NOVEL STRATEGIES FOR CARDIOPROTECTION AGAINST MYOCARDIAL INFARCTION BY USING A GENETIC AND A MULTI-TARGET APPROACH

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Table of Contents

LIST OF PUBLICATION

Articles relevant to the thesis:

I. Heger J*, Szabados T**, Brosinsky P, Bencsik P, Ferdinandy P, Schulz R. Sex Difference in Cardioprotection against Acute Myocardial Infarction in MAO-B Knockout Mice In Vivo.
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II. Szabados T*, Makkos A*, Ágg B, Benczik B, Brenner GG, Szabó M, Váradi B, Vörös I, Gömöri K, Varga VZ, Görbe A, Bencsik P*, Ferdinandy P*. Pharmacokinetics and pharmacodynamics of intravenous miR-125b* mimic: cardioprotection in a mouse model of acute myocardial infarction.
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Articles not related to the thesis:

I. Subantimicrobial Dose Doxycycline Worsens Chronic Arthritis-Induced Bone Microarchitectural Alterations in a Mouse Model: Role of Matrix Metalloproteinases?

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IF.: 5.92, D1

V. Chronic treatment with rofecoxib but not ischemic preconditioning of the myocardium ameliorates early intestinal damage following cardiac ischemia/reperfusion injury in rats.

László SB, Lázár B, Brenner GB, Makkos A, Balogh M, Al-Khrasani M, Hutka B, MohammadzadehA, Kemény Á, László T, Scheich B, **Szabados T**, Kenyeres É, Giricz Z, Bencsik P, Varga ZV, Novák J, Helyes Z, Ferdinandy P, Gyires K, Zádori ZS., 2020 Aug;178:114099. doi: 10.1016/j.bcp.2020.114099. Epub 2020 Jun 12.PMID: 32540483, Biochemical Pharmacology

IF.: 5.86, Q1

VI. Cardioprotective Effect of Novel Matrix Metalloproteinase Inhibitors.

Gömöri K, **Szabados T**, Kenyeres É, Pipis J, Földesi I, Siska A, Dormán G, Ferdinandy P, Görbe A, Bencsik P.; 2020 Sep 23;21(19):6990. doi: 10.3390/ijms21196990.; PMID: 32977437, International Journal of Molecular Sciences
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VII. Matrix Metalloproteinases Repress Hypertrophic Growth in Cardiac Myocytes.

Euler G, Locquet F, Kociszewska J, Osygus Y, Heger J, Schreckenberg R, Schlüter KD, Kenyeres É, **Szabados T**, Bencsik P, Ferdinandy P, Schulz R., 2021 Apr;35(2):353-365. Epub 2021 Jan 5.; PMID: 33400052, Cardiovascular Drugs and Therapy

IF.: 3.95, Q1

VIII. Ca2+/calmodulin-dependent protein kinase II and protein kinase G oxidation contributes to impaired sarcomeric proteins in hypertrophy model.

Gömöri K, Herwig M, Budde H, Hassoun R, Mostafi N, Zhazykbayeva S, Sieme M, Modi S, **Szabados T**, Pipis J, Farkas-Morvay N, Leprán I, Ágoston G, Baczkó I, Kovács Á, Mügge A, Ferdinandy P, Görbe A, Bencsik P, Hamdani N. 2022 Aug;9 Epub 2022 May 18. PMID: 35584900; ESC Heart Failure

IF.: 3.8, Q1

IX. Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) Deletion but Not Inhibition of Extracellular PCSK9 Reduces Infarct Sizes Ex Vivo but Not In Vivo.

Schreckenberg R, Wolf A, **Szabados T**, Gömöri K, Szabó IA, Ágoston G, Brenner G, Bencsik P, Ferdinandy P, Schulz R, Schlüter KD. 2022 Jun 10, PMID: 35742954; International Journal of Molecular Sciences

IF.: 5.6, D1

X. Altered Cellular Protein Quality Control System Modulates Cardiomyocyte Function in Volume Overload-Induced Hypertrophy.

Gömöri K, Herwig M, Hassoun R, Budde H, Mostafi N, Delalat S, Modi S, Begovic M, **Szabados T**, Pipis J, Farkas-Morvay N, Leprán I, Kovács Á, Mügge A, Ferdinandy P, Görbe A, Bencsik P, Hamdani N.; Antioxidants (Basel). 2022 Nov 8;11(11):2210; PMID: 36358581

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XI. Facile preparation of nickel-poor layered double hydroxides from mechanochemically pretreated gibbsite with a variety of interlamellar anions and their use as catalyst precursors for CO2 hydrogenation.

Szabados M, **Szabados T**, Mucsi R, Sápi A, Kónya Z, Kukovecz Á, Pálinkó I, Sipos P; Volume 157, January 2023, 112010, Materials Research Bulletin

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XII. Directed thermocatalytic CO2 reduction over NiAl4 layered double hydroxide precursors − activity and selectivity control using different interlayer anions.

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IF.: 7.2, D1

XIII. Cardioprotective efficacy of limb remote ischemic preconditioning in rats: discrepancy between meta-analysis and a three-centre in vivo study.

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. 2023 Jan 31:cvad024. doi: 10.1093/cvr/cvad024. Online ahead of print. PMID: 36718529, Cardiovascular Research

IF.: 10.4, D1

XIV. Identification of New, Translatable ProtectomiRs against Myocardial Ischemia/Reperfusion Injury and Oxidative Stress: The Role of MMP/Biglycan Signaling Pathways.

Szabados T, Molnár A, Kenyeres É, Gömöri K, Pipis J, Pósa B, Makkos A, Ágg B, Giricz Z, Ferdinandy P, Görbe A, Bencsik P. 2024 May 30;13(6):674. doi: 10.3390/antiox13060674. PMID: 38929113, Antioxidants (Basel)

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XV. Inhibition of MMP2 activity mitigates N-omega-nitro-l-arginine-methyl ester (l-NAME)-induced right heart failure.

Schreckenberg R, Schulz R, Itani N, Ferdinandy P, Bencsik P, **Szabados T**, Rohrbach S, Niemann B, Schlüter KD. 2024 Oct;76:103308. doi: 10.1016/j.redox.2024.103308. Epub 2024 Aug 15.PMID: 39167912, Redox Biology

IF.: 11.9, D1

XVI. Effects of SZV-2649, a new multiple ion channel inhibitor mexiletine analogue.

Mohammed ASA, Naveed M, **Szabados T**, Szatmári I, Lőrinczi B, Mátyus P, Czompa A, Orvos P, Husti Z, Hornyik T, Topal L, Déri S, Jost N, Virág L, Bencsik P, Baczkó I, Varró A., 2024 Oct 5;14(1):23188. doi: 10.1038/s41598-024-73576-5. PMID: 39369049, Scientific Reports

IF.: 3.9, Q1

XVII. IMproving Preclinical Assessment of Cardioprotective Therapies (IMPACT): a small animal acute myocardial infarction randomized-controlled multicenter study on the effect of ischemic preconditioning.

Hernandez-Resendiz S, Vilskersts R, Aluja D, Andreadou I, Bencsik P, Dambrova M, Efentakis P, Gao F, Giricz Z, Inserte J, Kelly-Laubscher R, Kiss A, Krieg T, Kwak BR, Lecour S, Lopaschuk G, Mączewski M, Waszkiewicz M, Oknińska M, Pagliaro P, Podesser B, Prag HA, Ruiz-Meana M, **Szabados T**, Zuurbier CJ, Ferdinandy P, Hausenloy DJ. 2025 Apr;120(2):335-346. doi: 10.1007/s00395-025-01102-3. PMID: 40072549, Basic Research in Cardiology

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LIST OF ABBREVIATIONS

AMI: Acute Myocardial Infarction

AAR: Area at Risk

cTn: Cardiac Troponin

CVD: Cardiovascular Disease

ECG: Electrocardiogram

ELISA: Enzyme-Linked Immunosorbent Assay

FDR: False Discovery Rate

GO: Gene Ontology

IPostC: Ischemic Postconditioning

IPreC: Ischemic Preconditioning

I/R: Ischemia/Reperfusion

IS: Infarct Size

KO: Knockout

LAD: Left Anterior Descending

MAO: Monoamine Oxidase

MAO-B: Monoamine Oxidase B

miRNA or miR: microRNA

PBS: Phosphate-Buffered Saline

PCI: Percutaneous Coronary Intervention

PoC: Proof of Concept

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

RISC: RNA-Induced Silencing Complex

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

SEM: Standard Error of the Mean

STEMI: ST-Elevation Myocardial Infarction

TTC: 2,3,5-Triphenyltetrazolium Chloride

U6 snRNA: U6 Small Nuclear RNA

WT: Wild Type

ABSTRACT

Novel Strategies for Cardioprotection Against Myocardial Infarction by Using a Genetic and a Multi-Target Approach

Myocardial infarction (MI) remains a leading cause of mortality worldwide. This thesis explores two innovative cardioprotection strategies: one based on genetic modification i.e. the cardiomyocyte-specific knockout (KO) of monoamine oxidase (MAO)-B, and the other applying a multi-target approach by microRNA (miRNA) therapy using microRNA-125b*mimic. Both approaches aim to reduce myocardial infarct size and improve cardiac outcomes following ischemia/reperfusion (I/R) injury. To investigate the effect of MAO-B knockout and miR-125b* mimic on acute myocardial infarction, we set up a clinically relevant open-chest mouse model of 45 minutes coronary occlusion followed by either 120 minutes or 24 hours of reperfusion, and determined myocardial infarct size (IS) using a standard Evans blue/TTC double staining. In case of MAO-B KO mice we found lower mortality in females but IS was only reduced in males. Intravenous administration of miR-125b* mimic significantly reduced IS as compared to vehicle and control miRNA groups. Both MAO-B KO and microRNA-125b* therapies have shown promising cardioprotective strategies by reducing IS and attenuating myocardial I/R injury, highlighting the potential of gene-based and multi-target therapies in the treatment of acute myocardial infarction.

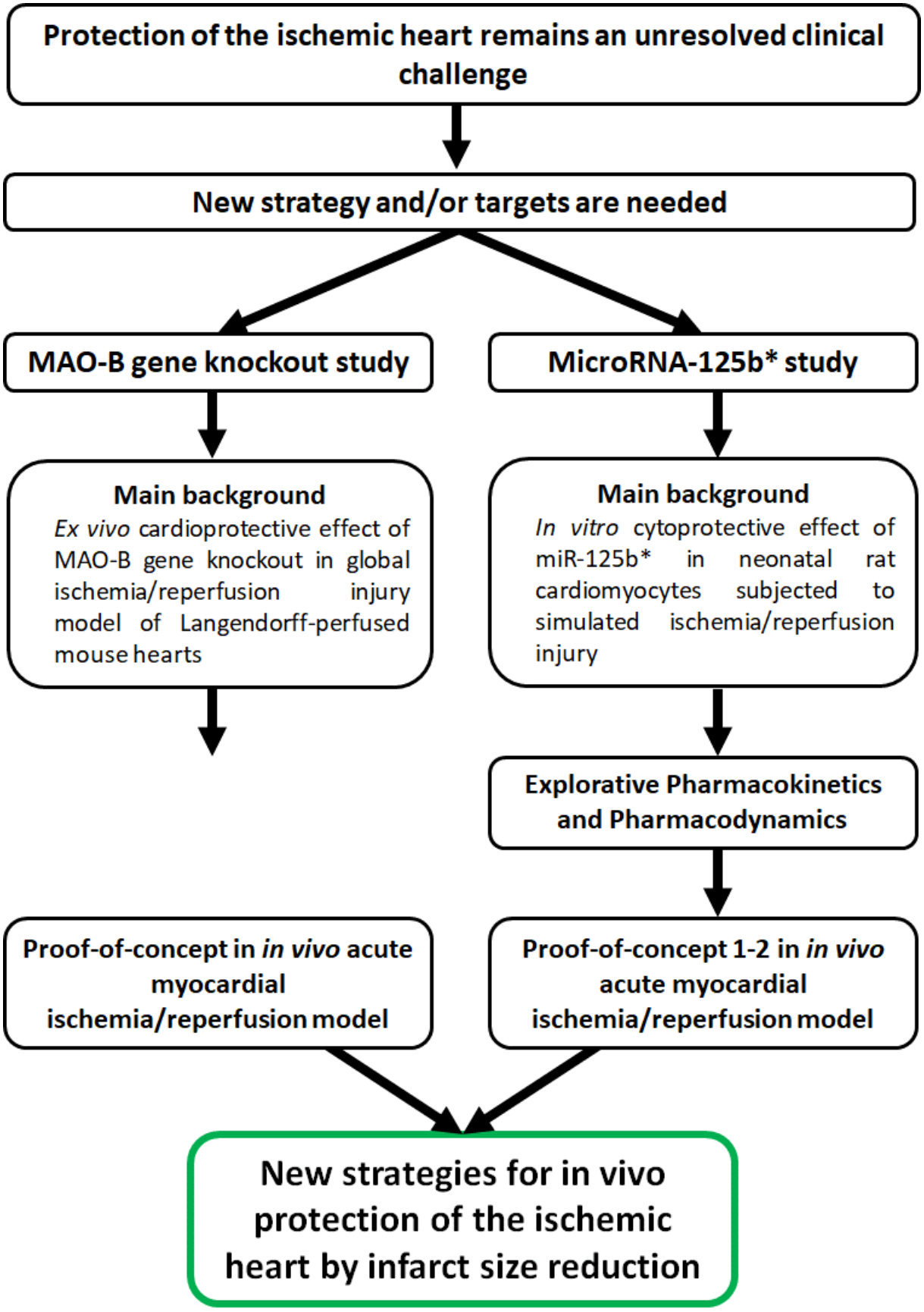

Figure 1. Graphical abstract of the content of the thesis.

INTRODUCTION

The history of recognition of myocardial infarction and ischemic damage as a disease entity

Acute myocardial infarction continues to be extensively researched, as cardiovascular diseases (CVD) are still the most common causes of mortality and morbidity (Nowbar et al., 2019; Timmis et al., 2022). Acute coronary syndrome is often the first clinical manifestation of CVD, which ultimately leads to a diagnosis of either acute myocardial infarction (AMI) or unstable angina (Byrne et al., 2023). To the best of our knowledge the first experimental infarction as coronary artery occlusion in a dog model was recorded in 1698 by a French physician Pierre Chirac, pioneer of experimental pathology of coronary vessels, who then observed that the dog's heart stopped beating in response to coronary artery ligation (Pierre, 1698). The earliest direct reference for the connection between a thrombus in the anterior descending coronary artery and myocardial infarction was described in 1859 by Malmsten, a Swedish researcher (Malmsten, 1859). In 1896, George Dock published a report on four autopsy cases of coronary occlusion due to thrombosis. One of those patients was diagnosed with myocardial infarction ante-mortem, and survived it for a week. The diagnosis was confirmed during the autopsy, and proven to have red thrombus in the left coronary artery (Dock, 1896). The development of electrocardiography by Willem Einthoven was met great success and became widespread in the early 20th century (Salam, 2019), which contributed to a growing number of researchers and clinicians to treat myocardial infarction as a disease that hold paramount significance instead of being a purely pathogenic condition. In 1925 Christian, H. A. et al. published the article entitled as "Coronary Thrombosis: An Easily Diagnosable Disease" (Christian, 1925). Despite advances in electrocardiographic understanding of the disease, predicting the prognosis of myocardial infarction, initially referred to as coronary thrombosis, remained challenging (Francis F. Rosenbaum, 1941). Interestingly, according to a diploma thesis by Joseph Frances Linsman, a physician at the University of Nebraska, in 1935, it was common knowledge that heart disease (including coronary thrombosis, heart failure, haemorrhage, arterial embolism and coronary artery disease) was the leading cause of death, and its mortality curve had been steadily rising for twenty years. Whether it was indeed the leading cause of death or not, it has generated an alarming amount of general interest among the laity and the medical doctors as well (Linsman, 1935).

Although growing body of literature began to accumulate, a definition and a proper nomenclature of heart diseases was lacking and needed to be defined clearly to avoid controversy and confusion. The first widely accepted definition of myocardial infarction was established by the working

groups of World Health Organization in the 1950-70s, which was primarily based on electrocardiography (ECG) and intended to use for epidemiological purposes (WHO, 1971). Based on the research of Peter R. Maroko and colleagues, a concept was proposed stating that the severity and the extent of myocardial ischemic injury following coronary occlusion depends on the balance between myocardial oxygen supply and demand (Maroko et al., 1971). This idea remains dominant in the present.

Despite the efforts of defining myocardial ischaemia, it seemed to remain an issue even at the end of the 20th century. In 1994 Hearse asked 33 cardiologists to define the term ischaemia, and the results confirmed the lack of a clear definition (Hearse, 1994). Gerd Heusch, a German physician took a critical view of the definition of oxygen supply and demand paradigm. In his opinion, myocardial oxygen supply can be measured, but the oxygen demand is only a virtual parameter, which cannot be measured, thus strong evidence of oxygen supply/demand imbalance cannot be demonstrated. He suggested that ischemia should simply be seen as reduced blood flow, and treatment should focus on improving the blood flow as much and as quickly as possible (Heusch, 2016).

The definition of myocardial infarction from an epidemiological perspective shifted to a more clinical point of view at the beginning of the 21st century, with the introduction of sensitive cardiac biomarkers. The current universal definition of myocardial injury and myocardial infarction are as follows: “The term of myocardial injury should be used when there is evidence of elevated cardiac troponin values (cTn) with at least one value above the 99th percentile upper reference limit (URL). The myocardial injury is considered acute if there is a rise and/or fall of cTn values. The term acute myocardial infarction should be used when there is acute myocardial injury with clinical evidence of acute myocardial ischaemia and with detection of a rise and/or fall of cTn values with at least one value above the 99th percentile URL and at least one of the following: symptoms of myocardial ischaemia; new ischaemic ECG changes; development of pathological Q waves; imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischaemic aetiology; identification of a coronary thrombus by angiography or autopsy” (Thygesen et al., 2019). Even this complex definition remains incomplete without additional context as it does not include all diagnostic criteria for each subtype (Types 1–5) of myocardial infarction. However, in preclinical research the definition of acute myocardial infarction is more freely interpreted.

Available treatments for acute myocardial infarction, challenges of cardioprotection strategies

For effective treatment of AMI, the primary goal is to alleviate ischemia and restore patency to the coronary vessels. Percutaneous coronary intervention (PCI) is the gold standard method in case of common type of MI, ST-Elevated Myocardial infarction (STEMI), but it is only effective within about 120 minutes time window from first medical contact. If timely PCI is not feasible, fibrinolysis with anticoagulation co-therapy should be initiated expeditiously (Byrne et al., 2023).

It is said that "time is muscle" during STEMI, meaning that prolonged ischemia leads to increased infarction and subsequent myocardial necrosis. Therefore, early reperfusion is critical and the only known way to effectively salvage the ischemic myocardium by limiting infarct size and subsequent ventricular remodelling (Mercuri et al., 2012). However, even with timely reperfusion the overall in-hospital mortality remained high (Menees et al., 2013), and in addition, more and more patients who survive the acute phase develop heart failure after MI (Heusch et al., 2014). The reasons might be complex, partly because of the increased number of patients with polypharmacy due to several cardiovascular co-morbidities (Nallamothu et al., 2015), or perhaps because reperfusion itself, which leads to irreversible damage to the myocardium and coronary circulation cannot be reduced beyond a certain limit due to the several confounding factors (Heusch & Gersh, 2017). This suggests that there is an utmost need for additional cardioprotective strategies beyond limiting the time of infarction. Cardioprotection has been defined as “all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage" (Kubler & Haass, 1996). Nowadays it refers to any intervention or agent that reduces infarct size following coronary occlusion and subsequent reperfusion. Various cardioprotective strategies (mechanical/non-pharmacological and pharmacological conditionings) were developed over the past decades to combat myocardial ischemia/reperfusion (I/R) injury resulting from AMI (Heusch & Gersh, 2017; Heusch et al., 2018). Ischemic conditioning techniques and many substances (extensively reviewed in (Heusch & Gersh, 2017)) have been shown to effectively reduce infarct size in numerous preclinical studies, some agents are still promising but the translation to clinical setting has been disappointing (Heusch et al., 2018). The challenges of translating preclinical findings into clinical practice, along with possible reasons for failures, are discussed in several recent publications in detail (Ferdinandy et al., 2023; Heusch, 2017; Heusch et al., 2018; Martin et al., 2022). The difficulty in preventing myocardial reperfusion injury may be largely due to an incomplete understanding of the underlying mechanisms. Nevertheless, since there are no available treatments on the market that specifically protects the ischemic heart, the development

of a new and effective therapy is of high importance, for which appropriate preclinical animal models are essential.

Role of oxidative stress in I/R injury and monoamine oxidase as a therapeutic target in the treatment of AMI

Even though early reperfusion is the only known way to effectively salvage ischemic myocardium, the return of oxygenated blood—and thus molecular oxygen—into the ischemic tissue results in the production of superoxide and other reactive oxygen species (ROS). The production of ROS at local low levels is a normal and important physiological phenomenon, maintaining cellular redox homeostasis and regulating transcription factors (Checa & Aran, 2020). However, the imbalance between pro-oxidants and antioxidants in favor of oxidants, termed as oxidative stress, is thought to have indirect detrimental effects through cell signaling dysfunction and modulation, as well as directly damaging nucleic acids, proteins, lipids, and carbohydrates, potentially leading to severe consequences in each living cell and biomolecule (Kalogeris et al., 2012). The extent and the role of each process of oxidative stress in the development of I/R injury is not yet clearly defined. There is a consensus that a significant portion of cell death resulting from myocardial reperfusion injury occurs during the initial minutes of reperfusion, which mainly occurring within the cardiomyocytes themselves (Hausenloy et al., 2017). In cardiomyocytes, mitochondria are the most prominent sites of ROS formation (Murphy, 2009) and are responsible for the majority of I/R-induced ROS production, mainly via the electron transport chain (Perrelli et al., 2011) and several enzymes. Mitochondrial ROS are also produced by monoamine oxidases (MAOs). According to the study by Anderson et al., MAO-induced ROS production measured in the atrial myocardium is 10-fold higher than that from the mitochondrial respiratory chain, highlighting MAO activity as a key determinant of myocardial redox balance (Anderson et al., 2014).

Role of the Monoamine oxidases in oxidative stress and I/R injury

Monoamine oxidases (MAO-A and MAO-B) are flavoproteins located in the outer membrane of mitochondria and are involved in the oxidative deamination of neurotransmitters and other biogenic amines (e.g. dietary tyramines), and produce hydrogen peroxide (H_2O_2), aldehydes and ammonia as necessary by-products. Both MAO-A and MAO-B are expressed in human myocardial tissue, and also in rodent hearts, however, MAO-A is more prevalent in rats, while MAO-B is more dominant in mice (Bugger & Pfeil, 2020). Although MAOs have clinically been associated with neurodegenerative diseases, they are also involved in the development of oxidative stress during myocardial I/R injury. One of the first direct evidence that oxidative stress is induced by MAO enzymes was provided by Bianchi and colleagues. They showed that administration of

serotonin (5-hydroxytryptamine [5-HT]) induced intracellular oxidative stress and cell apoptosis *in vitro* in adult rat cardiomyocytes, was prevented by the administration of pargyline, a relatively selective MAO-B inhibitor. They also demonstrated an *in vivo* cardioprotective effect of pargyline and clorgyline following acute myocardial infarction in rats. The administered MAO inhibitors reduced infarct size, myeloperoxidase activity (an indicator for inflammation), and the production of malondialdehyde (a marker for oxidative stress) (Bianchi et al., 2005). Under physiological conditions, the H_2O_2 by-product of MAOs is removed by endogenous scavengers. However, during I/R injury, antioxidant defence fails, and oxidative stress is further exaggerated by increased MAO activity likely due to the accumulation and higher availability of its substrates. This can lead to enhanced formation and accumulation of H_2O_2, contributing to oxidative stress (Deshwal et al., 2017). An Italian research group identified N-methylhistamine (the main metabolite of histamine) as a substrate of MAO, which is released mainly from synaptic terminals during I/R, and elicits cardiac MAO-induced ROS production (Costiniti et al., 2018). Most studies investigating the role of MAOs in cardiovascular diseases are focusing on MAO-A, but less is known about the role of MAO-B. The research group of Rainer Schulz in Germany investigated the effect of cardiomyocyte-specific deletion of MAO-B in an *ex vivo* global I/R model of Langendorff-perfused mouse hearts. They demonstrated that the absence of cardiomyocyte-specific MAO-B results in reduced infarct size, indicating an important contribution of MAO-B activity to acute reperfusion injury (Heger et al., 2021). However, the effect of cardiomyocyte-specific MAO-B deletion on infarct size in an *in vivo* model of acute myocardial infarction has never been investigated before.

MicroRNAs and their role as a therapeutic tool

The failure to translate preclinical results into clinical applications for the treatment of acute myocardial infarction, a multifactorial disease, is thought to be due to its complex origins. Researchers and clinicians suggest that triggering cardioprotection may require combining multitarget therapies (Davidson et al., 2019). To address multiple disease-related pathways simultaneously, microRNA-based RNA therapeutics offer a novel and promising approach. The first microRNA (miRNA, miR), lin-4, was identified in 1993 by the laboratories of Victor Ambros and Gary Ruvkun (Lee et al., 1993; Wightman et al., 1993). This discovery revealed a novel cellular post-transcriptional gene regulatory mechanism mediated by a non-protein-coding RNA. Their groundbreaking work was awarded the Nobel Prize in Physiology or Medicine in 2024. Since their discovery, the number of the identified miRNAs has been continuously increasing and

they proved to be highly conserved in the animal kingdom. Currently, in miRBAse (a microRNA database), more than 1,900 human miRNA sequences and annotations are available (miRBase).

MiRNAs are short, 18–25 nucleotide-long non-coding RNA sequences. The therapeutic application of miRNAs involves two primary strategies: silencing or enhancing endogenous miRNAs. MiRNA antagonists (antagomiRs) are engineered to be complementary to specific endogenous miRNAs, with the goal of inhibiting their activity (Bader et al., 2010). In contrast, miRNA mimics are synthetic double-stranded sequences designed to replicate the natural endogenous miRNAs, thereby restoring the function of downregulated or lost miRNA expression patterns and enhancing their regulatory roles (Cha et al., 2018). MiRNA mimics consist of a guide strand possessing the same sequence as its endogenous counterpart, and a not fully complementary passenger strand with 2-nt long 3′ overhang (Deleavey & Damha, 2012). They work similarly to the endogenous miRNAs by incorporating into the RNA-induced silencing complex (RISC), guiding the complex to complementary target mRNAs. The target mRNA is regulated either by degradation or translational inhibition (Bartel, 2009). MiRNA mimics are pleiotropic regulators, they can simultaneously target multiple genes (Krek et al., 2005), and a single mRNA can be regulated by multiple miRNAs, creating a complex regulatory network (Wilczynska & Bushell, 2015).

Role of microRNAs in cardiovascular system and diseases

MiRNAs are key regulators of numerous biological processes, including embryonic development, cell proliferation and differentiation, programmed cell death (apoptosis), and tumor formation (Ambros, 2004; Calin & Croce, 2006). In the cardiovascular system, miRNAs play a role in regulating cardiomyocyte growth and function, maintaining cardiac rhythm, modulating plaque formation, lipid metabolism, and promoting angiogenesis (Condorelli et al., 2014; Wagschal et al., 2015). MiRNAs are also involved in the regulation of oxidative stress and may contribute to cytoprotective mechanisms (Makkos et al., 2021). Altered expression of miRNAs has been linked to various diseases and pathophysiological conditions. Beside oncology, the most extensively researched area (Croce, 2009) is the cardiovascular field, including hypercholesterolemia (Varga et al., 2013), atherosclerosis, cardiac remodelling (Peters et al., 2020) and ischaemia/reperfusion injury (Varga et al., 2014).

MiR-125b family and microRNA-125b* as therapeutic target

An increasing number of studies have demonstrated that the miR-125 family play a pivotal role in various cellular processes and are implicated in numerous diseases, including malignancies and

cardiovascular diseases. The miR-125 family is a phylogenetically highly conserved group of mammalian miRNAs. Its members share an identical seed sequence, suggesting that they may target overlapping sets of mRNAs. The miR-125 family comprises three homologues: hsa-miR-125a, hsa-miR-125b-1, and hsa-miR-125b-2 (Cattane et al., 2019; Sun et al., 2013). MiR-125b* (also referred to as miR-125b-1-3p or miR-125b-2-3p, depending on the species of origin) is the partially complementary antisense, passenger strand of the matured miR-125b-5p (Varga et al., 2018). Without the three-letter prefix which designate the species of origin, the miRNA name does not denote an exact miRNA, but rather a collective name for miRNAs of possibly related origin that are more or less identical or only slightly different in origin across several species. The sequence of the matured miRNA-125b-5p is slightly different in various species, for example in mouse and rat and human the sequences are as follows: ACAAGUCAGGUUCUUGGGACCU; ACAAGUCAGGCUCUUGGGACCU; ACGGGUUAGGCUCUUGGGAGCU, respectively (https://mirbase.org/).

Our research group previously found that I/R injury significantly reduced the expression of miR-125b* compared to time-matched non-ischemic controls in an *ex vivo* rat model of 30 min of coronary occlusion followed by 120 min of reperfusion. The miRNA pattern was determined with microarray analysis from left ventricular samples from the risk zone (Varga et al., 2014). The potential cardioprotective role of miR-125b* was suggested by comparing its expression levels between I/R group and groups subjected to either ischemic preconditioning (IPreC) or ischemic postconditioning (IPostC), both of which significantly reduced infarct size in the same model. Notably, the expression of miR-125b* was upregulated by both IPreC and IPostC, counteracting the downregulation induced by I/R injury. These interventions appeared to inhibit the I/R-induced suppression of miR-125b* (see Figure 2).

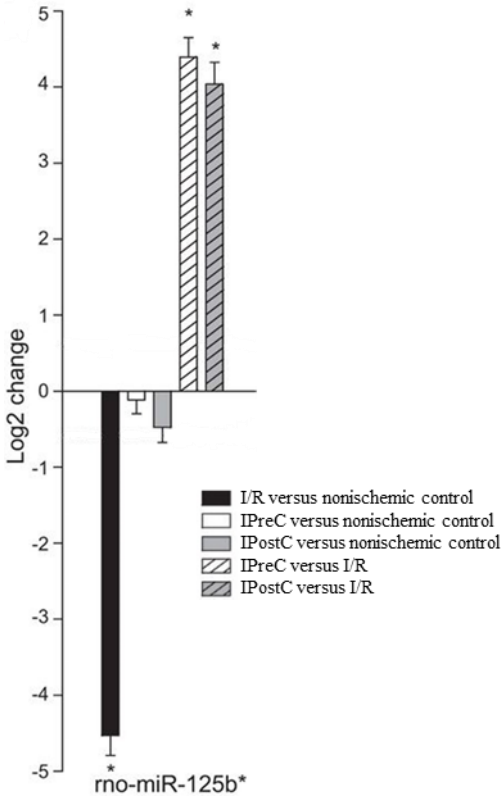

Figure 2. Altered expression of miR125b* by ischemia/reperfusion injury and ischemic pre- and postconditioning in a rat model of acute myocardial infarction. Adopted from Varga Z et al 2014 Am J Physiol Heart Circ Physiol.

In a subsequent step, the cytoprotective effect of miR-125b* was validated using a neonatal rat cardiac myocyte cell culture subjected to simulated I/R injury. Transfection of cardiac myocytes with miR-125b* enhanced the viability of these cells compared to cardiac myocytes transfected with cel-miR-67, which is commonly used as a negative control miRNA in the literature (Varga et al., 2014). Nevertheless, the cardioprotective effect of exogenous miR-125b* has not yet been investigated in an *in vivo* animal model of acute myocardial infarction.

OBJECTIVES

As there is no specific drug on the market approved for reducing infarct size, the development of a new therapeutic drug candidate protecting the heart against I/R injury is an unmet clinical need. Therefore, the aim of my doctoral work was to test two different cardioprotective strategies for infarct size reduction in *in vivo* rodent models. We conducted two parallel studies to test the following hypotheses:

1) to test the hypothesis that cardio-specific deletion of the MAO-B enzyme could lead to cardioprotection against myocardial ischemia/reperfusion injury using an *in vivo* cardiomyocyte-specific inducible MAO-B knockout mouse model. This research may support the potential repositioning of selegiline, a selective MAO-B inhibitor,- that is a safe and well- characterized drug currently available on the market for the treatment of Parkinson' disease.

2) to test the hypothesis that exogenous systemic administration of miR-125b* mimic could lead to cardioprotection by reducing myocardial infarct size in an *in vivo* mouse model of myocardial ischemia/reperfusion injury, and to characterize the pharmacokinetic and pharmacodynamic properties of this miRNA-based drug candidate.

MATERIALS AND METHODS

Ethical issues of animal studies

All our animal experiments were performed in accordance with the EU directive about the care and use of laboratory animals, published by the European Union (2010/63/EU). Methods were also approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority of Hungary), approval ID: XXVIII./171/2018.; on 24 January 2018) and permitted by the government (National Food Chain Safety Office and Animal Health Directorate of the Government Office for Pest County (PE/EA/1784-7/2017).

Animals

All mice were housed in individually ventilated cages (Sealsafe IVC system, Tecniplast S.p.a., Italy), which conform to the size recommendations of the Guide for the Care and Use of Laboratory Animals DHEW (NIH Publication No. 85–23, revised 1996) and EU Guidelines 63/2010. Cardiac-specific MAO-B-KO male and female mice were obtained from Justus Liebig University of Giessen in Germany, the C57BL/6 male mice were obtained from Charles River Laboratories. Animals were kept in a 12-h light/12-h dark cycle room with controlled temperature (22 ± 2 °C), while they had free access to filtered tap water and standard rodent chow (SAFE A40, Innovo Ltd., Isaszeg, Hungary) or standard rodent chow supplemented with 2% sucrose and 0.4 g/kg tamoxifen citrate (Altromin 1314P; MAO-B KO mice and their wild type littermates), without fasting before the experiments. The animals were acclimatized in the housing facility for at least 5 days prior to the start of the animal experiments. Litter placed beneath the mice was changed at least once a week.

Generation and genotyping the MAO-B KO mice

Cardiac-specific and tamoxifen-inducible double-transgenic MAO-B knockout mice (Myh6-MCreM_x_MAO-B$^{fl/fl}$) were generated in the Max Planck Institute for Heart and Lung Research in Bad Nauheim, Germany (for details see (Heger et al., 2021)). In brief, MAO-B targeting vector containing loxP-flanked MAO-B exon was electroporated into 129/B6 F1 hybrid embryonic stem cell line. Clones were injected into blastocysts of C57BL/6 mice and transferred into pseudopregnant C57Bl6/CBA F1 hybrid mice. The resulting chimeric mice were bred with an FLP (site specific recombinase)-deleter mouse strain creating an F1 generation that carries conditional mutant MAO-B. Then these mice were crossed with a mouse strain expressing a tamoxifen-inducible cardiac-specific CreERT2 gene controlled by Myh6-promotor (Myh6-MCreM). Cre recombinase-negative MAO-B$^{fl/fl}$ littermates were used as wild type. The genetic background of

the animals used in experiments were checked by PCR using ear biopsy. Heterozygous, homozygous and wild type mice were distinguished. In the experiments presented in the thesis, homozygous and negative control wild type mice were selected.

Summary of the protocols and experimental design of the two studies presented in the thesis

Cardiomyocyte specific MAO-B gene knockout study

Knockout of the MAO-B gene was induced by supplementing tamoxifen in rodent chow for two weeks, followed by a 10-week waiting period during which the animals were fed standard rodent chow. During these 10 weeks, cardiac MAO-B was degraded, while no new cardiac MAO-B was produced. After the 12-week pre-treatment period, myocardial ischemia/reperfusion injury was induced by 45 minutes of coronary occlusion and 120 minutes of reperfusion using the MAO-B knockout strain and its lean wild type controls. After the reperfusion period, infarct size was measured as the primary endpoint (Fig. 3).

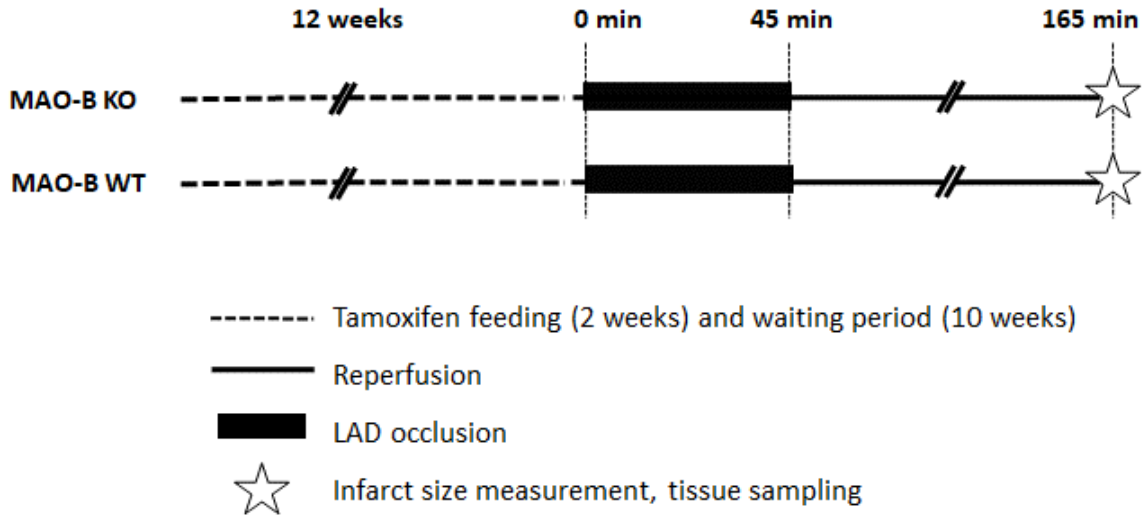

Figure 3. Protocol figure of MAO-B gene knockout study.

MiR-125b* study

The effect of exogenous microRNA-125b* mimic on infarct size (proof of concept; PoC-1 and -2) was measured in acute myocardial infarction model induced by 45-min coronary occlusion followed by 24-hour reperfusion using C57BL6 wild type mice (Fig. 4).

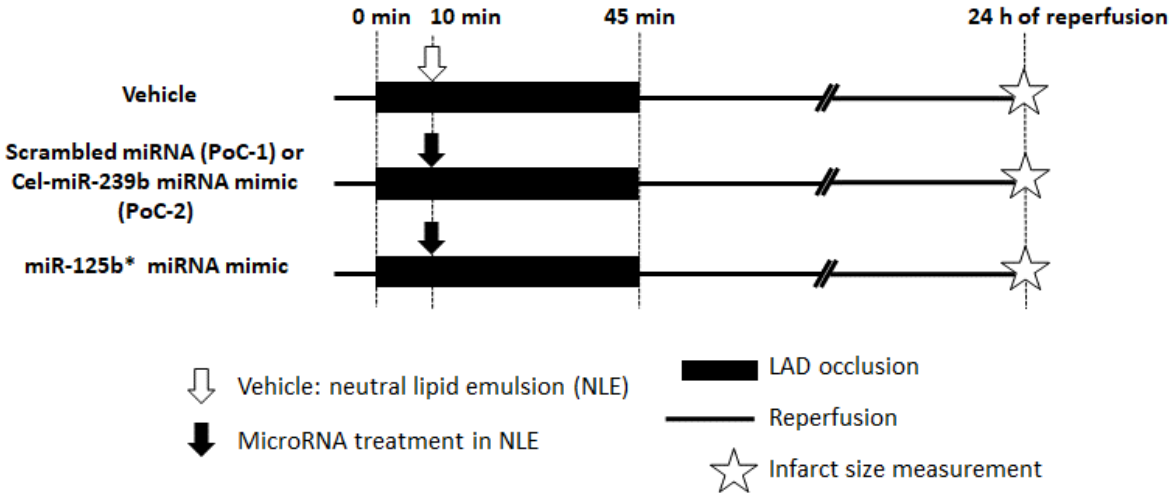

Figure 4. Protocol figure of PoC-1 and-2 of miR-125b* study.

Prior to the PoC study, pharmacokinetics and pharmacodynamics were carried out. Mice were treated either with 10 µg scrambled miRNA (randomly shuffled sequence of the miR-125b*) or 10 µg miR-125b* mimic or vehicle (neutral lipid emulsion). MiR-125b* expressions were measured in plasma and various tissue (myocardium, liver and kidney) samples, while the expression of selected targets of miR-125b* was measured from myocardium, at 1, 2, 4, 8 or 24 h after treatment (Fig. 5).

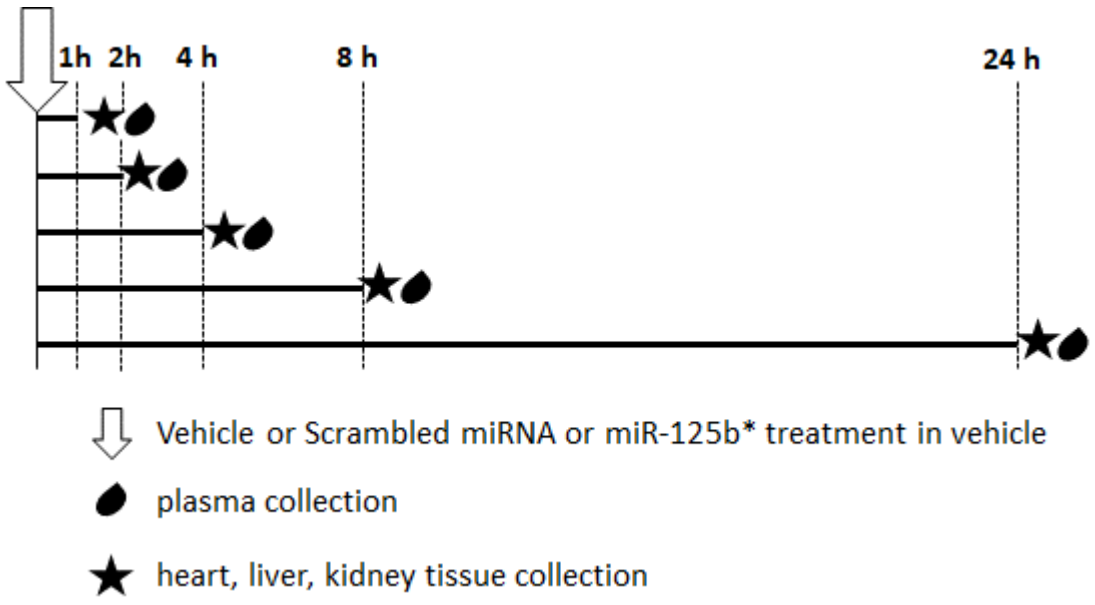

Figure 5. Protocol figure of pharmacokinetics and pharmacodynamics of miR125b* study.

The explorative pharmacokinetics and pharmacodynamics were followed by the PoC-1 study, using a scrambled miRNA, a miR-125b* mimic- and a vehicle-treated group. In PoC-1, a statistically non-significant infarct size reduction was detectable by both scrambled miRNA and

miR-125b* treated groups comparing to the vehicle group. We hypothesized that selecting an appropriate negative miRNA control group would result in a statistically significant reduction by miR125b*. Therefore, we repeated the PoC-1 study (PoC-2) using a different miRNA (cel-miR-239b) control group, all other parameters remained unchanged.

In vivo coronary occlusion protocol

Acute myocardial infarction was induced by external occlusion of the left descending coronary artery followed by the coronary artery reperfusion. In case of MAO-B KO infarction study 45-min ischemia was followed by 120-min reperfusion, in case of miR125b* PoC studies 45-min ischemia was followed by 24-hour reperfusion. Briefly, animals were anesthetized by intraperitoneal injection of 90 mg/kg body weight of sodium pentobarbital (Repose 50%, Le Vet. Pharma, Oudewater, The Netherlands). In case of the 24-hour surviving model, to provide analgesia, buprenorphine (0.05 mg/body weight) was subcutaneously administered (Bupaq multidose, Richter Pharma AG, Austria), shortly after the start of anaesthesia and at recovering following chest sewing. In case of the MAO-B KO infarction study, the mice were continuously anaesthetized, therefore analgesia was not applied. The depth of anaesthesia was monitored by pinching the tail or paws, and when necessary, maintained by administering a half dose of pentobarbital. Body temperature and body surface ECG were monitored during the experiments. Following orotracheal intubation and mechanical ventilation using room air (Model 845, Minivent ventilator), the chests of mice were opened between the 4th and 5th rib to visualize the left anterior descending coronary artery (LAD). To occlude the LAD, a suture was placed at its middle portion using an 8-0 Prolene (Prolene®, Ethicon) suture, and a small plastic cannula was looped and pulled tight above the coronary artery and released when reperfusion was induced. The presence of ischemia was confirmed by the appearance of ST-elevation and by the pale discolouration of the occluded ventricular area. In case of the miR-125b* PoC studies, scrambled miRNA, cel-miR-239b or miR-125b* mimic or vehicle was administered intravenously (i.v.) at the 10th min of coronary occlusion in a volume load of 300 µl as a slow bolus injection.

Protocol for determination of myocardial infarct size

At the end of the reperfusion period, infarct size was determined as described previously (Csonka et al., 2010). The LAD was re-occluded with previously inserted suture. While LAD was occluded, 2% Evans blue dye was injected which stained the whole heart to dark blue except the ischemic area, which remained unstained and thus delineating the area at risk. Thereafter, the heart was excised from the chest, the left ventricle was isolated and cut into 4-6 slices. The slices were washed in phosphate buffer then incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for

10 minutes. In living cells, the enzymatic activity of dehydrogenase enzymes leads to a reduction of TTC, resulting in the formation of a red formazan precipitation (Altman, 1976). In contrast, dead cells remain colourless or pale white, which was identified as infarct size. This double staining method (Evans blue and subsequent TTC staining) is considered a standard procedure for assessing the area at risk and infarct size. After the staining process, the slices were digitised and evaluated planimetrically using InfarctSize® software, in blinded fashion by two independent evaluators. The results show the consensus reached by comparing the assessments performed by the experts.

Determination of 17β-estradiol in the MAO-B KO study

Blood samples were collected immediately following the 120 min reperfusion from the apex of the heart into lithium heparin-coated tubes and were centrifuged for 15 min at 4°C at 1000 g. Plasma 17β-estradiol levels were measured using a 17 beta Estradiol ELISA Kit (ab108667, Abcam, Cambridge, UK) according to the manufacturer's instructions.

Preparation of microRNAs in the miR-125b* study

For the pharmacokinetics, pharmacodynamics and proof of concept of miR-125b* the following microRNAs were used: rno-miR-125b-2-3p miRIDIAN mimic referred to as miR-125b* mimic; scramble miRIDIAN control referred to as control scrambled miRNA; cel-miR-239b mimic referred to as non-targeting control cel-miR-239b miRNA mimic. All microRNAs were purchased from Horizon Discovery Ltd, in annealed, desalted and 2'-hydroxyl form.

The sequences of the guide and passenger strands of the above microRNAs are shown in Table 1.:

Name	Strand	Sequence
miR-125b* mimic	guide strand	5'-ACAAGUCAGGCUCUUGGGACCU-3'
	passenger strand	5'-GUCCCAAGAGCCUGACUUGUUU-3'
scrambled miRNA	guide strand	5'-UCACAACCUCCUAGAAAGAGUA-3'
	passenger strand	5'-CUCUUUCUAGGAGGUUGUGAUU-3'
cel-miR-239b mimic	guide strand	5'-UUUGUACUACACAAAAGUACUG-3'
	passenger strand	5'-GUACUUUUGUGUAGUACAAAUU-3'

Table 1. The sequences of microRNAs used in the presented study.

The microRNAs were re-suspended in RNase-free water and prepared for *in vivo* injection using 10x PBS and MaxSuppresor In Vivo RNA-LANCEr II neutral lipid emulsion (Bioo Scientific,

Cat. No. 3410-01) according to the manufacturer's instructions. Following five minutes in a sonication bath, 10 μg of microRNAs were prepared in a final volume of 300 μL per dose. Final dilution was prepared freshly and stored at 4°C up to maximum of 5 days.

Pharmacokinetics and pharmacodynamics of miR-125b*

For the pharmacokinetic and pharmacodynamics studies, a vehicle group, a scrambled miRNA treatment group and a miR-125b* treatment group were used. In the vehicle group, RNase-free water was used in place of the microRNA to standardise volumes. All treatments were administered intravenously through the tail vein. During preparation, the mice were placed in restraint devices, and heating was applied to their tails. Each mouse was injected with 300 μL emulsion containing 10 μg of scrambled miRNA or that of miR125b* mimic or only neutral lipid emulsion as vehicle. The single dose of 10 μg microRNA was chosen based on a publication, which showed that a mixture of miR-302b/c/-367 mimics administered at this concentration in mice could reach the heart with a peak cardiac level between 4 and 8 hours after injection (Tian et al., 2015).

After the administration of the miRNAs or the vehicle, blood was sampled at the following time points: 1, 2, 4, 8, and 24 hours. Blood was collected into K3-EDTA-containing tubes, and centrifuged immediately at 4°C at 2500 g force. Tissue samples were harvested following whole body perfusion with warm physiological saline. The obtained plasma and tissue samples were snap frozen in liquid nitrogen and stored at -80°C until further use.

Total RNA isolation and qRT-PCR measurement of miR-125b* gene expression

Total RNA was isolated from 25 mg heart, liver and kidney using Direct Zol RNA MiniPrep (Zymo Research, Irvine, CA, USA; Cat. No. #R2050) and from 200 μL plasma samples using Qiagen miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany; Cat. No. #217184) according to manufacturer's instructions. Complementary DNA was synthesized from 10 ng total RNA using the Qiagen miRCURY RT kit (Qiagen, Cat. No. #339340) according to the manufacturer's instructions. Quantitative real-time polymerase chain reactions (qRT-PCR) were performed on LightCycler® 480 II instrument using the miRCURY PCR assay kit (Qiagen, Cat. No. #339306). The polymerase reaction temperature was increased to 95°C for 2 min, and the targets were amplified and quantified in 45 cycles (denaturation: 10 s at 95°C; combined annealing/synthesis: 60 s at 56°C). Forward and reverse primers for miR-125b* were ordered from Qiagen (Cat. No. YP00205315). In case of tissue samples, U6 snRNA (Qiagen, Cat. No. YP00203907), and in case of plasma samples, miR-191-3p primers (Qiagen, Cat. No. YP00205175) were used as control

housekeeping genes. Relative gene expression values of miR-125b* were calculated with the $2^{-\Delta\Delta Ct}$ evaluation method.

Selection method and qRT-PCR measurement of miR-125b* target genes

To validate target engagement and thus the effect of intravenously administered miR-125b* on cardiac expression of target genes of mir125b*, we measured the relative gene expression levels of 5-selected targets (Fig. 6). We call this process as molecular pharmacodynamics. To find the possibly most relevant target genes, we used the miRTarBase version 4.5 (Huang et al., 2022) database, that collects experimentally validated miRNA-target gene interactions. In addition, to identify more possible interacting targets, we used two other *in silico* prediction based databases: miRDB version 5.0 (Wong & Wang, 2015) and TargetScan version 7.2 (McGeary et al., 2019). Genes based on strong miRNA-target interaction prediction were preselected. To further screen the results, and select the most relevant targets, an additional PubMed search was performed and heart-related genes were selected.

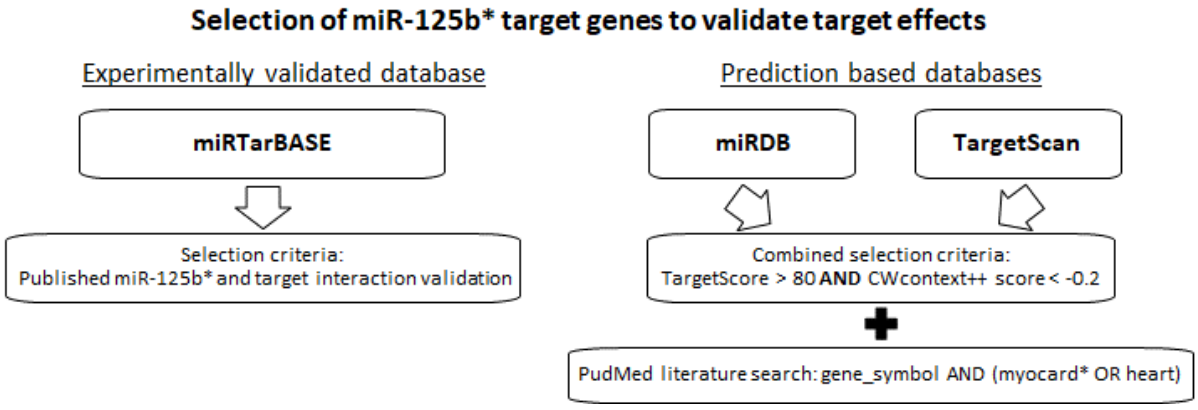

Figure 6. Workflow for the selection of the predicted target genes of miR-125b*.

Relative gene expressions of selected possible targets of miR-125b* were measured with qRT-PCR similarly to miR-125b* gene expression measurement. Complementary DNA was synthesized from 1 µg total RNA (Sensifast cDNA synthesis kit, Bioline) according to the manufacturer's instructions. For qRT-PCR, SensiFAST SYBR Green master mix (Bioline, London, UK; Cat No. #BIO-98005) and the appropriate forward and reverse primers were used (Merck Biosciences, Germany). Peptidylprolyl isomerase A was used as a housekeeping gene for the calculation of relative gene expression with the $2^{-\Delta\Delta Ct}$ method.

Target prediction for miR-125b*, scrambled miRNA and cel-miR-239b and gene set similarity analysis

For target prediction, guide strand or passenger strand, or by both strands were considered. Seed complementarity and sequence feature-based miRNA-like target predictions were performed using Perl scripts of the TargetScan algorithm (Agarwal et al., 2015), with minor adjustments and additional customizations (see details provided in the original article: (Szabados et al., 2025). The predicted results from TargetScan, using a total context++ score threshold of −0.2 (Kimura et al., 2019), were used as input database for miRNAtarget™ software (miRNAtarget™). This software was employed to construct miRNA-target interaction networks for scrambled miRNA and cel-miR-239b. Predicted targets of miR-125b* were then compared with the target predictions for cel-miR-239b and scrambled miRNA.

Gene-ontology analysis

Gene ontology analysis was performed to identify biological processes potentially affected by scrambled miRNA, cel-miR-239b mimic, or miR-125b* mimic sequences through their target genes. Over-representation analysis was performed using the clusterProfiler R package (v3.18.0) (Yu et al., 2012) with mouse annotations from GO release 2020-10-09 (Carbon, 2018). The analysis considered 'part_of' and 'is_a' relations, with all mouse genes as the background and significance set at FDR-adjusted $P \leq 0.05$. Relative leaf terms (most specific GO terms without enriched child terms) of miR-125b* were identified. Key terms related to heart, vessels, and arteries (heart, cardi*, vess*, aort*, arter*, ven*) were selected and visualized for all three sequences to highlight regulatory differences and mechanisms of action.

Statistical analyses

Statistical analyses were computed by using GraphPad Prism Software (version 9.1.2, GraphPad Software Inc., San Diego, CA, USA). Data are expressed as the mean ± standard error of the mean (SEM). Results for area at risk and infarct size were analysed using one-way ANOVA, followed by Fisher's LSD or Dunnett's post hoc tests when applicable. Data of pharmacokinetics and pharmacodynamics were not analysed using a statistical model due to the lack of at least five individual values. Data of β-estradiol levels were analysed using Two-Way ANOVA followed by Tukey's post hoc test. All-cause mortality of the three studies were analysed with Chi-square test. Statistical significance was accepted at values of $p < 0.05$.

RESULTS

Cardioprotection by infarct size reduction in cardiomyocyte-specific MAO-B KO and in miR-125b* treated mice

Mice were subjected to 45-min of coronary occlusion followed by either 120-min (in proof of concept of MAO-B KO study) or 24-hour of reperfusion (in miR-125b* proof of concept studies). Infarct size and area at risk were assessed at the end of reperfusion by standard Evans blue and TTC stainings. The size of the area at risk, as the area of the left ventricle affected by ischemia due to coronary occlusion, was comparable between the experimental groups in both studies and there were no differences between KO/miRNA-treated groups and wild-type/vehicle-treated groups, respectively (see panels "A" of Fig 7, 8 and 9).

MAO-B KO study

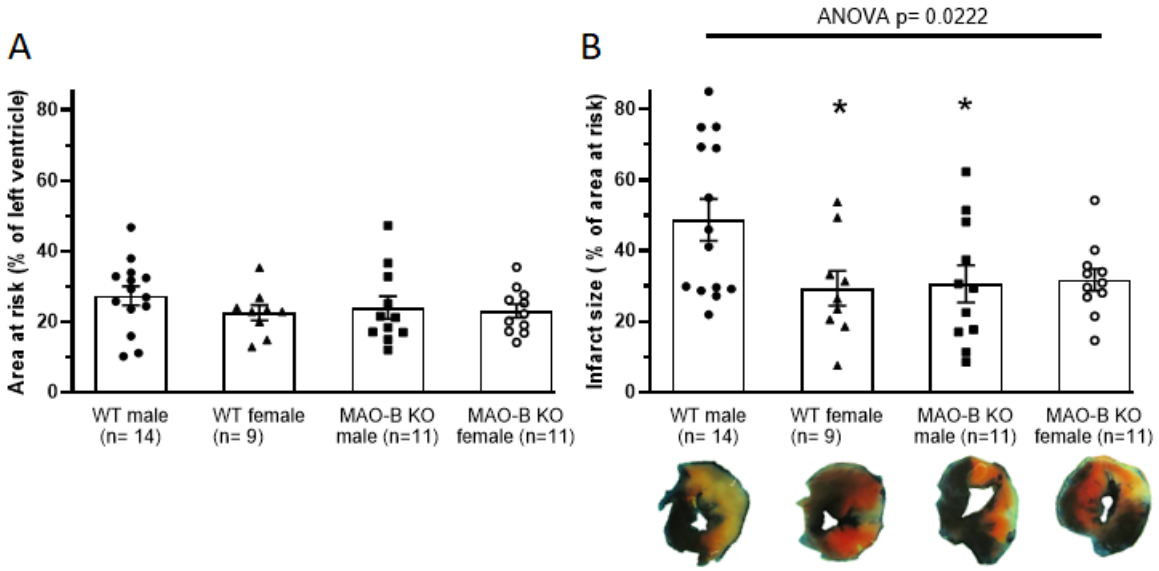

Figure 7. Results of the Proof of concept of MAO-B KO study.
Panel A shows the results of area at risk as the percentage of the left ventricle, delineated by the Evans blue staining. **Panel B** shows the infarct size as the percentage of the area at risk, detected by TTC staining. Representative images of the double-stained left ventricular slices are shown under panel B's corresponding groups. The term "n" refers to the number of values from independent individuals. Data are expressed as means ± S.E.M. One-Way ANOVA with uncorrected Fisher' LDS post hoc test; * $p < 0.05$ vs. WT male.

Male cardiomyocyte-specific MAO-B KO mice and female WT mice had significantly reduced infarct size compared to the WT male mice, while in female mice, cardiac MAO-B depletion did

not affect the infarct size. Interestingly, the infarct size observed in male MAO-B KO hearts was similar to that observed in both WT and KO female mice (see Fig. 7, Panel B).

Myocardial infarct size in miR-125b* proof of concept studies

The direct cardioprotective effect of miR-125b* mimic on myocardial infarct size was shown in two proof of concept studies (PoC-1, PoC-2). In PoC-1 study, we obtained a slight cardioprotective effect by both scrambled miRNA and miR-125b* mimic with a statistically non-significant reduction in infarct size (p=0.0749, see Fig. 8, Panel B).

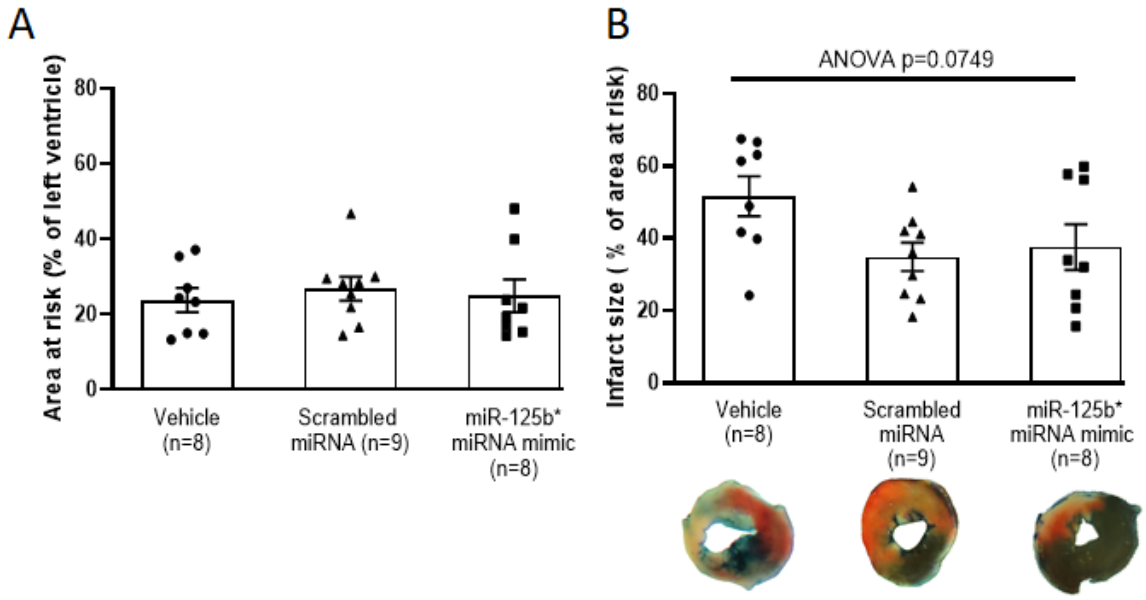

Figure 8. Results of the Proof of concept-1 of miR-125b* study.
Panel A shows the results from data of area at risk in the percentage of the left ventricle, delineated by the Evans blue staining. **Panel B** shows data from infarct size in the percentage of the area at risk, detected by TTC staining. Representative images of the double-stained left ventricular slices are shown under panel B' corresponding groups. The term "n" refers to the number of values from independent individuals. Data are expressed as means ± SEM. One-Way ANOVA analysis shows a slight decrease between infarct sizes without reaching the statistically significant level.

In PoC-2 study, we repeated the experiments conducted in PoC-1, with one modification. The scrambled miRNA was replaced with cel-miR-239b, a miRNA derived from Caenorhabditis elegans, a nematode lacking a circulatory system including a heart, which presumably served as a more suitable negative control. All other parameters and conditions remained consistent with those of PoC-1. In PoC-2 study, miR-125b* treatment led to a significantly reduced infarct size, while Cel-miR-239b treatment did not affect infarct size as compared to the vehicle group (see Fig. 9, Panel B).

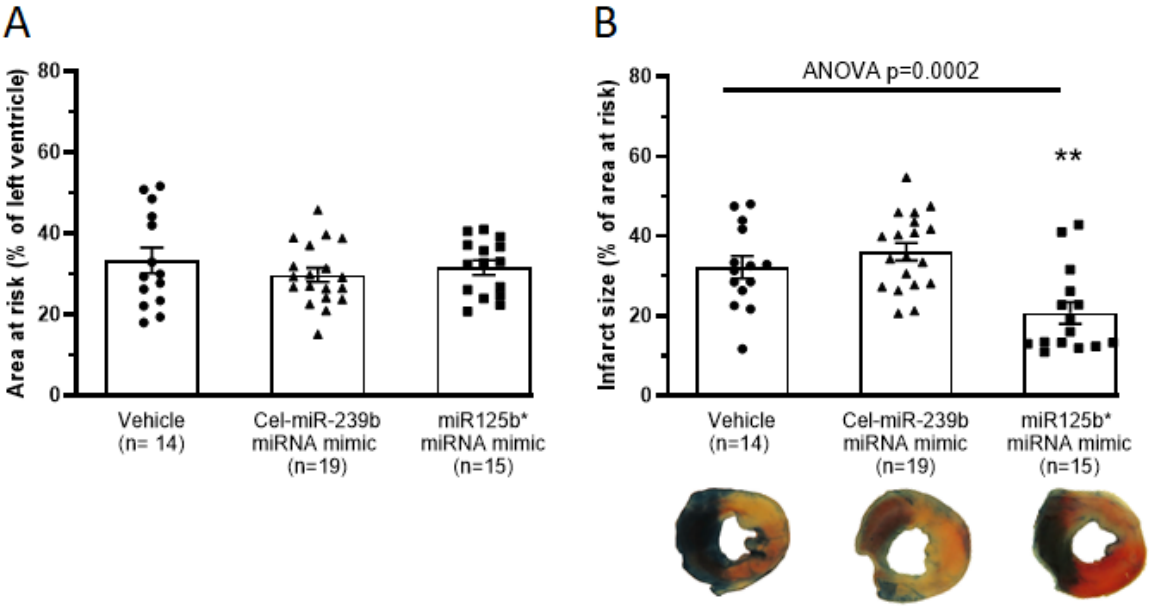

Figure 9. Results of the Proof of concept-2 of miR-125b* study.
Panel A shows the results from data of area at risk in the percentage of the left ventricle, delineated by the Evans blue staining. **Panel B** shows data from infarct size in the percentage of area at risk, detected by TTC staining. Representative images of the double-stained left ventricular slices are shown under panel B's corresponding groups. The term "n" refers to the number of values from independent individuals. Data are expressed as means ± SEM. One-Way ANOVA with Dunnett's post hoc test, ** $p < 0.01$ vs. vehicle.

All-cause mortality

All-cause mortality in MAO-B KO study

No significant difference in overall mortality was observed between wild-type (WT) and cardiomyocyte-specific MAO-B KO mice (see Table 2.). Nevertheless, a tendency toward higher mortality in males compared to females was noted when all males were compared to all female mice (p=0.103, Fisher's exact test).

Mortality of MAO-B KO study	WT male	WT female	MAO-B KO male	MAO-B KO female
Dead n, (%)	7 (29%)	3 (25%)	10 (43%)	1 (8%)
Survived n, (%)	17 (71%)	9 (75%)	13 (57%)	12 (92%)

Table 2. All-cause mortality in MAO B KO study.
Cardiomyocyte-specific MAO-B gene knockout did not affect mortality rate in ischemia/reperfusion model. Female mice tended to have a higher survival rate than that in male mice.

All-cause mortality in miR-125b* proof of concept studies

MiRNA treatment, including control miRNAs, did not affect the mortality rate in any experimental group either in PoC-1 or in PoC-2 (Table 3).

Mortality of miR-125b* study		Vehicle	Control miRNAs	miR-125b* mimic
PoC-1	Dead n, (%)	11 (44%)	10 (43.5%)	14 (56%)
	Survived n, (%)	14 (56%)	13 (56.5)	11 (44%)
PoC-2	Dead (n, %)	7 (28%)	5 (20%)	8 (32%)
	Survived (n, %)	18 (72%)	20 (80%)	17 (68%)

Table 3. All-cause mortality in miR-125b* PoC studies.

17β-estradiol measurement in MAO-B KO study

Due to the potential impact of female hormones, especially estrogens on myocardial I/R injury and/or MAO activity, we measured plasma levels of 17β-estradiol. We found that WT males had significantly higher 17β-estradiol levels compared to the other 3 groups, while there was no statistically significant difference between the other groups. (Table 4).

Plasma 17-β estradiol levels of MAO-B KO study		WT male	WT female	MAO-B KO male	MAO-B KO female
	Mean ± SEM (pg/mL)	27.9 ± 0.81 *	25.1 ± 0.96	23.7 ± 1.01	23.9 ± 0.81
	n	9	9	8	11

Table 4. Plasma levels of 17β-estradiol.

Explorative pharmacokinetics of miR-125b*

MiR-125b* mimic was administered intravenously to C57BL/6 mice via the tail vein in 300 uL volume load containing 10 ug of miRNA. Plasma and tissues (heart, liver, and kidney) were collected at 1, 2, 4, 8, and 24 hours following drug administration. Since the classical pharmacokinetic protocol, i.e. serial blood sampling from the same animal, is not feasible in mice due to their small body weight and limited blood volume, we performed pharmacokinetics using 5-6 mice at each time point. However, the final sample size in each group were between 3-6 (mostly due to technical issues), which is not suitable to perform a reliable statistical analysis, therefore, we call it as explorative pharmacokinetics and pharmacodynamics. Taking this limitation into account, the results are presented without explicitly stating statistical significance, nevertheless, we highlight marked differences.

Expression of miR-125b* in plasma and myocardium

The relative miR-125b* expression level in blood plasma exhibited a marked increase 1 and 2 hours after its injection, and 1 hour after its administration in myocardium as compared to the time-matched vehicle or scrambled miRNA groups. No marked difference was noted at other time points. The scrambled miRNA had no effect on miR-125b* expression at any time point (Fig. 10).

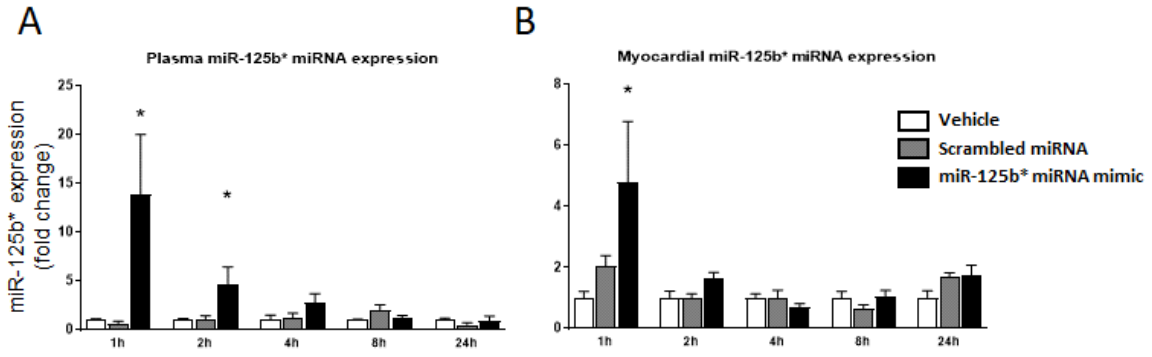

Figure 10. MicroRNA-125b* expression in plasma and myocardium measured after single 10 µg injection of scrambled miRNA or 10 µg of miR-125b* or vehicle administration.
Panel A shows the relative plasma levels of miR-125b* at 1, 2, 4, 8, and 24 hours after i.v. injection of vehicle or scrambled miRNA or miR-125b*. **Panel B** shows the relative myocardial expression of miR-125b* at 1, 2, 4, 8, and 24 hours after i.v. injection of vehicle or scrambled miRNA or miR-125b*. Asterisks indicate a marked increase without statistical significance vs. time-matched vehicle.

Expression of miR-125b* in the liver and kidney

The relative expression of hepatic miR-125b* showed a constant level after i.v. administration at the measured time points in the different groups except at 2 hours where a notable increase was observed. Renal miR-125b* expression showed relatively large standard errors, at 1 hour, miR-125b* group seems to be increased, and at 2 hours to be decreased followed by a gradual increase at 4, 8, and 24 hours, respectively, as compared to the time-matched vehicle group. Scrambled miRNA treatment appears to have a mild effect on renal miR-125b* expression (Fig. 11).

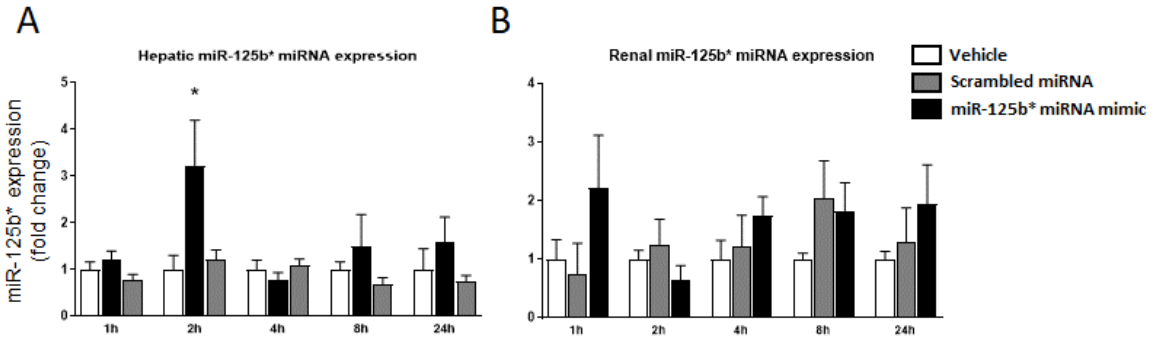

Figure 11. MicroRNA-125b* expression in liver and kidney measured after single 10 µg injection of scrambled miRNA or 10 µg of miR-125b* or vehicle administration.

Panel A shows the relative hepatic miR-125b* levels at 1, 2, 4, 8, and 24 hours after i.v. injection of vehicle or scrambled miRNA or miR-125b*. **Panel B** shows the relative renal miR-125b* levels at 1, 2, 4, 8, and 24 hours after i.v. injection of vehicle or scrambled miRNA or miR-125b*. Asterisk indicates a marked increase without statistical significance vs. time-matched vehicle.

Molecular pharmacodynamics of miR-125b*

To validate target engagement and investigate the molecular mechanism of the miR-125b* mimic through the altered expression of its target genes, we selected and measured the cardiac expression of five predicted gene targets. For target selection, we used an experimentally validated (miRTarBase), and two prediction-based (miRDB and TargetScan) databases. From miRTarBase, we selected two gene targets of miR-125b*, namely *Cyclin A2* (*Ccna2*) and *Eukaryotic elongation factor 2 kinase* (*Eef2k*) based on previous publication listed in miRTarBase database (Xu et al., 2016). From miRDB and TargetScan, the following cardiac-related gene targets were selected: *Calcium channel, voltage-dependent, beta 2 subunit* (*Cacnb2*), *Lysine (K)-specific demethylase 6A* (*Kdm6a*), and *Disabled 2, mitogen-responsive phosphoprotein* (*Dab2*).

The selected target gene's mRNA expressions were measured with qRT-PCR 1, 2, 4, 8 and 24 hours after a single intravenous administration of 10 μg of miRNA-125b* mimic or vehicle or scrambled miRNA. Gene expression of *Ccna2, Eef2k* and *Cacnb2* showed a marked reduction 8 hours after a single i.v. bolus of miR-125b* mimic compared to the time-matched vehicle, whereas mRNA expression levels of *Kdm6a* and *Dab2* did not differ significantly between groups at any time point. Scrambled miRNA also seemed to affect *Ccna2, Eef2k and Cacnb2* but to a much lesser extent and with greater standard deviation (Fig. 12).

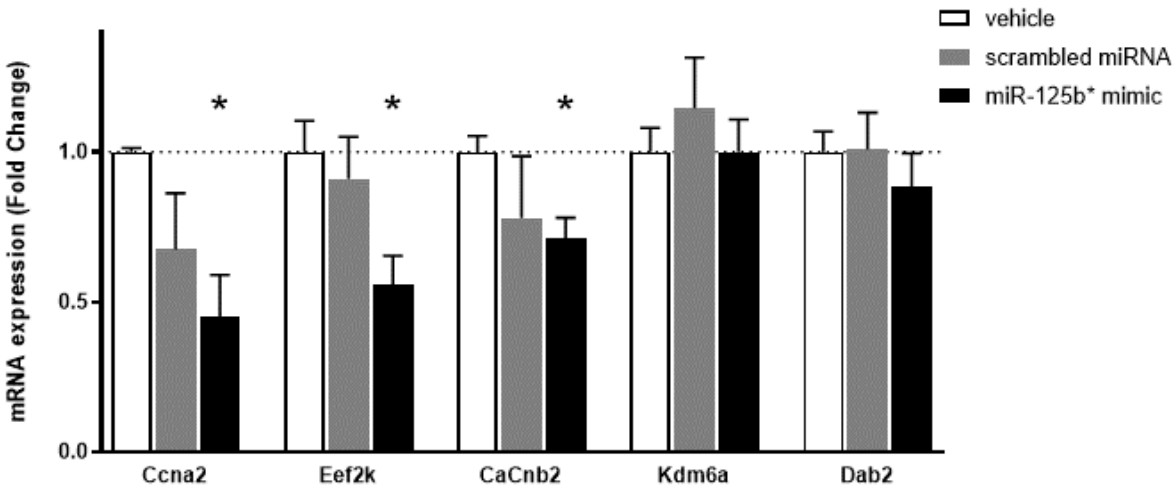

Figure 12. mRNA expression of the selected miR-125b* targets.
Target mRNAs were measured from heart samples at 8 hours after injection of a single 10 μg bolus of scrambled miRNA or 10 μg of miR-125b* or vehicle. Asterisks indicate a marked decrease without statistical significance vs. time-matched vehicle.

Target prediction for miR-125b*, scrambled miRNA and cel-miR-239b and gene set similarity analysis

In the PoC-1 study, we obtained a statistically non-significant infarct size reduction in both miR-125b* and scrambled miRNA-treated groups compared to the vehicle control. We assumed that the lack of statistically significant cardioprotection by infarct size reduction occurred due to the use of an inappropriate negative control group. We carried out a target prediction for miR-125b*, scrambled miRNA and cel-miR239b, then we analysed the gene set similarity between the groups. Using *in silico* miRNA-like target analysis, we have identified 11,086; 7,778, and 10,739 genes that could potentially be regulated by the scrambled miRNA, cel-miR-239b, and miR-125b*, respectively. In case of the scrambled miRNA, 7,223 predicted gene targets overlapped with the predicted targets of miR-125b*, while cel-miR-239b possess 5,320 overlapping predicted targets with miR-125b* (see Figure 13). In general, the less overlapping targets the control has, the lower the likelihood of undesired effects and the less overlapping targets may suggest lower similarity between the regulatory mechanisms of the two compared sequences.

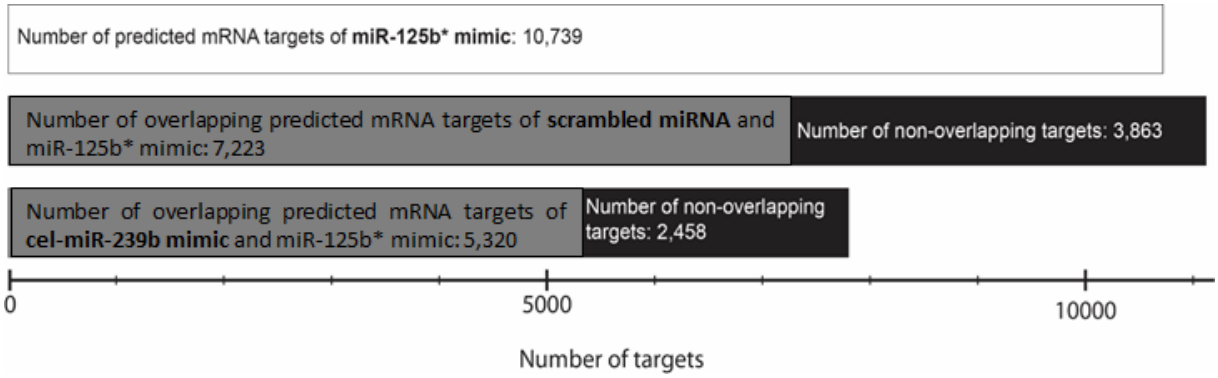

Figure 13. Scaled diagram of the results of target prediction for miR-125b*, scrambled miRNA and cel-miR-239b.
The white box presents the target number of miR-125b*. Dark grey boxes present the number of overlapping target numbers between miR-125b* and the control miRNAs. The black boxes present the numbers of non-overlapping target of scrambled miRNA and cel-miR-239b, respectively.

Gene ontology

We performed a gene ontology (GO) analysis to further interpret the outcome of the target prediction and gene set similarity analyses. GO analysis is used to identify biological processes or pathways that are significantly overrepresented (enriched) within specific gene sets. In our study, we focused the GO analysis on cardiovascular-related biological processes targeted by the miR-125b* mimic, and compared the results with those obtained from scrambled miRNA and cel-miR-239b mimic controls. The analysis revealed that miR-125b* potentially influence several

cardiovascular-related processes with high relevance such as atrioventricular canal development, cardiac pacemaker cells development, dorsal aorta morphogenesis, cardiac muscle cell proliferation, atrial action potential generation, vascular branching during blood vessel morphogenesis, and regulation of heart rate. When comparing the GO enrichment patterns, the scrambled miRNA showed higher similarity to the miR-125b* than that of the cel-miR-239b, which is in line with the results of the gene set similarity analysis (Fig. 14).

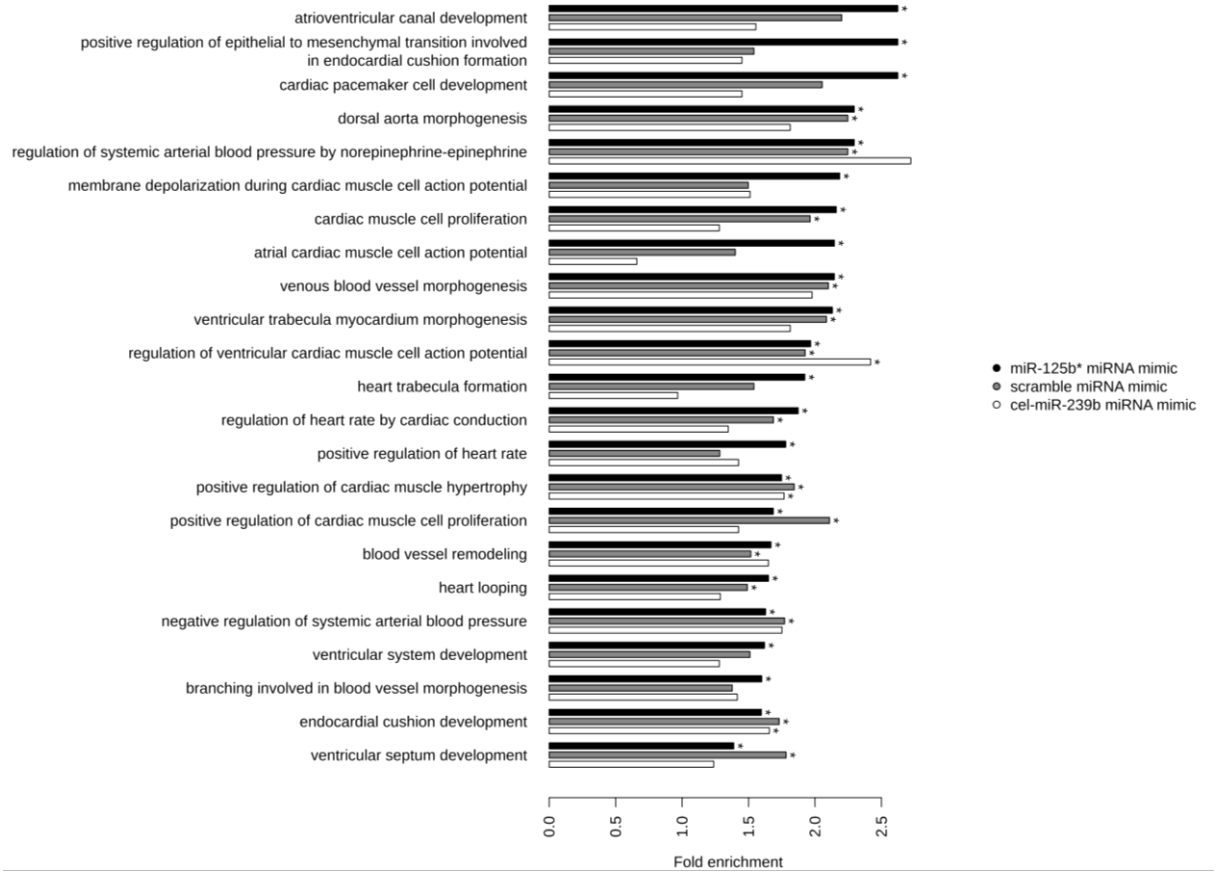

Figure 14. Gene Ontology enrichment analysis.
The results indicated that scrambled miRNA may influence several relevant cardiovascular biological processes, similarly to miR-125b*. In contrast, cel-miR-239b appears to be less likely involved in the regulation of these processes. Significant enrichment (False discovery rate ≥ 0.05) is indicated with an asterisk.

DISCUSSION

Summary

Heart failure as a consequence of myocardial infarction is one of the most common cause of death (Jenca et al., 2021), thus early treatment for MI is a key clinical strategy for improved prognosis. However, as effective treatment of acute myocardial infarction to decrease myocardial necrosis other than limiting the time of infarction is unavailable in the clinical practise (Heusch et al., 2014; Menees et al., 2013; Mercuri et al., 2012). This work focused on two cardioprotective strategies to reduce myocardial infarct size in *in vivo* acute coronary occlusion and reperfusion mouse models. The first cardioprotective approach involves a heart-specific knockout model of MAO-B enzyme, which is known to contribute to oxidative stress during I/R injury. Here, we demonstrated for the first time in the literature that cardiomyocyte-specific knockout of MAO-B (and presumably it's pharmacological inhibition as well) is cardioprotective in an *in vivo* model of acute myocardial infarction by reducing myocardial infarct size. We also showed that the cardioprotection was sex specific, in female mice the absence of cardiac MAO-B had no effect on infarct size. The second cardioprotective strategy that we investigated was a multi-target approach using exogenous delivery of miR-125b* mimic during AMI as a proof-of-concept study. We also described some pharmacokinetic and pharmacodynamic characteristics of the intravenous administration of miR-125b* mimic. We demonstrated for the first time that miR-125b* mimic protects the heart against AMI by reducing infarct size probably via targeting several genes such as *Cyclin 2A* (*Ccna2*) and *Eukaryotic elongation factor-2 kinase (Eef2k*) genes.

MAO-B KO study

After two weeks of oral tamoxifen treatment and a subsequent 10-week waiting period, when MAO-B enzyme has been downregulated in cardiac myocytes, an acute myocardial infarction was induced by 45 minutes of coronary occlusion followed by 120 minutes of reperfusion in wild-type (WT) and cardiomyocyte-specific MAO-B knockout (KO) mice.

To induce cardiac specific MAO-B gene knockout, a two-week feeding period with a tamoxifen citrate-containing chow followed by a 10-week of waiting period with a standard chow was used, based on a previously established protocol. The half-life of monoamine oxidases varies across tissues and species, and their expression generally increases with age. Based on the data provided by our collaborators, the estimated half-life of MAO-B in cardiac tissue is approximately 30 days, which was used to determine the 10-weeks long waiting period. This model resulted in an

approximately 80% reduction in MAO-B protein content in cardiomyocytes, measured by Western-blot analysis (Heger et al., 2021).

In the present study, subgroups were created based on sex and genotype. No significant differences were observed in phenotypic characteristics, including body weight, heart weight, intraoperative mortality and heart rate, between MAO-B KO and WT male and female animals. These findings are consistent with previous results from Heger et al., who showed no differences in left ventricular dimensions using the same genetic models (Heger et al., 2021). Heger and colleagues were also the first to demonstrate that cardiac-specific MAO-B deletion confers cardioprotection. Using an *ex vivo* Langendorff model with 45 minutes of global ischemia followed by 120 minutes of reperfusion, they observed a significant reduction in infarct size in the hearts of MAO-B KO as compared to the controls (Heger et al., 2021). Building on these findings, we further investigated the cardioprotective effect of MAO-B deletion in an *in vivo* mouse model of AMI. Our results revealed that the protective effect is sex-specific, as infarct size reduction was observed in male but not in female MAO-B KO mice. The percentage of the area at risks (AAR) was comparable and statistically identical in all groups, indicating that the extent of the left ventricular I/R injury was consistent. Additionally, we found that female WT mice exhibited smaller infarct size compared to their male counterparts. We hypothesize that the smaller infarct size observed in female WT mice may be due to the cardioprotective effects of estrogen, which appear not to be further enhanced by MAO-B deletion. It is important to note that our experiments were performed in young adult mice (~18-20 weeks old at the time of the surgery), which corresponds to the reproductive age in females. Multiple studies and reviews (da Silva et al., 2021; Hale et al., 1996; Sawada et al., 2000; Squadrito et al., 1997; Wexler & Greenberg, 1979) have demonstrated sex differences in myocardial ischemic tolerance, as well as the cardioprotective role of estrogens in this process. The cardioprotective effect of estrogens has also been reported in human studies (Barrett-Connor, 1997; Mauvais-Jarvis et al., 2020; Mehta et al., 2016). However, the mechanisms underlying estrogen-mediated cardioprotection remain only partially understood. Proposed mechanisms include direct effects on the vasculature through enhanced nitric oxide release, modulation of prostaglandin production, suppression of smooth muscle cell proliferation (Mehta et al., 2016), and transcriptional induction of antioxidant genes (da Silva et al., 2021). Given that estradiol can protect the heart against ischemic injury and may also influence MAO activity (Hernandez-Hernandez et al., 2019), we measured 17β-estradiol levels in plasma samples collected at the end of the 120-minute reperfusion period. We found that WT male mice exhibited significantly higher 17β-estradiol levels compared to MAO-B KO males. However,

cardioprotection in MAO-B KO males remained evident suggesting no direct link between the increased plasma 17β-estradiol levels and the observed infarct size reduction. Interestingly, estradiol levels in all groups of mice fell within or close to the physiological ranges reported in the literature. In young intact female mice, physiological 17β-estradiol serum level ranges from undetectable to approximately 47.22 ± 2.39 pg/ml, (Strom et al., 2012; Yan et al., 2017), while in intact males it is between 12.4 and 20.7 ± 3 pg/ml (Nilsson et al., 2015; Saito et al., 2009).

Reactive oxygen species (ROS) are known to play a central role in the development of ischemia/reperfusion injury. In the heart, the major sources of ROS formation are myocardial mitochondria, which are responsible for a significant proportion of this damage (Bugger & Pfeil, 2020). MAO activity is closely associated with ROS formation, as described by, among others, the discoverer of the MAO enzyme, Mary Lilias Christian Hare (Hare, 1928), and later confirmed by numerous studies (Anderson et al., 2014; Edmondson et al., 2009; Heger et al., 2021). Heger and colleagues showed that ROS production in mitochondria isolated from the hearts of MAO-B KO mice was significantly lower compared to mitochondria from control mice after addition of the MAO-B-specific substrate β-phenylethylamine (Heger et al., 2021).

The observed infarct size reduction in MAO-B KO mice is therefore likely due to decreased mitochondrial ROS generation. This finding is supported by the work of Inagaki et al., who demonstrated that pargyline, a selective MAO-B inhibitor (Magyar, 1993), significantly reduced hydroxyl radical production in rat hearts during both ischemia and reperfusion, and significantly reduced the subsequent cardiomyocyte injury indicated by significantly reduced myocardial interstitial myoglobin levels (Inagaki et al., 2016). Another MAO-B inhibitor, selegiline, has also been shown to be cardioprotective in a chronic heart failure rabbit model by reducing cardiac oxidative stress and myocyte apoptosis (Qin et al., 2003).

A substantial limitation of our study, in terms of clinical translatability, is the use of gene knockout technology. While this approach effectively models the absence of MAO-B in cardiomyocytes, it is not applicable to human therapy. However, given the cardioprotective effects of pargyline and selegiline, our results suggest the potential for drug repositioning of MAO-B inhibitors. Drug repositioning offers a time- and cost-efficient strategy for identifying new therapeutic applications for existing drugs that already have passed the safety assessments. MAO-B inhibitors (selegiline, rasagiline, safinamide) have passed the human safety tests and are used in the treatment of Parkinson's disease (Dezsi & Vecsei, 2017).

Although our results are promising, particularly in male mice, we did not observe infarct size reduction in females. The use of young animals may partly explain this, as older (perimenopausal or postmenopausal) female mice might exhibit different outcomes. Further studies in age-matched models are needed to confirm this hypothesis.

MiR-125b* study

Previously, microRNA-125b* (microRNA-125b-2-3p; miR-125b*) has been identified as a cardio-cytoprotective non-coding RNA in isolated rat neonatal cardiac myocytes subjected to simulated ischemia/reperfusion injury (Varga et al., 2014). Pharmacokinetic and pharmacodynamic properties of exogenously administered miR-125b* microRNA mimic and it's cardioprotective effect by infarct size reduction were assessed for the first time in the literature. After a single intravenous injection of 10 ug miR-125b* mimic, its expression was markedly increased in plasma and heart at 1 hour and remained slightly elevated in the plasma 2 hours after the injection. Potential molecular mechanism of miR-125b* mimic was assessed by target engagement validation. We found that the myocardial expression of three predicted mRNA targets of miR125b*, *Ccna2*, *Eef2k*, and *Cacnb2*, were markedly reduced 8 hours after the injection. Cardioprotective properties were investigated in two separate *in vivo* proof-of-concept (PoC) studies of acute myocardial infarction. In PoC-1 infarct size reduction by miR-125b* seemed convincing, however, the difference between the miR-125b*- and the vehicle treated group did not reach the statistically significant level, probably due to the surprising infarct size-decreasing trend of the non-optimal negative control (i.e. the scrambled miRNA) group. In a repeated series of experiments, PoC-2, an identical setup except for the negative control miRNA (i.e. cel-miR-239b) group, miR-125b* exerted a significant infarct size reduction, whereas the negative control miRNA did not show any protection as compared to the vehicle-treated group.

Explorative pharmacokinetics of miR-125b*

In an explorative pharmacokinetic study, we assessed the tissue distribution of the miR-125b* mimic. A single intravenous dose of 10 μg miR-125b* mimic was administered, and its relative expression levels were measured in plasma, heart, kidney, and liver samples 1, 2, 4, 8 or 24 hours after the injection. Compared to the time-matched vehicle or scrambled miRNA controls, a marked increase in miR-125b* expression was observed in plasma and myocardial tissue of the mimic-treated mice at 1 hour, which remained elevated in plasma 2 hours after the injection. Additionally, a marked increase was observed in the liver at 2 hours. As the final sample size did not reach n=5

at all time points and in all experimental groups, we did not report statistically significant differences, but rather highlighted possible biologically relevant changes.

Although the number of studies using microRNA-based therapeutics is rapidly increasing, data availability regarding the pharmacokinetics (and pharmacodynamics) of miRNAs remains strongly limited in the literature. Nevertheless, understanding these properties is essential for clinical drug development.

Our findings on the pharmacokinetics of miR-125b* show some similarity with those reported by Li and colleagues, who investigated the pharmacokinetics of the cardioprotective miRNA candidate, miR-144. In their study, a single intravenous dose of 8 mg/kg miR-144 was administered via the tail vein of mice. They have found a time-dependent distribution with a peak plasma level of miR-144 showing an over 2.5-fold increase 1 hour after its injection, which remained elevated for 7 days (~2-2.5-fold) compared to PBS-treated mice. In cardiac tissue, miR-144 reached a similar peak at 1 hour with an over 1.8-fold increase and remained elevated for 3 days after the injection (Li et al., 2016). A limitation of both experimental designs, including ours, is the use of healthy (intact) mice for pharmacokinetic analysis, which may not fully reflect the behavior of non-coding oligonucleotides in disease contexts such as ischemia/reperfusion. However, a study by Kwekkeboom and colleagues provided more detailed data on the distribution of antagomiR-214 (an oligonucleotide silencing miR-214): the fluorescence-labeled antagomiR was undetectable in the heart 30 min after its single i.v. injection at 0.75 mg/kg into healthy mice. However, when it was administered within the first 5 minutes of reperfusion following a 30-min coronary occlusion, its fluorescence intensity was pronounced in the heart at 30 min after injection and remained detectable for 24 hours. Fluorescence images revealed that antagomiR-214 was mostly taken up by the infarcted zone (Kwekkeboom et al., 2016).

Molecular pharmacodynamics of miR-125b*

To explore the possible molecular mechanisms of miR-125b* mimic, five of the predicted gene targets were selected for expression analysis. Among those, the mRNA levels of *Cyclin A2* (*Ccna2*), *Eukaryotic elongation factor-2 kinase* (*Eef2k*) and *Calcium channel, voltage-dependent, beta 2 subunit* (*Cacn2*) were markedly reduced at 8 hours following a single i.v. injection of miR-125b* mimic. These findings suggest that miR-125b* may exert its effects at least partly through the downregulation of genes involved in cell cycle regulation, protein synthesis control, and calcium signalling, all processes are critically involved in the pathophysiology of myocardial ischemia/reperfusion injury.

Ccna2

Cyclin A2 is a member of the cyclin protein family and is essential for regulating cell cycle by activating cyclin-dependent kinases (Loukil et al., 2015). The team of Chaudhry HW reported that transgenic mice with constitutive myocardial expression of *Cyclin A2*, as well as pigs injected with adenovirus expressing *Cyclin A2*, exhibited an enhanced cardiac repair following surgically induced myocardial infarction. This was characterized by new cardiomyocyte formation, reduced scarring, improved contractile function, and increased ejection fraction, demonstrating *Cyclin A2*'s potential to prevent post-MI heart failure (Cheng et al., 2007; Shapiro et al., 2014). These findings suggest an opposite role for *Cyclin A2* compared to our results, showing the need for further investigations to clarify this apparent contradiction.

Eef2k

Eukaryotic Elongation Factor-2 Kinase (*Eef2k*) acts as a calcium/calmodulin-dependent serine/threonine kinase that negatively regulates protein synthesis by phosphorylating and thus inactivating eukaryotic elongation factor 2 (eEF2), which is essential for protein synthesis (Ballard et al., 2021). Its role in oncology is well studied: its overexpression promotes tumor-cell survival in several different tumour types (Wang et al., 2017). Other than cancer, *Eef2k* has been linked to a range of human diseases, including cardiovascular diseases. Horman and colleagues demonstrated that myocardial ischemia activates AMPK, which in turn stimulates *Eef2k* activity, leading to increased eEF2 phosphorylation and suppression of protein synthesis in the ischemic heart. This energy-conserving response may protect cardiomyocytes during oxygen deprivation by reducing metabolic demand (Raemaekers et al., 2003). However, prolonged or excessive *Eef2k* activation could impair recovery by limiting the synthesis of repair proteins (Jeon, 2016). In our study, *Eef2k* mRNA expression was markedly reduced following miR-125b* mimic administration suggesting that transient inhibition of *Eef2k* might contribute to the cardioprotective effect by preserving translation capacity during reperfusion. Mitroshina and colleagues examined *Eef2k*, in primary hippocampal neuron–glia cultures under simulated ischemic conditions (hypoxia or glucose deprivation). They observed a 1.55–1.68-fold increase in *Eef2k* mRNA expression during the post-ischemic period, suggesting that ischemic stress induces *Eef2k* upregulation. Pharmacological inhibition of *Eef2k* in this model enhanced cell viability, suggesting a possible neuroprotective effect, however, it also impaired the functional activity of neuron-glial networks, suggesting a more complex role of *Eef2k* in ischemic injury (Mitroshina et al., 2021).

Cacnb2

Molina-Navarro and colleagues investigated gene expression changes in cardiac ion-channel components in dilated cardiomyopathy (DCM) and found that *Cacnb2* expression was

significantly downregulated in patients with DCM compared to healthy controls. *Cacnb2* is a crucial component of the L-type Ca^{2+} channel complex, which plays a central role in excitation–contraction coupling in cardiomyocytes. Reduced expression of *Cacnb2* may contribute to impaired calcium handling, a common feature observed in DCM linked to contractile dysfunction and arrhythmias (Molina-Navarro et al., 2013). Meissner and colleagues demonstrated that while *Cacnb2* is essential for embryonic survival, its loss in adult mice leads to moderate calcium channel dysfunction and impaired β-adrenergic responsiveness—both relevant to heart failure and arrhythmia risk (Meissner et al., 2011). To the best of our knowledge, *Cacnb2* has not been directly linked to myocardial ischemia–reperfusion injury. However, since calcium overload and dysregulated calcium handling are well-recognized contributors to reperfusion injury, *Cacnb2* could present a potentially relevant target for future research.

Cardioprotection by infarct size reduction

The cardioprotective potential of miR-125b* (microRNA-125b-2-3p) was initially suggested by its downregulation following myocardial ischemia–reperfusion injury and its counter-upregulated modulation by ischemic pre- and postconditioning. *In vitro* experiments in neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion injury confirmed its cytoprotective effect (Varga et al., 2014). In the present study, we extended these findings by demonstrating that intravenous administration of miR-125b* mimic during ischemia significantly reduced infarct size in an *in vivo* mouse model of acute myocardial infarction. Mice underwent 45 minutes of coronary occlusion followed by 24 hours of reperfusion, with infarct size quantified using Evans blue and TTC staining. The area at risk was comparable between groups. Two independent proof-of-concept studies were conducted: in PoC-1, both scrambled miRNA and miR-125b* showed a trend toward reduced infarct size without reaching a statistically significant difference. In PoC-2, which used cel-miR-239b as a more neutral negative control, miR-125b* treatment resulted in a statistically significant infarct size reduction compared to vehicle, which confirmed its direct cardioprotective effect, while cel-miR-239b did not affect infarct size.

Wang and colleagues demonstrated cardioprotective effects of miR-125b similar to our findings. They used both a lentiviral delivery and a transgenic overexpression of the 76-nucleotide precursor form of miR-125b (pre-miR-125b-1), which contains thymine in place of uracil and encodes both the mature miR-125b-5p (their miRNA of interest) and the miR-125b-2-3p sequence. They reported a significant reduction in infarct size after 45 min of ischemia followed by 4 hours of reperfusion, along with improved ejection fraction and fractional shortening 3 and 7 days after ischemia (Wang et al., 2014). Our study differs in several key aspects: we used the mature 22-

nucleotide miR-125b-2-3p (miR-125b*) sequence, and delivered it intravenously at a clinically relevant timepoint, during ischemia. Because the approach and the exact sequence of the miRNAs differ, Wang's results do not interfere the novelty of our findings, but rather they support and extend the evidence for cardioprotective potential of miR-125b family members.

The miR-125 family is a highly conserved cluster consisting of three homologs, miR-125a, miR-125b-1, and miR-125b-2. Members of this family are involved in various physiological and pathological processes, such as embryonic development (Kim et al., 2016), carcinogenesis (as tumor suppression and promotion (Wang et al., 2019), and cardiovascular diseases including myocardial I/R injury, heart failure and cardiac fibrosis, see detailed review by (Varga et al., 2018). MiR-125b* (also known as miR-125b-2-3p) is derived from the processing of the miR-125b-1 stem-loop precursor (Varga et al., 2018). Despite arising from distinct genomic loci, miR-125 family members share a conserved seed region (nucleotides 1–9), suggesting that they may regulate overlapping sets of target genes.

Many studies in the literature discuss the concordant as well as contradictory data regarding the protective role of miR-125b from different perspectives (Jia et al., 2016; Singh et al., 2020; Wu et al., 2023; Xiao et al., 2018), however, drawing straightforward conclusions about miR-125b remains challenging. The nomenclature of miRNAs has changed over time, making consistent and accurate identification challenging. Despite standardized nomenclature guidelines, there does not seem to be sufficient emphasis on their consistent use. Furthermore, many publications do not provide the exact sequence of the miRNAs of interest, which would be crucial for accurate identification, which is becoming increasingly important as the field rapidly evolving. This issue is further complicated by a shift in nomenclature conventions, transitioning from the "asterisk" system (e.g., miR-125b*) to suffixes denoting the -5p or -3p strands, which indicate the different maturation level of miRNAs generated from opposite arms of the same pre-miRNA. However, no straightforward rule exists for correlating the old “asterisk” system with the newer naming conventions across different species and miRNAs (Budak et al., 2016; Griffiths-Jones et al., 2006). Additionally, the miRBase database, which previously preserved records of the older nomenclature, is no longer accessible for such searches, further complicating efforts to accurately trace miRNA versions between systems. However, due to the highly conserved sequence origin, we can rely on the literature but at the same time caution should be taken when interpreting the results.

CONCLUSION

In this thesis, we explored two distinct cardioprotective strategies aimed to reduce myocardial infarct size in mouse models of acute myocardial infarction. Our findings provide new insights into both enzymatic and post-transcriptional regulation of myocardial ischemia–reperfusion injury.

Here, we demonstrated for the first time that cardiomyocyte-specific deletion of monoamine oxidase B (MAO-B) confers a significant infarct size reduction in male mice. This effect is likely mediated by the decreased production of mitochondrial reactive oxygen species, as MAO-B is already a known source of oxidative stress during ischemic injury. The cardioprotective effect was sex-specific and absent in female knockout mice, likely due to the already enhanced ischemic tolerance of the female heart—possibly mediated by estrogen. Although gene knockout is not directly translatable to human therapy, our results support the potential repurposing of clinically approved MAO-B inhibitors, such as selegiline and pargyline, as cardioprotective agents.

We established the cardioprotective efficacy of miR-125b* (miR-125b-2-3p) mimic administered systemically in an *in vivo* model of acute myocardial infarction. We showed that a single intravenous injection of miR-125b* during ischemia significantly reduced infarct size, likely through the downregulation of key target genes involved in cell cycle progression (*Ccna2*), translational control (*Eef2k*), and calcium handling (*Cacnb2*). Exploratory pharmacokinetic data confirmed a rapid cardiac uptake of the exogenous miRNA mimic, and pharmacodynamic profiling provided mechanistic support for its multi-target gene regulation. This study is the first to show the pharmacokinetics, tissue distribution, and infarct-size-limiting efficacy of miR-125b* mimic in an *in vivo* mouse model of AMI.

Together, the two approaches—one targeting mitochondrial oxidative stress upstream, the other modulating gene expression downstream—highlight the therapeutic potential of both enzyme inhibition and non-coding RNA delivery in myocardial infarction. While each model has its limitations, they offer complementary insights: MAO-B knockout revealed sex-specific effects and a mechanistic link to decreased ROS production, whereas miR-125b* provided a pharmacologically feasible, multi-target RNA-based strategy to improve cell survival, thereby to reduce myocardial infarct size.

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These findings contribute to the growing field of cardioprotective therapies and underscore the importance of both mechanism-specific and multi-targeted approaches. Future research should aim to validate these strategies in clinically relevant animal models, including aged or comorbid animals, and explore the translational potential of miRNA therapeutics and enzyme inhibitors in human cardiovascular diseases.

The new findings of my PhD thesis are the following:

1. Cardiomyocyte-specific deletion of monoamine oxidase B significantly reduces infarct size in male mice in an *in vivo* model of acute myocardial infarction.

2. The *in vivo* cardioprotective effect of monoamine oxidase B deletion is sex-specific and absent in female mice.

3. Single intravenous injection of 10 μg miR-125b* mimic markedly increases the expression of miR-125b* in plasma, heart, and kidney 1 hour, and in liver two hours after injection.

4. Single intravenous injection of 10 μg miR-125b* mimic can markedly decrease the cardiac expression of its target genes such as *Cyclin A2, Eukaryotic Elongation Factor-2 Kinase* and *Calcium channel, voltage-dependent, beta 2 subunit* 8 hours after injection.

5. Single intravenous injection of 10 μg miR-125b* mimic significantly reduces infarct size in an *in vivo* model of acute myocardial infarction in male mice.

6. Using a scrambled miRNA as an intended negative control without having previously established its non-targeting properties may mask the cardioprotective potential of the miRNA of interest. Therefore, when testing miRNA therapeutics *in vivo*, a preliminary determination and selection of an appropriate non-targeting control sequence is essential.

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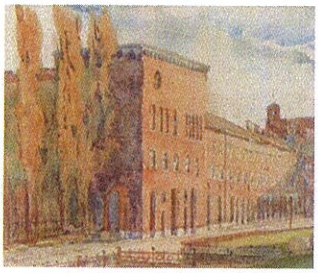

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