCTGAGCGATGTGCAGC	GCCATTGTCAAACACCTGCTGGAT	134	64
Rplp0	Human	GCGTCCTCGTGGAAGTGACA	GCATCTGCTTGGAGCCCACA	184	62
Rplp0	Mouse	TCCAGGCTTTGGGCATCA	CTTTATCAGCTGCACATCACTCAGA	74	60
Ctgf	Rat	GCGAGCCAACTGCCTGGTCC	GCGTCCGGATGCACTTTTTGCC	194	65
Ctgf	Human	GGAAGAGAACATTAAGAAGGGCA	TCCGGGACAGTTGTAATGGC	256	55
Ctgf	Mouse	AGCGGTGAGTCCTTCCAAAG	TTCCAGTCGGTAGGCAGCTA	222	61
Col1a1	Rat	TGGACCTCCGGCTCCTGCTC	TCGCACACAGCCGTGCCATT	172	65
Col1a1	Human	GTGGACACTACCCTCAAGAGC	AGTGGCACATCTTGAGGTCAC	109	60
Col1a1	Mouse	TCTCCACTCTTCTAGTTCCT	TTGGGTCATTTCCACATGC	226	62
Nppb	Rat	GACGGGCTGAGGTTGTTTTA	ACTGTGGCAAGTTTGTGCTG	195	58
Nppb	Human	CGCAAAATGGTCCTCTACAC	CCGTGGAAATTTTGTGCTC	298	55
Nppb	Mouse	TTTGGGCTGTAACGCACTGAA	TGTGGCAAGTTTGTGCTCCA	219	60
Col3a1	Rat	CTGTCCCGCGGAAGCACTGG	ATGTTCTGGGAGGCCCGGCT	178	65
Col3a1	Human	GACAGATGCTGGTGCTGAGAAG	TCTGAGGAAGGCCAGCTGTAC	127	61
Col3a1	Mouse	GCTCGAGGCAATGATGGT	ACCCTGCAGGTCCAACTTC	118	58

3.12. Cell surface area measurement

Treated cells were fixed with 2% paraformaldehyde in 1x PBS for 5 minutes at room temperature, followed by permeabilization with 0.2% Triton X-100 diluted in PBS (#T8787, Sigma, St. Louis, MO, USA) for 10 minutes at room temperature. Slides were then stained with phalloidin iFluor-594 (#ab176757, Abcam, Cambridge, UK) and DAPI (#D1306, Thermo Fisher Scientific, Waltham, MA, USA). Images were captured using a Leica LMD6 microscope (Leica Microsystems, Wetzlar, Germany). The surface area of 80-180 cells from eight independent fields per well was measured blinded using ImageJ software (version 1.8.0) (91).

3.13. Porcine model of acute myocardial infarction and ischemic conditioning

25-35 kg domestic female pigs were subjected to acute MI and ischemic conditioning stimuli in a previous study by Baranyai et al., as described previously (56, 92). The cardioprotective phenotype due to ischemic conditioning was determined by cardiac MRI, measuring infarct size, myocardial edema, and microvascular obstruction (56). Here, we collected left ventricular tissue samples that were snap-frozen in liquid nitrogen and stored at -80 °C until use. The following groups were utilized in the current study (Figure 4):

i) Sham-operated control group (Sham): a balloon catheter was inserted into the coronary artery but was not inflated.

ii) Ischemia-reperfusion group (AMI): MI was induced by occluding the LAD coronary artery for 90 minutes, followed by a 3-hour reperfusion.

iii) Ischemic preconditioning group (IPreC): three cycles of 5-minute I/R were applied before the 90-minute LAD artery occlusion, followed by a 3-hour reperfusion.

iv) Ischemic postconditioning group (IPostC): six cycles of 30-second I/R were applied at the onset of the 3-hour reperfusion immediately after the 90-minute LAD occlusion.

v) Ischemic remote perconditioning (RIPerC): four cycles of 5-minute I/R were induced in the hind limb during the final 40 minutes of the 90-minute LAD artery occlusion, followed by a 3-hour reperfusion.

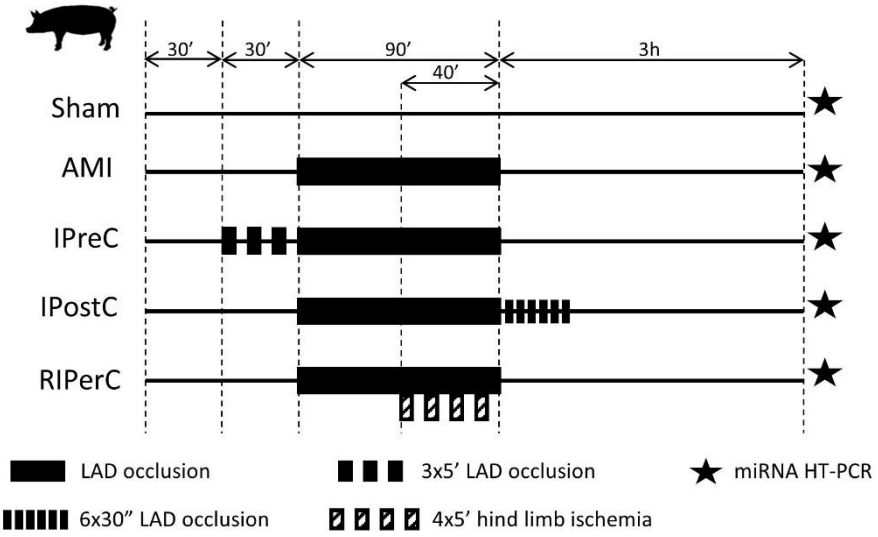


Figure 4. Treatment protocol of pigs subjected to closed-chest coronary artery occlusion/reperfusion and various ischemic conditioning protocols. Obtained from Nagy et al. (92) Sham: sham-operated control; AMI: acute myocardial infarction; IPreC: ischemic preconditioning; IPostC: ischemic postconditioning; RIPerC,: remote ischemic perconditioning).

3.14. MicroRNA isolation and high-throughput RT-PCR

miRNA was isolated from left ventricular tissue samples using the High Pure miRNA Isolation Kit (#05080576001, Roche Molecular Systems, Pleasanton, CA, USA) according to the manufacturer's protocol. MiRNA quantity was measured by NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). 1 μg of miRNA was reverse-transcribed to cDNA by the TaqMan™ MicroRNA Reverse Transcription Kit (#4366597, Applied Biosystems, Foster City, CA, USA). 4 ng of cDNA was used for each reaction with 10 pmol miRNA-specific primers and 1 μL LightCycler 1536 DNA Green Master

(#05573092001, Roche Molecular Systems, Pleasanton, CA, USA). All primers were purchased from Bioneer (Daedong, Korea). A total of 220 pig miRNAs were selected based on miRBase Release 20 (June 2013) (93), and further confirmation of their myocardial expression by a literature search in PubMed. The following search string was used for literature search in January 2016: "(pig OR swine OR porcine OR sus) AND (miRNA OR microRNA OR miR OR (micro AND RNA)) AND (heart OR cardi* OR myocardi*)". The LightCycler 1536 System (Roche Life Science, Basel, Switzerland) was used for performing the high-throughput RT-PCR with the following protocol: activation at 95 °C for 1 minute, then amplification by 60 cycles of 95 °C for 10 seconds, and 60 °C for 10 seconds, followed by final cooling to 40 °C for 10 seconds. MiRNA expression patterns were determined by the ΔΔCt method.

3.15. Selection of protectomiR candidates

Differential miRNA expression was identified using criteria of $\log_2$ fold changes ≥ 0.586 (upregulation) or ≤ -0.586 (downregulation) with p-values < 0.05 by Student's t-test. First, miRNAs differentially expressed in IPreC versus AMI were selected, followed by those altered in IPostC and RIPerC versus AMI. Overlapping miRNAs among all three cardioprotective conditioning protocols, IPreC, IPostC, and RIPerC, were identified as protectomiR candidates, assuming their expression changes participate in cardioprotection. During the *in vitro* validation experiments, miRNAs upregulated due to all three conditioning stimuli were modulated by miRNA mimics, while the downregulated miRNAs were suppressed by antagomiRs, simulating potential protective interventions.

3.16. Cross-species pig-rat-human microRNA sequence similarity

To determine the sequence orthologues of the selected protectomiR candidates in rats and humans, we used NCBI RNA BLAST and miRBase database (93). During the *in vitro* validation experiments in NRCMs, those protectomiR candidates were screened for cardiocytoprotection, which showed 100% sequence similarity across all three species.

3.17. MicroRNA transfection of cardiomyocytes

NRCM and AC16 cells were transfected with selected protectomiR candidates as we previously described (92). Briefly, 25 nM, 50 nM, or 100 nM of Dharmacon miRIDIAN microRNA Mimics or Hairpin Inhibitors (Horizon Discovery, Waterbeach, UK) were transfected by DharmaFECT1 transfection reagent (Horizon Discovery, Waterbeach,

UK) in antibiotic-free growth medium for 10 hours, followed by a 10-hour recovery phase without miRNA treatment. As a negative control, 50 nM of miRIDIAN microRNA Mimic (#CN-001000-01-05, Horizon Discovery, Waterbeach, UK) or Hairpin Inhibitor (#IN-001005-01-05, Horizon Discovery, Waterbeach, UK) Negative Control, a non-targeting miRNA from *Caenorhabditis elegans*, was used.

3.18. Simulated ischemia/reperfusion injury

Simulated ischemia/reperfusion (sI/R) injury was performed as we described previously (89, 92). Briefly, the cells were seeded onto 24-well or 96-well plates. 24 hours after seeding, the cell culture medium was changed to a hypoxic solution (119 mM NaCl, 5.4 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5 mM HEPES, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 20 mM Na-lactate and 0.1% BSA, pH 6.4) or a normoxic solution (125 mM NaCl, 5.4 mM KCl, 1.2 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 20 mM HEPES, 1.3 mM $MgSO_4$, 1 mM $CaCl_2$, 15 mM glucose, 5 mM taurine, 2.5 mM creatine-monohydrate, and 0.1% BSA, pH 7.4) as a control. To mimic ischemia, the hypoxia-treated cells were placed into a three-gas incubator in a hypoxic atmosphere (94% N_2, 5% CO_2, 1% O_2), while the control cells were placed in a standard CO_2 incubator. The length of the simulated ischemic period was 6 hours for the NRCM and NMCM cells, and 16 hours for the AC16, H9C2, HL-1 cell lines and hiPSC-CMs, based on our preliminary results and literature, in line with the recommendation of the European Society of Cardiology (ESC) Working Group Cellular Biology of the Heart, which suggests that the optimal *in vitro* setting for cardioprotection testing requires 50% cell death following I/R injury (58).

3.19. Cell viability measurements

To assess cell viability, the cell culture medium was removed, and the cells were washed twice with 1x DPBS. The cells were then incubated with 1 μM calcein-AM (#PK-CA707-80011, PromoCell, Heidelberg, Germany) for 30 min in a dark chamber. The fluorescence intensity was measured at 490 nm excitation and 520 nm emission using a VarioscanLUX plate-reader (Thermo Fisher Scientific, Waltham, MA, USA). In AC16 cells, following miRNA transfection experiments, the CellTiter-Glo Luminescent Viability Assay kit (#G7571, Promega, Madison, WI, USA) was applied according to the manufacturer's protocol.

3.20. MicroRNA target prediction, selection, and functional annotation

The targets of the endogenous sequences or porcine, rat and human miR-450 were predicted separately by miRNAtarget™ software (http://mirnatarget.com; Pharmahungary, Szeged, Hungary) (94-97), that integrates experimentally validated miRNA-target interactions from miRTarBase (version 9.0) (98), and predicted interactions from miRDB (version 6.0, with score > 80.0) (99, 100) and TargetScan (version 8.0) (101) databases. To identify targets expressed in the myocardium, we analyzed our RNA sequencing data from rat and human samples. We considered a target to be cardiac-expressed if it was detected in at least two left ventricular samples. The cardiac expression was shown as transcripts per million (TPM) units. We then identified the common predicted cardiac targets across all three species. Since target gene symbols may vary between species, ortholog information from Ensembl (release 111) was used to match genes across species. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted on all porcine and filtered rat and human targets. GO enrichment for biological processes was performed using the statistical overrepresentation test from the PANTHER Classification System (version 18.0) (102) via the Gene Ontology Consortium website (release 2024-01-17), applying default settings (103, 104). KEGG pathway enrichment was analyzed using DAVID (Database for Annotation, Visualization, and Integrated Discovery; Knowledgebase release v2024q1) (105, 106). We then identified the common GO biological processes and KEGG pathways across all three species.

3.21. Statistical analysis

All data are displayed as mean ± standard error of mean (SEM), except miRNA RT-PCR data, that is presented as mean ± standard deviation (SD), showing $\log_2$ changes. The unpaired Student's t-test was applied to compare two groups. To compare more than two groups, following testing for normal distribution, one-way analysis of variance (ANOVA) with Tukey's or Dunnett's multiple comparison test or Kruskal-Wallis test with Dunn's multiple comparisons test was applied to evaluate cell viability measurements. P-value <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

4. Results

4.1. Systematic comparison of cardiomyocyte models

4.1.1. Transcriptomic profile analysis of cardiomyocyte models

To systematically compare the gene expression profiles of the most commonly used cardiomyocyte models with their corresponding source tissues, we performed deep RNA sequencing. We evaluated the transcriptomic similarities between the different cellular models and adult cardiac tissues by analyzing the number of differentially expressed genes, conducting PCA, and hierarchical clustering. Our results showed that NRCM, NMCM, and hiPSC-CM cells exhibited a greater similarity to adult cardiac tissue samples compared to cell line models, regardless of their differentiation level (Figure 5).

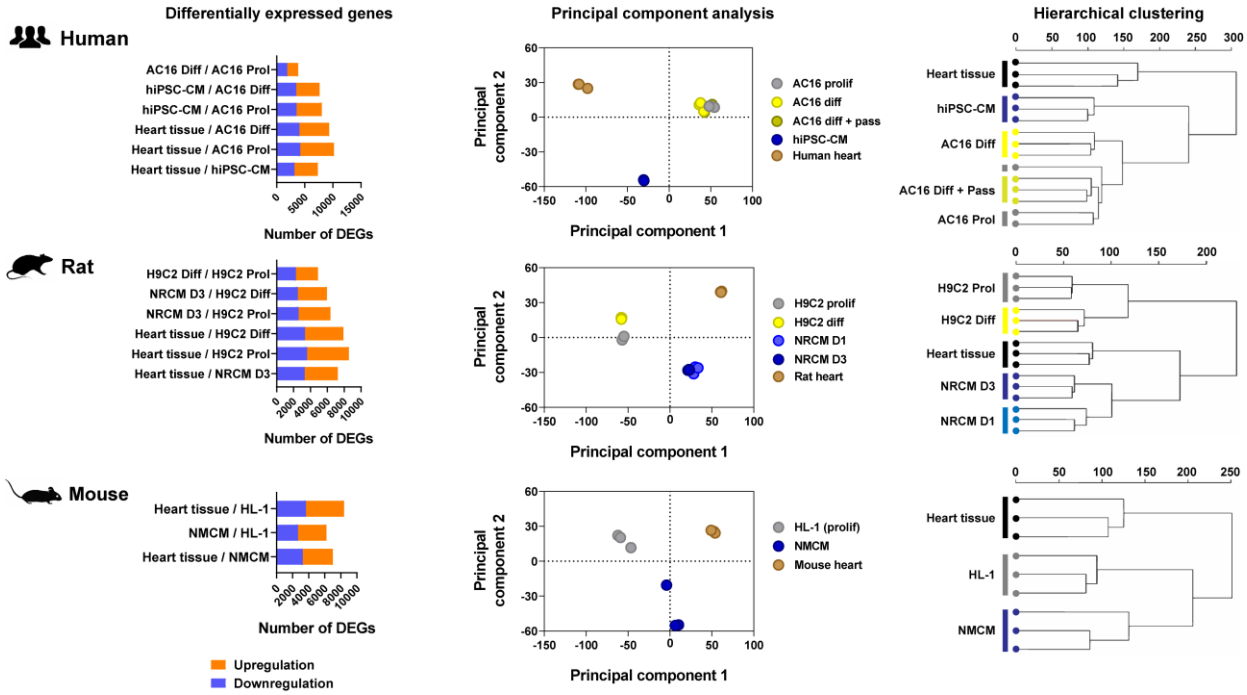


Figure 5. Transcriptomic analysis of human, rat, and mouse cardiomyocyte models and adult cardiac tissue. Whole transcriptome analysis of cellular models and cardiac tissues, including the number of differentially expressed genes (DEGs), principal component analysis (PCA), and hierarchical clustering. The plot displays the first two principal components, with dots colored according to their respective cell or tissue types. N = 3/group individual samples. Obtained from Onódi et al. (89).

To identify functional differences between cellular models and adult cardiac tissues, we selected GO terms relevant to cardiac functional parameters investigated in this study. Using our sequencing data, we calculated the signed cosine distances based on the differential expression of genes annotated to these GO terms (Figure 6). Our results

revealed that hiPSC-CMs are more similar to human cardiac tissues compared to proliferative or differentiated AC16 cells. However, both hiPSC-CMs and AC16 cells showed low expression of genes related to the inflammatory response. Furthermore, differentiation of AC16 cells towards a more mature cardiomyocyte did not lead to significant changes in the expression of functionally relevant genes. H9C2 cells and NRCMs demonstrated low functional similarity based on our transcriptomic data to rat myocardium, particularly in striated muscle cell development, inflammatory response, and skeletal muscle fiber development. In contrast, HL-1 cells and NMCMs showed greater similarity to murine cardiac tissue; however, genes associated with inflammatory response, endocardium development, and cardiac muscle cell differentiation exhibited less similar expression patterns in these models (Figure 6). Taken together, primary cells and hiPSC-CMs exhibit greater transcriptomic and functional similarity to adult cardiac tissues than cell line models.

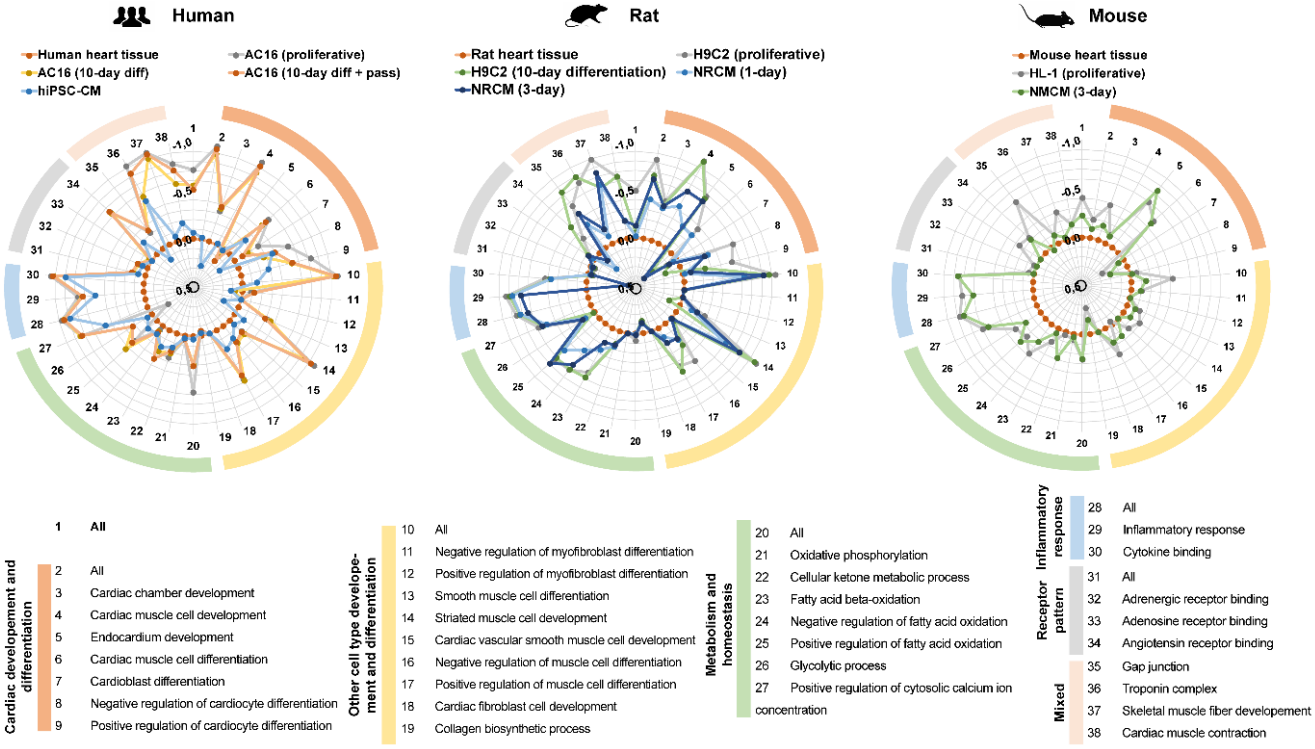
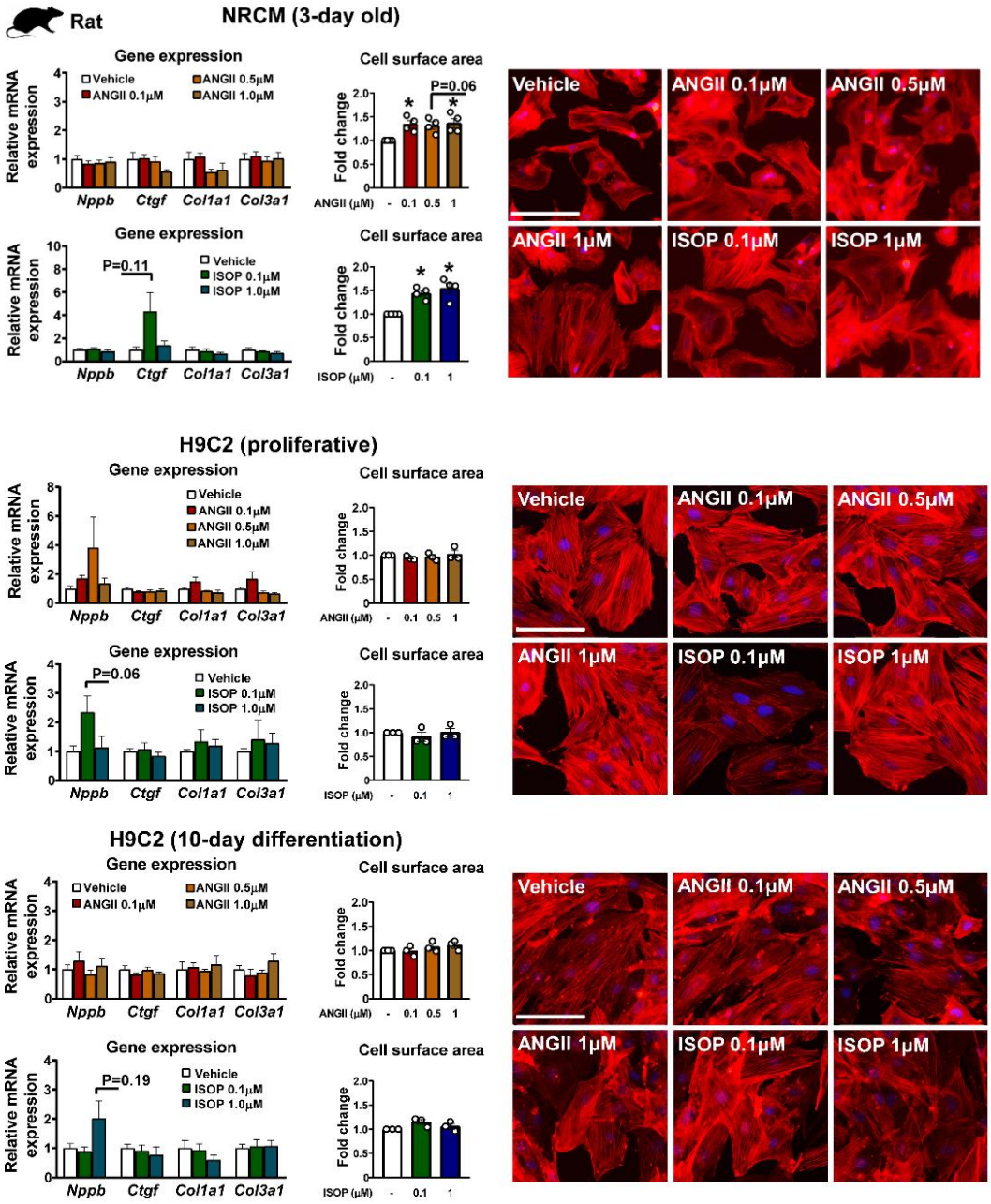


Figure 6. Radar plot illustrating the similarity of functional expression profiles based on GO terms between cardiac cell lines or primary cultures and their corresponding adult cardiac tissues across human, rat, and mouse species. In the radar plots, each concentric ring corresponds to a change in signed cosine distance, which quantifies the dissimilarity of functional expression vectors. The outermost ring represents the minimum value (−1.0), while the central point corresponds to +0.5. Negative and positive signed cosine distances indicate a relative

decrease or increase, respectively, in the expression of genes associated with the selected Gene Ontology terms. The reference baseline (zero) for this analysis is the corresponding heart tissue for each species. N = 3/group individual samples. Obtained from Onódi et al. (89)

4.1.2. Response of cardiomyocyte models to hypertrophic stimuli

Cardiac hypertrophy is a key adaptive response of cardiomyocytes to ischemic stress. Therefore, studying hypertrophic responses *in vitro* provides valuable insights into the underlying mechanism. Cell lines and primary NRCMs were treated with increasing doses of ANGII (100, 500, 1000 nM) or ISOP (100, 1000 nM) for 24 hours to examine their response to hypertrophic or adrenergic stimuli. Cell surface area measurements



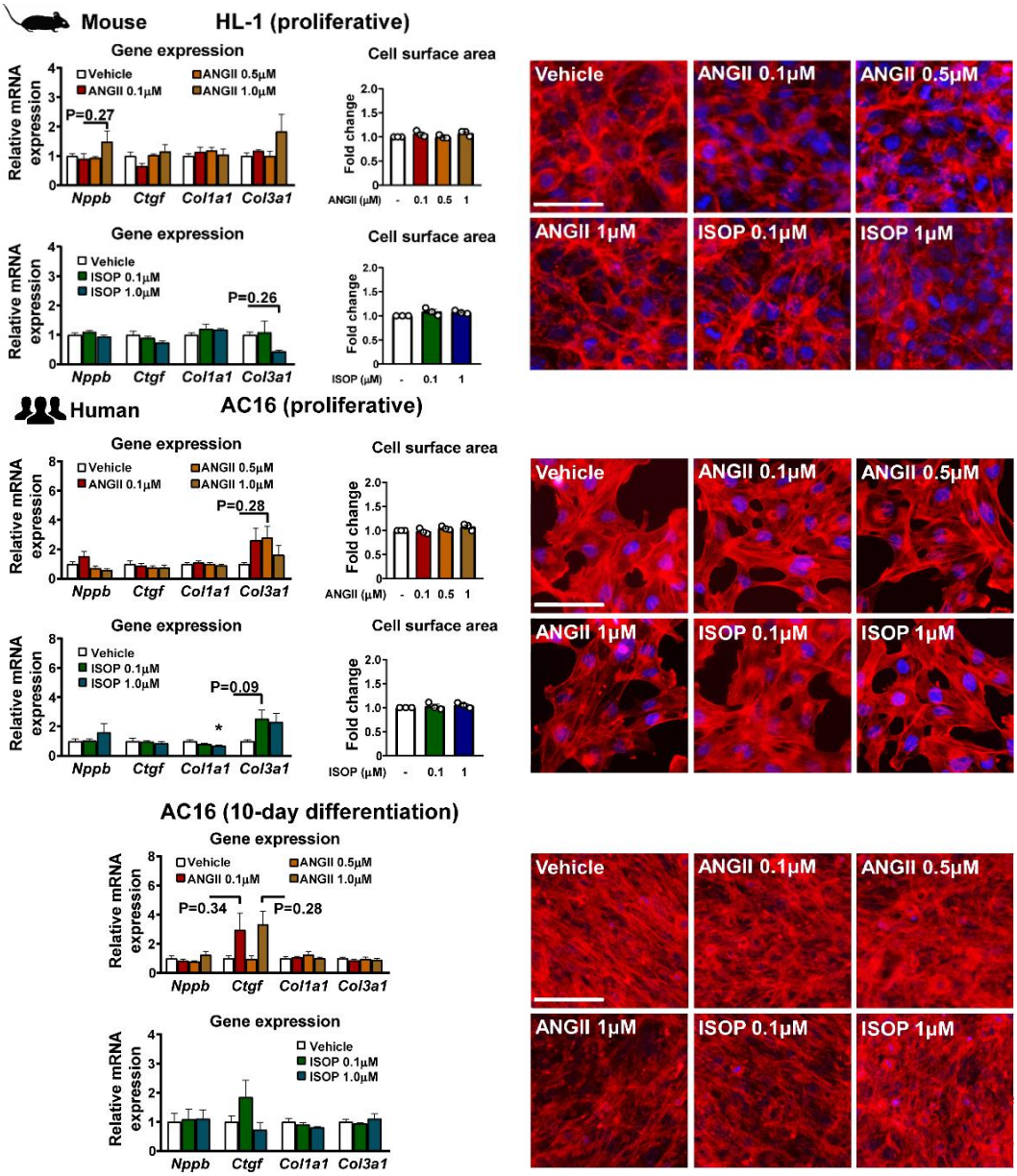


Figure 7. Changes in cell surface area and mRNA levels of hypertrophy markers (Nppb, Ctgf, Col1a1, and Col3a1) following angiotensin II or isoprenaline treatment. *p<0.05 vs corresponding control, one-way ANOVA followed by Tukey's post-hoc test; n=3-9/group individual samples per condition. Cells were stained with phalloidin (red) and DAPI (blue). Scale bar: 100 μm. Obtained from Onódi et al. (89)

revealed that only NRCMs responded to ANGII and ISOP treatment, while all other investigated cells showed no significant increase in cell surface area. The mRNA levels of widely-used hypertrophy markers *Nppb*, *Ctgf*, *Col1a1*, or *Col3a1* remained unchanged following ANGII or ISOP treatment in all cellular models (Figure 7).

4.1.3. Response of cardiomyocyte to simulated ischemia/reperfusion injury

sI/R injury is the most widely used experimental model for evaluating cardioprotective effects of novel drug targets. To compare the hypoxic tolerance of the cellular models investigated, we subjected primary isolated NRCM and NMCM cell to 6 hours of simulated ischemia, while cell lines and hiPSC-CMs were treated for 16 hours. In all experimental groups, the ischemic period was followed by a 2-hour reperfusion, after which cell viability was assessed. The duration of sI/R was selected in line with the recommendation of the ESC Working Group Cellular Biology of the Heart, which suggests aiming for approximately 50% cell death by the end of the protocol (58).

Table 3. Responder ratios of cardiomyocyte models to simulated ischemia/reperfusion injury. Responder ratio was calculated as a percentage of responder plates/all stimulated plate. Obtained from Onódi et al. (89)

Immortalized cell line		**Responder ratio**	**Primary cell culture**	**Responder ratio**	
		16H2H		**16H2H**	**6H2R**
AC16	proliferative	45,5%	**hiPSC-CM**	83.3%	0%
	differentiated	100%			
H9C2	proliferative	76.9%	**NRCM**	-	75%
	differentiated	100%			
HL-1	proliferative	17%	**NMCM**	-	50%

The responder ratio to sI/R was calculated as the percentage of successful sI/R experiments in which cell viability significantly decreased based on unpaired Student's t-test following sI/R treatment, relative to the total number of experiments performed using the same cellular model (Table 3). 6 hours of sI significantly reduced cell viability in primary NRCM and NMCM cell cultures, with responder ratios of 75% and 50%, respectively (Table 3, Figure 8). The same 6-hour protocol applied to hiPSC-CMs resulted in a 0% responder ratio; however, extending sI to 16 hours was able to decrease cell viability in 83.3% of the experiments (Table 3, Figure 8). Immortalized cell lines demonstrated a greater resistance to sI/R injury. Proliferative AC16 and H9C2 cells showed responder ratios of 45.5% and 76.9%, respectively (Table 3, Figure 8). Differentiation of AC16 and H9C2 cells resulted in an increased hypoxic sensitivity, as evidenced by responder ratios of 100% in both differentiated cell lines (Table 3, Figure 8). By contrast, murine HL-1 cardiomyocytes paradoxically exhibited a modest increase in cell viability after sI/R, with a responder ratio of only 17%, suggesting they possess

the greatest resistance to hypoxic stress among all cardiomyocyte models tested (Table 3, Figure 8).

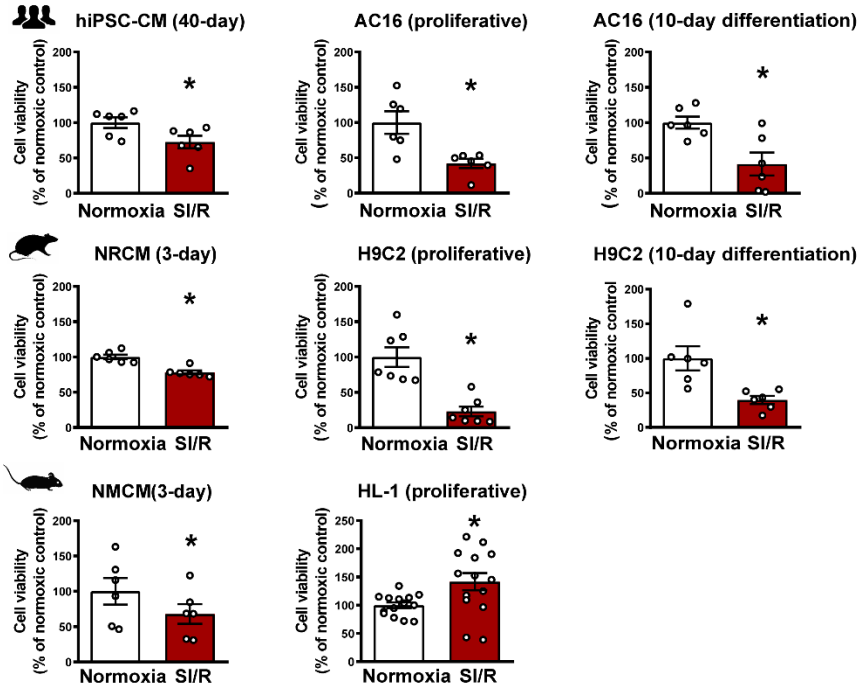


Figure 8. Cell viability of cardiomyocytes following simulated ischemia/reperfusion injury. Cell viability measurement of proliferative or differentiated cell lines and hiPSC-CMs after 16 hours, and primary NRCMs or NMCMs after 6 hours of simulated ischemia and 2 hours of reperfusion. Data is normalized to normoxic control (considered 100%) and expressed as mean ± SEM; *p<0.05 vs normoxia, Student's t-test, n=5-8/group independent experiments. Obtained from Onódi et al. (89)

4.2. Identification of novel cardioprotective miRNAs, protectomiRs

4.2.1. MicroRNA expression changes in a porcine model of acute myocardial infarction and ischemic conditioning stimuli

To identify novel cardioprotective miRNAs, we utilized a clinically relevant closed-chest porcine model of AMI combined with ischemic conditioning, and evaluated the miRNA expression changes associated with cardioprotection. Domestic pigs were subjected to 90 minutes of LAD coronary artery occlusion, followed by 3 hours of reperfusion to induce AMI as demonstrated in our previous study (56). This was combined with various ischemic conditioning protocols, IPreC, IPostC, and RIPerC to induce cardioprotection, show by the reduction in infarct size, microvascular obstruction or myocardial edema (56). At the end of reperfusion, the miRNA expression profiles were analyzed from left ventricular tissue samples across all experimental groups using high-throughput RT-PCR.

Out of 220 known porcine cardiac miRNAs assayed, the following numbers were detected in at least one myocardial sample: 186 in Sham, 196 in AMI, 199 in IPreC, 194 in IPostC, and 195 in RIPerC. Eight miRNAs could not be detected in any of the samples, namely miR-10a-3p, miR-24-2, miR-146a, miR-148a, miR-202-5p, miR-374a, miR-1277, and miR-1297.

The relative expression levels of 212 miRNAs were quantified across the comparisons of AMI versus Sham, IPreC versus AMI, IPostC versus AMI, and RIPerC versus AMI. Figure 9 shows the number of miRNAs that are significantly upregulated or downregulated, across one or more ischemic conditioning protocols compared to AMI, based on screening criteria predifined in Section 3.15. In the IPreC group, 29 miRNAs were significantly upregulated, while 28 were downregulated. IPostC resulted in 19 upregulated and 35 downregulated miRNAs, whereas RIPerC showed 33 upregulated and 35 downregulated miRNAs. Multiple miRNAs exhibited consistent changes in expression across various ischemic conditioning protocols, suggesting that these alterations are linked to cardioprotective effects that are not restricted to a single treatment method (Figure 9, intersections).

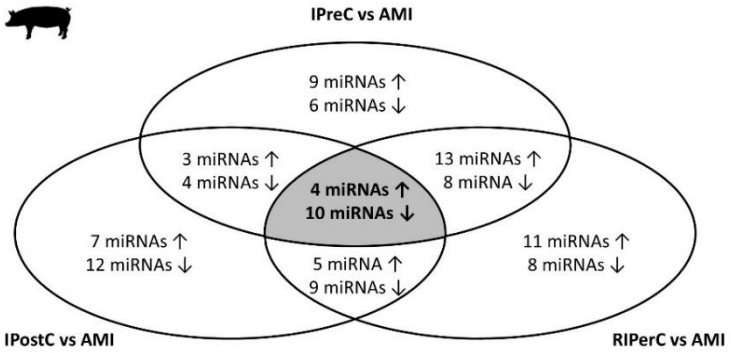


Figure 9. MicroRNA expression profile analysis of left ventricular samples from a porcine model of acute myocardial infarction and ischemic conditioning stimuli. Number of miRNAs linked to cardioprotection induced by ischemic preconditioning, postconditioning, and/or remote perconditioning, based on RT-PCR analysis. The three circles in the Venn diagram correspond to the miRNA expression profiles of each conditioning protocol compared to the ischemia/reperfusion (AMI) group. The numbers shown in the outer sections represent miRNAs uniquely associated with IPreC, IPostC, or RIPerC, while the overlapping areas indicate miRNAs exhibiting the same expression changes in two conditioning groups. The central overlap includes miRNAs commonly regulated by all three ischemic conditioning methods. AMI: ischemia/reperfusion; IPreC: ischemic preconditioning; IPostC: ischemic postconditioning; RIPerC: remote ischemic perconditioning. Obtained from Nagy et al. (92).

4.2.2. Selection of protectomiR candidates

miRNAs that were consistently modulated by all three ischemic conditioning protocols compared to AMI were identified as the most promising candidates for involvement in cardioprotection. Specifically, four miRNAs, miR-199a-3p, miR-450a, miR-450c-3p, and miR-451 were upregulated, while ten miRNAs, miR-181a, miR-339, miR-142, miR-193a-3p, miR-29a, miR-204a, miR-424-3p, miR-127, miR-34a, and miR-105-2 were downregulated. The detailed expression profiles of these 14 miRNAs across all comparisons are displayed in Figure 10.

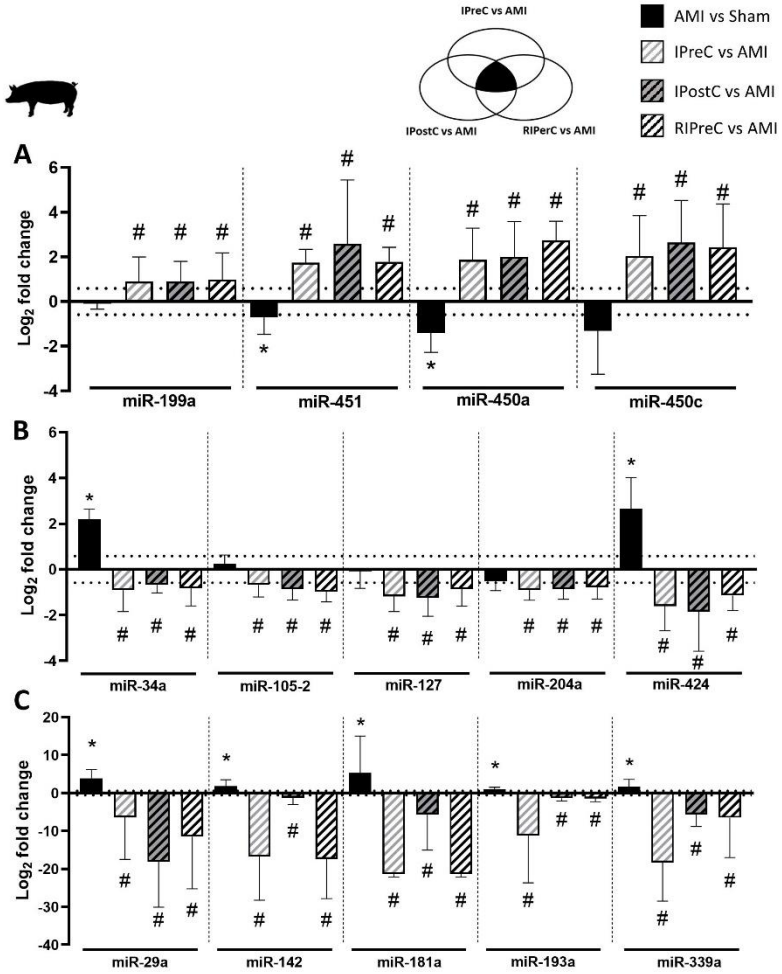


Figure 10. Analysis of the expression patterns of selected protectomiR candidate microRNAs associated with cardioprotection induced by ischemic preconditioning, postconditioning, and remote conditioning. (A) Four microRNAs (miR-199a, miR-450a, miR-450c-3p, and miR-451) were consistently upregulated across all three conditioning protocols (IPreC, IPostC, and RIPerC) compared to AMI. (B, C) Ten microRNAs (miR-29a, miR-34a, miR-105-2, miR-127, miR-142, miR-181a, miR-193a, miR-204a, miR-339a, and miR-424-3p) were downregulated across the same conditioning groups relative to AMI. N=3/group individual

samples; technical replicates: n=2. Results are presented as log_2 fold changes ± SD. Expression changes meeting the screening criteria had log_2 fold changes ≥ ±0.585 (indicated by the dashed lines) and *p<0.05 vs. Sham or #p<0.05 vs. AMI by Student's t-test. Sham: sham-operated control; AMI: ischemia/reperfusion; IPreC: ischemic preconditioning; IPostC: ischemic postconditioning; RIPerC: remote ischemic perconditioning. Obtained from Nagy et al. (92)

Based on whether their expression was increased or decreased, the protectomiR candidates were divided into two groups. Those miRNAs that were upregulated are considered protectomiRs when mimicked, while those that were downregulated are considered protectomiRs when inhibited by antagomiRs (Figure 11).

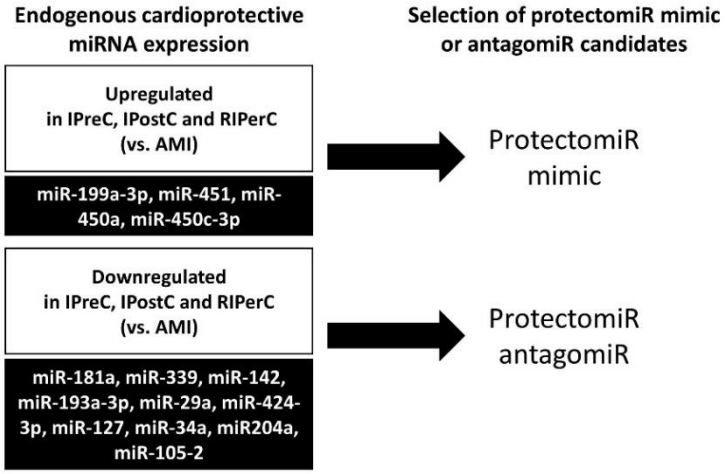


Figure 11. Selection of modulation strategies, using either protectomiR mimics or antagomiRs, based on the direction of miRNA expression changes observed after ischemic conditioning. Obtained from Nagy et al. (92)

4.2.3. Cross-species pig-rat-human miRNA sequence similarity assessment

We conducted *in vitro* validation in NRCM and AC16 cells; thus, we wanted to assess the sequence similarity of the protectomiR candidates to ensure that the selected miRNAs are relevant across species. Using the NCBI RNA Blast tool in conjunction with the miRBase database, we identified orthologous miRNAs of the investigated porcine sequences in rat and human species (Table 4). Of the 14 selected porcine miRNAs, 12 exhibited both rat and human orthologs with 100% sequence identity. However, the rat orthologue of miR-105-2 demonstrated less than 60% sequence similarity. Additionally, no rat orthologue was identified for miR-424-3p. Consequently, miR-105-2 and miR-424-3p were excluded from the validation experiments conducted in rat cardiomyocytes.

Table 4. Cross-species pig-rat-human miRNA orthologues. The first, second and third columns lists the name of the porcine, rat and human protectomiR candidates, respectively. The last two columns show the sequence similarity matching, and the sequence similarity ratios. Obtained from Nagy et al. (92)

Pig miRNA	Rat miRNA	Human miRNA	Pig-Rat-Human (ssc-rno-hsa) cross-species miRNA sequence matching	Sequence similarity ratio
ssc-miR-181a	rno-miR-181a-5p	hsa-miR-181a-5p	**ssc-miR: .acauucaacgcugucggugaguu** **||||||||||||||||||||||** **rno-miR: aacauucaacgcugucggugagu-** **||||||||||||||||||||||** **hsa-miR: aacauucaacgcugucggugagu-**	100%
ssc-miR-339	rno-miR-339-5p	hsa-miR-339-5p	**ssc-miR: ucccuguccuccaggagcuca--** **||||||||||||||||||||** **rno-miR: ucccuguccuccaggagcuca--** **||||||||||||||||||||** **hsa-miR: ucccuguccuccaggagcucacg**	100%
ssc-miR-142	rno-miR-142-5p	hsa-miR-142-5p	**ssc-miR: cauaaaguagaaagcacuacu** **||||||||||||||||||||** **rno-miR: cauaaaguagaaagcacuacu** **||||||||||||||||||||** **hsa-miR: cauaaaguagaaagcacuacu**	100%
miR-193a-3p	rno-miR-193a-3p	hsa-miR-193a-3p	**ssc-miR: aacuggccuacaaagucccagu** **||||||||||||||||||||** **rno-miR: aacuggccuacaaagucccagu** **||||||||||||||||||||** **hsa-miR: aacuggccuacaaagucccagu**	100%
ssc-miR-29a	rno-miR-29a-3p	hsa-miR-29a-3p	**ssc-miR: cuagcaccaucugaaaucgguua** **||||||||||||||||||||** **rno-miR: cuagcaccaucugaaaucgguua** **||||||||||||||||||||** **hsa-miR: -uagcaccaucugaaaucgguua**	100%
ssc-miR-127	rno-miR-127-3p	hsa-miR-127-3p	**ssc-miR: ucggauccgucugagcuuggcu** **||||||||||||||||||||||** **rno-miR: ucggauccgucugagcuuggcu** **||||||||||||||||||||||** **hsa-miR: ucggauccgucugagcuuggcu**	100%
ssc-miR-424-3p	no rat orthologue miRNA	hsa-miR-424-3p	**ssc-miR: caaaacgugaggcgcugcuau** **rno-miR: ----------** **hsa-miR: caaaacgugaggcgcugcuau**	porcine-human: 100%
ssc-miR-34a	rno-miR-34a-5p	hsa-miR-34a-5p	**ssc-miR: uggcagugucuuagcugguugu** **|||||||||||||||||||||||** **rno-miR: uggcagugucuuagcugguugu** **|||||||||||||||||||||||** **hsa-miR: uggcagugucuuagcugguugu**	100%
ssc-miR-204a	rno-miR-204-5p	hsa-miR-204-5p	**ssc-miR: uucccuuugucauccuaugccu** **||||||||||||||||||||||** **rno-miR: uucccuuugucauccuaugccu** **||||||||||||||||||||||** **hsa-miR: uucccuuugucauccuaugccu**	100%

ssc-miR-105-2	rno-miR-105	has-miR-105-5p	**ssc-miR: ucaaaugcucagacuccuug---** **-|||- |||||||| --- ||--** **rno-miR: -caagugcucagaugucuguggu** **hsa-miR: ucaaaugcucagacuccuguggu**	porcine-rat: < 60% porcine-human: 90%
ssc-miR-199a-3p	rno-miR-199a-3p	hsa-miR-199a-3p	**ssc-miR: acaguagucugcacauugguua** **||||||||||||||||||||** **rno-miR: acaguagucugcacauugguua** **||||||||||||||||||||** **hsa-miR: acaguagucugcacauugguua**	100%
ssc-miR-451	rno-miR-451-5p	hsa-miR-451a	**ssc-miR: aaaccguuaccauuacugaguu** **|||||||||||||||||||** **rno-miR: aaaccguuaccauuacugaguu** **|||||||||||||||||||** **hsa-miR: aaaccguuaccauuacugaguu**	100%
miR-450a	rno-miR-450b-5p	hsa-miR-450a-5p	**ssc-miR: uuuugcgauguguuccuaaua-** **|||||||||||||||||||** **rno-miR: uuuugcgauguguuccuaaua-** **|||||||||||||||||||** **hsa-miR: uuuugcgauguguuccuaauau**	100%
miR-450c-3p	rno-miR-450a-3p	hsa-miR-450a-1-3p	**ssc-miR: -auugggaacauuuugcauucgu** **|||||||||||||||||--** **rno-miR: uauugggaacauuuugcauaa-** **|||||||||||||||||--** **hsa-miR: -auugggaacauuuugcauguau**	100%

4.2.4. Cardioprotective effect of protectomiRs in neonatal rat cardiomyocytes and AC16 cells following simulated ischemia/reperfusion

To assess the cardiocytoprotective effects of the protectomiR candidates, NRCMs were transfected with either miRNA mimics or antagomiRs at 25, 50, or 100 nM concentration, and then subjected to sI/R injury, followed by cell viability measurement. Transfection with two protectomiR mimics at 25 nM, rno-miR-450a-3p and rno-miR-451-5p, significantly improved cardiomyocyte survival following sI/R (93.71 ± 3.47% and 84.84 ± 2.61%, respectively) compared to cells transfected with a negative control mimic under the same sI/R conditions (Figure 12). In contrast, transfection with other miRNA mimics (rno-miR-199a-3p and rno-miR-450b-5p) or antagomiRs targeting rno-miR-29a-3p, rno-miR-34a-5p, rno-miR-127-3p, rno-miR-142-5p, rno-miR-181a-5p, rno-miR-193a-3p, rno-miR-204-5p, and rno-miR-339-5p did not result in a significant increase in cell viability after sI/R (Figure 12 and 13). Notably, the antagomiRs of rno-miR-29a-3p, rno-miR-34a-5p, rno-miR-127-3p, and rno-miR-142-5p, as well as the rno-miR-450b-5p mimic, appeared to exacerbate sI/R-induced injury at 50 or 100 nM concentrations (Figure 12 and 13).

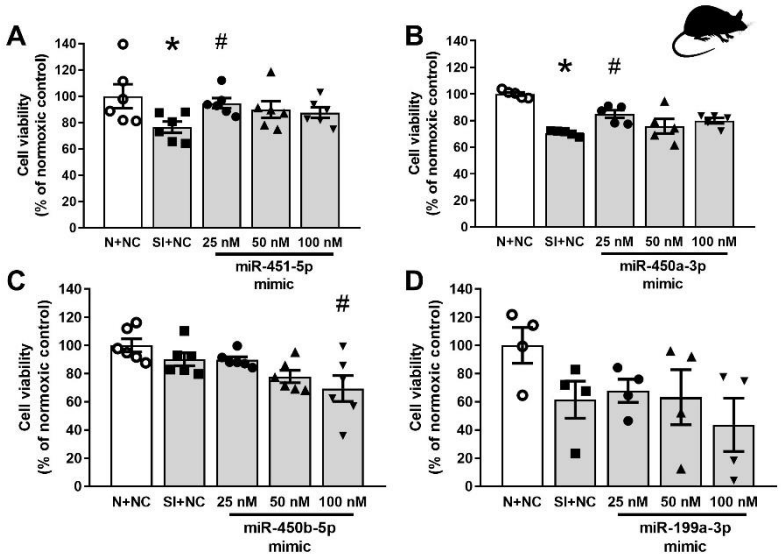


Figure 12. Effect of protectomiR mimics on cell survival following simulated ischemia/reperfusion in primary neonatal rat cardiomyocytes. Cell viability is presented as a percentage relative to the normoxic group transfected with negative control mimic. Transfection with (A) miR-451-5p and (B) miR-450a-3p mimics significantly improved cardiomyocyte survival. (C) miR-450b-5p mimic transfection exhibited a harmful effect, while (D) miR-199a-3p mimic had no significant impact on cell viability after sI/R injury. N=6 for miR-451-5p and miR-450b-5p, N=5 for miR-450a-3p, and N=4 for miR-199a-3p per treatment group, technical replicates n=6-12, values are mean ± SEM, *p < 0.05 vs. N+NC, #p < 0.05 vs. SI+NC one-way ANOVA with Dunnett's post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test. N: normoxia, SI: simulated ischemia, NC: negative control (92).

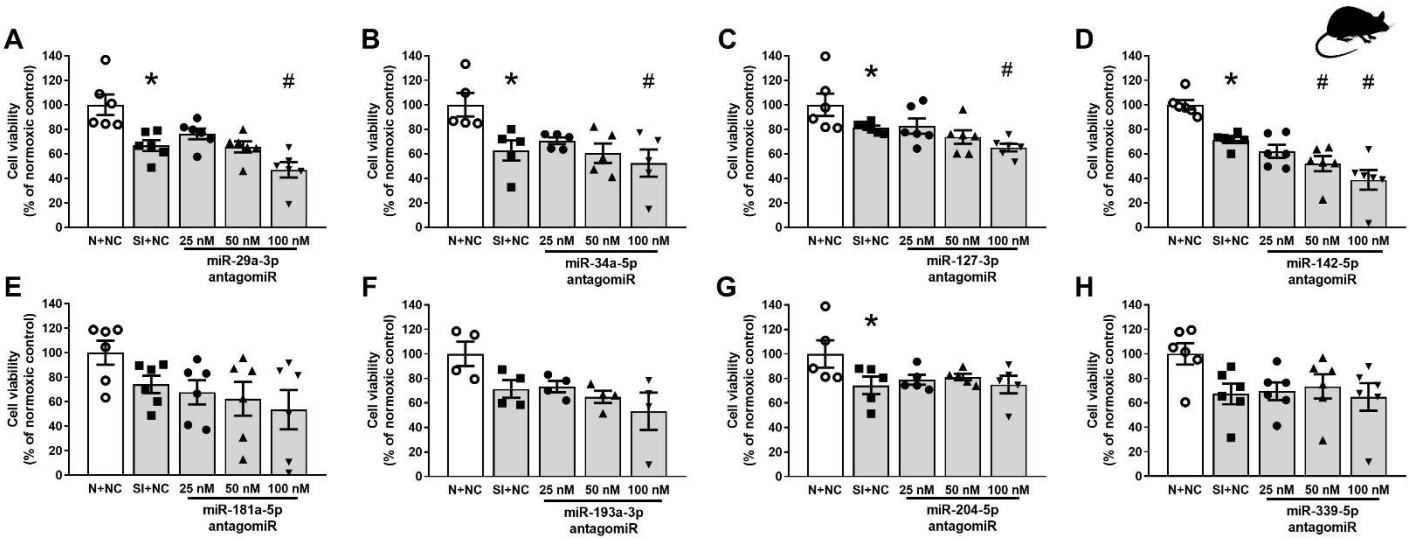


Figure 13. Effect of protectomiR antagomiRs on cell survival following simulated ischemia/reperfusion in primary neonatal rat cardiomyocytes. Cell viability is presented as a percentage relative to the normoxic group transfected with negative control antagomiR. Transfection with antagomiRs targeting (A) miR-29a-3p, (B) miR-34a-5p, (C) miR-127-3p, and

(D) miR-142-5p resulted in cell viability reduction. In contrast, antagomiRs against (E) miR-181a-5p, (F) miR-193a-3p, (G) miR-204-3p, and (H) miR-339-5p had no significant impact on cardiomyocyte viability after sI/R. N=6 for miR-29a-3p, miR-127-3p, miR-142-5p, miR-181a-5p, and miR-339-5p; N=5 for miR-34a-5p and miR-204-3p; and N=4 for miR-193a-3p per treatment group, with 6-12 technical replicates, values are mean ± SEM, *p < 0.05 vs. N+NC, #p < 0.05 vs. SI+NC, one-way ANOVA with Dunnett's post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test. N: normoxia, SI: simulated ischemia, NC: negative control (92).

To further confirm the cardiocytoprotective effects of miR-450a and miR-451 mimics, we transfected the human cardiomyocyte cell line AC16 at 25, 50, and 100 nM and subjected them to sI/R, followed by assessing cell viability. Consistent with our findings in NRCMs, transfection with the miR-450a mimic at 25 nM significantly enhanced AC16 cell survival following sI/R injury compared to cells transfected with a negative control miRNA, showing 87.10 ± 5.03% viability. In contrast, miR-451 mimic transfection did not alleviate sI/R-induced cell death in AC16 cells (Figure 14).

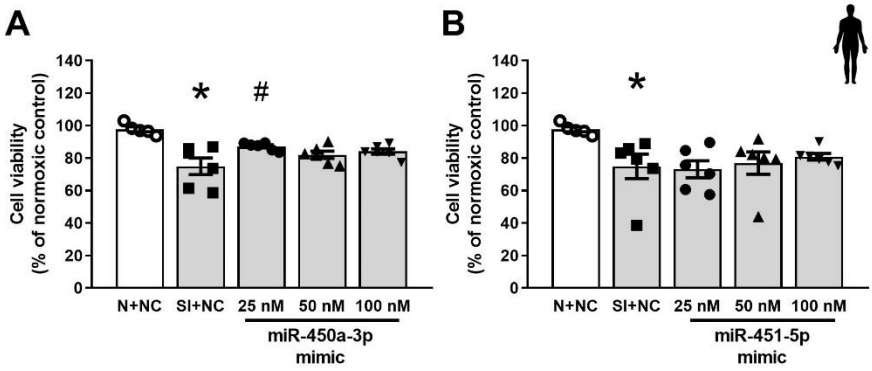


Figure 14. Effect of miR-450a-3p and miR-451-5p protectomiR mimics on cell survival following simulated ischemia/reperfusion in AC16 cardiomyocytes. Cell viability is presented as a percentage relative to the normoxic group transfected with negative control mimic. (A) Transfection with miR-450a-3p at 25 nM significantly enhanced cell survival following 16 hours of ischemia and 2 hours of reperfusion. (B) In contrast, miR-451-5p mimic did not show cardiocytoprotective effects under the same conditions. N=6 per treatment group, with 6-12 technical replicates, values are mean ± SEM, *p < 0.05 vs. N+NC, #p < 0.05 vs. SI+NC, one-way ANOVA with Dunnett's post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test. N: normoxia, SI: simulated ischemia, NC: negative control (92).

4.2.5. Functional analysis of miR-450a target genes

To uncover the potential molecular mechanisms underlying the cardioprotective effects of miR-450a, a target prediction of endogenous miR-450a across pigs, rats, and humans

was conducted. We then assessed the functional relevance of these targets in cardiac tissue using GO and KEGG pathway enrichment analyses.

Our predictions identified 3987, 4279, and 8328 mRNA targets of miR-450a in pigs, rats, and humans, respectively. Based on our RNA sequencing data from rat and human left ventricular samples, we filtered these predictions to include only genes expressed in the myocardium, resulting in 2682 rat and 6099 human cardiac-expressed target genes. We identified 607 common predicted target genes of miR-450a in pigs, rats, and humans. The top 50 genes were selected from this common set based on their target prediction strength score in humans and are presented in Table 5.

Table 5. Top 50 predicted target genes of miR-450a across pigs, rats, and humans, ranked by TargetScan strength in humans. Obtained from Nagy et al. (92)

Target Symbol	Pig			Rat			Human		
	mirdb strength	targetscan strength	mirtarbase strength	mirdb strength	targetscan strength	mirtarbase strength	mirdb strength	targetscan strength	mirtarbase strength
SMAD2	0.000	-0.767	0	0.000	-0.552	0	84.927	-12.588	0
VPS35	0.000	-0.667	0	0.000	-0.220	0	0.000	-4.929	0
PPM1L	0.000	-4.051	0	0.000	-2.658	0	86.291	-4.574	1
AP1M1	0.000	-0.476	0	0.000	-0.245	0	0.000	-4.207	0
TNRC6A	0.000	-0.456	0	0.000	-0.488	0	0.000	-4.188	0
AAK1	0.000	-1.032	0	0.000	-1.856	0	89.394	-4.185	0
DNAJC10	0.000	-0.539	0	0.000	-0.670	0	0.000	-4.080	1
RNF169	0.000	-2.258	0	0.000	-0.224	0	0.000	-4.070	0
RPS6KA5	0.000	-0.585	0	0.000	-0.842	0	0.000	-3.974	0
HS2ST1	0.000	-0.590	0	0.000	-0.424	0	0.000	-3.940	0
CIITA	0.000	-1.407	0	0.000	-0.606	0	0.000	-3.521	0
MTHFD2	0.000	-0.549	0	0.000	-0.694	0	86.302	-3.495	0
GNL3L	0.000	-0.207	0	0.000	-0.202	0	0.000	-3.433	1
RGMA	0.000	-0.365	0	0.000	-0.785	0	0.000	-3.418	0
GPC6	0.000	-1.071	0	0.000	-1.227	0	98.925	-3.415	0
ZBTB25	0.000	-0.533	0	0.000	-0.524	0	0.000	-3.388	0
FAM78B	0.000	-0.569	0	0.000	-2.708	0	0.000	-3.357	0
MED28	0.000	-0.690	0	0.000	-0.233	0	0.000	-3.316	1
CBX5	0.000	-2.626	0	0.000	-0.527	0	0.000	-3.300	0
EIF5A2	0.000	-0.599	0	0.000	-0.823	0	0.000	-3.290	0
DCTN5	0.000	-0.710	0	0.000	-1.394	0	0.000	-3.277	0
KIAA1958	0.000	-0.577	0	0.000	-1.033	0	0.000	-3.257	0
PDK3	0.000	-0.970	0	0.000	-0.724	0	0.000	-3.235	0
ATE1	0.000	-1.741	0	0.000	-0.226	0	0.000	-3.232	0
GULP1	0.000	-1.960	0	0.000	-0.592	0	80.527	-3.219	0
GGCX	0.000	-0.377	0	0.000	-0.237	0	0.000	-3.148	1
CEP57L1	0.000	-0.532	0	0.000	-0.615	0	0.000	-3.136	0
TNPO1	0.000	-0.273	0	0.000	-0.697	0	0.000	-3.113	0
DAPK2	0.000	-0.443	0	0.000	-0.502	0	0.000	-3.026	0

DGKH	0.000	-1.005	0	0.000	-0.345	0	92.304	-3.000	0
PAIP2B	0.000	-2.599	0	0.000	-1.410	0	0.000	-2.941	0
APBB2	0.000	-0.793	0	0.000	-1.272	0	0.000	-2.918	0
GPATCH2	0.000	-2.269	0	0.000	-1.153	0	85.990	-2.868	0
NABP1	0.000	-1.054	0	0.000	-0.568	0	0.000	-2.837	0
CPPED1	0.000	-0.988	0	0.000	-0.849	0	0.000	-2.748	0
DBT	0.000	-0.444	0	0.000	-2.082	0	0.000	-2.708	0
SNX19	0.000	-1.444	0	0.000	-0.843	0	0.000	-2.665	0
CHN2	0.000	-0.928	0	0.000	-0.614	0	0.000	-2.657	0
EXOSC2	0.000	-0.512	0	0.000	-0.755	0	0.000	-2.610	0
SPRTN	0.000	-2.332	0	0.000	-0.207	0	90.994	-2.575	0
RRM2B	0.000	-1.286	0	0.000	-1.228	0	0.000	-2.538	0
CCDC50	0.000	-1.158	0	0.000	-0.210	0	0.000	-2.490	0
NFIC	0.000	-0.610	0	0.000	-0.682	0	0.000	-2.485	0
PAQR3	0.000	-0.526	0	0.000	-0.724	0	0.000	-2.473	0
CHD9	0.000	-0.615	0	0.000	-0.636	0	90.685	-2.473	0
ARHGEF18	0.000	-1.034	0	0.000	-0.522	0	80.171	-2.472	0
SOD2	0.000	-1.225	0	0.000	-0.457	0	0.000	-2.472	0
TOR1AIP2	0.000	-0.472	0	0.000	-0.975	0	0.000	-2.440	1
ORAI2	0.000	-0.594	0	0.000	-2.019	0	0.000	-2.433	0
CADM1	0.000	-1.068	0	0.000	-2.634	0	0.000	-2.425	0

Subsequent GO enrichment analysis of the predicted cardiac targets of miR-450a in each species revealed 829 significantly enriched terms in pigs, 1161 in rats, and 1023 in humans. Among these, 421 GO terms were shared across all three species and were ranked according to their fold enrichment values in humans. The top 50 of these shared GO terms are listed in Table 6, with the complete dataset available in our published work (92). KEGG pathway analysis identified 56, 55, and 56 significantly enriched pathways in pigs, rats, and humans, respectively, with 13 pathways common across all species (Table 7).

Table 6. Top 50 of the shared, significantly enriched GO terms across pigs, rats, and humans, ranked by their fold enrichment values in humans. Obtained from Nagy et al. (92) FDR: false discovery rate, GO: gene ontology.

GO biological process	Fold enrichment (Pig)	FDR (Pig)	Fold enrichment (Rat)	FDR (Rat)	Fold enrichment (Human)	FDR (Human)
regulation of alternative mRNA splicing, via spliceosome (GO:0000381)	2.28	0.009	2.17	0.045	1.88	0.002
regulation of stress fiber assembly (GO:0051492)	1.81	0.047	1.98	0.029	1.81	0.000
regulation of actomyosin structure organization (GO:0110020)	1.79	0.045	2.04	0.009	1.8	0.000
nuclear envelope organization (GO:0006998)	2.13	0.026	2.42	0.029	1.72	0.014
vesicle localization (GO:0051648)	1.69	0.023	2.23	0.000	1.71	0.000

negative regulation of translation (GO:0017148)	1.72	0.045	2.19	0.001	1.7	0.000
regulation of translational initiation (GO:0006446)	1.93	0.037	2.6	0.001	1.67	0.003
negative regulation of amide metabolic process (GO:0034249)	1.93	0.001	2.08	0.001	1.67	0.000
regulation of mRNA splicing, via spliceosome (GO:0048024)	2.15	0.000	2.49	0.000	1.67	0.000
cytoskeleton-dependent intracellular transport (GO:0030705)	1.62	0.019	1.82	0.005	1.66	0.000
protein-containing complex localization (GO:0031503)	1.69	0.014	1.92	0.001	1.65	0.000
establishment of vesicle localization (GO:0051650)	1.69	0.031	2.29	0.000	1.65	0.000
protein import into nucleus (GO:0006606)	1.78	0.018	2.17	0.001	1.64	0.001
nuclear transport (GO:0051169)	1.61	0.004	1.78	0.001	1.63	0.000
nucleocytoplasmic transport (GO:0006913)	1.62	0.003	1.8	0.001	1.62	0.000
import into nucleus (GO:0051170)	1.82	0.010	2.14	0.001	1.62	0.001
nucleobase-containing compound transport (GO:0015931)	1.78	0.000	1.71	0.014	1.61	0.000
peptidyl-serine phosphorylation (GO:0018105)	1.84	0.009	2.32	0.001	1.61	0.000
regulation of RNA splicing (GO:0043484)	2.03	0.000	2.24	0.000	1.61	0.000
regulation of mRNA processing (GO:0050684)	2.06	0.000	2.41	0.000	1.59	0.001
vesicle-mediated transport in synapse (GO:0099003)	1.75	0.007	2.27	0.000	1.59	0.000
synaptic vesicle cycle (GO:0099504)	1.78	0.008	2.23	0.000	1.58	0.000
Golgi vesicle transport (GO:0048193)	1.63	0.001	1.72	0.002	1.57	0.000
mRNA processing (GO:0006397)	1.57	0.000	1.94	0.000	1.56	0.000
regulation of translation (GO:0006417)	1.63	0.000	1.81	0.000	1.56	0.000
mRNA metabolic process (GO:0016071)	1.56	0.000	1.86	0.000	1.56	0.000
peptidyl-serine modification (GO:0018209)	1.79	0.008	2.32	0.001	1.56	0.000
positive regulation of type I interferon production (GO:0032481)	1.87	0.040	2.11	0.027	1.55	0.046
regulation of RNA stability (GO:0043487)	1.76	0.003	1.65	0.042	1.55	0.000
regulation of amide metabolic process (GO:0034248)	1.77	0.000	1.85	0.000	1.54	0.000
vesicle fusion (GO:0006906)	1.79	0.045	2.26	0.004	1.53	0.018
post-transcriptional regulation of gene expression (GO:0010608)	1.61	0.000	1.77	0.000	1.53	0.000
protein-containing complex disassembly (GO:0032984)	1.63	0.038	1.97	0.003	1.53	0.002
regulation of mRNA catabolic process (GO:0061013)	1.82	0.000	1.64	0.046	1.53	0.000
protein localization to plasma membrane (GO:0072659)	1.62	0.009	2.12	0.000	1.53	0.000
regulation of cellular response to stress (GO:0080135)	1.54	0.000	1.47	0.007	1.53	0.000
organelle membrane fusion (GO:0090174)	1.82	0.034	2.21	0.005	1.53	0.015
intracellular protein transport (GO:0006886)	1.6	0.000	2	0.000	1.52	0.000
cell-matrix adhesion (GO:0007160)	1.82	0.006	1.95	0.009	1.52	0.003
intracellular receptor signaling pathway (GO:0030522)	1.55	0.047	1.87	0.003	1.52	0.000

protein localization to nucleus (GO:0034504)	1.69	0.005	2.02	0.000	1.52	0.000
developmental cell growth (GO:0048588)	1.79	0.045	1.98	0.005	1.52	0.013
regulation of mRNA metabolic process (GO:1903311)	1.81	0.000	1.94	0.000	1.52	0.000
cell-substrate adhesion (GO:0031589)	1.62	0.014	1.94	0.001	1.51	0.000
phosphatidylinositol metabolic process (GO:0046488)	1.66	0.010	2.01	0.001	1.51	0.002
intracellular transport (GO:0046907)	1.57	0.000	1.93	0.000	1.51	0.000
positive regulation of mRNA metabolic process (GO:1903313)	1.86	0.006	1.93	0.012	1.51	0.005
protein localization to cell periphery (GO:1990778)	1.64	0.002	1.96	0.000	1.51	0.000
regulation of small GTPase mediated signal transduction (GO:0051056)	1.49	0.041	2.27	0.000	1.5	0.000
vacuole organization (GO:0007033)	1.62	0.012	1.7	0.030	1.49	0.000

Table 7. Shared, significantly enriched KEGG pathways across pigs, rats, and humans, ranked by their Fold enrichment values in humans. Obtained from Nagy et al. (92) FDR: false discovery rate.

KEGG Terms	Fold Enrichment (Pig)	FDR (Pig)	Fold Enrichment (Rat)	FDR (Rat)	Fold Enrichment (Human)	FDR (Human)
Adherens junction	1.820	0.017	2.018	0.010	1.536	0.022
Phosphatidylinositol signaling system	1.726	0.023	2.018	0.010	1.505	0.022
EGFR tyrosine kinase inhibitor resistance	1.831	0.023	2.182	0.009	1.494	0.034
Proteoglycans in cancer	1.464	0.023	1.760	0.003	1.424	0.002
Focal adhesion	1.402	0.043	1.878	0.001	1.408	0.004
Autophagy - animal	1.507	0.026	1.667	0.015	1.374	0.022
Sphingolipid signaling pathway	1.631	0.023	1.807	0.015	1.360	0.046
Hepatitis B	1.584	0.017	2.030	0.001	1.342	0.033
mTOR signaling pathway	1.452	0.046	1.777	0.010	1.314	0.045
Axon guidance	1.482	0.025	1.551	0.034	1.280	0.048
Pathways in cancer	1.408	0.001	1.396	0.008	1.275	0.001
Regulation of actin cytoskeleton	1.485	0.017	1.784	0.001	1.248	0.047
PI3K-Akt signaling pathway	1.296	0.041	1.626	0.001	1.237	0.023

5. Discussion

This doctoral thesis primarily focuses on the research area that supports the development of preclinical experimental cell-based models, thereby providing an opportunity to raise the standard of preclinical drug development. Drug development for the treatment of ischemic heart disease has long been a major area of interest for our research group, and this work represents an important milestone in this line of research.

In this thesis, we aimed to systematically compare commonly used cardiomyocyte models and identify novel protectomiRs in a clinically relevant porcine model of acute MI combined with ischemic conditioning. We demonstrated that immortalized cardiac cell lines (human AC16, murine HL-1, rat H9C2) differ considerably from their original tissues or primary cardiomyocytes. Differentiation of H9C2 and AC16 cells induced only moderate changes, with some cardiac markers increasing, but skeletal muscle traits also becoming more prominent. Both proliferative and differentiated cell lines failed to respond to short-term hypertrophic or ischemic stimuli, indicating their limited relevance to *in vivo* conditions. Using a closed-chest porcine model of acute MI, we identified 14 miRNAs consistently regulated by different cardioprotective ischemic conditioning protocols. Among these, miR-450a mimic showed a direct cardioprotective effect *in vitro* in NRCMs and AC16 cells. This is the first evidence linking miR-450a to ischemic conditioning-induced cardioprotection in a clinically relevant large animal model, highlighting its potential as a therapeutic candidate.

5.1. Comparison of cardiomyocyte models

Animal models are frequently used in drug screening and disease modeling, but they raise ethical concerns and involve high costs. Consequently, alternative methods such as computational modeling, cell-based platforms and advanced 3D cell culture models are increasingly favored for high-throughput screening and assessing drug safety during the preclinical phase of drug development as supported by the recently approved Modernization Act 2.0 by the US Food and Drug Administration (107). The implementation of these alternatives aligns with the 3R principles (Replacement, Reduction, Refinement) (108). Immortalized cardiac cell lines, primary isolated cardiomyocytes, and stem cell-derived cardiomyocytes, particularly of human origin, are emerging as valuable substitutes for animal models. However, it is important to note that results observed *in vitro* do not always replicate *in vivo*; for example, some

cardioprotective drugs and targets effective in cell studies have not shown benefits in animal or clinical models (109-112). Therefore, a thorough understanding of both the advantages and limitations of cardiac cell lines is essential.

5.1.1. Transcriptomic profiles of cardiomyocyte models

To date, only a limited number of studies have provided detailed analyses of the key characteristics and gene expression profiles of cell lines and primary isolated cardiomyocytes. Through comparative transcriptomic analysis, we revealed that immortalized cardiac cell lines differ fundamentally in their cardiac and metabolic profiles from both the original tissue and primary cell cultures. These gene expression changes can significantly influence the results of *in vitro* studies using these cell lines. For the murine HL-1 and human AC16 cells, no analyses have been conducted that compare their gene expression profiles to cardiac tissue. The detailed genetic and molecular characterization of the widely used cardiomyocyte cell line H9C2 was published only recently. This study showed that undifferentiated H9C2 cells retain key cardiac features, demonstrated by the expression of several myosin genes and other cardiomyocyte-specific markers. However, the expression of natriuretic peptides, which are abundant markers in mature cardiomyocytes, is absent in H9C2 cells, potentially limiting their use in studies focused on heart failure or hypertrophy (113). H9C2 cells were also shown to have a different pattern of equilibrative nucleoside transporter expression compared to cardiomyocytes, which limits their suitability for drug screening in adenosine pharmacology (114). Our findings highlight the necessity of thoroughly characterizing each cell line before employing it as an *in vitro* cardiomyocyte model.

5.1.2. Impact of cell line differentiation on cellular characteristics

A major finding of our study is that differentiation of H9C2 and AC16 cells using all-trans retinoic acid protocols significantly alters their gene expression profiles; however, the differentiated cells continue to present a distinct gene expression pattern from primary cardiac cells and adult heart tissue. Additionally, differentiation extends the culture period by at least one week, and limited data is available on the functional properties of these cells. Previous reports revealed that differentiation of H9C2 cells induces an increased expression of cardiac-specific markers and signaling pathways, and results in metabolic remodeling (64, 65). On the other hand, we confirmed that differentiated H9C2 cells acquire skeletal muscle-like features, consistently with previous observations (115). In

addition, multiple studies concluded that differentiation of H9C2 cells may occur in only a subset of cells, resulting in a heterogeneous cell population, which suggests that careful consideration is required when using this experimental model (116, 117). In our study, the differentiation of AC16 cells showed a minimal enhancement of cardiac-specific gene expression. However, a proteomic analysis of differentiated AC16 cells, conducted after the publication of our study, revealed the expression of several cardiac-specific markers and transcription factors, as well as non-cardiac-specific myosin proteins, in the differentiated cells (118). Taken together, differentiation protocols only partially induce cardiac-like gene expression in H9C2 and AC16 cells, their reliability is limited as accurate models for cardiac research.

5.1.3. Ischemic tolerance of cardiomyocyte models

Only a few studies have examined the hypoxic sensitivity of various cell lines in detail. These investigations have identified significant differences between cell lines and primary cultures, particularly regarding their metabolism and mitochondrial function, which may explain the wide variation in hypoxic sensitivity observed in our sI/R experiments. Genes involved in key molecular pathways related to hypoxia, such as oxidative phosphorylation, HIF-1 signaling, and mitochondrial respiration, were shown to be consistently expressed in H9C2 cells and in an MI rat model (119). Similarly, hypoxia induces changes in the expression of genes encoding calcium ion channels, mitochondrial function, and key signaling pathways in proliferative AC16 cells (120). In this study, we used different ischemic period lengths for primary cells and cell lines according to the recommendation of the ESC Working Group Cellular Biology of the Heart to make them comparable (58). Our results showed that the responder ratio of H9C2 and AC16 cells to sI/R was moderate but comparable to that of primary cardiomyocytes, suggesting that these cell lines are suitable models for I/R injury. Differentiation further increased the hypoxic sensitivity of AC16 and H9C2 cells in our study; however, another study reported that AC16 cells become less responsive to hypoxia following differentiation (118). Consistent with our findings, H9C2 cells have been shown to exhibit greater sensitivity to hypoxia than HL-1 cells, along with higher mitochondrial function (63). HL-1 cells are derived from atrial cardiomyocytes, which contain fewer mitochondria with irregular distribution, lower oxidative activity, and reduced ATP synthesis compared to ventricular cardiomyocytes (121-124). These results suggest that

the HL-1 line may not serve as an ideal cellular model for I/R injury. In contrast, NRCMs demonstrate mitochondrial reorganization and sarcolemmal changes after a short period of ischemia, closely mirroring *in vivo* observations (125). Additionally, NRCMs display a stable phenotype and contractile function during H/R comparable to *in situ* hearts during I/R, making them a suitable model for I/R injury (126).

5.1.4. Response of cardiomyocytes to hypertrophic stimuli

The ability of cardiomyocyte cultures to respond to hypertrophic and other damaging stimuli is a critical characteristic, especially when these models are used for drug screening. In our study, cardiac cell lines showed no definitive response to hypertrophic stimuli, as indicated by measurements of various markers and cell surface area following angiotensin II and isoprenaline treatment. These findings are in contrast with earlier studies using similar concentrations and durations (127, 128). However, the literature is inconsistent, with some reports failing to detect increases in cell size or hypertrophy-related gene expression under comparable conditions (129, 130). Notably, higher doses (above 1 μM) and longer exposure times (48 hours) have also been utilized to achieve hypertrophic responses (131-133). This suggests that methodological variations, such as treatment dosage, duration, and the choice of endpoints, can influence the hypertrophic responsiveness of both cell lines and primary cultures. Given the considerable variability and lack of standardized protocols in the field, it is recommended to conduct experiments with sufficiently large sample sizes, multiple endpoints, and to validate cellular findings in complementary animal models.

5.1.5. Characteristics of hiPSC-derived cardiomyocytes

This study highlights that commonly used cell lines poorly represent adult heart biology in most aspects. Among the tested models, hiPSC-CMs are the closest to myocardial gene expression; however, they are still not widely used in the literature. Despite commercial availability, challenges such as diverse cell origins, variable differentiation protocols, and the immature phenotype of hiPSC-CMs limit the accurate modeling of mature heart tissue and *in vivo* translation (69, 134-136). Our transcriptomic analysis showed that hiPSC-CMs exhibited greater similarity to adult cardiac tissue and primary cells, even without additional maturation, in line with previous data (137). However, their metabolic immaturity was evident in our sI/R experiments, where hiPSC-CMs showed increased resistance to hypoxia compared to primary cardiomyocytes, likely due to a higher

glycolysis rate compared to adult cardiomyocytes (138). Previous works suggest that with further differentiation can improve the metabolic maturity of hiPSC-CMs (139-142). This suggests that hiPSC-CMs represent a valuable tool for cardiac disease modeling and drug screening, although additional refinement may be required.

5.2. Identification and selection of protectomiR candidates

To identify miRNAs involved in cardioprotection, protectomiRs, we compared the miRNA expression profiles in cardiac tissue subjected to ischemic conditioning versus I/R injury in a porcine MI model. Porcine MI models are valuable for translational research to validate therapies before conducting clinical studies since their cardiac anatomy, hemodynamics, and the timing and progression of MI closely resemble those of humans (6, 143). Although the pig genome is less well annotated than those of humans or mice, high-throughput omics tools are available for pigs (144). Our porcine model showed that IPreC reduced IS, while IPostC and RIPerC alleviated microvascular obstruction and edema (56). Building on previous findings from a rat model (84), we analyzed 220 known pig miRNAs, using an unbiased high-throughput method. Consistent with earlier reports, miR-29b and miR-199a expression changed significantly (145, 146). However, miR-133a and miR-146b levels were not changed, in contrast to a previous study, likely due to the differences in experimental protocols (145). By focusing on miRNAs linked to reduced myocardial injury after ischemic preconditioning and also involved in vascular protection during post- and remote conditioning, we narrowed down 14 protectomiR candidates. These included mimics of miR-199a, miR-450a, miR-450c, and miR-451, and antagomiRs targeting miR-29a, miR-34a, miR-105-2, miR-127, miR-142, miR-181a, miR-193a, miR-204a, miR-339a, and miR-424-3p, selected based on their regulation patterns. Given the translational relevance of porcine models, this study provides a valuable resource for identifying miRNA targets that mediate cardiac and vascular protection during ischemic injury, with potential for therapeutic development.

5.2.1. In vitro validation of the cardioprotective effect of protectomiRs

To validate the cardioprotective effects of selected mimic and antagomiR protectomiRs, we used a well-established *in vitro* NRCM I/R model (147, 148), chosen for its high transfection efficiency (84, 149). Although adult pig ventricular cardiomyocytes are physiologically more relevant, technical challenges make them less suitable for such studies (149-151). To address species differences, we selected protectomiRs with 100%

cross-species sequence orthology between pig and rat miRNAs. Transfection with miR-450a and miR-451 mimics significantly improved cell survival after sI/R. While miR-451 is known for hypoxia-related roles and cardioprotection in rodent and human studies (96, 152-155), it did not protect human cardiomyocytes from sI/R in our model. The cardioprotective role of miR-450a had not been previously explored. Previous studies conducted that miR-450a is enriched in exosomes from the pericardial fluid of patients with atrial fibrillation, and its influence on cardiac fibroblast activity was confirmed in primary rat cells (156, 157). In contrast, studies in mouse embryonic fibroblasts showed that miR-450a-3p overexpression promoted apoptosis and inhibited proliferation by downregulating Bub1 protein (158). Our study is the first to demonstrate a cardioprotective role for miR-450a in rat and human cardiomyocytes, indicating its potential involvement in ischemic conditioning-induced protection. However, further validation in *in vivo* models, including those with relevant comorbidities, is necessary to better reflect clinical conditions.

Other protectomiR mimics and antagomiRs showed no cardioprotective effects in our model. For instance, miR-29a and miR-34a antagomiRs were neutral or even toxic when transfected at higher doses, despite previous reports of their inhibition reducing infarct size and cardiomyocyte apoptosis in rodents (159-163). Both miR-142 and miR-181a were downregulated after ischemic conditioning in our samples, but their inhibition did not protect primary rat cardiomyocytes from sI/R injury. This contrasts with previous reports showing improved cell viability or reduced apoptosis following their inhibition in hypoxia-treated H9C2 cells (164-166). However, high concentrations of miR-142 inhibitor were found to increase apoptosis and reduce viability, suggesting dose-dependent toxicity, which aligns with our results (167). miR-193a inhibition had no effect on cell survival in our system, in contrast to findings in H9C2 cells (168). miR-204 expression was downregulated in our samples, but its inhibition did not enhance viability in NRCMs after sI/R. In contrast, miR-204 mimics have been shown to provide cardioprotection in rat and mouse I/R models (169-171). The role of miR-127-3p in cardioprotection is unexplored, but its expression is reduced in hypoxic mouse hearts (152). This may explain why high-dose miR-127 antagomiR decreased cardiomyocyte viability in our study. miR-339, which targets mainly cell death genes, is known to be upregulated after ischemia in human hearts (172). Consistently, we found its expression

decreased after cardioprotective ischemic conditioning in porcine hearts. Finally, our study is the first to demonstrate expression changes in miR-450b-5p and miR-105 after ischemic conditioning, although their protective roles were not confirmed by our study.

5.2.2. mRNA targets of miR-450a and their role in cardioprotection

To explore the mechanism behind miR-450a-induced cardioprotection, we analyzed its target genes and their potential functions across pigs, rats, and humans. Several of the top 50 predicted target genes of miR-450a are linked to key processes related to cardioprotection, such as apoptosis, redox signaling, DNA damage response, and autophagy. SMAD Family Member 2 (SMAD2) is a regulator in transforming growth factor beta (TGFβ) signaling (173); Death Associated Protein Kinase 2 (DAPK2) regulates apoptosis and is targeted by miR-133a to reduce I/R injury (174, 175); while Superoxide Dismutase 2 (SOD2) is a key player in redox signaling and cardioprotection against I/R damage (176). DNA damage response involves Ring Finger Protein 169 (RNF169) and SprT-Like N-Terminal Domain (SPRTN) genes (177, 178), while autophagy is modulated by G Protein Nucleolar 3 Like (GNL3L), Arginyltransferase 1 (ATE1), and Coiled-Coil Domain Containing 50 (CCDC50) genes, the latter also influencing type I interferon responses (179). The Eukaryotic Translation Initiation Factor 5A2 (EIF5A2) gene is induced by hypoxia in cancer cells, but has an unknown role in cardiac cells (180). These findings suggest that miR-450a likely exerts its protective effects through the regulation of multiple cellular processes.

Our GO analysis identified 421 biological processes regulated by miR-450a across pigs, rats, and humans. Among the top 100 enriched terms in humans were mRNA processing, splicing, and vesicle transport, key mechanisms of miRNA function (181, 182). miR-450a targets also influence stress fiber assembly and actomyosin organization, important for cardiac structure and function (183-185). Our results suggest that miR-450a may regulate TGFβ signaling, which has pleiotropic effects in the infarcted heart (186). Our findings indicate that miR-450a influences apoptotic signaling and DNA damage response, suggesting that its cardioprotective effect may result from modulating these pathways. While apoptosis is a primary cause of myocardial cell death, efforts to inhibit it clinically have faced significant challenges (187). miR-450a also regulates type I interferon production based on our GO analysis. Since MI activates the type I interferon response in cardiac macrophages, potentially worsening cardiac damage, miR-450a may reduce

cardiac inflammation by suppressing this pathway (188). Additionally, type I interferon signaling influences phosphoinositide 3-kinase (PI3K) activity, which in turn activates the mechanistic target of the rapamycin (mTOR) signaling pathway (189). Our KEGG analysis also showed miR-450a targets involved in PI3K and mTOR signaling across species. PI3K has a key role in MI and post-MI remodeling (190), while mTOR activation may reduce apoptosis early after ischemia, whereas its inhibition promotes autophagy beneficial for heart repair (191). miR-450a might also impact sphingolipid signaling, which is important in the pathophysiology of CVDs (192). Taken together, miR-450a seems to modulate multiple interconnected pathways that support cardiomyocyte survival, although further studies are required to fully elucidate the underlying molecular mechanisms.

5.3. Limitations

Our study has some limitations. Transcriptome analysis was performed on whole heart tissue rather than isolated adult cardiomyocytes, resulting in RNA samples containing non-cardiomyocyte RNA as well. We used only XCL-1-derived hiPSC-CMs, which do not capture the high variability of hiPSC-derived cardiac cells. While analyzing porcine myocardial miRNA expression, only 220 known pig miRNAs were examined instead of small RNA sequencing. In addition, the cell type-specific expression of miRNAs was not determined due to the lack of porcine miRNA databases. A further limitation is that while miRNA expression was measured in porcine samples, cytoprotective validation was performed primarily in rat cardiomyocytes for ethical and technical reasons. However, our selection for *in vitro* testing was limited to miRNAs with 100% sequence similarity between rats and pigs. NRCMs were used due to their translational relevance and transfection efficiency, while AC16 cells were used due to their human relevance. Additionally, only miRNAs consistently altered by all three conditioning stimuli were tested, possibly overlooking other relevant protectomiR candidates. Finally, in our experimental setup, miRNAs were administered before simulated ischemia to enable effective transfection of living cardiac myocytes, although this timing does not reflect a clinically relevant design.

5.4. Future plans

Building on our promising results, our goal is to further explore the therapeutic potential of miR-450a in preclinical studies, ultimately leading toward its clinical application for

treating myocardial I/R injury. The next step involves determining the optimal timing and dosing of miR-450a in an *in vivo* pharmacokinetic study. Subsequently, we aim to assess the pharmacodynamic effects of miR-450a in mice to validate the effective target inhibition *in vivo*. As a proof-of-concept, we will evaluate the potential of miR-450a to reduce infarct size in a mouse model of myocardial I/R. Upon successful validation, we will proceed to test the efficacy of the miR-450a-based therapeutic in a porcine MI model to generate the preclinical data required for approval of a subsequent clinical trial. These plans will advance our understanding of the therapeutic potential of miR-450a and enhance its translation into clinical trials for myocardial I/R injury.

6. Conclusions

Although individual *in vitro* cardiomyocyte models have been characterized, a comprehensive comparison was lacking in the literature. These models are valuable tools for testing cardioprotective agents against ischemia/reperfusion injury, with miRNAs emerging as promising therapeutic candidates. Here, we aimed to systematically compare cardiomyocyte models, identify novel cardioprotective miRNAs (protectomiRs) in a porcine myocardial infarction model, validate their effects *in vitro*, and explore their underlying molecular mechanisms for cardioprotection.

In summary, we demonstrated that cardiac cell lines, such as the rat H9C2, murine HL-1, and human AC16, offer valuable tools for cardiovascular research but have limitations that can affect experimental outcomes. Therefore, selecting the most suitable model requires careful consideration of the specific features of each cell line. In contrast, primary cell cultures, especially human iPSC-derived cardiomyocytes, are often more suitable for *in vitro* studies because they closely resemble adult cardiac tissue. However, their limited availability restricts their use for applications like high-throughput drug screening. Thus, additional studies are needed to validate findings from *in vitro* experiments and accurately translate them to *in vivo* conditions.

By analyzing the cardiac miRNA expression profile of a clinically relevant porcine model of acute myocardial infarction, we identified 14 protectomiRs, that were consistently regulated by ischemic preconditioning, postconditioning and remote ischemic perconditioning.

Among these protectomiRs, miR-450a and miR-451 mimics demonstrated direct cardiocytoprotective effects in an *in vitro* model of I/R injury in neonatal rat cardiomyocytes. Additionally, miR-450a showed cytoprotective effects in the human AC16 cell line subjected to sI/R.

The mechanism of action of miR-450a is likely mediated through multiple cardioprotective pathways, as suggested by its predicted targets and their functional analysis. However, further studies are needed to experimentally confirm these mechanisms.

In conclusion, miR-450a is a promising candidate for further development as a therapeutic agent, with the potential to fulfill the unmet need for effective cardioprotective treatments.

7. Summary

IHD is the leading cause of death globally (1). While coronary intervention preserves heart tissue, reperfusion can worsen cardiac damage through I/R injury (9). Although ischemic conditioning showed promising results in preclinical studies, its clinical translation was not successful (19, 22). Improving the translation of cardioprotective therapies requires a better understanding of molecular mechanisms, improved *in vitro* cardiac models, and the development of multitargeted therapies like miRNAs that may modulate complex conditions such as I/R injury (22, 73).

This thesis aims to 1) compare commonly used rat, mouse, and human cardiomyocyte models with mature cardiac tissues to identify their limitations, 2) analyze miRNA expression changes induced by ischemic conditioning in a porcine acute MI model, 3) validate the cytoprotective effects of selected protectomiRs *in vitro*, and 4) explore the molecular mechanisms underlying their cardioprotective effects.

Cardiac cell lines (H9C2, AC16, HL-1) were cultured and differentiated, then compared to cardiac tissue, primary cardiomyocytes, and hiPSC-CMs through RNA sequencing for transcriptomic profiling. To assess their response to hypertrophic stimuli, cells were treated with angiotensin II or isoprenaline. Hypoxic tolerance was evaluated by subjecting cells to sI/R. Cell lines showed a lower cardiac marker expression than reference tissues, a less mature cardiac gene expression profile, and a distinct response to sI/R injury and hypertrophic stimuli compared to primary cells, regardless of their differentiation status.

Through the analysis of miRNA expression changes in cardiac tissues from a porcine model subjected to various ischemic conditioning treatments compared to ischemia alone, 14 protectomiR candidates were identified.

Selected protectomiRs were validated in sI/R models using isolated NRCMs and AC16 cells. Among these, miR-450a demonstrated cardioprotective effects in both rat and human cardiomyocytes.

The target prediction, GO and KEGG pathway analyses revealed that miR-450a targets multiple mRNAs across pigs, rats, and humans, which are linked to cardioprotective molecular pathways.

In conclusion, cardiac cell lines have limited similarity to primary cells, highlighting their moderate translational value. We identified miR-450a as a promising candidate for the development of cardioprotective therapies.

8. References

1. Naghavi M, Ong KL, Aali A, Ababneh HS, Abate YH, Abbafati C, Abbasgholizadeh R, Abbasian M, Abbasi-Kangevari M, Abbastabar H, Abd ElHafeez S, Abdelmasseh M, Abd-Elsalam S, Abdelwahab A, Abdollahi M, Abdollahifar M-A, Abdoun M, Abdulah DM, Abdullahi A, Abebe M, Abebe SS, Abedi A, Abegaz KH, Abhilash ES, Abidi H, Abiodun O, Aboagye RG, Abolhassani H, Abolmaali M, Abouzid M, Aboye GB, Abreu LG, Abrha WA, Abtahi D, Abu Rumeileh S, Abualruz H, Abubakar B, Abu-Gharbieh E, Abu-Rmeileh NME, Aburuz S, Abu-Zaid A, Accrombessi MMK, Adal TG, Adamu AA, Addo IY, Addolorato G, Adebiyi AO, Adekanmbi V, Adepoju AV, Adetunji CO, Adetunji JB, Adeyeoluwa TE, Adeyinka DA, Adeyomoye OI, Admass BAA, Adnani QES, Adra S, Afolabi AA, Afzal MS, Afzal S, Agampodi SB, Agasthi P, Aggarwal M, Aghamiri S, Agide FD, Agodi A, Agrawal A, Agyemang-Duah W, Ahinkorah BO, Ahmad A, Ahmad D, Ahmad F, Ahmad MM, Ahmad S, Ahmad S, Ahmad T, Ahmadi K, Ahmadzade AM, Ahmed A, Ahmed A, Ahmed H, Ahmed LA, Ahmed MS, Ahmed MS, Ahmed MB, Ahmed SA, Ajami M, Aji B, Akara EM, Akbarialiabad H, Akinosoglou K, Akinyemiju T, Akkaif MA, Akyirem S, Al Hamad H, Al Hasan SM, Alahdab F, Alalalmeh SO, Alalwan TA, Al-Aly Z, Alam K, Alam M, Alam N, Al-amer RM, Alanezi FM, Alanzi TM, Al-Azzam S, Albakri A, Albashtawy M, AlBataineh MT, Alcalde-Rabanal JE, Aldawsari KA, Aldhaleei WA, Aldridge RW, Alema HB, Alemayohu MA, Alemi S, Alemu YM, Al-Gheethi AAS, Alhabib KF, Alhalaiqa FAN, Al-Hanawi MK, Ali A, Ali A, Ali L, Ali MU, Ali R, Ali S, Ali SSS, Alicandro G, Alif SM, Alikhani R, Alimohamadi Y, Aliyi AA, Aljasir MAM, Aljunid SM, Alla F, Allebeck P, Al-Marwani S, Al-Maweri SAA, Almazan JU, Al-Mekhlafi HM, Almidani L, Almidani O, Alomari MA, Al-Omari B, Alonso J, Alqahtani JS, Alqalyoobi S, Alqutaibi AY, Al-Sabah SK, Altaany Z, Altaf A, Al-Tawfiq JA, Altirkawi KA, Aluh DO, Alvis-Guzman N, Alwafi H, Al-Worafi YM, Aly H, Aly S, Alzoubi KH, Amani R, Amare AT, Amegbor PM, Ameyaw EK, Amin TT, Amindarolzarbi A, Amiri S, Amirzade-Iranaq MH, Amu H, Amugsi DA, Amusa GA, Ancuceanu R, Anderlini D, Anderson DB, Andrade PP, Andrei CL, Andrei T, Angus C, Anil A, Anil S, Anoushiravani A, Ansari H, Ansariadi A, Ansari-Moghaddam A, Antony CM, Antriyandarti E, Anvari D, Anvari S, Anwar S, Anwar SL, Anwer R, Anyasodor AE, Aqeel M, Arab JP, Arabloo J, Arafat M, Aravkin AY, Areda D, Aremu A, Aremu O,

Ariffin H, Arkew M, Armocida B, Arndt MB, Ärnlöv J, Arooj M, Artamonov AA, Arulappan J, Aruleba RT, Arumugam A, Asaad M, Asadi-Lari M, Asgedom AA, Asghariahmadabad M, Asghari-Jafarabadi M, Ashraf M, Aslani A, Astell-Burt T, Athar M, Athari SS, Atinafu BTT, Atlaw HW, Atorkey P, Atout MMdW, Atreya A, Aujayeb A, Ausloos M, Avan A, Awedew AF, Aweke AM, Ayala Quintanilla BP, Ayatollahi H, Ayuso-Mateos JL, Ayyoubzadeh SM, Azadnajafabad S, Azevedo RMS, Azzam AY, B DB, Babu AS, Badar M, Badiye AD, Baghdadi S, Bagheri N, Bagherieh S, Bah S, Bahadorikhalili S, Bahmanziari N, Bai R, Baig AA, Baker JL, Bako AT, Bakshi RK, Balakrishnan S, Balasubramanian M, Baltatu OC, Bam K, Banach M, Bandyopadhyay S, Banik PC, Bansal H, Bansal K, Barbic F, Barchitta M, Bardhan M, Bardideh E, Barker-Collo SL, Bärnighausen TW, Barone-Adesi F, Barqawi HJ, Barrero LH, Barrow A, Barteit S, Barua L, Basharat Z, Bashiri A, Basiru A, Baskaran P, Basnyat B, Bassat Q, Basso JD, Basting AVL, Basu S, Batra K, Baune BT, Bayati M, Bayileyegn NS, Beaney T, Bedi N, Beghi M, Behboudi E, Behera P, Behnoush AH, Behzadifar M, Beiranvand M, Bejarano Ramirez DF, Béjot Y, Belay SA, Belete CM, Bell ML, Bello MB, Bello OO, Belo L, Beloukas A, Bender RG, Bensenor IM, Beran A, Berezvai Z, Berhie AY, Berice BN, Bernstein RS, Bertolacci GJ, Bettencourt PJG, Beyene KA, Bhagat DS, Bhagavathula AS, Bhala N, Bhalla A, Bhandari D, Bhangdia K, Bhardwaj N, Bhardwaj P, Bhardwaj PV, Bhargava A, Bhaskar S, Bhat V, Bhatti GK, Bhatti JS, Bhatti MS, Bhatti R, Bhutta ZA, Bikbov B, Bishai JD, Bisignano C, Bisulli F, Biswas A, Biswas B, Bitaraf S, Bitew BD, Bitra VR, Bjørge T, Boachie MK, Boampong MS, Bobirca AV, Bodolica V, Bodunrin AO, Bogale EK, Bogale KA, Bohlouli S, Bolarinwa OA, Boloor A, Bonakdar Hashemi M, Bonny A, Bora K, Bora Basara B, Borhany H, Borzutzky A, Bouaoud S, Boustany A, Boxe C, Boyko EJ, Brady OJ, Braithwaite D, Brant LC, Brauer M, Brazinova A, Brazo-Sayavera J, Breitborde NJK, Breitner S, Brenner H, Briko AN, Briko NI, Britton G, Brown J, Brugha T, Bulamu NB, Bulto LN, Buonsenso D, Burns RA, Busse R, Bustanji Y, Butt NS, Butt ZA, Caetano dos Santos FL, Calina D, Cámera LA, Campos LA, Campos-Nonato IR, Cao C, Cao Y, Capodici A, Cárdenas R, Carr S, Carreras G, Carrero JJ, Carugno A, Carvalheiro CG, Carvalho F, Carvalho M, Castaldelli-Maia JM, Castañeda-Orjuela CA, Castelpietra G, Catalá-López F, Catapano AL, Cattaruzza MS, Cederroth CR, Cegolon L, Cembranel F, Cenderadewi M, Cercy KM, Cerin E, Cevik M, Chadwick J, Chahine Y, Chakraborty C, Chakraborty PA, Chan JSK,

Chan RNC, Chandika RM, Chandrasekar EK, Chang C-K, Chang J-C, Chanie GS, Charalampous P, Chattu VK, Chaturvedi P, Chatzimavridou-Grigoriadou V, Chaurasia A, Chen AW, Chen A-T, Chen CS, Chen H, Chen MX, Chen S, Cheng C-Y, Cheng ETW, Cherbuin N, Cheru WA, Chien J-H, Chimed-Ochir O, Chimoriya R, Ching PR, Chirinos-Caceres JL, Chitheer A, Cho WCS, Chong B, Chopra H, Choudhari SG, Chowdhury R, Christopher DJ, Chukwu IS, Chung E, Chung E, Chung E, Chung S-C, Chutiyami M, Cindi Z, Cioffi I, Claassens MM, Claro RM, Coberly K, Cogen RM, Columbus A, Comfort H, Conde J, Cortese S, Cortesi PA, Costa VM, Costanzo S, Cousin E, Couto RAS, Cowden RG, Cramer KM, Criqui MH, Cruz-Martins N, Cuadra-Hernández SM, Culbreth GT, Cullen P, Cunningham M, Curado Mp, Dadana S, Dadras O, Dai S, Dai X, Dai Z, Dalli LL, Damiani G, Darega Gela J, Das JK, Das S, Das S, Dascalu AM, Dash NR, Dashti M, Dastiridou A, Davey G, Dávila-Cervantes CA, Davis Weaver N, Davletov K, De Leo D, de Luca K, Debele AT, Debopadhaya S, Degenhardt L, Dehghan A, Deitesfeld L, Del Bo C, Delgado-Enciso I, Demessa BH, Demetriades AK, Deng K, Deng X, Denova-Gutiérrez E, Deravi N, Dereje N, Dervenis N, Dervišević E, Des Jarlais DC, Desai HD, Desai R, Devanbu VGC, Dewan SMR, Dhali A, Dhama K, Dhimal M, Dhingra S, Dhulipala VR, Dias da Silva D, Diaz D, Diaz MJ, Dima A, Ding DD, Ding H, Dinis-Oliveira RJ, Dirac MA, Djalalinia S, Do THP, do Prado CB, Doaei S, Dodangeh M, Dodangeh M, Dohare S, Dokova KG, Dolecek C, Dominguez R-MV, Dong W, Dongarwar D, D'Oria M, Dorostkar F, Dorsey ER, dos Santos WM, Doshi R, Doshmangir L, Dowou RK, Driscoll TR, Dsouza HL, Dsouza V, Du M, Dube J, Duncan BB, Duraes AR, Duraisamy S, Durojaiye OC, Dwyer-Lindgren L, Dzianach PA, Dziedzic AM, E'Mar AR, Eboreime E, Ebrahimi A, Echieh CP, Edinur HA, Edvardsson D, Edvardsson K, Efendi D, Efendi F, Effendi DE, Eikemo TA, Eini E, Ekholuenetale M, Ekundayo TC, El Sayed I, Elbarazi I, Elema TB, Elemam NM, Elgar FJ, Elgendy IY, ElGohary GMT, Elhabashy HR, Elhadi M, El-Huneidi W, Elilo LT, Elmeligy OAA, Elmonem MA, Elshaer M, Elsohaby I, Emeto TI, Engelbert Bain L, Erkhembayar R, Esezobor CI, Eshrati B, Eskandarieh S, Espinosa-Montero J, Esubalew H, Etaee F, Fabin N, Fadaka AO, Fagbamigbe AF, Fahim A, Fahimi S, Fakhri-Demeshghieh A, Falzone L, Fareed M, Farinha CSeS, Faris MEM, Faris PS, Faro A, Fasanmi AO, Fatehizadeh A, Fattahi H, Fauk NK, Fazeli P, Feigin VL, Feizkhah A, Fekadu G, Feng X, Fereshtehnejad S-M, Feroze AH, Ferrante D, Ferrari AJ, Ferreira N, Fetensa G, Feyisa BR, Filip I, Fischer F,

Flavel J, Flood D, Florin BT, Foigt NA, Folayan MO, Fomenkov AA, Foroutan B, Foroutan M, Forthun I, Fortuna D, Foschi M, Fowobaje KR, Francis KL, Franklin RC, Freitas A, Friedman J, Friedman SD, Fukumoto T, Fuller JE, Fux B, Gaal PA, Gadanya MA, Gaidhane AM, Gaihre S, Gakidou E, Galali Y, Galles NC, Gallus S, Ganbat M, Gandhi AP, Ganesan B, Ganiyani MA, Garcia-Gordillo MA, Gardner WM, Garg J, Garg N, Gautam RK, Gbadamosi SO, Gebi TG, Gebregergis MW, Gebrehiwot M, Gebremeskel TG, Georgescu SR, Getachew T, Gething PW, Getie M, Ghadiri K, Ghahramani S, Ghailan KY, Ghasemi M-R, Ghasempour Dabaghi G, Ghasemzadeh A, Ghashghaee A, Ghassemi F, Ghazy RM, Ghimire A, Ghoba S, Gholamalizadeh M, Gholamian A, Gholamrezanezhad A, Gholizadeh N, Ghorbani M, Ghorbani Vajargah P, Ghoshal AG, Gill PS, Gill TK, Gillum RF, Ginindza TG, Girmay A, Glasbey JC, Gnedovskaya EV, Göbölös L, Godinho MA, Goel A, Golchin A, Goldust M, Golechha M, Goleij P, Gomes NGM, Gona PN, Gopalani SV, Gorini G, Goudarzi H, Goulart AC, Goulart BNG, Goyal A, Grada A, Graham SM, Grivna M, Grosso G, Guan S-Y, Guarducci G, Gubari MIM, Gudeta MD, Guha A, Guicciardi S, Guimarães RA, Gulati S, Gunawardane DA, Gunturu S, Guo C, Gupta AK, Gupta B, Gupta MK, Gupta M, Gupta RD, Gupta R, Gupta S, Gupta VB, Gupta VK, Gupta VK, Gurmessa L, Gutiérrez RA, Habibzadeh F, Habibzadeh P, Haddadi R, Hadei M, Hadi NR, Haep N, Hafezi-Nejad N, Hailu A, Haj-Mirzaian A, Halboub ES, Hall BJ, Haller S, Halwani R, Hamadeh RR, Hameed S, Hamidi S, Hamilton EB, Han C, Han Q, Hanif A, Hanifi N, Hankey GJ, Hanna F, Hannan MA, Haque MN, Harapan H, Hargono A, Haro JM, Hasaballah AI, Hasan I, Hasan MT, Hasani H, Hasanian M, Hashi A, Hasnain MS, Hassan I, Hassanipour S, Hassankhani H, Haubold J, Havmoeller RJ, Hay SI, He J, Hebert JJ, Hegazi OE, Heidari G, Heidari M, Heidari-Foroozan M, Helfer B, Hendrie D, Herrera-Serna BY, Herteliu C, Hesami H, Hezam K, Hill CL, Hiraike Y, Holla R, Horita N, Hossain MM, Hossain S, Hosseini M-S, Hosseinzadeh H, Hosseinzadeh M, Hosseinzadeh Adli A, Hostiuc M, Hostiuc S, Hsairi M, Hsieh VC-r, Hsu RL, Hu C, Huang J, Hultström M, Humayun A, Hundie TG, Hussain J, Hussain MA, Hussein NR, Hussien FM, Huynh H-H, Hwang B-F, Ibitoye SE, Ibrahim KS, Iftikhar PM, Ijo D, Ikiroma AI, Ikuta KS, Ikwegbue PC, Ilesanmi OS, Ilic IM, Ilic MD, Imam MT, Immurana M, Inamdar S, Indriasih E, Iqhrammullah M, Iradukunda A, Iregbu KC, Islam MR, Islam SMS, Islami F, Ismail F, Ismail NE, Iso H, Isola G, Iwagami M, Iwu CCD, Iyamu IO, Iyer M, J LM, Jaafari J,

Jacob L, Jacobsen KH, Jadidi-Niaragh F, Jafarinia M, Jafarzadeh A, Jaggi K, Jahankhani K, Jahanmehr N, Jahrami H, Jain N, Jairoun AA, Jaiswal A, Jamshidi E, Janko MM, Jatau AI, Javadov S, Javaheri T, Jayapal SK, Jayaram S, Jebai R, Jee SH, Jeganathan J, Jha AK, Jha RP, Jiang H, Jin Y, Johnson O, Jokar M, Jonas JB, Joo T, Joseph A, Joseph N, Joshua CE, Joshy G, Jozwiak JJ, Jürisson M, K V, Kaambwa B, Kabir A, Kabir Z, Kadashetti V, Kadir DH, Kalani R, Kalankesh LR, Kalankesh LR, Kaliyadan F, Kalra S, Kamal VK, Kamarajah SK, Kamath R, Kamiab Z, Kamyari N, Kanagasabai T, Kanchan T, Kandel H, Kanmanthareddy AR, Kanmiki EW, Kanmodi KK, Kannan S S, Kansal SK, Kantar RS, Kapoor N, Karajizadeh M, Karanth SD, Karasneh RA, Karaye IM, Karch A, Karim A, Karimi SE, Karimi Behnagh A, Kashoo FZ, Kasnazani QHA, Kasraei H, Kassebaum NJ, Kassel MB, Kauppila JH, Kaur N, Kawakami N, Kayode GA, Kazemi F, Kazemian S, Kazmi TH, Kebebew GM, Kebede AD, Kebede F, Keflie TS, Keiyoro PN, Keller C, Kelly JT, Kempen JH, Kerr JA, Kesse-Guyot E, Khajuria H, Khalaji A, Khalid N, Khalil AA, Khalilian A, Khamesipour F, Khan A, Khan A, Khan G, Khan I, Khan IA, Khan MN, Khan M, Khan MJ, Khan MAB, Khan ZA, Khan suheb MZ, Khanmohammadi S, Khatab K, Khatami F, Khatatbeh H, Khatatbeh MM, Khavandegar A, Khayat Kashani HR, Khidri FF, Khodadoust E, Khorgamphar M, Khormali M, Khorrami Z, Khosravi A, Khosravi MA, Kifle ZD, Kim G, Kim J, Kim K, Kim MS, Kim YJ, Kimokoti RW, Kinzel KE, Kisa A, Kisa S, Klu D, Knudsen AKS, Kocarnik JM, Kochhar S, Kocsis T, Koh DSQ, Kolahi A-A, Kolves K, Kompani F, Koren G, Kosen S, Kostev K, Koul PA, Koulmane Laxminarayana SL, Krishan K, Krishna H, Krishna V, Krishnamoorthy V, Krishnamoorthy Y, Krohn KJ, Kuate Defo B, Kucuk Bicer B, Kuddus MA, Kuddus M, Kuitunen I, Kulimbet M, Kulkarni V, Kumar A, Kumar A, Kumar H, Kumar M, Kumar R, Kumari M, Kumie FT, Kundu S, Kurmi OP, Kusnali A, Kusuma D, Kwarteng A, Kyriopoulos I, Kyu HH, La Vecchia C, Lacey B, Ladan MA, Laflamme L, Lagat AK, Lager ACJ, Lahmar A, Lai DTC, Lal DK, Lalloo R, Lallukka T, Lam H, Lám J, Landrum KR, Lanfranchi F, Lang JJ, Langguth B, Lansingh VC, Laplante-Lévesque A, Larijani B, Larsson AO, Lasrado S, Lassi ZS, Latief K, Latifinaibin K, Lauriola P, Le NHH, Le TTT, Le TDT, Ledda C, Ledesma JR, Lee M, Lee PH, Lee SW, Lee SWH, Lee W-C, Lee YH, LeGrand KE, Leigh J, Leong E, Lerango TL, Li M-C, Li W, Li X, Li Y, Li Z, Ligade VS, Likaka ATM, Lim L-L, Lim SS, Lindstrom M, Linehan C, Liu C, Liu G, Liu J, Liu R, Liu S, Liu X, Liu X, Llanaj E, Loftus MJ, López-Bueno R, Lopukhov PD, Loreche AM,

Lorkowski S, Lotufo PA, Lozano R, Lubinda J, Lucchetti G, Lugo A, Lunevicius R, Ma ZF, Maass KL, Machairas N, Machoy M, Madadizadeh F, Madsen C, Madureira-Carvalho ÁM, Maghazachi AA, Maharaj SB, Mahjoub S, Mahmoud MA, Mahmoudi A, Mahmoudi E, Mahmoudi R, Majeed A, Makhdoom IF, Malakan Rad E, Maled V, Malekzadeh R, Malhotra AK, Malhotra K, Malik AA, Malik I, Malta DC, Mamun AA, Mansouri P, Mansournia MA, Mantovani LG, Maqsood S, Marasini BP, Marateb HR, Maravilla JC, Marconi AM, Mardi P, Marino M, Marjani A, Martinez G, Martinez-Guerra BA, Martinez-Piedra R, Martini D, Martini S, Martins-Melo FR, Martorell M, Marx W, Maryam S, Marzo RR, Masaka A, Masrie A, Mathieson S, Mathioudakis AG, Mathur MR, Mattumpuram J, Matzopoulos R, Maude RJ, Maugeri A, Maulik PK, Mayeli M, Mazaheri M, Mazidi M, McGrath JJ, McKee M, McKowen ALW, McLaughlin SA, McPhail SM, Mechili EA, Medina JRC, Mediratta RP, Meena JK, Mehra R, Mehrabani-Zeinabad K, Mehrabi Nasab E, Mekene Meto T, Meles GG, Mendez-Lopez MAM, Mendoza W, Menezes RG, Mengist B, Mentis A-FA, Meo SA, Meresa HA, Meretoja A, Meretoja TJ, Mersha AM, Mesfin BA, Mestrovic T, Mettananda KCD, Mettananda S, Meylakhs P, Mhlanga A, Mhlanga L, Mi T, Miazgowski T, Micha G, Michalek IM, Miller TR, Mills EJ, Minh LHN, Mini GK, Mir Mohammad Sadeghi P, Mirica A, Mirijello A, Mirrakhimov EM, Mirutse MK, Mirzaei M, Misganaw A, Mishra A, Misra S, Mitchell PB, Mithra P, Mittal C, Mobayen M, Moberg ME, Mohamadkhani A, Mohamed J, Mohamed MFH, Mohamed NS, Mohammad-Alizadeh-Charandabi S, Mohammadi S, Mohammadian-Hafshejani A, Mohammadifard N, Mohammed H, Mohammed H, Mohammed M, Mohammed S, Mohammed S, Mohan V, Mojiri-Forushani H, Mokari A, Mokdad AH, Molinaro S, Molokhia M, Momtazmanesh S, Monasta L, Mondello S, Moni MA, Moodi Ghalibaf A, Moradi M, Moradi Y, Moradi-Lakeh M, Moradzadeh M, Moraga P, Morawska L, Moreira RS, Morovatdar N, Morrison SD, Morze J, Mosser JF, Motappa R, Mougin V, Mouodi S, Mousavi P, Mousavi SE, Mousavi Khaneghah A, Mpolya EA, Mrejen M, Mubarik S, Muccioli L, Mueller UO, Mughal F, Mukherjee S, Mulita F, Munjal K, Murillo-Zamora E, Musaigwa F, Musallam KM, Mustafa A, Mustafa G, Muthupandian S, Muthusamy R, Muzaffar M, Myung W, Nagarajan AJ, Nagel G, Naghavi P, Naheed A, Naik GR, Naik G, Nainu F, Nair S, Najmuldeen HHR, Nakhostin Ansari N, Nangia V, Naqvi AA, Narasimha Swamy S, Narayana AI, Nargus S, Nascimento BR, Nascimento GG, Nasehi S, Nashwan AJ, Natto ZS, Nauman J, Naveed

M, Nayak BP, Nayak VC, Nazri-Panjaki A, Ndejjo R, Nduaguba SO, Negash H, Negoi I, Negoi RI, Negru SM, Nejadghaderi SA, Nejjari C, Nena E, Nepal S, Ng M, Nggada HA, Nguefack-Tsague G, Ngunjiri JW, Nguyen AH, Nguyen DH, Nguyen HTH, Nguyen PT, Nguyen VT, Niazi RK, Nielsen KR, Nigatu YT, Nikolouzakis TK, Nikoobar A, Nikoomanesh F, Nikpoor AR, Ningrum DNA, Nnaji CA, Nnyanzi LA, Noman EA, Nomura S, Noreen M, Noroozi N, Norrving B, Noubiap JJ, Novotney A, Nri-Ezedi CA, Ntaios G, Ntsekhe M, Nuñez-Samudio V, Nurrika D, Nutor JJ, Oancea B, Obamiro KO, Oboh MA, Odetokun IA, Odogwu NM, O'Donnell MJ, Oduro MS, Ofakunrin AOD, Ogunkoya A, Oguntade AS, Oh I-H, Okati-Aliabad H, Okeke SR, Okekunle AP, Okonji OC, Olagunju AT, Olaiya MT, Olatubi MI, Oliveira GMM, Olufadewa II, Olusanya BO, Olusanya JO, Oluwafemi YD, Omar HA, Omar Bali A, Omer GL, Ondayo MA, Ong S, Onwujekwe OE, Onyedibe KI, Ordak M, Orisakwe OE, Orish VN, Ortega-Altamirano DV, Ortiz A, Osman WMS, Ostroff SM, Osuagwu UL, Otoiu A, Otstavnov N, Otstavnov SS, Ouyahia A, Ouyang G, Owolabi MO, Ozten Y, P A MP, Padron-Monedero A, Padubidri JR, Pal PK, Palicz T, Palladino C, Palladino R, Palma-Alvarez RF, Pan F, Pan H-F, Pana A, Panda P, Panda-Jonas S, Pandi-Perumal SR, Pangaribuan HU, Panos GD, Panos LD, Pantazopoulos I, Pantea Stoian AM, Papadopoulou P, Parikh RR, Park S, Parthasarathi A, Pashaei A, Pasovic M, Passera R, Pasupula DK, Patel HM, Patel J, Patel SK, Patil S, Patoulias D, Patthipati VS, Paudel U, Pazoki Toroudi H, Pease SA, Peden AE, Pedersini P, Pensato U, Pepito VCF, Peprah EK, Peprah P, Perdigão J, Pereira M, Peres MFP, Perianayagam A, Perico N, Pestell RG, Pesudovs K, Petermann-Rocha FE, Petri WA, Pham HT, Philip AK, Phillips MR, Pierannunzio D, Pigeolet M, Pigott DM, Pilgrim T, Piracha ZZ, Piradov MA, Pirouzpanah S, Plakkal N, Plotnikov E, Podder V, Poddighe D, Polinder S, Polkinghorne KR, Poluru R, Ponkilainen VT, Porru F, Postma MJ, Poudel GR, Pourshams A, Pourtaheri N, Prada SI, Pradhan PMS, Prakasham TN, Prasad M, Prashant A, Prates EJS, Prieto Alhambra D, Priscilla T, Pritchett N, Purohit BM, Puvvula J, Qasim NH, Qattea I, Qazi AS, Qian G, Qiu S, Qureshi MF, Rabiee Rad M, Radfar A, Radhakrishnan RA, Radhakrishnan V, Raeisi Shahraki H, Rafferty Q, Raggi A, Raghav PR, Raheem N, Rahim F, Rahim MJ, Rahimi-Movaghar V, Rahman MM, Rahman MHU, Rahman M, Rahman MA, Rahmani AM, Rahmani S, Rahmanian V, Rajaa S, Rajput P, Rakovac I, Ramasamy SK, Ramazanu S, Rana K, Ranabhat CL, Rancic N, Rane A, Rao CR, Rao IR, Rao M, Rao SJ, Rasali DP, Rasella D, Rashedi S, Rashedi

V, Rashidi M-M, Rasouli-Saravani A, Rasul A, Rathnaiah Babu G, Rauniyar SK, Ravangard R, Ravikumar N, Rawaf DL, Rawaf S, Rawal L, Rawassizadeh R, Rawlley B, Raza RZ, Razo C, Redwan EMM, Rehman FU, Reifels L, Reiner Jr RC, Remuzzi G, Reyes LF, Rezaei M, Rezaei N, Rezaei N, Rezaeian M, Rhee TG, Riaz MA, Ribeiro ALP, Rickard J, Riva HR, Robinson-Oden HE, Rodrigues CF, Rodrigues M, Roever L, Rogowski ELB, Rohloff P, Romadlon DS, Romero-Rodríguez E, Romoli M, Ronfani L, Roshandel G, Roth GA, Rout HS, Roy N, Roy P, Rubagotti E, Ruela GdA, Rumisha SF, Runghien T, Rwegerera GM, Rynkiewicz A, S N C, Saad AMA, Saadatian Z, Saber K, Saber-Ayad MM, SaberiKamarposhti M, Sabour S, Sacco S, Sachdev PS, Sachdeva R, Saddik B, Saddler A, Sadee BA, Sadeghi E, Sadeghi E, Sadeghian F, Saeb MR, Saeed U, Safaeinejad F, Safi SZ, Sagar R, Saghazadeh A, Sagoe D, Saheb Sharif-Askari F, Saheb Sharif-Askari N, Sahebkar A, Sahoo SS, Sahoo U, Sahu M, Saif Z, Sajid MR, Sakshaug JW, Salam N, Salamati P, Salami AA, Salaroli LB, Saleh MA, Salehi S, Salem MR, Salem MZY, Salimi S, Samadi Kafil H, Samadzadeh S, Samargandy S, Samodra YL, Samy AM, Sanabria J, Sanna F, Santomauro DF, Santos IS, Santric-Milicevic MM, Sao Jose BP, Sarasmita MA, Saraswathy SYI, Saravanan A, Saravi B, Sarikhani Y, Sarkar T, Sarmiento-Suárez R, Sarode GS, Sarode SC, Sarveazad A, Sathian B, Sathish T, Satpathy M, Sayeed A, Sayeed MA, Saylan M, Sayyah M, Scarmeas N, Schaarschmidt BM, Schlaich MP, Schlee W, Schmidt MI, Schneider IJC, Schuermans A, Schumacher AE, Schutte AE, Schwarzinger M, Schwebel DC, Schwendicke F, Šekerija M, Selvaraj S, Senapati S, Senthilkumaran S, Sepanlou SG, Serban D, Sethi Y, Sha F, Shabany M, Shafaat A, Shafie M, Shah NS, Shah PA, Shah SM, Shahabi S, Shahbandi A, Shahid I, Shahid S, Shahid W, Shahsavari HR, Shahwan MJ, Shaikh A, Shaikh MA, Shakeri A, Shalash AS, Sham S, Shamim MA, Shams-Beyranvand M, Shamshad H, Shamsi MA, Shanawaz M, Shankar A, Sharfaei S, Sharifan A, Sharifi-Rad J, Sharma R, Sharma S, Sharma U, Sharma V, Shastry RP, Shavandi A, Shayan M, Shehabeldine AME, Sheikh A, Sheikhi RA, Shen J, Shetty A, Shetty BSK, Shetty PH, Shi P, Shibuya K, Shiferaw D, Shigematsu M, Shin M-J, Shin YH, Shiri R, Shirkoohi R, Shitaye NA, Shittu A, Shiue I, Shivakumar KM, Shivarov V, Shokraneh F, Shokri A, Shool S, Shorofi SA, Shrestha S, Shuval K, Siddig EE, Silva JP, Silva LMLR, Silva S, Simpson CR, Singal A, Singh A, Singh BB, Singh G, Singh J, Singh NP, Singh P, Singh S, Sinha DN, Sinto R, Siraj MS, Sirota SB, Sitas F, Sivakumar S, Skryabin VY, Skryabina AA, Sleet DA, Socea B, Sokhan

A, Solanki R, Solanki S, Soleimani H, Soliman SSM, Song S, Song Y, Sorensen RJD, Soriano JB, Soyiri IN, Spartalis M, Spearman S, Sreeramareddy CT, Srivastava VK, Stanaway JD, Stanikzai MH, Stark BA, Starnes JR, Starodubova AV, Stein C, Stein DJ, Steinbeis F, Steiner C, Steinmetz JD, Steiropoulos P, Stevanović A, Stockfelt L, Stokes MA, Stortecky S, Subramaniyan V, Suleman M, Suliankatchi Abdulkader R, Sultana A, Sun HZ, Sun J, Sundström J, Sunkersing D, Sunnerhagen KS, Swain CK, Szarpak L, Szeto MD, Szócska M, Tabaee Damavandi P, Tabarés-Seisdedos R, Tabatabaei SM, Tabatabaei Malazy O, Tabatabaeizadeh S-A, Tabatabai S, Tabish M, Tadakamadla J, Tadakamadla SK, Taheri Abkenar Y, Taheri Soodejani M, Taiba J, Takahashi K, Talaat IM, Talukder A, Tampa M, Tamuzi JL, Tan K-K, Tandukar S, Tang H, Tang HK, Tarigan IU, Tariku MK, Tariqujjaman M, Tarkang EE, Tavakoli Oliaee R, Tavangar SM, Taveira N, Tefera YM, Temsah M-H, Temsah RMH, Teramoto M, Tesler R, Teye-Kwadjo E, Thakur R, Thangaraju P, Thankappan KR, Tharwat S, Thayakaran R, Thomas N, Thomas NK, Thomson AM, Thrift AG, Thum CCC, Thygesen LC, Tian J, Tichopad A, Ticoalu JHV, Tillawi T, Tiruye TY, Titova MV, Tonelli M, Topor-Madry R, Toriola AT, Torre AE, Touvier M, Tovani-Palone MR, Tran JT, Tran NM, Trico D, Tromans SJ, Truyen TTTT, Tsatsakis A, Tsegay GM, Tsermpini EE, Tumurkhuu M, Tung K, Tyrovolas S, Uddin SMN, Udoakang AJ, Udoh A, Ullah A, Ullah I, Ullah S, Ullah S, Umakanthan S, Umeokonkwo CD, Unim B, Unnikrishnan B, Unsworth CA, Upadhyay E, Urso D, Usman JS, Vahabi SM, Vaithinathan AG, Valizadeh R, Van de Velde SM, Van den Eynde J, Varga O, Vart P, Varthya SB, Vasankari TJ, Vasic M, Vaziri S, Vellingiri B, Venketasubramanian N, Verghese NA, Verma M, Veroux M, Verras G-I, Vervoort D, Villafañe JH, Villanueva GI, Vinayak M, Violante FS, Viskadourou M, Vladimirov SK, Vlassov V, Vo B, Vollset SE, Vongpradith A, Vos T, Vujcic IS, Vukovic R, Wafa HA, Waheed Y, Wamai RG, Wang C, Wang N, Wang S, Wang S, Wang Y, Wang Y-P, Waqas M, Ward P, Wassie EG, Watson S, Watson SLW, Weerakoon KG, Wei MY, Weintraub RG, Weiss DJ, Westerman R, Whisnant JL, Wiangkham T, Wickramasinghe DP, Wickramasinghe ND, Wilandika A, Wilkerson C, Willeit P, Wilson S, Wojewodzic MW, Woldegebreal DH, Wolf AW, Wolfe CDA, Wondimagegene YA, Wong YJ, Wongsin U, Wu A-M, Wu C, Wu F, Wu X, Wu Z, Xia J, Xiao H, Xie Y, Xu S, Xu W-D, Xu X, Xu YY, Yadollahpour A, Yamagishi K, Yang D, Yang L, Yano Y, Yao Y, Yaribeygi H, Ye P, Yehualashet SS, Yesiltepe M, Yesuf SA, Yezli S, Yi S, Yigezu A, Yiğit A, Yiğit V,

Yip P, Yismaw MB, Yismaw Y, Yon DK, Yonemoto N, Yoon S-J, You Y, Younis MZ, Yousefi Z, Yu C, Yu Y, Yuh FH, Zadey S, Zadnik V, Zafari N, Zakham F, Zaki N, Zaman SB, Zamora N, Zand R, Zangiabadian M, Zar HJ, Zare I, Zarrintan A, Zeariya MGM, Zeinali Z, Zhang H, Zhang J, Zhang J, Zhang L, Zhang Y, Zhang Z-J, Zhao H, Zhong C, Zhou J, Zhu B, Zhu L, Ziafati M, Zielińska M, Zitoun OA, Zoladl M, Zou Z, Zuhlke LJ, Zumla A, Zweck E, Zyoud SH, Wool EE, Murray CJL. Global burden of 288 causes of death and life expectancy decomposition in 204 countries and territories and 811 subnational locations, 1990–2021: a systematic analysis for the Global Burden of Disease Study 2021. The Lancet. 2024;403(10440):2100-32.

2. Wang W, Hu M, Liu H, Zhang X, Li H, Zhou F, Liu Y-M, Lei F, Qin J-J, Zhao Y-C, Chen Z, Liu W, Song X, Huang X, Zhu L, Ji Y-X, Zhang P, Zhang X-J, She Z-G, Yang J, Yang H, Cai J, Li H. Global Burden of Disease Study 2019 suggests that metabolic risk factors are the leading drivers of the burden of ischemic heart disease. Cell Metabolism. 2021;33(10):1943-56.e2.

3. Wang Y, Li Q, Bi L, Wang B, Lv T, Zhang P. Global trends in the burden of ischemic heart disease based on the global burden of disease study 2021: the role of metabolic risk factors. BMC Public Health. 2025;25(1):310.

4. Timmis A, Aboyans V, Vardas P, Townsend N, Torbica A, Kavousi M, Boriani G, Huculeci R, Kazakiewicz D, Scherr D, Karagiannidis E, Cvijic M, Kapłon-Cieślicka A, Ignatiuk B, Raatikainen P, De Smedt D, Wood A, Dudek D, Van Belle E, Weidinger F, Societies ENC. European Society of Cardiology: the 2023 Atlas of Cardiovascular Disease Statistics. European heart journal. 2024;45(38):4019-62.

5. Heusch G, Gersh BJ. The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. European heart journal. 2016;38(11):774-84.

6. Heusch G. Myocardial ischaemia-reperfusion injury and cardioprotection in perspective. Nat Rev Cardiol. 2020;17(12):773-89.

7. Frangogiannis NG. Pathophysiology of Myocardial Infarction. Comprehensive Physiology. 2015;5(4):1841-75.

8. Sutton MGSJ, Sharpe N. Left Ventricular Remodeling After Myocardial Infarction. Circulation. 2000;101(25):2981-8.

9. Frantz S, Hundertmark MJ, Schulz-Menger J, Bengel FM, Bauersachs J. Left ventricular remodelling post-myocardial infarction: pathophysiology, imaging, and novel therapies. European heart journal. 2022;43(27):2549-61.

10. Byrne RA, Rossello X, Coughlan JJ, Barbato E, Berry C, Chieffo A, Claeys MJ, Dan G-A, Dweck MR, Galbraith M, Gilard M, Hinterbuchner L, Jankowska EA, Jüni P, Kimura T, Kunadian V, Leosdottir M, Lorusso R, Pedretti RFE, Rigopoulos AG, Rubini Gimenez M, Thiele H, Vranckx P, Wassmann S, Wenger NK, Ibanez B, Group ESD. 2023 ESC Guidelines for the management of acute coronary syndromes: Developed by the task force on the management of acute coronary syndromes of the European Society of Cardiology (ESC). European heart journal. 2023;44(38):3720-826.

11. Neumann F-J, Sousa-Uva M, Ahlsson A, Alfonso F, Banning AP, Benedetto U, Byrne RA, Collet J-P, Falk V, Head SJ, Jüni P, Kastrati A, Koller A, Kristensen SD, Niebauer J, Richter DJ, Seferović PM, Sibbing D, Stefanini GG, Windecker S, Yadav R, Zembala MO, Group ESD. 2018 ESC/EACTS Guidelines on myocardial revascularization. European heart journal. 2018;40(2):87-165.

12. Doenst T, Haverich A, Serruys P, Bonow RO, Kappetein P, Falk V, Velazquez E, Diegeler A, Sigusch H. PCI and CABG for Treating Stable Coronary Artery Disease. JACC. 2019;73(8):964-76.

13. Scheldeman L, Sinnaeve P, Albers GW, Lemmens R, Van de Werf F. Acute myocardial infarction and ischaemic stroke: differences and similarities in reperfusion therapies-a review. European heart journal. 2024;45(30):2735-47.

14. Capodanno D, Angiolillo DJ. Personalised antiplatelet therapies for coronary artery disease: what the future holds. European heart journal. 2023;44(32):3059-72.

15. Pavasini R, Biscaglia S, Kunadian V, Hakeem A, Campo G. Coronary artery disease management in older adults: revascularization and exercise training. European heart journal. 2024;45(31):2811-23.

16. Barzegar M, Kaur G, Gavins FNE, Wang Y, Boyer CJ, Alexander JS. Potential therapeutic roles of stem cells in ischemia-reperfusion injury. Stem Cell Research. 2019;37:101421.

17. Spath NB, Mills NL, Cruden NL. Novel cardioprotective and regenerative therapies in acute myocardial infarction: a review of recent and ongoing clinical trials. Future Cardiol. 2016;12(6):655-72.

18. Galli M, Niccoli G, De Maria G, Brugaletta S, Montone RA, Vergallo R, Benenati S, Magnani G, D'Amario D, Porto I, Burzotta F, Abbate A, Angiolillo DJ, Crea F. Coronary microvascular obstruction and dysfunction in patients with acute myocardial infarction. Nat Rev Cardiol. 2024;21(5):283-98.
19. Hausenloy DJ, Yellon DM. The therapeutic potential of ischemic conditioning: an update. Nat Rev Cardiol. 2011;8(11):619-29.
20. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986;74(5):1124-36.
21. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. American journal of physiology Heart and circulatory physiology. 2003;285(2):H579-88.
22. Ferdinandy P, Andreadou I, Baxter GF, Bøtker HE, Davidson SM, Dobrev D, Gersh BJ, Heusch G, Lecour S, Ruiz-Meana M, Zuurbier CJ, Hausenloy DJ, Schulz R. Interaction of Cardiovascular Nonmodifiable Risk Factors, Comorbidities and Comedications With Ischemia/Reperfusion Injury and Cardioprotection by Pharmacological Treatments and Ischemic Conditioning. Pharmacological reviews. 2023;75(1):159-216.
23. Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, Aupetit J-F, Bonnefoy E, Finet G, André-Fouët X, Ovize M. Postconditioning the Human Heart. Circulation. 2005;112(14):2143-8.
24. Thibault H, Piot C, Staat P, Bontemps L, Sportouch C, Rioufol G, Cung TT, Bonnefoy E, Angoulvant D, Aupetit J-F, Finet G, André-Fouët X, Macia JC, Raczka F, Rossi R, Itti R, Kirkorian G, Derumeaux G, Ovize M. Long-Term Benefit of Postconditioning. Circulation. 2008;117(8):1037-44.
25. Freixa X, Bellera N, Ortiz-Pérez JT, Jiménez M, Paré C, Bosch X, De Caralt TM, Betriu A, Masotti M. Ischaemic postconditioning revisited: lack of effects on infarct size following primary percutaneous coronary intervention. European heart journal. 2012;33(1):103-12.
26. Tarantini G, Favaretto E, Marra MP, Frigo AC, Napodano M, Cacciavillani L, Giovagnoni A, Renda P, De Biasio V, Plebani M, Mion M, Zaninotto M, Isabella G,

Bilato C, Iliceto S. Postconditioning during coronary angioplasty in acute myocardial infarction: the POST-AMI trial. Int J Cardiol. 2012;162(1):33-8.

27. Engstrøm T, Kelbæk H, Helqvist S, Høfsten DE, Kløvgaard L, Clemmensen P, Holmvang L, Jørgensen E, Pedersen F, Saunamaki K, Ravkilde J, Tilsted HH, Villadsen A, Aarøe J, Jensen SE, Raungaard B, Bøtker HE, Terkelsen CJ, Maeng M, Kaltoft A, Krusell LR, Jensen LO, Veien KT, Kofoed KF, Torp-Pedersen C, Kyhl K, Nepper-Christensen L, Treiman M, Vejlstrup N, Ahtarovski K, Lønborg J, Køber L. Effect of Ischemic Postconditioning During Primary Percutaneous Coronary Intervention for Patients With ST-Segment Elevation Myocardial Infarction: A Randomized Clinical Trial. JAMA Cardiol. 2017;2(5):490-7.

28. Sayour NV, Brenner GB, Makkos A, Kiss B, Kovácsházi C, Gergely TG, Aukrust SG, Tian H, Zenkl V, Gömöri K, Szabados T, Bencsik P, Heinen A, Schulz R, Baxter GF, Zuurbier CJ, Vokó Z, Ferdinandy P, Giricz Z. Cardioprotective efficacy of limb remote ischaemic preconditioning in rats: discrepancy between a meta-analysis and a three-centre in vivo study. Cardiovascular research. 2023;119(6):1336-51.

29. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation. 1993;87(3):893-9.

30. Crimi G, Pica S, Raineri C, Bramucci E, De Ferrari GM, Klersy C, Ferlini M, Marinoni B, Repetto A, Romeo M, Rosti V, Massa M, Raisaro A, Leonardi S, Rubartelli P, Oltrona Visconti L, Ferrario M. Remote ischemic post-conditioning of the lower limb during primary percutaneous coronary intervention safely reduces enzymatic infarct size in anterior myocardial infarction: a randomized controlled trial. JACC Cardiovasc Interv. 2013;6(10):1055-63.

31. White SK, Frohlich GM, Sado DM, Maestrini V, Fontana M, Treibel TA, Tehrani S, Flett AS, Meier P, Ariti C, Davies JR, Moon JC, Yellon DM, Hausenloy DJ. Remote Ischemic Conditioning Reduces Myocardial Infarct Size and Edema in Patients With ST-Segment Elevation Myocardial Infarction. JACC: Cardiovascular Interventions. 2015;8(1, Part B):178-88.

32. Prunier F, Angoulvant D, Saint Etienne C, Vermes E, Gilard M, Piot C, Roubille F, Elbaz M, Ovize M, Bière L, Jeanneteau J, Delépine S, Benard T, Abi-Khalil W, Furber A. The RIPOST-MI study, assessing remote ischemic perconditioning alone or in

combination with local ischemic postconditioning in ST-segment elevation myocardial infarction. Basic research in cardiology. 2014;109(2):400.

33. Yellon DM, Ackbarkhan AK, Balgobin V, Bulluck H, Deelchand A, Dhuny MR, Domah N, Gaoneadry D, Jagessur RK, Joonas N, Kowlessur S, Lutchoo J, Nicholas JM, Pauvaday K, Shamloll O, Walker JM, Hausenloy DJ. Remote Ischemic Conditioning Reduces Myocardial Infarct Size in STEMI Patients Treated by Thrombolysis. Journal of the American College of Cardiology. 2015;65(25):2764-5.

34. Bøtker HE, Kharbanda R, Schmidt MR, Bøttcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sørensen HT, Redington AN, Nielsen TT. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. Lancet. 2010;375(9716):727-34.

35. Hausenloy DJ, Kharbanda RK, Møller UK, Ramlall M, Aarøe J, Butler R, Bulluck H, Clayton T, Dana A, Dodd M, Engstrom T, Evans R, Lassen JF, Christensen EF, Garcia-Ruiz JM, Gorog DA, Hjort J, Houghton RF, Ibanez B, Knight R, Lippert FK, Lønborg JT, Maeng M, Milasinovic D, More R, Nicholas JM, Jensen LO, Perkins A, Radovanovic N, Rakhit RD, Ravkilde J, Ryding AD, Schmidt MR, Riddervold IS, Sørensen HT, Stankovic G, Varma M, Webb I, Terkelsen CJ, Greenwood JP, Yellon DM, Bøtker HE, Junker A, Kaltoft A, Madsen M, Christiansen EH, Jakobsen L, Carstensen S, Kristensen SD, Thim T, Pedersen KM, Korsgaard MT, Iversen A, Jørgensen E, Joshi F, Pedersen F, Tilsted HH, Alzuhairi K, Saunamäki K, Holmvang L, Ahlehof O, Sørensen R, Helqvist S, Mark BL, Villadsen AB, Raungaard B, Thuesen L, Christiansen MK, Freeman P, Jensen SE, Skov CS, Aziz A, Hansen HS, Ellert J, Veien K, Pedersen KE, Hansen KN, Ahlehoff O, Cappelen H, Wittrock D, Hansen PA, Ankersen JP, Hedegaard KW, Kempel J, Kaus H, Erntgaard D, Pedersen DM, Giebner M, Hansen TMH, Radosavljevic-Radovanovic M, Prodanovic M, Savic L, Pejic M, Matic D, Uscumlic A, Subotic I, Lasica R, Vukcevic V, Suárez A, Samaniego B, Morís C, Segovia E, Hernández E, Lozano I, Pascual I, Vegas-Valle JM, Rozado J, Rondán J, Avanzas P, del Valle R, Padrón R, García-Castro A, Arango A, Medina-Cameán AB, Fente AI, Muriel-Velasco A, Pomar-Amillo Á, Roza CL, Martínez-Fernández CM, Buelga-Díaz C, Fernández-Gonzalo D, Fernández E, Díaz-González E, Martinez-González E, Iglesias-Llaca F,

Viribay FM, Fernández-Mallo FJ, Hermosa FJ, Martínez-Bastida G, Goitia-Martín J, Vega-Fernández JL, Tresguerres JM, Rodil-Díaz JA, Villar-Fernández L, Alberdi L, Abella-Ovalle L, de la Roz M, Fernández-Carral MF-C, Naves MC, Peláez MC, Fuentes MD, García-Alonso M, Villanueva MJ, Vinagrero MS, Vázquez-Suárez M, Martínez-Valle M, Nonide M, Pozo-López M, Bernardo-Alba P, Galván-Núñez P, Martínez-Pérez PJ, Castro R, Suárez-Coto R, Suárez-Noriega R, Guinea R, Quintana RB, de Cima S, Hedrera SA, Laca SI, Llorente-Álvarez S, Pascual S, Cimas T, Mathur A, McFarlane-Henry E, Leonard G, Veerapen J, Westwood M, Colicchia M, Prossora M, Andiapen M, Mohiddin S, Lenzi V, Chong J, Francis R, Pine A, Jamieson-Leadbitter C, Neal D, Din J, McLeod J, Roberts J, Polokova K, Longman K, Penney L, Lakeman N, Wells N, Hopper O, Coward P, O'Kane P, Harkins R, Guyatt S, Kennard S, Orr S, Horler S, Morris S, Walvin T, Snow T, Cunnington M, Burd A, Gowing A, Krishnamurthy A, Harland C, Norfolk D, Johnstone D, Newman H, Reed H, O'Neill J, Greenwood J, Cuxton J, Corrigan J, Somers K, Anderson M, Burtonwood N, Bijsterveld P, Brogan R, Ryan T, Kodoth V, Khan A, Sebastian D, Gorog D, Boyle G, Shepherd L, Hamid M, Farag M, Spinthakis N, Waitrak P, De Sousa P, Bhatti R, Oliver V, Walshe S, Odedra T, Gue Y, Kanji R, Ryding A, Ratcliffe A, Merrick A, Horwood C, Sarti C, Maart C, Moore D, Dockerty F, Baucutt K, Pitcher L, Ilsley M, Clarke M, Germon R, Gomes S, Clare T, Nair S, Staines J, Nicholson S, Watkinson O, Gallagher I, Nelthorpe F, Musselwhite J, Grosser K, Stimson L, Eaton M, Heppell R, Turney S, Horner V, Schumacher N, Moon A, Mota P, O'Donnell J, Panicker AS, Musa A, Tapp L, Krishnamoorthy S, Ansell V, Ali D, Hyndman S, Banerjee P, Been M, Mackenzie A, McGregor A, Hildick-Smith D, Champney F, Ingoldby F, Keate K, Bennett L, Skipper N, Gregory S, Harfield S, Mudd A, Wragg C, Barmby D, Grech E, Hall I, Middle J, Barker J, Fofie J, Gunn J, Housley K, Cockayne L, Weatherlley L, Theodorou N, Wheeldon N, Fati P, Storey RF, Richardson J, Iqbal, Javid, Adam Z, Brett S, Agyemang M, Tawiah C, Hogrefe K, Raju P, Braybrook C, Gracey J, Waldron M, Holloway R, Burunsuzoglu S, Sidgwick S, Hetherington S, Beirnes C, Fernandez O, Lazar N, Knighton A, Rai A, Hoare A, Webb I, Breeze J, Martin K, Andrews M, Patale S, Bennett A, Smallwood A, Radford E, Cotton J, Martins J, Wallace L, Milgate S, Munir S, Metherell S, Cottam V, Massey I, Copestick J, Delaney J, Wain J, Sandhu K, Emery L, Butler R, Hall C, Bucciarelli-Ducci C, Besana R, Hussein J, Bell S, Gill A, Bales E, Polwarth G, East C, Smith I, Oliveira J, Victor S, Woods S, Hoole S,

Ramos A, Sevillano A, Nicholson A, Solieri A, Redman E, Byrne J, Joyce J, Riches J, Davies J, Allen K, Saclot L, Ocampo M, Vertue M, Christmas N, Koothoor R, Gamma R, Alvares W, Pepper S, Kobson B, Reeve C, Malik I, Chester E, Saunders H, Mojela I, Smee J, Davies J, Davies N, Clifford P, Dias P, Kaur R, Moreira S, Ahmad Y, Tomlinson L, Pengelley C, Bidle A, Spence S, Al-Lamee R, Phuyal U, Abbass H, Bose T, Elliott R, Foundun A, Chung A, Freestone B, Lee DK, Elshiekh DM, Pulikal G, Bhatre G, Douglas J, Kaeng L, Pitt M, Watkins R, Gill S, Hartley A, Lucking A, Moreby B, Darby D, Corps E, Parsons G, De Mance G, Fahrai G, Turner J, Langrish J, Gaughran L, Wolyrum M, Azkhalil M, Bates R, Given R, Kharbanda R, Douthwaite R, Lloyd S, Neubauer S, Barker D, Dana A, Suttling A, Turner C, Smith C, Longbottom C, Ross D, Cunliffe D, Cox E, Whitehead H, Hudson K, Jones L, Drew M, Chant N, Haworth P, Capel R, Austin R, Howe S, Smith T, Hobson A, Strike P, Griffiths H, Anantharam B, Jack P, Thornton E, Hodgson A, Jennison A, McSkeane A, Smith B, Shaw C, Leathers C, Armstrong E, Carruthers G, Simpson H, Smith J, Hodierne J, Kelly J, Barclay J, Scott K, Gregson L, Buchanan L, McCormick L, Varma M, Kelsall N, McArthy R, Taylor R, Thompson R, Shelton R, Moore R, Tomlinson S, Thambi S, Cooper T, Oakes T, Deen Z, Relph C, prentice S, Hall L, Dillon A, Meadows D, Frank E, Markham-Jones H, Thomas I, Gale J, Denman J, Gale J, O'Connor J, Hindle J, Jackson-Lawrence K, Warner K, Lee K, Upton R, Elston R, Lee S, Venugopal V, Finch A, Fleming C, Whiteside C, Pemberton C, Wilkinson C, Sebastian D, Riedel E, Giuffrida G, Burnett G, Spickett H, Glen J, Brown J, Thornborough L, Pedley L, Morgan M, Waddington N, Brennan O, More R, Brady R, Preston S, Loder C, Vlad I, Laurence J, Smit A, Dimond K, Hayes M, Paddy L, Crause J, Amed N, Kaur-Babooa P, Rakhit R, Kotecha T, Fayed H, Francis R, Pavlidis A, Prendergast B, Clapp B, Perara D, Atkinson E, Ellis H, Wilson K, Gibson K, Smith M, Khawaja MZ, Sanchez-Vidal R, Redwood S, Jones S, Tipping A, Oommen A, Hendry C, Fath-Orboubadi DRF, Phillips H, Kolakaluri L, Sherwood M, Mackie S, Aleti S, Charles T, Roy L, Henderson R, Stables R, Redwood S, Marber M, Berry A, Redington A, Thygesen K, Andersen HR, Berry C, Copas A, Meade T, Kelbæk H, Bueno H, von Weitzel-Mudersbach P, Andersen G, Ludman A, Cruden N, Topic D, Mehmedbegovic Z, de la Hera Galarza JM, Robertson S, Van Dyck L, Chu R, Astarci J, Jamal Z, Hetherington D, Collier L. Effect of remote ischaemic conditioning on clinical outcomes

in patients with acute myocardial infarction (CONDI-2/ERIC-PPCI): a single-blind randomised controlled trial. The Lancet. 2019;394(10207):1415-24.

36. Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. J Clin Invest. 2005;115(5):1232-40.

37. Morris JL, Zaman AG, Smyllie JH, Cowan JC. Nitrates in myocardial infarction: influence on infarct size, reperfusion, and ventricular remodelling. British Heart Journal. 1995;73(4):310-9.

38. Siddiqi N, Neil C, Bruce M, MacLennan G, Cotton S, Papadopoulou S, Feelisch M, Bunce N, Lim PO, Hildick-Smith D, Horowitz J, Madhani M, Boon N, Dawson D, Kaski JC, Frenneaux M, investigators N, Siddiqi N, Neil C, Bruce M, MacLennan G, Cotton S, Dawson D, Frenneaux M, Singh S, Schwarz K, Jagpal B, Metcalfe M, Stewart A, Hannah A, Awsan N, Broadhurst P, Hogg D, Garg D, Slattery E, Davidson T, McDonald A, McPherson G, Kaski J-C, Lim PO, Brown S, Papadopoulou SA, Gonzalvez F, Roy D, Firoozi S, Bogle R, Roberts E, Rhodes J, Hildick-Smith D, de Belder A, Cooter N, Bennett L, Horowitz J, Rajendran S, Dautov R, Black M, Jansen E, Boon N, Struthers A, Toff W, Dargie H, Lang C, Nightingale P. Intravenous sodium nitrite in acute ST-elevation myocardial infarction: a randomized controlled trial (NIAMI). European heart journal. 2014;35(19):1255-62.

39. Janssens SP, Bogaert J, Zalewski J, Toth A, Adriaenssens T, Belmans A, Bennett J, Claus P, Desmet W, Dubois C, Goetschalckx K, Sinnaeve P, Vandenberghe K, Vermeersch P, Lux A, Szelid Z, Durak M, Lech P, Zmudka K, Pokreisz P, Vranckx P, Merkely B, Bloch KD, Van de Werf F, investigators N. Nitric oxide for inhalation in ST-elevation myocardial infarction (NOMI): a multicentre, double-blind, randomized controlled trial. European heart journal. 2018;39(29):2717-25.

40. Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J, Robert D, Ovize M. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. Journal of molecular and cellular cardiology. 2005;38(2):367-74.

41. Skyschally A, Schulz R, Heusch G. Cyclosporine A at reperfusion reduces infarct size in pigs. Cardiovasc Drugs Ther. 2010;24(1):85-7.

42. Hausenloy D, Kunst G, Boston-Griffiths E, Kolvekar S, Chaubey S, John L, Desai J, Yellon D. The effect of cyclosporin-A on peri-operative myocardial injury in adult patients undergoing coronary artery bypass graft surgery: a randomised controlled clinical trial. Heart. 2014;100(7):544-9.

43. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, André-Fouët X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med. 2008;359(5):473-81.

44. Cung T-T, Morel O, Cayla G, Rioufol G, Garcia-Dorado D, Angoulvant D, Bonnefoy-Cudraz E, Guérin P, Elbaz M, Delarche N, Coste P, Vanzetto G, Metge M, Aupetit J-F, Jouve B, Motreff P, Tron C, Labeque J-N, Steg PG, Cottin Y, Range G, Clerc J, Claeys MJ, Coussement P, Prunier F, Moulin F, Roth O, Belle L, Dubois P, Barragan P, Gilard M, Piot C, Colin P, Poli FD, Morice M-C, Ider O, Dubois-Randé J-L, Unterseeh T, Breton HL, Béard T, Blanchard D, Grollier G, Malquarti V, Staat P, Sudre A, Elmer E, Hansson MJ, Bergerot C, Boussaha I, Jossan C, Derumeaux G, Mewton N, Ovize M. Cyclosporine before PCI in Patients with Acute Myocardial Infarction. New England Journal of Medicine. 2015;373(11):1021-31.

45. Ottani F, Latini R, Staszewsky L, La Vecchia L, Locuratolo N, Sicuro M, Masson S, Barlera S, Milani V, Lombardi M, Costalunga A, Mollichelli N, Santarelli A, De Cesare N, Sganzerla P, Boi A, Maggioni AP, Limbruno U. Cyclosporine A in Reperfused Myocardial Infarction: The Multicenter, Controlled, Open-Label CYCLE Trial. Journal of the American College of Cardiology. 2016;67(4):365-74.

46. Wu Y, Liu H, Wang X. Cardioprotection of pharmacological postconditioning on myocardial ischemia/reperfusion injury. Life Sci. 2021;264:118628.

47. Heusch G. Critical Issues for the Translation of Cardioprotection. Circulation research. 2017;120(9):1477-86.

48. Buja LM. Pathobiology of Myocardial Ischemia and Reperfusion Injury: Models, Modes, Molecular Mechanisms, Modulation, and Clinical Applications. Cardiology in Review. 2023;31(5):252-64.

49. Bøtker HE, Hausenloy D, Andreadou I, Antonucci S, Boengler K, Davidson SM, Deshwal S, Devaux Y, Di Lisa F, Di Sante M, Efentakis P, Femminò S, García-Dorado

D, Giricz Z, Ibanez B, Iliodromitis E, Kaludercic N, Kleinbongard P, Neuhäuser M, Ovize M, Pagliaro P, Rahbek-Schmidt M, Ruiz-Meana M, Schlüter KD, Schulz R, Skyschally A, Wilder C, Yellon DM, Ferdinandy P, Heusch G. Practical guidelines for rigor and reproducibility in preclinical and clinical studies on cardioprotection. Basic research in cardiology. 2018;113(5):39.

50. Michael LH, Entman ML, Hartley CJ, Youker KA, Zhu J, Hall SR, Hawkins HK, Berens K, Ballantyne CM. Myocardial ischemia and reperfusion: a murine model. Am J Physiol. 1995;269(6 Pt 2):H2147-54.

51. Guo Y, Flaherty MP, Wu WJ, Tan W, Zhu X, Li Q, Bolli R. Genetic background, gender, age, body temperature, and arterial blood pH have a major impact on myocardial infarct size in the mouse and need to be carefully measured and/or taken into account: results of a comprehensive analysis of determinants of infarct size in 1,074 mice. Basic research in cardiology. 2012;107(5):288.

52. Heimburger RF. Injection into pericardial sac and ligation of coronary artery of the rat. Archives of Surgery. 1946;52(6):677-89.

53. Baker JE, Konorev EA, Gross GJ, Chilian WM, Jacob HJ. Resistance to myocardial ischemia in five rat strains: is there a genetic component of cardioprotection? American journal of physiology Heart and circulatory physiology. 2000;278(4):H1395-400.

54. Zhai P, Eurell TE, Cotthaus R, Jeffery EH, Bahr JM, Gross DR. Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats. American journal of physiology Heart and circulatory physiology. 2000;279(6):H2766-75.

55. Wang SQ, Chen TY, Wang L, Cheng HX, Li HB, He CQ, Fu CY, Wei Q. Cardiac rehabilitation in porcine models: Advances in therapeutic strategies for ischemic heart disease. Zool Res. 2025;46(3):576-607.

56. Baranyai T, Giricz Z, Varga ZV, Koncsos G, Lukovic D, Makkos A, Sárközy M, Pávó N, Jakab A, Czimbalmos C, Vágó H, Ruzsa Z, Tóth L, Garamvölgyi R, Merkely B, Schulz R, Gyöngyösi M, Ferdinandy P. In vivo MRI and ex vivo histological assessment of the cardioprotection induced by ischemic preconditioning, postconditioning and remote conditioning in a closed-chest porcine model of reperfused acute myocardial infarction: importance of microvasculature. Journal of translational medicine. 2017;15(1):67.

57. Lindsey ML, Bolli R, Canty JM, Du X-J, Frangogiannis NG, Frantz S, Gourdie RG, Holmes JW, Jones SP, Kloner RA, Lefer DJ, Liao R, Murphy E, Ping P, Przyklenk K, Recchia FA, Schwartz Longacre L, Ripplinger CM, Van Eyk JE, Heusch G. Guidelines for experimental models of myocardial ischemia and infarction. American Journal of Physiology-Heart and Circulatory Physiology. 2018;314(4):H812-H38.

58. Lecour S, Bøtker HE, Condorelli G, Davidson SM, Garcia-Dorado D, Engel FB, Ferdinandy P, Heusch G, Madonna R, Ovize M, Ruiz-Meana M, Schulz R, Sluijter JP, Van Laake LW, Yellon DM, Hausenloy DJ. ESC working group cellular biology of the heart: position paper: improving the preclinical assessment of novel cardioprotective therapies. Cardiovascular research. 2014;104(3):399-411.

59. Liu SJ. Characterization of functional capacity of adult ventricular myocytes in long-term culture. Int J Cardiol. 2013;168(3):1923-36.

60. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. Experimental Cell Research. 1976;98(2):367-81.

61. Claycomb WC, Lanson NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proceedings of the National Academy of Sciences. 1998;95(6):2979-84.

62. Davidson MM, Nesti C, Palenzuela L, Walker WF, Hernandez E, Protas L, Hirano M, Isaac ND. Novel cell lines derived from adult human ventricular cardiomyocytes. Journal of molecular and cellular cardiology. 2005;39(1):133-47.

63. Kuznetsov AV, Javadov S, Sickinger S, Frotschnig S, Grimm M. H9c2 and HL-1 cells demonstrate distinct features of energy metabolism, mitochondrial function and sensitivity to hypoxia-reoxygenation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2015;1853(2):276-84.

64. Branco AF, Pereira SP, Gonzalez S, Gusev O, Rizvanov AA, Oliveira PJ. Gene Expression Profiling of H9c2 Myoblast Differentiation towards a Cardiac-Like Phenotype. PLOS ONE. 2015;10(6):e0129303.

65. Pereira SL, Ramalho-Santos J, Branco AF, Sardão VA, Oliveira PJ, Carvalho RA. Metabolic Remodeling During H9c2 Myoblast Differentiation: Relevance for In Vitro Toxicity Studies. Cardiovascular Toxicology. 2011;11(2):180-90.

66. Pálóczi J, Szántai Á, Kobolák J, Bock I, Ruivo E, Kiss B, Gáspár R, Pipis J, Ocsovszki I, Táncos Z, Fehér A, Dinnyés A, Onódi Z, Madonna R, Ferdinandy P, Görbe A. Systematic analysis of different pluripotent stem cell-derived cardiac myocytes as potential testing model for cardiocytoprotection. Vascular pharmacology. 2020;133-134:106781.

67. Pálóczi J, Varga ZV, Apáti Á, Szebényi K, Sarkadi B, Madonna R, De Caterina R, Csont T, Eschenhagen T, Ferdinandy P, Görbe A. Exogenous Nitric Oxide Protects Human Embryonic Stem Cell-Derived Cardiomyocytes against Ischemia/Reperfusion Injury. Oxidative Medicine and Cellular Longevity. 2016;2016(1):4298945.

68. Wang L, Nguyen T, Rosa-Garrido M, Zhou Y, Cleveland DC, Zhang J. Comparative analysis of the cardiomyocyte differentiation potential of induced pluripotent stem cells reprogrammed from human atrial or ventricular fibroblasts. Front Bioeng Biotechnol. 2023;11:1108340.

69. Karakikes I, Ameen M, Termglinchan V, Wu JC. Human induced pluripotent stem cell-derived cardiomyocytes: insights into molecular, cellular, and functional phenotypes. Circulation research. 2015;117(1):80-8.

70. Robertson C, Tran DD, George SC. Concise Review: Maturation Phases of Human Pluripotent Stem Cell-Derived Cardiomyocytes. Stem Cells. 2013;31(5):829-37.

71. Portal L, Martin V, Assaly R, d'Anglemont de Tassigny A, Michineau S, Berdeaux A, Ghaleh B, Pons S. A Model of Hypoxia-Reoxygenation on Isolated Adult Mouse Cardiomyocytes:Characterization, Comparison With Ischemia-Reperfusion, and Application to the Cardioprotective Effect of Regular Treadmill Exercise. Journal of Cardiovascular Pharmacology and Therapeutics. 2013;18(4):367-75.

72. Caporali A, Anwar M, Devaux Y, Katare R, Martelli F, Srivastava PK, Pedrazzini T, Emanueli C. Non-coding RNAs as therapeutic targets and biomarkers in ischaemic heart disease. Nature Reviews Cardiology. 2024;21(8):556-73.

73. Shah AM, Giacca M. Small non-coding RNA therapeutics for cardiovascular disease. European heart journal. 2022;43(43):4548-61.

74. Seyhan AA. Trials and Tribulations of MicroRNA Therapeutics. International Journal of Molecular Sciences. 2024;25(3):1469.

75. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843-54.

76. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993;75(5):855-62.
77. Li SC, Chan WC, Hu LY, Lai CH, Hsu CN, Lin WC. Identification of homologous microRNAs in 56 animal genomes. Genomics. 2010;96(1):1-9.
78. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. Nature Reviews Drug Discovery. 2012;11(11):860-72.
79. Boon RA, Dimmeler S. MicroRNAs in myocardial infarction. Nature Reviews Cardiology. 2015;12(3):135-42.
80. Poller W, Dimmeler S, Heymans S, Zeller T, Haas J, Karakas M, Leistner DM, Jakob P, Nakagawa S, Blankenberg S, Engelhardt S, Thum T, Weber C, Meder B, Hajjar R, Landmesser U. Non-coding RNAs in cardiovascular diseases: diagnostic and therapeutic perspectives. European heart journal. 2018;39(29):2704-16.
81. Bauersachs J, Solomon SD, Anker SD, Antorrena-Miranda I, Batkai S, Viereck J, Rump S, Filippatos G, Granzer U, Ponikowski P, de Boer RA, Vardeny O, Hauke W, Thum T. Efficacy and safety of CDR132L in patients with reduced left ventricular ejection fraction after myocardial infarction: Rationale and design of the HF-REVERT trial. European Journal of Heart Failure. 2024;26(3):674-82.
82. Foinquinos A, Batkai S, Genschel C, Viereck J, Rump S, Gyöngyösi M, Traxler D, Riesenhuber M, Spannbauer A, Lukovic D, Weber N, Zlabinger K, Hašimbegović E, Winkler J, Fiedler J, Dangwal S, Fischer M, de la Roche J, Wojciechowski D, Kraft T, Garamvölgyi R, Neitzel S, Chatterjee S, Yin X, Bär C, Mayr M, Xiao K, Thum T. Preclinical development of a miR-132 inhibitor for heart failure treatment. Nature Communications. 2020;11(1):633.
83. Täubel J, Hauke W, Rump S, Viereck J, Batkai S, Poetzsch J, Rode L, Weigt H, Genschel C, Lorch U, Theek C, Levin AA, Bauersachs J, Solomon SD, Thum T. Novel antisense therapy targeting microRNA-132 in patients with heart failure: results of a first-in-human Phase 1b randomized, double-blind, placebo-controlled study. European heart journal. 2021;42(2):178-88.
84. Varga ZV, Zvara A, Faragó N, Kocsis GF, Pipicz M, Gáspár R, Bencsik P, Görbe A, Csonka C, Puskás LG, Thum T, Csont T, Ferdinandy P. MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning:

protectomiRs. American journal of physiology Heart and circulatory physiology. 2014;307(2):H216-27.

85. Li J, Rohailla S, Gelber N, Rutka J, Sabah N, Gladstone RA, Wei C, Hu P, Kharbanda RK, Redington AN. MicroRNA-144 is a circulating effector of remote ischemic preconditioning. Basic research in cardiology. 2014;109(5):423.

86. He B, Xiao J, Ren A-J, Zhang Y-F, Zhang H, Chen M, Xie B, Gao X-G, Wang Y-W. Role of miR-1 and miR-133a in myocardial ischemic postconditioning. Journal of Biomedical Science. 2011;18(1):22.

87. Duan X, Ji B, Wang X, Liu J, Zheng Z, Long C, Tang Y, Hu S. Expression of MicroRNA-1 and MicroRNA-21 in Different Protocols of Ischemic Conditioning in an Isolated Rat Heart Model. Cardiology. 2012;122(1):36-43.

88. Kim HW, Mallick F, Durrani S, Ashraf M, Jiang S, Haider KH. Concomitant activation of miR-107/PDCD10 and hypoxamir-210/Casp8ap2 and their role in cytoprotection during ischemic preconditioning of stem cells. Antioxidants & redox signaling. 2012;17(8):1053-65.

89. Onódi Z, Visnovitz T, Kiss B, Hambalkó S, Koncz A, Ágg B, Váradi B, Tóth V, Nagy RN, Gergely TG, Gergő D, Makkos A, Pelyhe C, Varga N, Reé D, Apáti Á, Leszek P, Kovács T, Nagy N, Ferdinandy P, Buzás EI, Görbe A, Giricz Z, Varga ZV. Systematic transcriptomic and phenotypic characterization of human and murine cardiac myocyte cell lines and primary cardiomyocytes reveals serious limitations and low resemblances to adult cardiac phenotype. Journal of molecular and cellular cardiology. 2022;165:19-30.

90. Varga ZV, Pipicz M, Baán JA, Baranyai T, Koncsos G, Leszek P, Kuśmierczyk M, Sánchez-Cabo F, García-Pavía P, Brenner GJ, Giricz Z, Csont T, Mendler L, Lara-Pezzi E, Pacher P, Ferdinandy P. Alternative Splicing of NOX4 in the Failing Human Heart. Frontiers in physiology. 2017;8:935.

91. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671-5.

92. Nagy RN, Makkos A, Baranyai T, Giricz Z, Szabó M, Kravcsenko-Kiss B, Bereczki Z, Ágg B, Puskás LG, Faragó N, Schulz R, Gyöngyösi M, Lukovic D, Varga ZV, Görbe A, Ferdinandy P. Cardioprotective microRNAs (protectomiRs) in a pig model

of acute myocardial infarction and cardioprotection by ischaemic conditioning: MiR-450a. British Journal of Pharmacology. 2025;182(2):396-416.

93. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res. 2019;47(D1):D155-d62.

94. Ágg B, Baranyai T, Makkos A, Vető B, Faragó N, Zvara Á, Giricz Z, Veres DV, Csermely P, Arányi T, Puskás LG, Varga ZV, Ferdinandy P. MicroRNA interactome analysis predicts post-transcriptional regulation of ADRB2 and PPP3R1 in the hypercholesterolemic myocardium. Scientific reports. 2018;8(1):10134.

95. Bencsik P, Kiss K, Ágg B, Baán JA, Ágoston G, Varga A, Gömöri K, Mendler L, Faragó N, Zvara Á, Sántha P, Puskás LG, Jancsó G, Ferdinandy P. Sensory Neuropathy Affects Cardiac miRNA Expression Network Targeting IGF-1, SLC2a-12, EIF-4e, and ULK-2 mRNAs. Int J Mol Sci. 2019;20(4).

96. Makkos A, Ágg B, Varga ZV, Giricz Z, Gyöngyösi M, Lukovic D, Schulz R, Barteková M, Görbe A, Ferdinandy P. Molecular Network Approach Reveals Rictor as a Central Target of Cardiac ProtectomiRs. Int J Mol Sci. 2021;22(17).

97. Heger J, Partsch S, Harjung C, Varga ZV, Baranyai T, Weiß J, Kremer L, Locquet F, Leszek P, Ágg B, Benczik B, Ferdinandy P, Schulz R, Euler G. YB-1 Is a Novel Target for the Inhibition of α-Adrenergic-Induced Hypertrophy. Int J Mol Sci. 2023;25(1).

98. Huang HY, Lin YC, Cui S, Huang Y, Tang Y, Xu J, Bao J, Li Y, Wen J, Zuo H, Wang W, Li J, Ni J, Ruan Y, Li L, Chen Y, Xie Y, Zhu Z, Cai X, Chen X, Yao L, Chen Y, Luo Y, LuXu S, Luo M, Chiu CM, Ma K, Zhu L, Cheng GJ, Bai C, Chiang YC, Wang L, Wei F, Lee TY, Huang HD. miRTarBase update 2022: an informative resource for experimentally validated miRNA-target interactions. Nucleic Acids Res. 2022;50(D1):D222-d30.

99. Liu W, Wang X. Prediction of functional microRNA targets by integrative modeling of microRNA binding and target expression data. Genome Biol. 2019;20(1):18.

100. Chen Y, Wang X. miRDB: an online database for prediction of functional microRNA targets. Nucleic Acids Res. 2020;48(D1):D127-d31.

101. McGeary SE, Lin KS, Shi CY, Pham TM, Bisaria N, Kelley GM, Bartel DP. The biochemical basis of microRNA targeting efficacy. Science. 2019;366(6472).

102. Thomas PD, Ebert D, Muruganujan A, Mushayahama T, Albou LP, Mi H. PANTHER: Making genome-scale phylogenetics accessible to all. Protein Sci. 2022;31(1):8-22.

103. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25-9.

104. Aleksander SA, Balhoff J, Carbon S, Cherry JM, Drabkin HJ, Ebert D, Feuermann M, Gaudet P, Harris NL, Hill DP, Lee R, Mi H, Moxon S, Mungall CJ, Muruganugan A, Mushayahama T, Sternberg PW, Thomas PD, Van Auken K, Ramsey J, Siegele DA, Chisholm RL, Fey P, Aspromonte MC, Nugnes MV, Quaglia F, Tosatto S, Giglio M, Nadendla S, Antonazzo G, Attrill H, Dos Santos G, Marygold S, Strelets V, Tabone CJ, Thurmond J, Zhou P, Ahmed SH, Asanitthong P, Luna Buitrago D, Erdol MN, Gage MC, Ali Kadhum M, Li KYC, Long M, Michalak A, Pesala A, Pritazahra A, Saverimuttu SCC, Su R, Thurlow KE, Lovering RC, Logie C, Oliferenko S, Blake J, Christie K, Corbani L, Dolan ME, Drabkin HJ, Hill DP, Ni L, Sitnikov D, Smith C, Cuzick A, Seager J, Cooper L, Elser J, Jaiswal P, Gupta P, Jaiswal P, Naithani S, Lera-Ramirez M, Rutherford K, Wood V, De Pons JL, Dwinell MR, Hayman GT, Kaldunski ML, Kwitek AE, Laulederkind SJF, Tutaj MA, Vedi M, Wang SJ, D'Eustachio P, Aimo L, Axelsen K, Bridge A, Hyka-Nouspikel N, Morgat A, Aleksander SA, Cherry JM, Engel SR, Karra K, Miyasato SR, Nash RS, Skrzypek MS, Weng S, Wong ED, Bakker E, Berardini TZ, Reiser L, Auchincloss A, Axelsen K, Argoud-Puy G, Blatter MC, Boutet E, Breuza L, Bridge A, Casals-Casas C, Coudert E, Estreicher A, Livia Famiglietti M, Feuermann M, Gos A, Gruaz-Gumowski N, Hulo C, Hyka-Nouspikel N, Jungo F, Le Mercier P, Lieberherr D, Masson P, Morgat A, Pedruzzi I, Pourcel L, Poux S, Rivoire C, Sundaram S, Bateman A, Bowler-Barnett E, Bye AJH, Denny P, Ignatchenko A, Ishtiaq R, Lock A, Lussi Y, Magrane M, Martin MJ, Orchard S, Raposo P, Speretta E, Tyagi N, Warner K, Zaru R, Diehl AD, Lee R, Chan J, Diamantakis S, Raciti D, Zarowiecki M, Fisher M, James-Zorn C, Ponferrada V, Zorn A, Ramachandran S, Ruzicka L, Westerfield M. The Gene Ontology knowledgebase in 2023. Genetics. 2023;224(1).

105. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
106. Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, Lane HC, Imamichi T, Chang W. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). Nucleic Acids Res. 2022;50(W1):W216-w21.
107. Han JJ. FDA Modernization Act 2.0 allows for alternatives to animal testing. Artificial Organs. 2023;47(3):449-50.
108. Aske KC, Waugh CA. Expanding the 3R principles: More rigour and transparency in research using animals. EMBO Rep. 2017;18(9):1490-2.
109. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, Ohman P, Frederich R, Wiviott SD, Hoffman EB, Cavender MA, Udell JA, Desai NR, Mosenzon O, McGuire DK, Ray KK, Leiter LA, Raz I. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. N Engl J Med. 2013;369(14):1317-26.
110. Ma Y, Wang J, Wang C, Zhang Q, Xu Y, Liu H, Xiang X, Ma J. DPP-4 inhibitor anagliptin protects against hypoxia-induced cytotoxicity in cardiac H9C2 cells. Artif Cells Nanomed Biotechnol. 2019;47(1):3823-31.
111. Pang L, Cai Y, Tang EH, Yan D, Kosuru R, Li H, Irwin MG, Ma H, Xia Z. Cox-2 Inhibition Protects against Hypoxia/Reoxygenation-Induced Cardiomyocyte Apoptosis via Akt-Dependent Enhancement of iNOS Expression. Oxid Med Cell Longev. 2016;2016:3453059.
112. Brenner GB, Makkos A, Nagy CT, Onódi Z, Sayour NV, Gergely TG, Kiss B, Görbe A, Sághy É, Zádori ZS, Lázár B, Baranyai T, Varga RS, Husti Z, Varró A, Tóthfalusi L, Schulz R, Baczkó I, Giricz Z, Ferdinandy P. Hidden Cardiotoxicity of Rofecoxib Can be Revealed in Experimental Models of Ischemia/Reperfusion. Cells. 2020;9(3).
113. Liehr T, Kankel S, Hardt KS, Buhl EM, Noels H, Keller DT, Schröder-Lange SK, Weiskirchen R. Genetic and Molecular Characterization of H9c2 Rat Myoblast Cell Line. Cells. 2025;14(7):502.
114. Leung GPH, Tse C-M, Man RYK. Characterization of adenosine transport in H9c2 cardiomyoblasts. International Journal of Cardiology. 2007;116(2):186-93.

115. Ménard C, Pupier S, Mornet D, Kitzmann M, Nargeot J, Lory P. Modulation of L-type Calcium Channel Expression during Retinoic Acid-induced Differentiation of H9C2 Cardiac Cells *. Journal of Biological Chemistry. 1999;274(41):29063-70.
116. Patten VA, Chabaesele I, Sishi B, Vuuren DV. Cardiomyocyte differentiation : experience and observations from 2 laboratories. SA Heart. 2017;14(2):96-107.
117. Campero-Basaldua C, Herrera-Gamboa J, Bernal-Ramírez J, Lopez-Moran S, Luévano-Martínez L-A, Alves-Figueiredo H, Guerrero G, García-Rivas G, Treviño V. The retinoic acid response is a minor component of the cardiac phenotype in H9c2 myoblast differentiation. BMC Genomics. 2023;24(1):431.
118. Alan L, Opletalova B, Hayat H, Markovic A, Hlavackova M, Vrbacky M, Mracek T, Alanova P. Mitochondrial metabolism and hypoxic signaling in differentiated human cardiomyocyte AC16 cell line. American Journal of Physiology-Cell Physiology. 2025;328(5):C1571-C85.
119. Niu N, Miao H, Ren H. Transcriptome Analysis of Myocardial Ischemic-Hypoxic Injury in Rats and Hypoxic H9C2 Cells. ESC Heart Fail. 2024;11(6):3775-95.
120. Sharma D, Karuna, Gupta H, Gupta A, Kumari M, Varshney R, Meena RC. Identification and validation of hypoxia-responsive signature pathways in human cardiomyocytes. 3 Biotech. 2025;15(4):103.
121. Eimre M, Paju K, Pelloux S, Beraud N, Roosimaa M, Kadaja L, Gruno M, Peet N, Orlova E, Remmelkoor R, Piirsoo A, Saks V, Seppet E. Distinct organization of energy metabolism in HL-1 cardiac cell line and cardiomyocytes. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2008;1777(6):514-24.
122. Saks V, Guzun R, Timohhina N, Tepp K, Varikmaa M, Monge C, Beraud N, Kaambre T, Kuznetsov A, Kadaja L, Eimre M, Seppet E. Structure-function relationships in feedback regulation of energy fluxes in vivo in health and disease: mitochondrial interactosome. Biochim Biophys Acta. 2010;1797(6-7):678-97.
123. Anmann T, Guzun R, Beraud N, Pelloux S, Kuznetsov AV, Kogerman L, Kaambre T, Sikk P, Paju K, Peet N, Seppet E, Ojeda C, Tourneur Y, Saks V. Different kinetics of the regulation of respiration in permeabilized cardiomyocytes and in HL-1 cardiac cells: Importance of cell structure/organization for respiration regulation. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2006;1757(12):1597-606.

124. Monge C, Beraud N, Tepp K, Pelloux S, Chahboun S, Kaambre T, Kadaja L, Roosimaa M, Piirsoo A, Tourneur Y, Kuznetsov AV, Saks V, Seppet E. Comparative analysis of the bioenergetics of adult cardiomyocytes and nonbeating HL-1 cells: respiratory chain activities, glycolytic enzyme profiles, and metabolic fluxesThis article is one of a selection of papers from the NATO Advanced Research Workshop on Translational Knowledge for Heart Health (published in part 2 of a 2-part Special Issue). Canadian Journal of Physiology and Pharmacology. 2009;87(4):318-26.

125. Musters RP, Post JA, Verkleij AJ. The isolated neonatal rat-cardiomyocyte used in an in vitro model for 'ischemia'. I. A morphological study. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1991;1091(3):270-7.

126. Yamashita N, Nishida M, Hoshida S, Kuzuya T, Hori M, Taniguchi N, Kamada T, Tada M. Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning. J Clin Invest. 1994;94(6):2193-9.

127. Siti HN, Jalil J, Asmadi AY, Kamisah Y. Rutin Modulates MAPK Pathway Differently from Quercetin in Angiotensin II-Induced H9c2 Cardiomyocyte Hypertrophy. Int J Mol Sci. 2021;22(10).

128. Zong J, Wu QQ, Zhou H, Zhang JY, Yuan Y, Bian ZY, Deng W, Dai J, Li FF, Xu M, Fang Y, Tang QZ. 3,3'-Diindolylmethane attenuates cardiac H9c2 cell hypertrophy through 5'-adenosine monophosphate-activated protein kinase-α. Mol Med Rep. 2015;12(1):1247-52.

129. Watkins SJ, Borthwick GM, Arthur HM. The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. In Vitro Cell Dev Biol Anim. 2011;47(2):125-31.

130. Alfarano C, Sartiani L, Nediani C, Mannucci E, Mugelli A, Cerbai E, Raimondi L. Functional coupling of angiotensin II type 1 receptor with insulin resistance of energy substrate uptakes in immortalized cardiomyocytes (HL-1 cells). Br J Pharmacol. 2008;153(5):907-14.

131. Ye S, Luo W, Khan ZA, Wu G, Xuan L, Shan P, Lin K, Chen T, Wang J, Hu X, Wang S, Huang W, Liang G. Celastrol Attenuates Angiotensin II-Induced Cardiac Remodeling by Targeting STAT3. Circulation research. 2020;126(8):1007-23.

132. Lim S, Lee ME, Jeong J, Lee J, Cho S, Seo M, Park S. sRAGE attenuates angiotensin II-induced cardiomyocyte hypertrophy by inhibiting RAGE-NFκB-NLRP3 activation. Inflamm Res. 2018;67(8):691-701.

133. Luna-Figueroa E, Bernal-Ramírez J, Vázquez-Garza E, Huerta-Arcos L, García-Rivas G, Contreras-Torres FF. Angiotensin II-Induced Hypertrophy in H9c2 Cells Reveals Severe Cytotoxicity of Graphene Oxide. ACS Omega. 2025;10(7):7327-37.

134. Denning C, Borgdorff V, Crutchley J, Firth KS, George V, Kalra S, Kondrashov A, Hoang MD, Mosqueira D, Patel A, Prodanov L, Rajamohan D, Skarnes WC, Smith JG, Young LE. Cardiomyocytes from human pluripotent stem cells: From laboratory curiosity to industrial biomedical platform. Biochim Biophys Acta. 2016;1863(7 Pt B):1728-48.

135. Tu C, Chao BS, Wu JC. Strategies for Improving the Maturity of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. Circulation research. 2018;123(5):512-4.

136. Lee Y-K, Ng K-M, Lai W-H, Chan Y-C, Lau Y-M, Lian Q, Tse H-F, Siu C-W. Calcium Homeostasis in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. Stem Cell Reviews and Reports. 2011;7(4):976-86.

137. Pavlovic BJ, Blake LE, Roux J, Chavarria C, Gilad Y. A Comparative Assessment of Human and Chimpanzee iPSC-derived Cardiomyocytes with Primary Heart Tissues. Scientific reports. 2018;8(1):15312.

138. Hidalgo A, Glass N, Ovchinnikov D, Yang SK, Zhang X, Mazzone S, Chen C, Wolvetang E, Cooper-White J. Modelling ischemia-reperfusion injury (IRI) in vitro using metabolically matured induced pluripotent stem cell-derived cardiomyocytes. APL Bioeng. 2018;2(2):026102.

139. Lundy SD, Zhu W-Z, Regnier M, Laflamme MA. Structural and Functional Maturation of Cardiomyocytes Derived from Human Pluripotent Stem Cells. Stem Cells and Development. 2013;22(14):1991-2002.

140. Kamakura T, Makiyama T, Sasaki K, Yoshida Y, Wuriyanghai Y, Chen J, Hattori T, Ohno S, Kita T, Horie M, Yamanaka S, Kimura T. Ultrastructural Maturation of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes in a Long-Term Culture. Circulation Journal. 2013;77(5):1307-14.

141. Horikoshi Y, Yan Y, Terashvili M, Wells C, Horikoshi H, Fujita S, Bosnjak ZJ, Bai X. Fatty Acid-Treated Induced Pluripotent Stem Cell-Derived Human Cardiomyocytes Exhibit Adult Cardiomyocyte-Like Energy Metabolism Phenotypes. Cells. 2019;8(9):1095.
142. Bekhite MM, González Delgado A, Menz F, Kretzschmar T, Wu JMF, Bekfani T, Nietzsche S, Wartenberg M, Westermann M, Greber B, Schulze PC. Longitudinal metabolic profiling of cardiomyocytes derived from human-induced pluripotent stem cells. Basic research in cardiology. 2020;115(4):37.
143. Huang CK, Kafert-Kasting S, Thum T. Preclinical and Clinical Development of Noncoding RNA Therapeutics for Cardiovascular Disease. Circulation research. 2020;126(5):663-78.
144. Chilukoti RK, Lendeckel J, Darm K, Bukowska A, Goette A, Sühling M, Utpatel K, Peters B, Homuth G, Völker U, Wolke C, Scharf C, Lendeckel U. Integration of "omics" techniques: Dronedarone affects cardiac remodeling in the infarction border zone. Experimental biology and medicine (Maywood, NJ). 2018;243(11):895-910.
145. Baars T, Skyschally A, Klein-Hitpass L, Cario E, Erbel R, Heusch G, Kleinbongard P. microRNA expression and its potential role in cardioprotection by ischemic postconditioning in pigs. Pflugers Archiv : European journal of physiology. 2014;466(10):1953-61.
146. Rane S, He M, Sayed D, Vashistha H, Malhotra A, Sadoshima J, Vatner DE, Vatner SF, Abdellatif M. Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. Circulation research. 2009;104(7):879-86.
147. Gorbe A, Giricz Z, Szunyog A, Csont T, Burley DS, Baxter GF, Ferdinandy P. Role of cGMP-PKG signaling in the protection of neonatal rat cardiac myocytes subjected to simulated ischemia/reoxygenation. Basic research in cardiology. 2010;105(5):643-50.
148. Makkos A, Szántai Á, Pálóczi J, Pipis J, Kiss B, Poggi P, Ferdinandy P, Chatgilialoglu A, Görbe A. A Comorbidity Model of Myocardial Ischemia/Reperfusion Injury and Hypercholesterolemia in Rat Cardiac Myocyte Cultures. Frontiers in physiology. 2019;10:1564.

149. Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. Journal of molecular and cellular cardiology. 2011;51(3):288-98.

150. Gadeberg HC, Bond RC, Kong CH, Chanoit GP, Ascione R, Cannell MB, James AF. Heterogeneity of T-Tubules in Pig Hearts. PLoS One. 2016;11(6):e0156862.

151. Voigt N, Pearman CM, Dobrev D, Dibb KM. Methods for isolating atrial cells from large mammals and humans. Journal of molecular and cellular cardiology. 2015;86:187-98.

152. Chouvarine P, Legchenko E, Geldner J, Riehle C, Hansmann G. Hypoxia drives cardiac miRNAs and inflammation in the right and left ventricle. Journal of molecular medicine (Berlin, Germany). 2019;97(10):1427-38.

153. Scrimgeour NR, Wrobel A, Pinho MJ, Høydal MA. microRNA-451a prevents activation of matrix metalloproteinases 2 and 9 in human cardiomyocytes during pathological stress stimulation. American journal of physiology Cell physiology. 2020;318(1):C94-c102.

154. Lv XW, He ZF, Zhu PP, Qin QY, Han YX, Xu TT. miR-451-3p alleviates myocardial ischemia/reperfusion injury by inhibiting MAP1LC3B-mediated autophagy. Inflamm Res. 2021;70(10-12):1089-100.

155. Wang X, Zhu H, Zhang X, Liu Y, Chen J, Medvedovic M, Li H, Weiss MJ, Ren X, Fan GC. Loss of the miR-144/451 cluster impairs ischaemic preconditioning-mediated cardioprotection by targeting Rac-1. Cardiovascular research. 2012;94(2):379-90.

156. Liu L, Chen Y, Shu J, Tang CE, Jiang Y, Luo F. Identification of microRNAs enriched in exosomes in human pericardial fluid of patients with atrial fibrillation based on bioinformatic analysis. J Thorac Dis. 2020;12(10):5617-27.

157. Liu L, Luo F, Lei K. Exosomes Containing LINC00636 Inhibit MAPK1 through the miR-450a-2-3p Overexpression in Human Pericardial Fluid and Improve Cardiac Fibrosis in Patients with Atrial Fibrillation. Mediators of Inflammation. 2021;2021:9960241.

158. Luo M, Weng Y, Tang J, Hu M, Liu Q, Jiang F, Yang D, Liu C, Zhan X, Song P, Bai H, Li B, Shi Q. MicroRNA-450a-3p represses cell proliferation and regulates embryo development by regulating Bub1 expression in mouse. PLoS One. 2012;7(10):e47914.

159. Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, Kaluza D, Tréguer K, Carmona G, Bonauer A, Horrevoets AJG, Didier N, Girmatsion Z, Biliczki P, Ehrlich JR, Katus HA, Müller OJ, Potente M, Zeiher AM, Hermeking H, Dimmeler S. MicroRNA-34a regulates cardiac ageing and function. Nature. 2013;495(7439):107-10.
160. Hua CC, Liu XM, Liang LR, Wang LF, Zhong JC. Targeting the microRNA-34a as a Novel Therapeutic Strategy for Cardiovascular Diseases. Front Cardiovasc Med. 2021;8:784044.
161. Wu Z, Cheng S, Wang S, Li W, Liu J. C-MYC ameliorates ventricular remodeling of myocardial infarction rats via binding to the promoter of microRNA-29a-3p to facilitate TET2 expression. Int J Cardiol. 2022.
162. Yang Y, Cheng HW, Qiu Y, Dupee D, Noonan M, Lin YD, Fisch S, Unno K, Sereti KI, Liao R. MicroRNA-34a Plays a Key Role in Cardiac Repair and Regeneration Following Myocardial Infarction. Circulation research. 2015;117(5):450-9.
163. Ye Y, Hu Z, Lin Y, Zhang C, Perez-Polo JR. Downregulation of microRNA-29 by antisense inhibitors and a PPAR-gamma agonist protects against myocardial ischaemia-reperfusion injury. Cardiovascular research. 2010;87(3):535-44.
164. Zhan L, Lei S, Li W, Zhang Y, Wang H, Shi Y, Tian Y. Suppression of microRNA-142-5p attenuates hypoxia-induced apoptosis through targeting SIRT7. Biomed Pharmacother. 2017;94:394-401.
165. Hao P, Cao X, Zhu Z, Gao C, Chen Y, Qi D. Effects of miR-181a targeting XIAP gene on apoptosis of cardiomyocytes induced by hypoxia/reoxygenation and its mechanism. J Cell Biochem. 2018.
166. Qi M, He L, Ma X, Li Z. MiR-181a-5p is involved in the cardiomyocytes apoptosis induced by hypoxia–reoxygenation through regulating SIRT1. Bioscience, Biotechnology, and Biochemistry. 2020;84(7):1353-61.
167. Du J, Yang ST, Liu J, Zhang KX, Leng JY. Silence of LncRNA GAS5 Protects Cardiomyocytes H9c2 against Hypoxic Injury via Sponging miR-142-5p. Mol Cells. 2019;42(5):397-405.
168. Ren L, Chen S, Liu W, Hou P, Sun W, Yan H. Downregulation of long non-coding RNA nuclear enriched abundant transcript 1 promotes cell proliferation and inhibits cell apoptosis by targeting miR-193a in myocardial ischemia/reperfusion injury. BMC Cardiovasc Disord. 2019;19(1):192.

169. Qiu R, Li W, Liu Y. MicroRNA-204 protects H9C2 cells against hypoxia/reoxygenation-induced injury through regulating SIRT1-mediated autophagy. Biomed Pharmacother. 2018;100:15-9.
170. Rong J, Pan H, He J, Zhang Y, Hu Y, Wang C, Fu Q, Fan W, Zou Q, Zhang L, Tang Y, Peng X, Wang P, Xiang Y, Peng J, Liu Z, Zheng Z. Long non-coding RNA KCNQ1OT1/microRNA-204-5p/LGALS3 axis regulates myocardial ischemia/reperfusion injury in mice. Cell Signal. 2020;66:109441.
171. Wang LZ, Xi JN, Liu TJ, Zhang ZY, Zhang P. MiR-204 reduces apoptosis in rats with myocardial infarction by targeting SIRT1/p53 signaling pathway. Eur Rev Med Pharmacol Sci. 2020;24(23):12306-14.
172. Saddic LA, Chang T-W, Sigurdsson MI, Heydarpour M, Raby BA, Shernan SK, Aranki SF, Body SC, Muehlschlegel JD. Integrated microRNA and mRNA responses to acute human left ventricular ischemia. Physiological Genomics. 2015;47(10):455-62.
173. Tzavlaki K, Moustakas A. TGF-β Signaling. Biomolecules. 2020;10(3).
174. Geering B. Death-associated protein kinase 2: Regulator of apoptosis, autophagy and inflammation. Int J Biochem Cell Biol. 2015;65:151-4.
175. Li S, Xiao FY, Shan PR, Su L, Chen DL, Ding JY, Wang ZQ. Overexpression of microRNA-133a inhibits ischemia-reperfusion-induced cardiomyocyte apoptosis by targeting DAPK2. J Hum Genet. 2015;60(11):709-16.
176. Chen CL, Zhang L, Jin Z, Kasumov T, Chen YR. Mitochondrial redox regulation and myocardial ischemia-reperfusion injury. American journal of physiology Cell physiology. 2022;322(1):C12-c23.
177. Poulsen M, Lukas C, Lukas J, Bekker-Jensen S, Mailand N. Human RNF169 is a negative regulator of the ubiquitin-dependent response to DNA double-strand breaks. J Cell Biol. 2012;197(2):189-99.
178. Vaz B, Popovic M, Newman JA, Fielden J, Aitkenhead H, Halder S, Singh AN, Vendrell I, Fischer R, Torrecilla I, Drobnitzky N, Freire R, Amor DJ, Lockhart PJ, Kessler BM, McKenna GW, Gileadi O, Ramadan K. Metalloprotease SPRTN/DVC1 Orchestrates Replication-Coupled DNA-Protein Crosslink Repair. Mol Cell. 2016;64(4):704-19.
179. Hou P, Yang K, Jia P, Liu L, Lin Y, Li Z, Li J, Chen S, Guo S, Pan J, Wu J, Peng H, Zeng W, Li C, Liu Y, Guo D. A novel selective autophagy receptor, CCDC50, delivers

K63 polyubiquitination-activated RIG-I/MDA5 for degradation during viral infection. Cell Res. 2021;31(1):62-79.

180. Li Y, Fu L, Li JB, Qin Y, Zeng TT, Zhou J, Zeng ZL, Chen J, Cao TT, Ban X, Qian C, Cai Z, Xie D, Huang P, Guan XY. Increased expression of EIF5A2, via hypoxia or gene amplification, contributes to metastasis and angiogenesis of esophageal squamous cell carcinoma. Gastroenterology. 2014;146(7):1701-13.e9.

181. Correia de Sousa M, Gjorgjieva M, Dolicka D, Sobolewski C, Foti M. Deciphering miRNAs' Action through miRNA Editing. Int J Mol Sci. 2019;20(24).

182. Sluijter JPG, Davidson SM, Boulanger CM, Buzás EI, de Kleijn DPV, Engel FB, Giricz Z, Hausenloy DJ, Kishore R, Lecour S, Leor J, Madonna R, Perrino C, Prunier F, Sahoo S, Schiffelers RM, Schulz R, Van Laake LW, Ytrehus K, Ferdinandy P. Extracellular vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology. Cardiovascular research. 2018;114(1):19-34.

183. Song XW, Li Q, Lin L, Wang XC, Li DF, Wang GK, Ren AJ, Wang YR, Qin YW, Yuan WJ, Jing Q. MicroRNAs are dynamically regulated in hypertrophic hearts, and miR-199a is essential for the maintenance of cell size in cardiomyocytes. J Cell Physiol. 2010;225(2):437-43.

184. van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(48):18255-60.

185. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007;316(5824):575-9.

186. Frangogiannis NG. Transforming growth factor-β in myocardial disease. Nat Rev Cardiol. 2022;19(7):435-55.

187. Teringova E, Tousek P. Apoptosis in ischemic heart disease. Journal of translational medicine. 2017;15(1):87.

188. King KR, Aguirre AD, Ye YX, Sun Y, Roh JD, Ng RP, Jr., Kohler RH, Arlauckas SP, Iwamoto Y, Savol A, Sadreyev RI, Kelly M, Fitzgibbons TP, Fitzgerald KA,

Mitchison T, Libby P, Nahrendorf M, Weissleder R. IRF3 and type I interferons fuel a fatal response to myocardial infarction. Nat Med. 2017;23(12):1481-7.

189. Lekmine F, Sassano A, Uddin S, Smith J, Majchrzak B, Brachmann SM, Hay N, Fish EN, Platanias LC. Interferon-gamma engages the p70 S6 kinase to regulate phosphorylation of the 40S S6 ribosomal protein. Exp Cell Res. 2004;295(1):173-82.

190. Walkowski B, Kleibert M, Majka M, Wojciechowska M. Insight into the Role of the PI3K/Akt Pathway in Ischemic Injury and Post-Infarct Left Ventricular Remodeling in Normal and Diabetic Heart. Cells. 2022;11(9).

191. Kaldirim M, Lang A, Pfeiler S, Fiegenbaum P, Kelm M, Bönner F, Gerdes N. Modulation of mTOR Signaling in Cardiovascular Disease to Target Acute and Chronic Inflammation. Front Cardiovasc Med. 2022;9:907348.

192. Borodzicz-Jażdżyk S, Jażdżyk P, Łysik W, Cudnoch-Jędrzejewska A, Czarzasta K. Sphingolipid metabolism and signaling in cardiovascular diseases. Front Cardiovasc Med. 2022;9:915961.

9. Bibliography of the candidate's publications

9.1. The publications of the candidate involved in the current thesis

1. **Nagy RN**, Makkos A, Baranyai T, Giricz Z, Szabó M, Kravcsenko-Kiss B, Bereczki Z, Ágg B, Puskás LG, Faragó N, Schulz R, Gyöngyösi M, Lukovic D, Varga ZV, Görbe A, Ferdinandy P. Cardioprotective microRNAs (protectomiRs) in a pig model of acute myocardial infarction and cardioprotection by ischaemic conditioning: MiR-450a. British Journal of Pharmacology. 2025;182(2):396-416.
2. Onódi Z, Visnovitz T, Kiss B, Hambalkó S, Koncz A, Ágg B, Váradi B, Tóth V, **Nagy RN**, Gergely TG, Gergő D, Makkos A, Pelyhe C, Varga N, Reé D, Apáti Á, Leszek P, Kovács T, Nagy N, Ferdinandy P, Buzás EI, Görbe A, Giricz Z, Varga ZV. Systematic transcriptomic and phenotypic characterization of human and murine cardiac myocyte cell lines and primary cardiomyocytes reveals serious limitations and low resemblances to adult cardiac phenotype. Journal of molecular and cellular cardiology. 2022;165:19-30.

9.2. The publications of the candidate not involved in the current thesis

1. Fodor E, **Nagy RN**, Nógrádi A, Toovey S, Kamal MA, Vadász P, Bencsik P, Görbe A, Ferdinandy P. An Observational Study on the Pharmacokinetics of Oseltamivir in Lactating Influenza Patients. Clin Pharmacol Ther. 2024;115(2):318-23.
2. Sayour NV, Tóth V, **Nagy RN**, Vörös I, Gergely TG, Onódi Z, Nagy N, Bödör C, Váradi B, Ruppert M, Radovits T, Bleckwedel F, Zelarayán LC, Pacher P, Ágg B, Görbe A, Ferdinandy P, Varga ZV. Droplet Digital PCR Is a Novel Screening Method Identifying Potential Cardiac G-Protein-Coupled Receptors as Candidate Pharmacological Targets in a Rat Model of Pressure-Overload-Induced Cardiac Dysfunction. Int J Mol Sci. 2023;24(18).
3. Gergely TG, Brenner GB, **Nagy RN**, Sayour NV, Makkos A, Kovácsházi C, Tian H, Schulz R, Giricz Z, Görbe A, Ferdinandy P. Effects of Bempedoic Acid in Acute Myocardial Infarction in Rats: No Cardioprotection and No Hidden Cardiotoxicity. Int J Mol Sci. 2023;24(2).

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