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Pre-clinical investigation of cardioprotective therapies for acute myocardial ischemia/reperfusion-injury and for pressure-overload-induced chronic heart failure

PhD thesis

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List of Abbreviations

AAR: area at risk

AMI: acute myocardial infarction

CABG: coronary artery bypass grafting surgery

CI: confidence interval

CVD: cardiovascular disease

ddPCR: droplet digital polymerase chain reaction

DNA: deoxy ribonucleic acid

ECG: electrocardiogram

GPCR: G-protein-coupled receptor

HF: heart failure

HFpEF: heart failure with preserved ejection fraction

HFrEF: heart failure with reduced ejection fraction

I/R: ischemia/reperfusion

IHD: ischaemic heart disease

IPC: cardiac ischemic preconditioning

IS: infarct size

LAD: left anterior descending coronary artery

LVA/PWTd: left ventricular anterior/posterior wall thickness in diastole

LVCO: left ventricular cardiac output

LVEF: left ventricular ejection fraction

LVES/DV/D: left ventricular end-systolic/diastolic volume/diameter

LVSV: left ventricular stroke volume

MAP: mean arterial blood pressure

MD: mean difference

MVO: microvascular obstruction

NRCM: neonatal rat cardiomyocyte

PCI: percutaneous coronary intervention

PLAX: parasternal long-axis view

PSAX: parasternal short-axis view

RIPC: remote ischemic preconditioning

RNA: ribonucleic acid

RWT: relative wall thickness

SEM: standard error of the mean

TTC: triphenyltetrazolium chloride

1. INTRODUCTION

1.1. Disease burden of acute myocardial infarction and heart failure

Despite the continuous improvement of preventive, diagnostic, and therapeutic approaches for cardiovascular diseases (CVDs), this disease group is the leading cause of mortality and disability worldwide. As estimated by the Global Burden of Cardiovascular Diseases Study (1990-2019), the number of deaths for total CVDs has reached 18.6 million, and disability-adjusted life years has risen to 400 million person-years in 2019, both measures of disease burden showing a steady increase for the last 3 decades, irrespective of the region [1].

When broken down into causes of CVD, ischaemic heart disease (IHD) accounts for nearly 50% of all CVD-related deaths and disability, consisting principally of acute myocardial infarction (AMI) (Figure 1.). AMI is defined as an irreversible injury of the heart arising from prolonged and severe ischaemia of the myocardial tissue, typically caused by the occlusion of a coronary artery on the basis of plaque erosion [2]. AMI is estimated to affect 197 million people worldwide, with a five-year mortality rate of 12.5%, resulting in being a leading cause of global mortality *per se* [3].

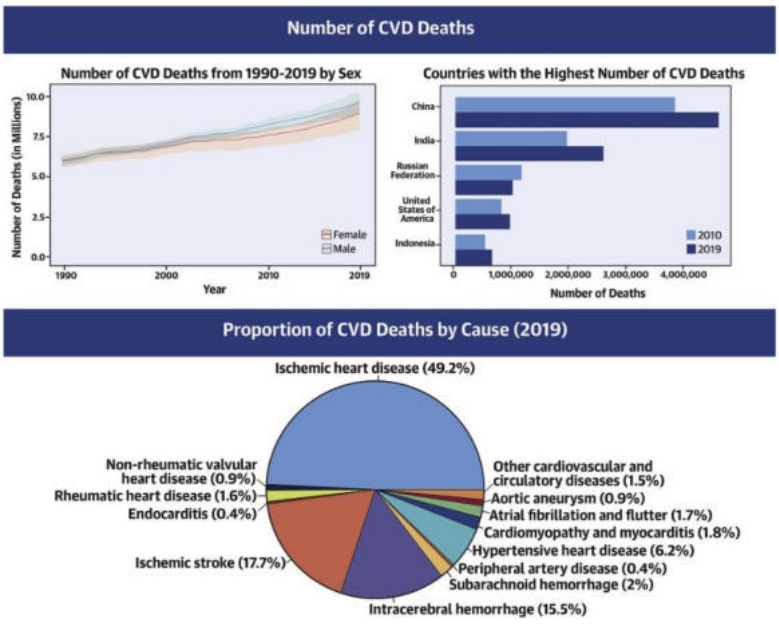


Figure 1. Main results from the Global Burden of Cardiovascular Diseases Study 2019. **CVD:** cardiovascular disease. *Figure was adapted without modifications from: Gregory A. Roth et al., JACC, 2020* [1].

The Global Burden of Disease Study did not account for heart failure (HF) as a primary *cause* of CVD, as HF is rather a *consequence* of a large array of cardiovascular (e.g. IHD, hypertension, valvular heart disease), and non-cardiovascular (e.g. cardiotoxic medications, kidney disease, infections) aetiologies [4]. According to the universal definition of HF, this disease is a complex clinical syndrome caused by structural and/or functional abnormalities of the heart, with a large variety of underlying cardiac or non-cardiac aetiologies [5]. HF has been estimated to affect more than 64 million people globally with a steady increase in prevalence, a stable incidence rate, and with a five-year mortality rate of ≥50% even with the current guideline-directed medical therapies (Figure 2.), making this disease also a pivotal cause of global mortality and hospitalization [6].

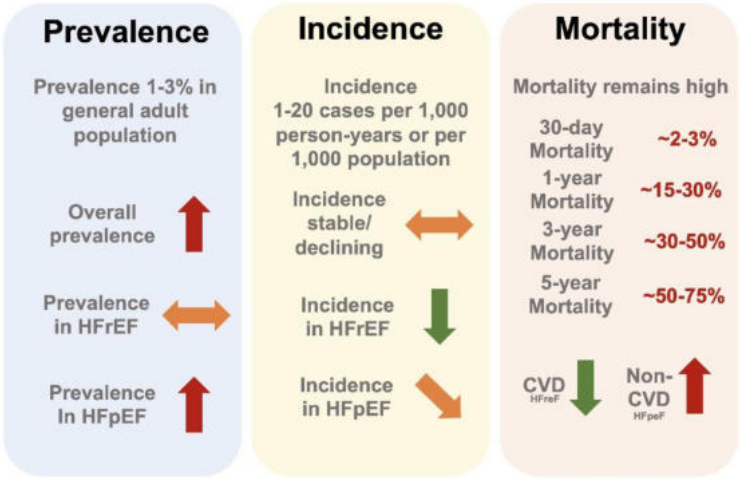


Figure 2. Main results from the Global Burden of Heart Failure Study showing a steady increase in the number of overall prevalence, a stable incidence, and very high mortality rates over the course of heart failure. **HFrEF:** heart failure with reduced ejection fraction. **HFpEF:** heart failure with preserved ejection fraction. **CVD:** cardiovascular disease. *Figure was adapted without modifications from: Gianluigi Savarese et al., Cardiovasc. Res., 2022* [6].

Overall, there is still an unmet need for novel pharmacological or non-pharmacological therapeutic interventions to prevent, and decrease cardiac damage, and thereby, to improve the survival and quality of life of patients with AMI and HF. Thus, a deeper understanding of the pathophysiology and identifying and testing novel modifiable targets are essential. To achieve this goal, reproducible pre-clinical studies with a high

translational value are of paramount importance, as some of these investigations have profoundly shaped our current treatment pathways in both AMI and HF. Although the number of pre-clinical investigations with the above aim continue to rise intensively, their reproducibility and translatability need to be improved [7].

1.2. Role of pre-clinical experiments in identifying therapeutic targets and strategies for myocardial ischemia/reperfusion-injury

The first studies to test various factors influencing myocardial infarct size (IS), and thus, contributing to the development of the hypothesis that revascularization of the occluded coronary limits infarction expansion, have been conducted in the pre-clinical setting [8]. Also, studies identifying that revascularisation salvages ischemic, but viable myocardium only in a limited time window, have been performed in dogs [9]. These pre-clinical investigations served as a solid basis for the current approach to treating AMI by timely revascularization in the clinical setting.

Although timely revascularization – e.g. by primary percutaneous coronary intervention (PCI) – is currently the only effective strategy to rescue the myocardial tissue from further ischaemic damage in AMI, this approach induces an additional component of the cardiac injury, defined as reperfusion injury. This effect has long been debated whether it contributes to and to what extent to the harmful consequences of myocardial infarction [10]. However, for the past two decades, pre-clinical studies showing that myocardial infarct size (IS) could be reduced by interventions implemented during early reperfusion provided undisputed proof for the presence of myocardial injury caused not only by ischemia, but also by reperfusion [11].

Beside the increase in myocardial IS, reperfusion injury has also been linked (i) to myocardial stunning - a reversible mechanical dysfunction of the heart, (ii) to ventricular arrhythmias, which are relatively treatable, and finally (iii) to the no-reflow phenomenon, as a consequence of microvascular occlusion (MVO) caused by microembolism of the capillaries by cellular debris and dissolving microthrombi [12]. Given the fact that myocardial IS and the presence or extent of MVO are strong and independent predictors for all-cause mortality and/or major adverse cardiovascular events [13,14], cardioprotective approaches aiming to reduce myocardial ischemia/reperfusion (I/R) injury (i.e. IS and

MVO), as an adjunct to revascularization, are urgently needed to improve life expectancies of patients with AMI.

Several cardioprotective strategies have long been demonstrated to exert IS- and MVO-limiting effects in animal models of acute myocardial I/R-injury. Of these approaches, ischemic conditioning strategies are amongst the most investigated non-pharmacological cardioprotective methods. These are exerted by short-term, non-lethal I/R cycles that can be applied either on the affected coronary artery or on a remote organ (termed as *local*, or *remote* ischemic conditioning, respectively), and either before, during, or after the index myocardial ischemia (termed as *pre-*, *per-*, or *post*conditioning, respectively) (Figure 3.) [15].

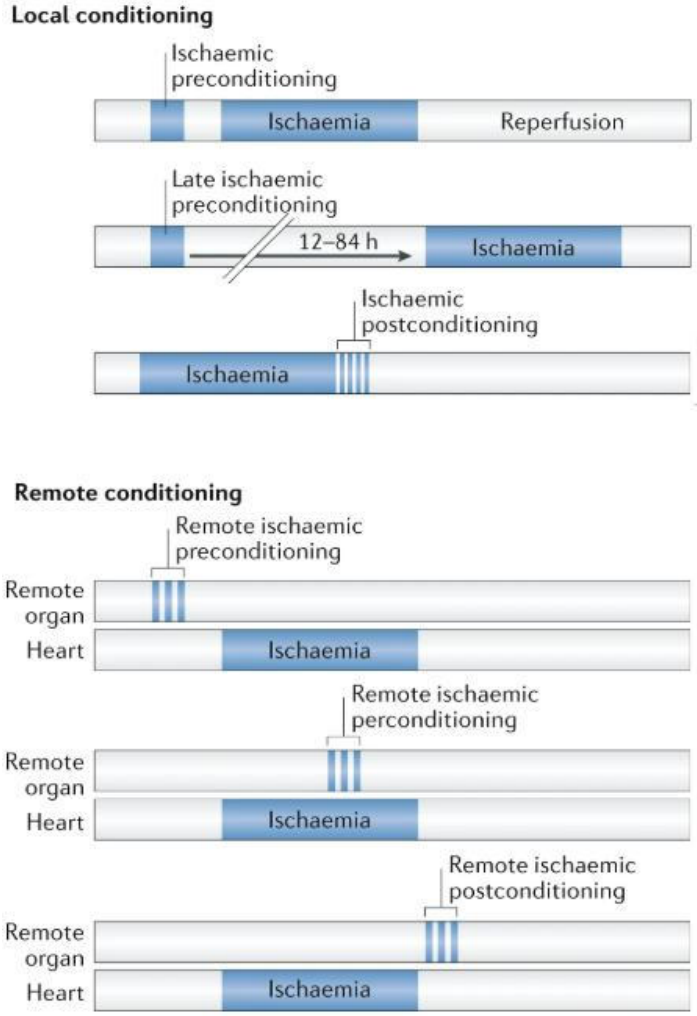


Figure 3. Overview of cardioprotective ischemic conditioning strategies. *Figure was adapted without modifications from: Gerd Heusch, Nat. Rev. Cardiol, 2020* [10].

For patients undergoing AMI (with unknown onset of myocardial index ischemia), or coronary artery bypass grafting (CABG) surgery (with known onset of myocardial index ischemia), remote ischemic conditioning could theoretically be a clinically relevant, easy-to-perform, and cheap approach for exerting cardioprotection, as only cyclic inflation (causing ischemia) and deflation (causing reperfusion) of a blood pressure cuff applied on an extremity would be needed before or during myocardial ischemia (e.g. in CABG or AMI, respectively) [16].

Although a substantial amount of pre-clinical, and smaller-scale clinical studies have demonstrated cardioprotective efficacy of remote ischemic conditioning almost unanimously [17], several robust clinical trials have challenged the translatability of these results. For instance, in the randomized, controlled CONDI-2/ERIC-PPCI trial that included AMI patients (n=5401) undergoing PCI, remote ischemic *per*conditioning failed to reduce major adverse cardiovascular events [18]. In addition, not only the primary endpoints were unaffected by in this trial, but also surrogate, soft endpoints (i.e. myocardial IS or MVO) remained unchanged after remote ischemic *per*conditioning, as assessed by cardiac magnetic resonance imaging [19].

Neutral effects of remote ischemic *pre*conditioning (RIPC) have also been shown in patients undergoing elective CABG surgery in the ERICCA (n=1612) and RIPHeart (n=1403) trials, the two most prominent phase III trials investigating efficacy of RIPC [20,21].

Overall, this translational failure has raised reasonable doubts about the real cardioprotective efficacy of remote ischemic conditioning approaches, for which the research community divided the possible underlying causes into two main parts, i.e. clinical and pre-clinical aspects. For the clinical studies, lack of Phase II "dose-finding" clinical trials (i.e. in which the number and duration of remote or in situ ischemic conditioning cycles are defined) is pointed out to play a principal role in the translational failure of cardioprotective interventions [17]. On the other hand, for the pre-clinical studies, failure for reproducibility (i.e. achieving similar results in another lab) and lack of rigor (e.g. lack of randomization, blinded evaluation) are mentioned as major causes of false-positive results, also substantially contributing to unsuccessful translation. In addition, the EU-Cardioprotection COST Action members have set out a guideline (IMPACT

Criteria) for improving the pre-clinical investigation of cardioprotective interventions in myocardial I/R-injury. As stated in this guideline, the very first step is to assess the efficacy (i.e. reduction in IS and MVO) of a cardioprotective strategy in a reductionist pre-clinical model of small animals without known confounding factors (e.g. diabetes, hypertension, dyslipidemia) [22]. These studies would not only validate the cardioprotective efficacy of an investigated strategy, but novel pathophysiological, or possible cardioprotective mechanisms could also be identified. Moreover, these studies would serve as a basis for further studies complicated by human-relevant co-morbidities and/or co-medications contributing to a successful translation.

To this end, we aimed to establish a RIPC protocol that is of robust and reproducible cardioprotective efficacy in rat models of acute myocardial I/R-injury. We sought to test the cardioprotective efficacy of various limb RIPC protocols by assessing the effect of (i) the number of remote I/R cycles, (ii) the method of causing limb ischemia, (iii) the effector organ mass (i.e. the involvement of one or both limbs), and (iv) the duration of myocardial ischemia on the cardioprotective efficacy. These studies were performed in three study centres in Hungary and the Netherlands to avoid sources of systematic bias (stemming e.g. from the operating team, or the housing environment). The experiments were carried out in an individually designed, randomized and blinded manner.

To ascertain the most commonly used methods and overall results of similarly designed studies for comparing those to our experiments, we performed a systematic review and meta-analysis.

1.3. Role of pre-clinical experiments in identifying therapeutic targets and strategies for heart failure

In parallel with the field of AMI, basic research has facilitated the achievement of the current treatment strategies for HF as well. For instance, before beta blockers were available, Eugene Braunwald and colleagues demonstrated that the blockade of the adrenergic synapses (by guanethidine) greatly reduced the cardiac output response to exercise [8]. This landmark study has shed light on the crucial role of the adrenergic nervous system in HF patients, in whom elevated circulating levels of noradrenaline, a key adrenergic neurotransmitter, were observed, suggesting an overactivity of the adrenergic system in this disease [23]. Later, it became clear that the overactivation of the adrenergic

system was a maladaptive response and intensified the severity of heart failure. Based on these results, beta-adrenergic receptor blockade was tested among HF patients with positive outcomes and became a pillar of current medical therapy in this disease [4].

Another great example of the invaluable importance of basic research in the field of HF may be the pre-clinical path leading to the development of neprilysin inhibitors. In one of the earliest studies, infusion of rats with supernatants of atrial myocardial homogenates has been shown to increase urine volume and ion excretion [24]. This pivotal study facilitated the identification of natriuretic peptides, a group of molecules excreted during cardiomyocyte stretch (caused by congestion), and exerting numerous beneficial effects on the circulatory system (e.g. natriuresis) [25]. Later, an enzyme that eliminates natriuretic peptides, currently known as neprilysin, was identified in rat kidney brush border membranes [26], and has been shown that its expression and activity became increased in rat models of severe HF [27]. This cluster of pre-clinical investigations has led to the development of neprilysin inhibitors, the effect of which has been tested in patients with HF, showing a striking clinical benefit [28]. Finally, neprilysin inhibitor (in combination with an angiotensin receptor blocker) became one of the pillars of the current medical therapy in HF patients [29].

Overall, basic research and pre-clinical studies were essential in identifying key pathophysiological mechanisms and therapeutic targets for HF. This led to successful implementation into the clinical reality and consequently great benefits for patients. The progress made in basic cardiovascular research has led to a remarkable increase in the number of possible therapeutic targets for HF. Nevertheless, only a handful of novel drugs have been developed and successfully implemented in the clinical world [30]. To improve the successful translation of basic research results into clinical testing, systematic target screening approaches became increasingly needed in basic research by "omic" techniques (e.g. transcriptomics or proteomics) [31].

However, target screening is only one of the very first steps toward creating an entirely new drug from discovery to approval. This is a time-consuming and resource-intensive process with a low success rate, particularly in the field of cardiology [32,33]. Nonetheless, *repurposing* already approved drugs for new indications – also known as drug *repositioning* – offers a strategy to mitigate risks, costs, and time associated with drug

development, as these medications have already undergone clinical trials (Phases I-III) with established safety profiles [33,34]. This "shortcut" has been successful in the cases of SGLT2 inhibitors (repurposed from type two diabetes to HF) [35] or sildenafil (repurposed from pulmonary and systemic hypertension to erectile dysfunction) [36].

In the realm of drug repositioning, the extensive family of G-protein-coupled receptors (GPCRs) presents a rich pool of potential candidates for novel HF treatments [37], as GPCRs are deeply involved in both the normal functioning of the heart and the pathological pathways of HF. Some GPCRs, such as ß-adrenergic receptors and angiotensin-II receptor type 1, are already targeted by drugs approved for HF treatment in clinical practice (e.g. by beta receptor blockers and angiotensin receptor blockers, respectively) [38]. Additionally, various other GPCRs have demonstrated significant roles in HF within pre-clinical models, showing promise for translation into clinical applications [39]. However, to date, no systematic molecular screening in the field of HF has been accomplished to identify molecules that could be targets for drug repositioning. Using this approach, an already approved drug (with a known safety profile and known side effects) would be tested for a new possible indication in HF, effectively saving effort and time in the field of drug development and implementation.

We hypothesized that cardiac GPCRs are differentially expressed in failing vs. healthy rat hearts, and that some of these GPCRs may be targets for drug repositioning. We aimed to screen for such GPCR targets by the gold standard deep RNA sequencing, and by droplet digital polymerase chain reaction (ddPCR). We then aimed to test the efficacy of a modulator of a selected GPCR in an in vitro model of cardiomyocyte hypertrophy.

2. OBJECTIVES

The main objectives of this doctoral work are to establish pre-clinical approaches aiming to enhance reproducibility, and thus, translatability in studies (i) investigating the cardioprotective efficacy of RIPC in acute myocardial I/R-injury, or (ii) identifying novel GPCR targets for HF.

2.1. Objectives for Study no. 1:

In Study no. 1, we aimed to investigate and optimize the efficacy of RIPC, a known cardioprotective strategy to reduce acute myocardial I/R-injury, as follows:

1. To test the cardioprotective efficacy of various RIPC protocols in an *in vivo* rat model of acute myocardial I/R-injury conducted in three study centres, in an individually designed, randomized and blinded manner.
2. To perform a systematic review and meta-analysis on similar studies, in order to assess that our in vivo methods, and the IS-limiting effects of RIPC achieved in our in vivo study were in accordance with the findings of previous publications.

2.2. Objectives for Study no. 2:

In Study no. 2, we aimed to identify and test novel pharmacological targets for HFrEF, both of them performed on well-established rat models of disease, as follows:

1. To investigate the differential expression of GPCR genes of pressure-overload-induced cardiac dysfunction vs. healthy rat hearts by bulk deep RNA sequencing and by ddPCR.
2. To select cardiac GPCRs for further investigation based on the following criteria: (i) only those GPCRs that were found to be significantly differentially expressed by bulk deep RNA sequencing and by ddPCR, (ii) of these, GPCRs that show significant correlation in expression measured by bulk deep RNA sequencing and by ddPCR, (iii) of these, GPCRs that have never been investigated in the context of cardiac dysfunction before, and have commercially available pharmacological modulators.

3. METHODS

Sections written in italics and put within parentheses are quotations from my own published papers and included by the permission of the respective publishing groups, as indicated in the appendix of the current doctoral thesis [4041].

3.1. Ethical approval, animal housing and pre-defined exclusion criteria

All the investigations performed in the Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary comply with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Investigations were compliant with local directives and approved by The Animal Ethics Committees at Semmelweis University, Budapest (PE/EA/1784-7/2017, and PEI/001/2374-4/2015). Experiments performed by collaborators in University of Szeged, Hungary, and in the Academic Medical Centre, University of Amsterdam (The Netherlands), investigations were compliant with local directives and approved by The Animal Ethics Committees at University of Szeged, Szeged, and by the Animal Ethics Committee of the Academic Medical Centre, Amsterdam, respectively. All studies were conducted in accordance with the ARRIVE 2.0 guidelines[42].

In the Budapest and Szeged study centres, animals were obtained from Toxi Coop Zrt. (Budapest or Dunakeszi, Hungary). In the Amsterdam study centre, animals were obtained from Charles River Laboratories, Germany. For all animal studies, young (3-10 weeks old), healthy, male Wistar rats were housed under standard conditions of 25±2°C, constant humidity, constant 12 h light-dark cycles, and were allowed free access to a standard rodent chow diet and tap water ad libitum. Before any intervention, at least five days of acclimatization period was applied. No fasting was applied before surgeries. For all in vivo studies, animals were randomly assigned to each experimental group.

Animals were excluded from further analyses if iatrogenic death or severe complications occurred during or after surgery. The cause of death was classified as either irreversible ventricular fibrillation, pulseless electrical activity, or severe bradycardia (<150 beats per minute). After suspecting life threatening events during monitoring, attempts were made

to resuscitate animals by tapping or flicking the chest, followed by chest compressions at a regular, near-physiological frequency. If the life threatening event was irreversible within 5 min, the animal was considered to be dead and excluded from further analysis

3.2. Methods for Study no. 1

3.2.1. Study design

"Methodological settings regarding RIPC timing, RIPC occlusion/reperfusion protocol, involved limbs, and techniques of RIPC occlusions, as well as methods of anesthesia, and length of cardiac index ischemia were established based on a non-systematic review of the literature performed in April 2018. Animals were randomized sequentially into experimental groups at each individual centre. Coronary ligation and RIPC/sham maneuvers were performed by independent operators, leading to a blinded application of RIPC, and results were evaluated in a blinded manner at all three study centres. Study design and protocols are illustrated in Figure 4.

At the Budapest study centre (Figure 4/A), a total of 67 animals were subjected to 30 min index myocardial ischemia followed by 120 min reperfusion. The control group (CON-C, n=15) did not receive ischemic conditioning. The positive control group (IPC-C, n=19) was subjected to cardiac ischemic preconditioning (IPC), elicited by 3 cycles of 5 min left anterior descending coronary artery (LAD) occlusion followed by 5 min reperfusion prior to index myocardial ischemia. UNIRIC-C group (n=16) received 3 cycles of unilateral RIC, and BIRIC-C group (n=17) received 4 cycles of bilateral RIC by 5 min clamping of femoral artery and vein followed by 5 min hind limb reperfusion before index myocardial ischemia (indicated as "-C" in group name).

At the Szeged study centre (Figure 4/B), a total of 29 animals were subjected to 30 min myocardial ischemia followed by 120 min reperfusion. Similar to the study centre of Budapest, the control group (CON-T, n=6) did not receive ischemic conditioning, and the positive control group (IPC-T, n=8) was subjected to IPC according to the same protocol. BIRIC-T group (n=15) received 4 cycles of bilateral RIC before index myocardial ischemia by 5 min tightening of a tourniquet on the proximal part of both hind limbs followed by 5 min reperfusion induced by loosening of the tourniquet (indicated as "-T" in group names).

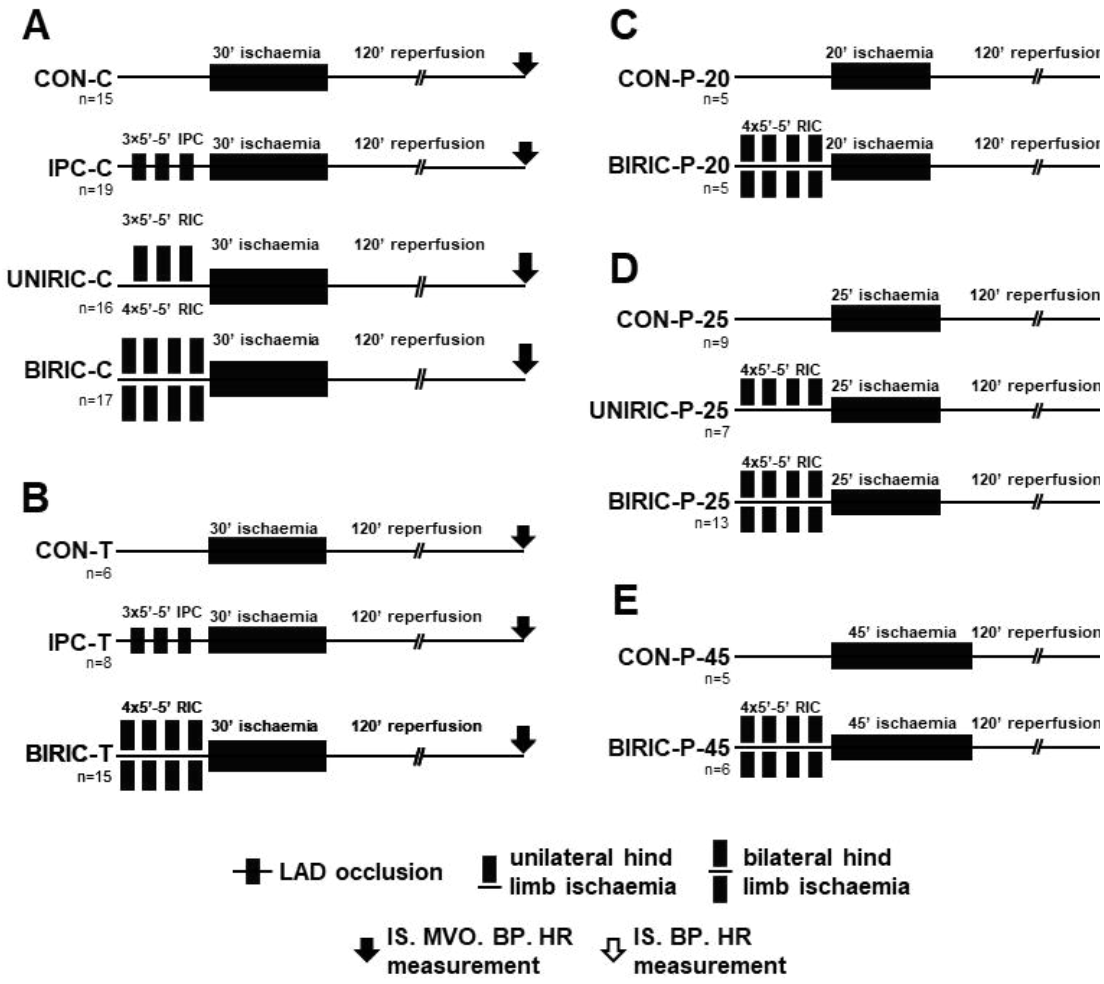


Figure 4. Experimental protocols of myocardial ischemia/reperfusion-injury and various ischemic conditioning methods in rats. A: Budapest study centre - hind limb ischemia and reperfusion by clamping femoral artery and vein. **B:** Szeged study centre - hind limb ischemia and reperfusion by tightening and loosening of a tourniquet. **C, D, E:** experimental protocol with various durations of myocardial ischemia in Amsterdam study centre - hind limb ischemia and reperfusion by using pressure cuff. Initial group sizes (*n*), as number of animals are shown under the corresponding groups. **LAD:** left anterior descending coronary artery, **RIC:** remote ischaemic conditioning, **CON:** control, **IPC:** ischaemic preconditioning, **UNIRIC:** unilateral RIC, **BIRIC:** bilateral RIC, **IS:** infarct size, **MVO:** microvascular obstruction, **BP:** blood pressure, **HR:** heart rate. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

At the Amsterdam study centre (Figure 4/C, 4/D, and 4/E), a total of 50 animals were subjected either to 20, or 25, or 45 min of myocardial index ischemia (indicated as "-20" or "-25" or "-45" in group names) followed by 120 min reperfusion. Control groups (CON-P-20, n=5; CON-P-25, n=9; and CON-P-45, n=5) did not receive ischemic conditioning. UNIRIC-P-25 group (n=7) was subjected to unilateral RIC, whereas BIRIC-P-20 (n=5), BIRIC-P-25 (n=13) and BIRIC-P-45 (n=6) groups were subjected to bilateral RIC by 4 cycles of 5 min inflation of pressure cuffs to 240 mmHg, applied on the proximal part of one or both hind limbs, followed by 5 min reperfusion by deflating pressure cuffs (indicated as "–P" in group names).

At the Budapest and Szeged study centres, stabilization before applying myocardial ischemia was 40 min, whereas at the Amsterdam study centre, stabilization time was 60 min. The time between the end of the last RIPC, or local IPC stimulus and the myocardial index ischemia was 5 min at all study centres. At all study centres, the presence of hind limb ischemia was verified by apparent pallor during ischemia and pronounced hyperemia after reperfusion. Following the 120-min myocardial reperfusion, animals were sacrificed humanely under anesthesia, and hearts were excised for further analysis. The primary endpoint was myocardial infarct size as a percentage of area at risk (IS/AAR) and secondary endpoints were microvascular obstruction (MVO) and I/R-induced arrhythmias."

3.2.2. Rat model of acute myocardial ischemia/reperfusion-injury and remote ischemic preconditioning

"At the Budapest and Szeged study centres, experimental animals were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (60 mg/kg body weight; Produlab Pharma, Raamsdonksweer, The Netherlands), and anesthesia was maintained by supplying half dose pentobarbital i.p. as required when plantar reflex could be elicited through regular paw pinch monitoring. After orotracheal intubation, rats were ventilated with a rodent ventilator (Ugo-Basile, Gemonio, Italy) with room air at a volume of 6.2 mL/kg and frequency of 69 ± 3 breaths/min.

In the Amsterdam study centre, anesthesia was induced by i.p. injection of pentobarbital sodium (80 mg/kg body weight; Euthasol 20%, Produlab Pharma, Raamsdonksweer, The Netherlands) and maintained by continuous tail vein i.v. infusion at a rate of 30mg/kg body weight/h. Following intubation, animals were pressure-control ventilated with 35%

oxygen in room air at a frequency of 65 breaths/min. Plantar reflex was monitored regularly for depth of anesthesia.

The following vital parameters were monitored throughout the whole protocol in each study centre: surface electrocardiogram (ECG) was recorded using standard needle limb electrodes (AD Instruments, Bella Vista, Australia); mean arterial blood pressure (MAP) was measured directly by carotid artery cannulation; core body temperature was recorded and maintained by rectal thermometer and heating pad (Harvard Apparatus, Holliston, Massachusetts) in Hungary, and by heating pad plus heating lamp in Amsterdam. Body temperature was maintained at physiological temperature (range 37.0-37.5 °C). At the Amsterdam study centre, right jugular vein was cannulated for administration of saline with 20 mM sodium bicarbonate at a rate of 10 ml/kg/h.

Myocardial I/R-injury was induced after left minimally invasive thoracotomy. Hearts were exposed and 5-0 prolene sutures (Ethicon, Johnson & Johnson, Budapest, Hungary) were placed around the proximal part of the LAD, and reversible myocardial ischemia was induced by tightening a snare around the LAD. At all study centres the presence of myocardial ischemia was confirmed by the appearance of ST-segment changes, I/R-induced arrhythmias, and visible pallor of the myocardial regions distal to the occlusion.

After various durations of LAD occlusion, 120 min of reperfusion was induced by relieving the snare. Reperfusion was confirmed by ST-segment normalization, occurrence of early reperfusion arrhythmias, and conspicuous hyperemia of the reperfused cardiac region. To prevent coagulation, heparin (Budapest study centre: i.p. 100 U/kg; Szeged study centre: i.v. 100 U/kg; Amsterdam study centre: i.v. 25 U/animal) was administered either within 5 minutes before the beginning of limb ischemia, at the end of LAD ischemia, and at the end of reperfusion (centres in Hungary) or at start of operation only (centre in Amsterdam)."

3.2.3. Infarct Size Measurement

"After 120 min of reperfusion, hearts were excised under deep anaesthesia and immediately perfused retrogradely through the ascending aorta with oxygenated Krebs-Henseleit solution at 37 °C on a Langendorff apparatus. After 2 min of equilibration time, the LAD was reoccluded and the area at risk (AAR) was negatively stained by retrogradely perfusing Evans blue dye through the ascending aorta. Hearts were beating

during dye injection. Viable myocardial tissue was assessed by incubation of 2 mm-thick cardiac slices in 1% triphenyltetrazolium chloride (TTC) at 37 °C. Hearts were not frozen prior to TTC staining. After weighing and scanning of cardiac slices, infarct sizes (as proportions of AARs), and AARs (as proportions of total left ventricular areas) were measured with computer planimetry by independent and blinded investigators using InfarctSize software (version 2.4b, Pharmahungary Group, Budapest, Hungary) or SigmaScan Pro 5 (Amsterdam lab)."

3.2.4. Microvascular Obstruction Measurement

"MVO was measured in Budapest and Szeged study centres. Retrogradely perfused hearts were stained with Thioflavine-S fluorescent dye immediately prior to the administration of Evans blue dye. Heart slices were put into a dark chamber and high-resolution photos were taken under UV light. The size of MVO was estimated by computer planimetry using ImageJ software (version 1.51j8, NIH, USA) and expressed as the proportion of the total left ventricular area."

3.2.5. Arrhythmia Analysis

"The severity and duration of I/R-induced arrhythmias were analyzed by independent investigators in a blinded fashion. Continuous ECG records of each animal were scored according to the Lambeth conventions and quantified as previously described by Curtis et al. [43,44]*. To increase the time resolution of the occurrence of arrhythmias, each of the ECG records was divided into five-minute intervals and every interval was individually scored according to the most severe arrhythmia type using the above-mentioned scoring system."*

3.2.6. Systematic review

"We performed a systematic review aiming to verify that the study parameters of the current in vivo studies are in good alignment with previously published in vivo rat studies of acute myocardial I/R-injury showing cardioprotection by limb RIPC. We assessed the reporting frequency of methodological parameters and their values. The systematic review was not registered to PROSPERO.

The systematic literature search was performed in accordance with the PRISMA guidelines 31, and was conducted on 23 April 2021 by N.V.S., Huimin Tian, and Viktória Zenkl. Two different search terms were used to identify articles of interest in PubMed

*(*details are available in the supplementary of the original article[40]*). Further studies were identified by consulting with experts in the field.*

*PICOS approach was used to define study eligibility criteria, aiming to find original research articles investigating cardioprotection by limb RIPC compared to control (sham procedure or no treatment) in in vivo rat models of acute myocardial I/R-injury, measuring IS/AAR by TTC staining (*details are available in the supplementary of the original article[40]*).*

Articles were excluded according to the following criteria: in vivo myocardial I/R-injury was not performed; RIPC was not performed; RIPC was not elicited by limb I/R; RIPC and myocardial I/R-injury were performed in separate animals; no IS/AAR measurement was performed by TTC staining; article was not available in English; article was published before 1993, the year of first publication on RIPC. Reviews and editorial letters were also excluded.

After excluding duplicates, titles and abstracts were screened for eligibility criteria, followed by full-text analysis. The study selection process is summarized on Figure 5. Assessment of eligibility was performed independently in a standardized, unblinded fashion by Huimin Tian and Viktória Zenkl, and was peer reviewed by N.V.S. Disagreements between reviewers were resolved by consensus, or by consulting with senior authors.

*Using a predefined data sheet, data extraction was performed by Huimin Tian and Viktória Zenkl, and was peer reviewed by N.V.S. Disagreements between reviewers were resolved by consensus, or by consulting with senior authors. A total of 56 data items were collated, containing items derived from the ARRIVE guidelines[42], and an extensive list of methodological parameters, i.e., animal and housing characteristics, perioperative measures and monitoring, interventional details regarding RIPC and MI, and endpoints additional to IS/AAR. 48 of these data items were additionally investigated as follows: for every included study, each data item was scored individually in a binary manner by giving either 0 if not reported, or 1 if reported. The sum of the individual reported data items per study divided by the total number of reportable data items, and the number of studies reporting on each individual data item divided by the total number studies were calculated (*details are available in the supplementary of the original article[40]*).”*

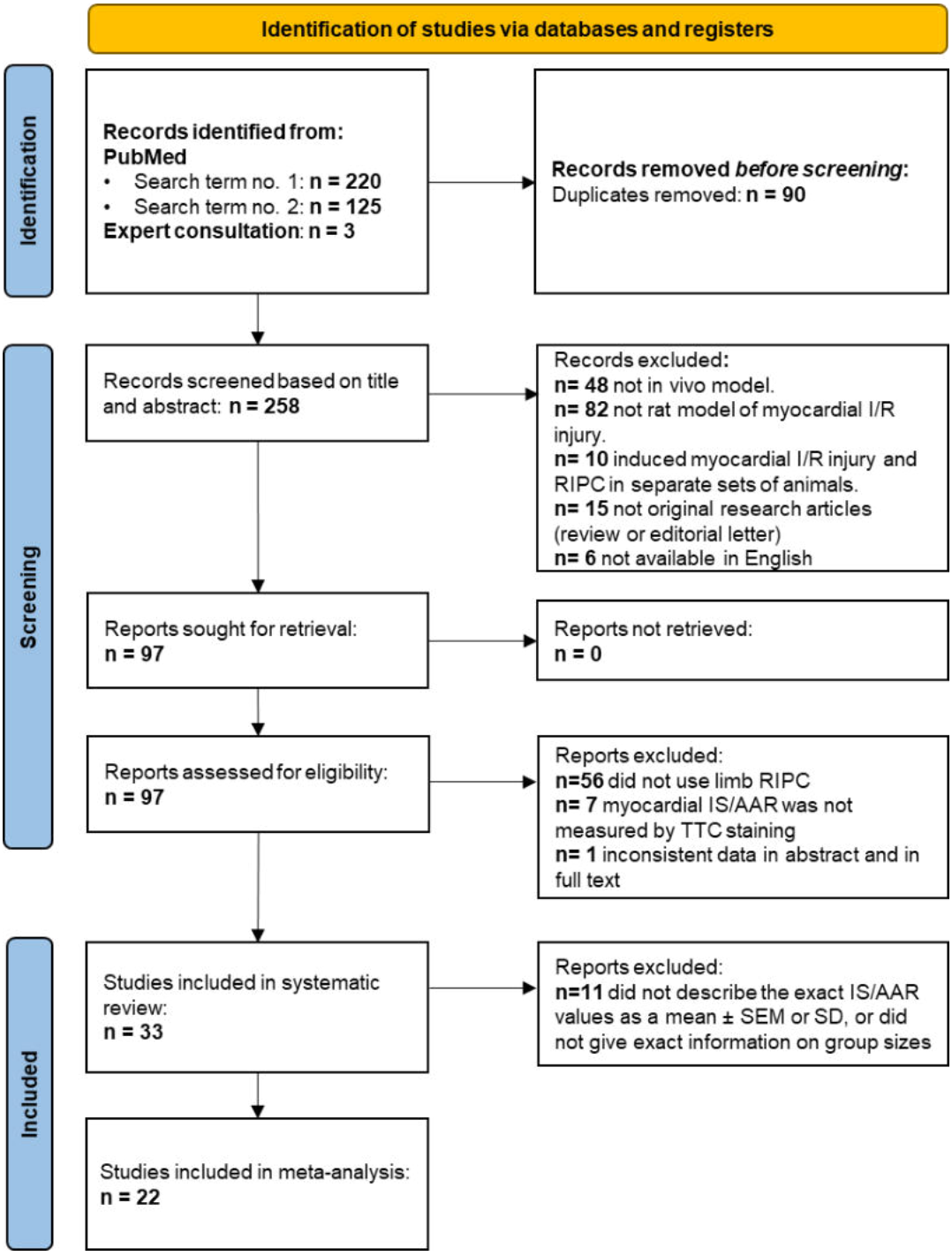


Figure 5. Flow chart of the study selection process. A total of 348 studies was identified by systematic literature search. After excluding 90 duplicates. a total for 225 studies were excluded after title and abstract. and full text screening, resulting in 33 studies included in the systematic review, and 22 articles in the meta-analysis. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

3.2.7. Meta-analysis and risk of bias measurement

"The aim of the meta-analysis was to determine the overall cardioprotective efficacy of RIPC and its correlation with the number of reported data items, as well as to assess publication bias. The primary outcome of the current meta-analysis was defined as the unstandardized, weighted mean differences (MD) between IS/AAR% of the RIPC and control groups. MD was used as all data extracted from the included studies were presented in the same units (IS/AAR%), and measured in a similar manner, i.e. by TTC staining. Articles not describing the exact IS/AAR% as mean ± SEM or mean ± SD, or lacking exact description of group sizes were excluded from the meta-analysis.

For the independent comparisons, effect sizes as MDs, and the 95% confidence intervals (CI) were used. Heterogeneity was assessed by the I2, τ2 statistics and test of heterogeneity. As the included studies were found to be highly heterogeneous, random-effects DerSimonian-Laird model was used for the analysis. To test the robustness of the current meta-analysis, sensitivity was analyzed by re-performing the meta-analysis using normalized mean difference (NMD, the mean difference divided by the mean value in the control group).

To assess whether the number of reported data items influences the effect size, a random-effects meta-regression was performed. Publication bias was assessed by visual interpretation of the funnel plot for asymmetry, the use of Egger's regression test for assessing small study effects, and by non-parametric trim-and-fill analysis."

3.3. Methods for Study no. 2

3.3.1. Rat model of transverse aortic constriction induced heart failure

"Animals were randomly assigned to sham (SHAM) or transverse aortic constriction (TAC) surgery. During the surgery, body temperature was continuously monitored and maintained at 37 °C. Pain reflex was monitored by pinching the toes every 5-10 minutes. Under isoflurane anaesthesia (5 V/V% isoflurane for induction and 1.5-2 V/V% isoflurane for maintenance after orotracheal intubation, 100% O2), after removal of the chest hair and disinfecting the surgical area, a left anterolateral thoracotomy was performed in the 2nd intercostal space under surgical stereomicroscope. After the partial removal of the thymus, the aortic arch between the brachiocephalic trunk and the left common carotid artery was identified by atraumatically dissecting the surrounding connective tissue. The aorta was constricted to the external size of a 21-gauge needle in

the TAC animals. SHAM animals underwent the same procedure without the completion of the aortic constriction. Thereafter, thorax was closed by suturing the 2nd and 3rd ribs followed by the suturing of the skin. To prevent postoperative pain and dehydration, tramadol and physiological saline (10 mg/kg in 0.5 mL) was injected subcutaneously before the animals regained consciousness. After a median follow-up of 15 weeks, echocardiography was performed and animals were euthanized by terminal arterial blood collection under deep anaesthesia, followed by cardiac sample collection for further analyses."

3.3.2. Echocardiography

*"An echocardiographic imaging unit (Vivid i; GE Healthcare, Waukesha, WI) with a 13-MHz linear probe (GE 12L-RS; GE Healthcare) at a constant frame rate of 218 frames/sec was used for echocardiographic measurements. Animals were anaesthetized by isoflurane (5 V/V% isoflurane for induction and 1.5-2 V/V% isoflurane for maintenance through a nose cone, 100% O2) and were placed onto a heating pad in a supine position to maintain body temperature at 37 °C. Chest hair was removed to obtain an optimal acoustic window. Echocardiographic cines were taken in 2D parasternal long-axis (PLAX) and short-axis (PSAX) views. Left ventricular end-systolic and end-diastolic volumes were derived from the rotational volumes of the left ventricular trace at diastole and systole, around the long axis line of the spline (LVESV, LVEDV respectively), obtained from the PLAX view in B-mode acquisition. Left ventricular stroke volume (LVSV) was calculated as LVEDV-LVESV. Left ventricular ejection fraction (LVEF) was calculated as [LVSV/LVEDV]*100. Left ventricular cardiac output (LVCO) was calculated as [heart rate*LVSV]/1000. Left ventricular end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively), as well as left ventricular anterior and posterior wall thicknesses in diastole (LVAWTd and LVPWTd, respectively) were obtained from the PSAX view in M-mode acquisition at midpapillary level. Relative wall thickness (RWT) was calculated as 2*LVPWTd/LVEDD. LVMass was calculated according to the modified cubic formula as 1.04*{[(LVEDD + LVAWd + LVPWd)*3 − LVIDd*3]*0.8 + 0.6}. Cines were obtained and analysed (EchoPAC; GE Healthcare) in a blinded fashion by a single operator."*

3.3.3. RNA isolation

"Total RNA was isolated from rat hearts by using a chloroform/isopropanol precipitation method. Briefly, Qiazol® (Qiagen, The Netherlands) was added to each sample and homogenized with TissueLyser (Qiagen, The Netherlands). Homogenates were then centrifuged, and from the clean upper phase, DNA and protein were precipitated with chloroform, followed by precipitation of the total RNA using isopropanol. Pellets were washed four times with 75% ethanol (vWR, PA, USA), then total RNA was resuspended in nuclease-free water. Finally, RNA concentrations for each sample were determined by spectrophotometry (Implen Nanophotometer® N60, München, Germany)."

3.3.4. RNA sequencing and bioinformatic analysis

"The RNA Integrity Numbers and RNA concentration were determined by RNA ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, USA) and RNA HS Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

For mRNA-Seq library construction, NEXTFLEX® Rapid Directional RNA-Seq Kit 2.0 with Poly(A) Beads 2.0 (PerkinElmer, Waltham, MA, USA) was applied according to the manufacturer's protocol. The quality and quantity of the library were determined by using High Sensitivity DNA1000 ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, USA) and dsDNA HS Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Pooled libraries were diluted to 1.6 pM for 2x80 bp paired-end sequencing with 150-cycle High Output v2 Kit on the NextSeq 500 Sequencing System (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

During preprocessing of raw sequencing data by Cutadapt (version 3.0) adapter sequences, poly(A) tails and bases with a Phred score below 30 were trimmed, reads below a length of 19 nt were filtered out [45,46]*. Quality of reads was checked by FastQC (version 0.11.8) and MultiQC (version 1.7) softwares* [47]*. Alignment and an-notation of reads were performed by Hisat2 (version 2.0.4) and featureCounts (version 2.0.0), respectively using Ensembl Rnor 6.0. reference genome and annotation* [48,49]*. Sequence alignment map (SAM) files were converted to binary form by Samtools (version 1.9)* [50,51]*. Differential expression analysis and calculation of transcripts per million (TPM) was conducted in R environment (version 3.2.3) with the usage of DESeq2 (version 1.10.1)*

package [52]*. P values of Wald tests were adjusted by Benja-mini-Hochberg method due to multiple comparisons. After the completion of whole transcriptome sequencing and bioinformatic analysis, data was screened for GPCRs."*

3.3.5. Droplet digital PCR

"Screening and quantitative assessment of cardiac GPCR expression were performed by droplet digital polymerase chain reaction (QX200 Droplet Digital PCR System; Bio-Rad Laboratories, CA, USA) using a pre-designed assay kit allowing for the measurement of 288 GPCRs (PrimePCR Pathway Plate, 96 well; GPCR Tier 1-2-3 R96, Rat; Bio-Rad Laboratories, CA, USA). Absolute quantification of the target molecules was performed using water-oil emulsion droplet technology. Briefly, each sample was fractionated into 15-20.000 droplets by QX200 Droplet Generator (DG32 Automated Droplet Generator Cartridges, Automated Droplet Generation Oil for EvaGreen; Bio-Rad Laboratories, CA, USA), and PCR amplification of the template molecules occurs in each individual droplet. Detection of gene expression measured by QX Droplet Reader System using ddPCR Droplet Reader Oil (Bio-Rad Laboratories, CA, USA). cDNA was synthesized from 4 µg total RNA by iScript Advanced cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's protocol. cDNA was further diluted 20× with RNAse-free water. All reactions were carried out using QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, CA, USA) and 100 ng of input cDNA. Level of GPCR gene expression, i.e. copies/µL was quantified by QuantaSoftTM Analysis Pro (version 1.0.596, Bio-Rad Laboratories, CA, USA)."

3.3.6. Neonatal rat cardiomyocyte model of hypertrophy

"In vitro model of cardiomyocyte hypertrophy was performed as described earlier [53]*. Briefly, primary neonatal rat cardiomyocytes (NRCM) were isolated from neonatal rats of both sexes (post-partum days 1-2). After disinfection by 70% ethanol, animals were euthanized by cervical dislocation, followed by the excision of the hearts, which were then transferred into phosphate-buffered saline (PBS, pH 7.2). Then, ventricles were separated and gently minced by using fine forceps, followed by a digestion in 0.25% trypsin solution (5 mL per heart) at 37 °C for 90 min. Cell suspension was then centrifuged at 300 g at 4 °C for 15 min, supernatant was discarded, and pellets were re-suspended in growth medium (glucose and glutamine-rich Dulbecco's MEM [10-014-CV, Corning Inc, Corning, NY, USA] supplemented with 10% fetal bovine serum, 1% L-*

glutamine [25030081, Life Technologies Corporation, CA, USA] and 1% antibiotic/antimycotic solution [30004CI, Corning Inc, NY, USA]) and plated onto 6-well plates (1.0-1.2 × 106 cells/well, in 2 mL growth medium) to eliminate fibroblasts at 37 °C for 25 min (pre-plating step). Cells of the supernatant were then re-plated onto fresh 24-well plates onto coverslips (1.0-1.2 × 105 cells/well, in 1 mL growth medium), and were kept in 5% CO2 incubator at 37 °C. On the following day, medium was changed to a fresh medium.

To achieve hypertrophy of NRCM cells, medium was changed again to a fresh medium at the second day after the isolation, and cells were treated with 1 μM of angiotensin-II (ANG-II; A9525, Sigma, St. Louis, MO, USA) in DMSO, as vehicle. For control treatment, cells received DMSO only. ANG-II treated cells received either no additional treatment, or AL-8810 (a selective inhibitor for Ptgfr; A3846, Sigma, St. Louis, MO, USA) in DMSO at the concentrations of 1 μM or 10 μM. The volume of the vehicle was equal in all the groups. 24 hours after start of treatment, cells were fixed with 2% paraformaldehyde in 1×PBS for 5 min at room temperature, then permeabilized with 0.2% Triton-X (Sigma, St. Louis, MO, USA) for 10 min. Slides were then stained with phalloidin iFluor-594 (ab176757, Abcam, Cambridge, UK) and DAPI, and images were taken by Leica LMD6 microscope. The surface area of at least 150 cells from 6 independent and random fields was measured by two blinded and independent experimenters using the ImageJ software. The average cell surface area of all measured cells in a treatment group was used as one data point, and each data point represents one biological replicate. We pre-defined the following exclusion criteria: (i) if the ANG-II treatment increased the cell surface area by <5%, and (ii) if cell viability was <90%."

3.3.7. RNA Scope® In Situ Hybridization Assay

"The in situ hybridization assay was performed on the cross section slides of the ventricles harvested from mouse heart samples using RNA Scope® Multiplex Fluorescent Kit v2 according to the manufacturer's instructions (Advanced Cell Diagnostics Pharma Assay Services, Newark, CA, United States). Briefly, formalin-fixed paraffin-embedded tissue sections were baked for 1 h at 60 °C, and then deparaffinized. Endogenous HRP activity was blocked with hydrogen peroxide (catalog number: 322335) treatment for 10 min at room temperature. Target retrieval was performed for 15 min at 100 °C, followed by Protease Plus (catalog number: 322331) treatment for 15 min at 40

°C. Probes were then hybridized for 2 h at 40 °C (3-plex Positive Control Probe-Mm (catalog number: 320881), 3-plex Negative Control Probe (catalog number: 320871), Mm-Ptgfr-O1 (catalog number: 501841, accession no.: NM_008966.3), Mm-Vim-C2 (catalog number: 457961-C2, accession no.: NM_011701.4), Mm-Cd68 (catalog number: 316611, accession no.: NM_009853.1), Mm-Cd68-C3 (catalog number: 316611-C3, accession no.: NM_009853.1), Mm-Pecam1-C2 (catalog number: 316721-C2, accession no.: NM_001032378.1), Mm-Ryr2-C2 (catalog number: 479981-C2, accession no.: NM_023868.2), and Mm-Tagln-C2 (catalog number: 480331-C2, accession no.: NM_011526.5)). Cell type-specific markers were used to identify cardiomyocytes with a probe recognizing the mRNA of Ryanodine receptor 2 (RYR2), endothelial cells with a probe recognizing the mRNA of platelet endothelial cell adhesion molecule 1 (PECAM-1, a.k.a CD31), fibroblast cells with a probe recognizing the mRNA of Vimentin (VIM), smooth muscle cells with a probe recognizing the mRNA of transgelin (TAGLN), and macrophages with a probe recognizing the mRNA of cluster of differentiation 68 (CD68), respectively. Afterwards RNA Scope amplification was performed followed by signal development with TSA fluorophores (TSA-Cy3, TSA-FITC, Akoya Biosciences, Marlborough, MA, United States). Nuclei were counterstained with DAPI (catalog number: 323108) and mounted with Prolong Gold Antifade Reagent (catalog number: 9071S, Cell Signaling Technology, Danvers, MA, United States). Specific RNA staining signal was identified as red/green dots. Fluorescent signals were detected by a Leica DMI8 Confocal microscope (Leica, Wetzlar, Germany)."

3.4. Statistical analysis

All data were generated from at least four independent experiments. Continuous data are presented as mean ± standard error of the mean (SEM). Discrete values are shown as median ± 25%-75% interquartile range. Statistical analysis was performed using GraphPad Prism (version 8.0.1). A p value of <0.05 was considered significant. Normal distribution of data was tested by Shapiro-Wilk normality test. For comparisons between two groups, either parametric two-tailed Student's t-test, or nonparametric Mann–Whitney U-test was performed. For comparison of multiple groups to each other, either one-way ANOVA followed by Fisher's LSD post hoc test, or Kruskal-Wallis test, followed by Dunn's post hoc test was used. For comparison of multiple groups to one control group, one-way ANOVA followed by Dunett's post hoc test was used. The post

hoc tests were conducted only if F in ANOVA test achieved $p < 0.05$ and there was no significant variance in homogeneity. For correlation analysis of two continuous variables, Spearman's rho (R) was computed. For comparing counts between two groups, Chi-square test was used. ROUT analysis was performed to identify outliers, with Q value = 1%. For the meta-analysis, the Egger's regression test, and the non-parametric trim-and-fill analysis, STATA 16.1 software was used.

4. RESULTS

Sections written in italics and put within parentheses are quotations from my own published papers and used with the permission of the respective publishing groups, as indicated in the appendix of the current doctoral thesis [4041].

4.1. Results for Study no. 1

4.1.1. Limb RIPC does not affect myocardial infarct size in the current in vivo rat studies

"We aimed to establish a limb RIPC protocol in an in vivo rat model of myocardial I/R-injury with an IS-limiting efficacy similar to that of the literature, as a first step of further studies in our laboratories. Studies were conducted in three study centres, and were designed and performed independently in an individually blinded and randomized fashion, with local variations in experimental parameters and techniques in the three study centres consistent with the range of approaches and variations recorded in the published literature.

In the in vivo experiments animals were excluded from further evaluation either due to death during the experiment, unsuccessful recording of ECG during the whole protocol, lack of ST-segment elevation or depression during myocardial ischemia, or technical failure at Evans blue staining (1 animal in CON-C group; 7 animals in IPC-C group; 1 animal in BIRIC-C group; 1 animal from CON-P-25; 1 animal from CON-P-45 group). Animals were excluded from the IS/AAR measurement, but not from the arrhythmia analysis due to death after randomization (1 animal in CON-C group; 2 animals in IPC-C group; 2 animals in UNIRIC-C group; 2 animals in BIRIC-C group; 1 animal in CON-T group; 1 animal in BIRIC-T group; 1 animal in BIRIC-P-45 group). Mortality rates (as % of group sizes after exclusion) did not differ significantly between experimental groups or study centres (Table 1.).

Table 1. Mortality rates shown as % of initial group sizes. Neither IPC nor RIPC affected mortality rates in any setting**.** Chi-square test was applied for each experimental group. **CON:** control, **IPC:** ischemic preconditioning, **UNIRIC:** unilateral RIC, **BIRIC:** bilateral RIC, **RIC:** remote ischemic conditioning. Group sizes (n), as number of animals are shown under the corresponding column. *Table was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

Experimental group	Initial group sizes (*n*)	Mortality (% of initial group sizes)
CON-C	15	7.14%
IPC-C	19	16.67%
UNIRIC-C	16	12.5%
BIRIC-C	17	12.5%
CON-T	6	16.67%
IPC-T	8	0%
BIRIC-T	15	6.67%
CON-P-20	5	0%
BIRIC-P-20	5	0%
CON-P-25	9	0%
UNIRIC-P-25	7	0%
BIRIC-P-25	13	0%
CON-P-45	5	0%
BIRIC-P-45	6	16.67%

IS/AAR was measured to explore the cardioprotective effects of different ischemic conditioning protocols. Sizes of the area distal to LAD occlusion (i.e. AAR) did not differ

significantly between corresponding groups, except for IPC-C group, where AAR showed a significant decrease compared to CON-C group (Table 2.).

Table 2. Area at risks as % of LV areas, and infarct sizes as % of AARs and infarct sizes as % of LV areas. Results are presented as mean ± SEM. In cases of ≥3 groups, one-way ANOVA, uncorrected Fisher's LSD post-hoc tests were used. * $p<0.05$ vs. CON, ¤ $p<0.05$ vs UNIRIC, # $p<0.05$ vs. BIRIC. In cases of 2 groups, unpaired t-tests were used. **AAR:** area at risk, **LV:** left ventricle, **CON:** control, **IPC:** ischemic preconditioning, **UNIRIC:** unilateral RIC, **BIRIC:** bilateral RIC, **RIC:** remote ischemic conditioning. Group sizes (*n*), as number of animals are shown under the corresponding column. *Table was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

Experimental group	Group sizes (*n*)	AAR (% of LV area)	IS/AAR (%)	IS/LV (%)
CON-C	13	42.99 ± 2.0	58.15 ± 2.14	25.03 ± 1.56
IPC-C	10	35.68 ± 2.8*#	23.45 ± 1.48*¤#	8.24 ± 0.55*¤#
UNIRIC-C	14	40.07 ± 1.9	53.12 ± 4.11	21.78 ± 2.63
BIRIC-C	14	43.80 ± 1.9	55.41 ± 3.60	24.31 ± 1.79
CON-T	5	37.43 ± 3.8	43.43 ± 5.08	17.60 ± 2.16
IPC-T	8	39.17 ± 8.1	20.56 ± 3.91*#	8.25 ± 2.05*#
BIRIC-T	14	42.37 ± 4.1	44.87 ± 5.84	23.17 ± 2.61
CON-P-20	5	31.59 ± 6.1	48.31 ± 10.46	16.40 ± 4.93
BIRIC-P-20	5	32.09 ± 4.5	56.27 ± 7.07	17.60 ± 3.23
CON-P-25	8	30.48 ± 3.7	59.60 ± 7.25	17.13 ± 3.28
UNIRIC-P-25	7	34.97 ± 4.3	55.10 ± 6.60	17.96 ± 1.82
BIRIC-P-25	13	31.00 ± 2.2	61.84 ± 3.71	19.83 ± 1.58
CON-P-45	4	34.93 ± 3.4	80.34 ± 5.33	26.72 ± 3.13
BIRIC-P-45	5	36.90 ± 3.8	72.29 ± 2.70	27.03 ± 2.89

At the Budapest study centre, RIPC performed by cyclic clamping and releasing of femoral vessels either uni- or bilaterally did not reduce IS/AAR (53.12±4.11% and 55.41±3.60% in UNIRIC-C and BIRIC-C groups, respectively), whereas, in the positive

control IPC-C group, IS/AAR showed a significant reduction compared to CON-C group (23.45±1.48% and 58.15±2.14%, respectively) (Figure 6A, Table 2).

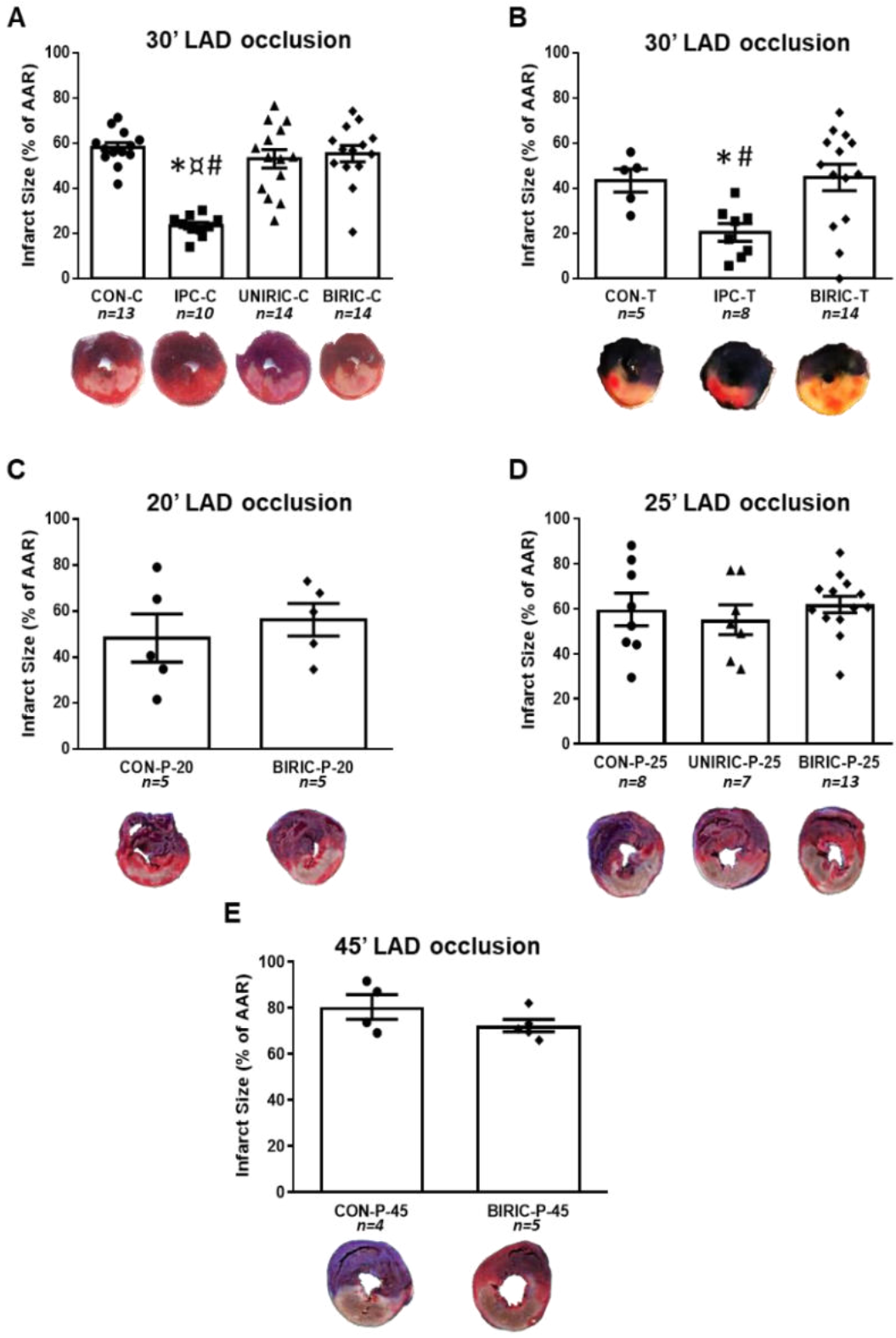


Figure 6. Myocardial infarct sizes as % of area at risk (IS/AAR). Results are presented as mean ± SEM. **A:** Budapest study centre, one-way ANOVA, uncorrected Fisher's LSD post-hoc test. **B:** Szeged study centre, one-way ANOVA, uncorrected Fisher's LSD post-hoc test. **C, D, E:** Amsterdam study centre, experimental protocol with various durations of myocardial ischaemia, in cases of **C** and **E,** unpaired *t*-test. In case of **D** one-way ANOVA, uncorrected Fisher's LSD post-hoc test, * $p<0.05$ vs. CON, ¤ $p<0.05$ vs UNIRIC, # $p<0.05$ vs. BIRIC. **LAD:** left anterior descending coronary artery, **CON:** control, **IPC:** ischemic preconditioning, **UNIRIC:** Unilateral RIC, **BIRIC:** Bilateral RIC, **RIC:** remote ischemic conditioning. Group sizes (*n*), as number of animals are shown under the corresponding groups. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

At the Szeged study centre, RIPC affected by cyclic tightening and loosening of bilateral tourniquets (in BIRIC-T group) did not decrease IS/AAR, whereas, in the positive control IPC-T group, approximately 53% relative decrease in IS/AAR was shown as compared to CON-T (44.87±5.84%, 20.56±3.91%, and 43.43±5.08% in the BIRIC-T, IPC-T and CON-T groups, respectively) (Figure 6B, Table 2).

*At the Amsterdam study centre, RIC was elicited by cyclic inflation and deflation of unilateral or bilateral pressure cuffs applied on hind limbs. Neither unilateral RIC (in UNIRIC-P-20, UNIRIC-P-25, UNIRIC-P-45 groups) nor bilateral RIC (in BIRIC-P-25 group) influenced IS/AAR when performed before either 20, or 25, or 45 min of myocardial ischemia compared to corresponding controls (CON-P-20, CON-P-25, and CON-P-45, respectively) (Figure 6C, 6D, and 6E, Table 2). Further, RIPC did not decrease cardiac necroenzyme levels compared to CON at the Amsterdam study centre (*details are available in the supplementary of the original article[40]*). IS/LV data of all studies show similar results to that of IS/AAR data (Table 2)."*

4.1.2. Limb RIPC does not affect microvascular obstruction and arrhythmia scores in the current in vivo rat studies

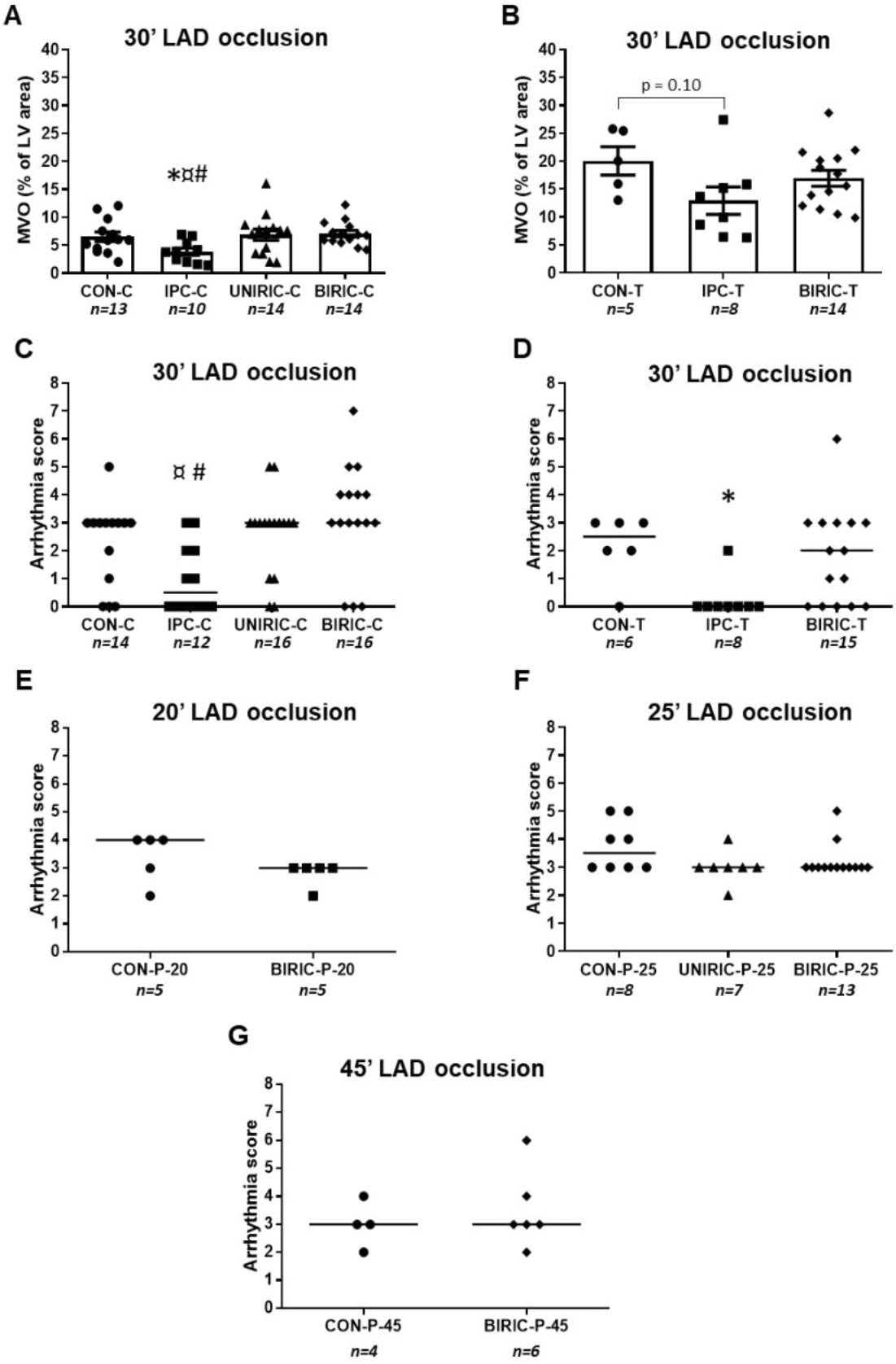
"To further examine the severity of myocardial I/R-injury, the extent of MVO was measured at the Budapest and Szeged study centres. While the positive control IPC-C significantly decreased the extent of MVO and in IPC-T MVO tended to be lower than in control groups, none of the different RIC protocols used in any of the study centres

showed a reduction in MVO as compared to corresponding control groups (Figure 7A and 7B.).

To measure the effect of different ischemic conditioning protocols on cardiac I/R-induced arrhythmias during myocardial ischemia and early reperfusion, arrhythmia analysis was performed according to the Lambeth conventions. At the Budapest study centre, cardiac arrhythmias were not significantly reduced in UNIRIC-C and BIRIC-C groups compared to the CON-C group. In the positive control IPC-C group, arrhythmia scores were significantly lower when compared to UNIRIC-C and BIRIC-C groups and tended to be lower when compared to CON-C (Figure 7C). At the Szeged study centre, the occurrence, severity, and duration of cardiac arrhythmias of BIRIC-T group did not differ significantly from that of CON-T group, whereas in the positive control IPC-T group, arrhythmia scores showed significant reduction as compared to CON-T (Figure 7D). In Amsterdam, arrhythmia scores of UNIRIC-P-20, UNIRIC-P-25, BIRIC-P-25, and UNIRIC-P-45 groups showed no significant difference in comparison with corresponding control groups (CON-P-20, CON-P-25, and CON-P-45, respectively) (Figure 7E, 7F, and 7G).

*To increase the time resolution of arrhythmias, arrhythmia scores were calculated in each 5 min interval of the entire ischemic period and the first 15 min of reperfusion. None of the RIC protocols at any study centre showed a significant difference in arrhythmia scores in any 5-min interval when either median or mean values were compared to corresponding control groups. However, cardiac arrhythmias in IPC-C group were significantly lower in several intervals as compared to either CON-C or UNIRIC-C or BIRIC-C (*data not shown, details are available in the supplementary of the original article[40]*).*

MAP and HR data throughout the whole protocol are available in the supplementary of the original article [40]*."*


A
30' LAD occlusion
MVO (% of LV area)
*¤#
CON-C n=13
IPC-C n=10
UNIRIC-C n=14
BIRIC-C n=14
B
30' LAD occlusion
MVO (% of LV area)
p = 0.10
CON-T n=5
IPC-T n=8
BIRIC-T n=14
C
30' LAD occlusion
Arrhythmia score
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CON-C n=14
IPC-C n=12
UNIRIC-C n=16
BIRIC-C n=16
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30' LAD occlusion
Arrhythmia score
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CON-T n=6
IPC-T n=8
BIRIC-T n=15
E
20' LAD occlusion
Arrhythmia score
CON-P-20 n=5
BIRIC-P-20 n=5
F
25' LAD occlusion
Arrhythmia score
CON-P-25 n=8
UNIRIC-P-25 n=7
BIRIC-P-25 n=13
G
45' LAD occlusion
Arrhythmia score
CON-P-45 n=4
BIRIC-P-45 n=6

Figure 7. Microvascular obstruction and arrhythmia scores. In case of MVO, results are presented as mean ± SEM; in case of arrhythmia scores, results are presented as median. **A:** MVO at study centre of Budapest, one-way ANOVA, uncorrected Fisher's LSD post-hoc test. **B:** MVO at study centre of Szeged, one-way ANOVA, uncorrected Fisher's LSD post-hoc test, * $p<0.05$ vs. CON, ¤ $p<0.05$ vs. UNIRIC, # $p<0.05$ vs. BIRIC. **C:** Arrhythmia scores at study centre of Budapest, Kruskal-Wallis test, multiple comparisons, Dunn's post hoc test. **D:** Arrhythmia scores at study centre of Szeged, Kruskal-Wallis test, multiple comparisons, Dunn's post hoc test. **E, F, G:** Arrhythmia scores at study centre of Amsterdam with various durations of myocardial ischaemia. In cases of **E** and **G**, Mann-Whitney test, in case of **F**, Kruskal-Wallis test, multiple comparisons, Dunn's post hoc test. **MVO:** microvascular obstruction, **LV:** left ventricle, LAD: left anterior descending coronary artery, **CON:** control, **IPC:** ischemic preconditioning, **UNIRIC:** unilateral RIC, **BIRIC:** bilateral RIC, **RIC:** remote ischemic conditioning. Group sizes (*n*), as number of animals are shown under the corresponding groups. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

4.1.3. Systematic review evidences no difference between the most often reported methodological settings in the literature and the methods used in our in vivo study

"In order to identify methodological differences and possible methodological confounding factors underlying the neutral cardioprotective results of limb RIPC seen in the current in vivo experiments, we performed a systematic review of the literature and evaluated the reporting frequencies of key methodological settings. Accordingly, a total of 348 articles were identified by the two search algorithms on PubMed and by consulting with experts, followed by the removal of 90 duplicates. A sum of 258 articles were investigated for eligibility criteria. 161 articles were excluded by title and abstract screening, and an additional 64 articles were excluded by full-text screening, resulting in a total of 33 articles included in the systematic review(45-77)*. Causes of exclusion at each level of eligibility investigation is summarized in Figure 5*. Details are available in the supplementary of the original article [40].

Out of 33 studies investigating the cardioprotective effect of limb RIPC in in vivo rat models of myocardial I/R-injury, all studies used male animals, as in our experiments.

Fifteen studies used Wistar, 17 studies used Sprague-Dawley, and 1 study used Zucker strain. Since no clear preference of animal strain was seen in the reviewed studies, the use of Wistar rats in our experiments may not be considered as a significant methodological variation.

Rat models of comorbidity were used in 12% of studies: acute or chronic hyperglycemia, hypercholesterolemia, or uremia was modelled in 6%, 3%, and 3% of publications, respectively. 64% of studies investigated the effects of different drugs on the cardioprotective effect of RIPC, either given to interrogate signal transduction, or to investigate cardioprotective effects additive to RIPC. Cardioprotective effects of different ischemic conditioning protocols were compared in 27% of studies. In our in vivo model, we used healthy and young rats without any comorbidities or comedications to avoid their known confounding effect, and at the Budapest and Szeged study centres, IPC was used as a positive control.

67% of studies used pentobarbital as anesthetic, 9% used chloral hydrate, 9% used volatile agents (isoflurane or sevoflurane), and 18% used other types of anesthetic. 6% of studies used either mixed anesthesia, or compared the effect of different anesthetics. In our in vivo experiments, we used pentobarbital anesthesia in all three centres, as reported by the majority of the reviewed studies. However, high heterogeneity of the induction or maintenance doses and administration sites was found.

Out of 33 studies, 15 publications reported on the use of room air ventilation, and 5 described the use of supplementary oxygen; however, the remaining 13 studies did not report on the type of respiratory gas. At the Budapest and Szeged study centres, room air ventilation was used, and in Amsterdam study centre, oxygen supplementation was applied, with a respiratory rate and volume similar to published studies.

All studies induced myocardial I/R-injury by occluding and releasing the LAD (sometimes named as left coronary artery in the rat). In 45.5% of studies, durations of myocardial index ischemia and reperfusion were 30 min and 120 min, respectively, and 6% of studies used recovery models of myocardial I/R-injury using a 24h reperfusion model. As at the Amsterdam study centre the experiments were designed to assess the effect of myocardial ischemia duration on RIPC efficacy, short (20 min), commonly reported (25 min), and long (45 min) durations were used, whereas at the Budapest and Szeged study centres the

most commonly applied, 30 min myocardial ischemia was performed. All study groups were subjected to 120 min reperfusion in our experiments, as reported in 82% of the reviewed studies.

The following methodological characteristics of limb RIPC were investigated by our systematic review: number of RIPC cycles, number of limbs involved, limb ischemia duration, limb reperfusion duration, and the technique of establishing limb ischemia. 21% of studies used 1 cycle, 42% used 3 cycles, 33% used 4 cycles, and 6% used three times daily 3 cycles of limb RIPC. 45.5% of studies used unilateral limb ischemia, 45.5% used bilateral limb ischemia, but 9% did not report on the number of limbs involved in RIPC. 82% of studies used 5 min limb ischemia followed by 5 min limb reperfusion, 3% used 10 min limb ischemia followed by 10 min limb reperfusion, and 15% used 15 min limb ischemia followed by 10 min limb reperfusion. 52% of studies established limb ischemia by invasive surgical methods; 30% used non-invasive methods of which 12% were conducted by uncontrolled tightening of the limb using tourniquet, and the remaining 18% used external pressure cuffs. However, 18% of studies did not give precise information on the technique of RIPC.

For the assessment of IS/AAR%, 9 of the 33 studies reported on ex vivo retrograde perfusion of the hearts with the AAR-staining dye, 19 studies stained the hearts in vivo, and 5 studies did not exactly describe the staining method of AAR. As the Langendorff method is frequently used for staining the AAR, here we also used the ex vivo retrograde perfusion method.

During the data collection process other study parameters were found to be reported with a lower frequency, but if reported, a remarkable heterogeneity between the studies was identified. Therefore, we decided to assess the reporting frequencies of study parameters, based on whether a given data item was reported or not (Figure 8).”

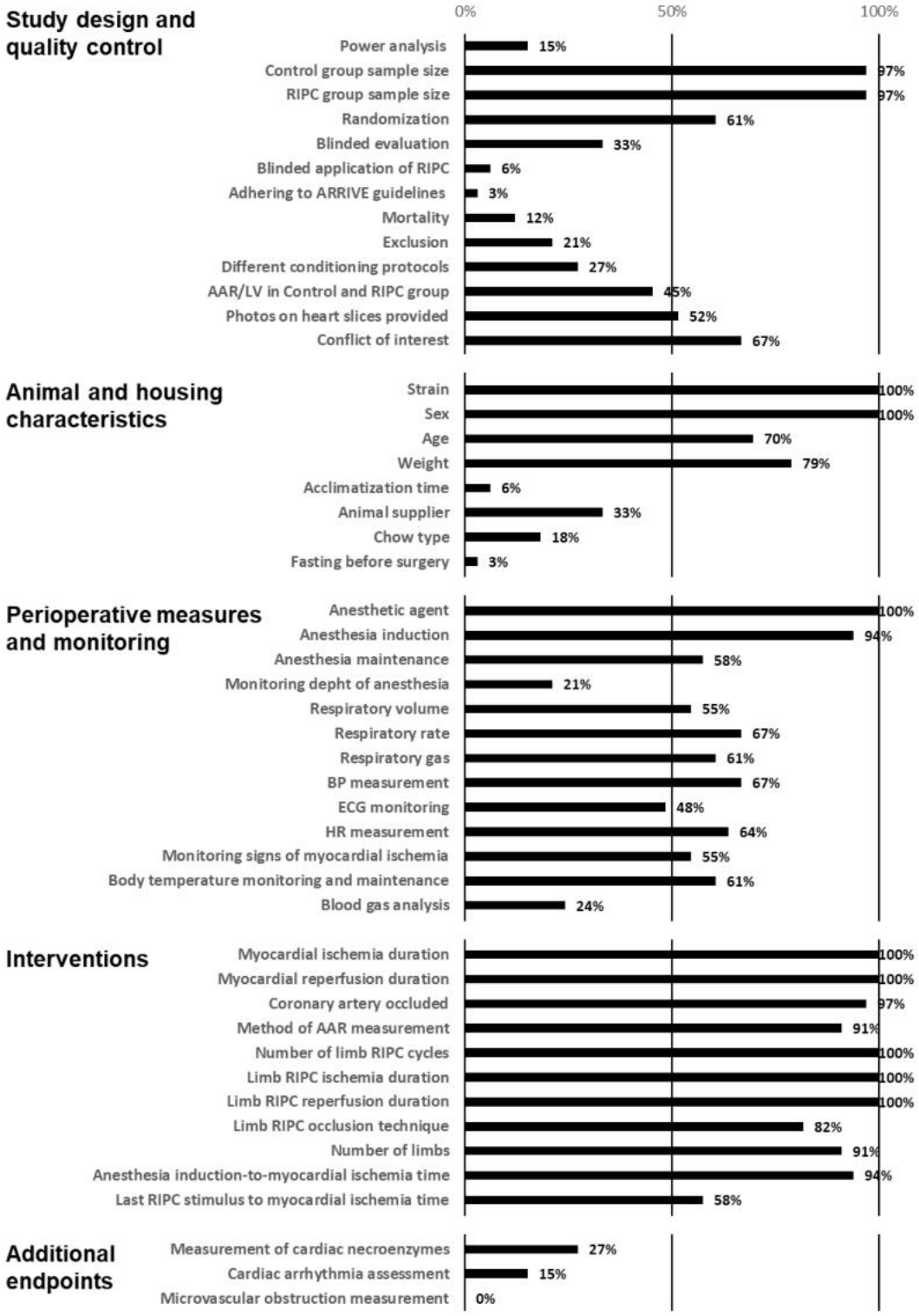

Figure 8. Frequencies of reporting a parameter by category. Number of studies reporting on the certain parameters are expressed as a percentage of all included studies. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

4.1.4. Systematic review identifies insufficient reporting in a high proportion of in vivo rat studies on cardioprotective effects of limb RIPC

"To enable measurement of the overall reporting of the reviewed studies, the number of reported items of each study was assessed as described in the Methods section, Systematic review sub-section.

All study characteristics collected according to the data items, as well as the number of reported data items in each included study, are available in the supplementary of the original article [40]. *The median of the number of reported items was 28 out of 48 (inter-quartile range: 24-31), and the number of reported data items did not increase in correlation with the publication date (Figure 9A).*

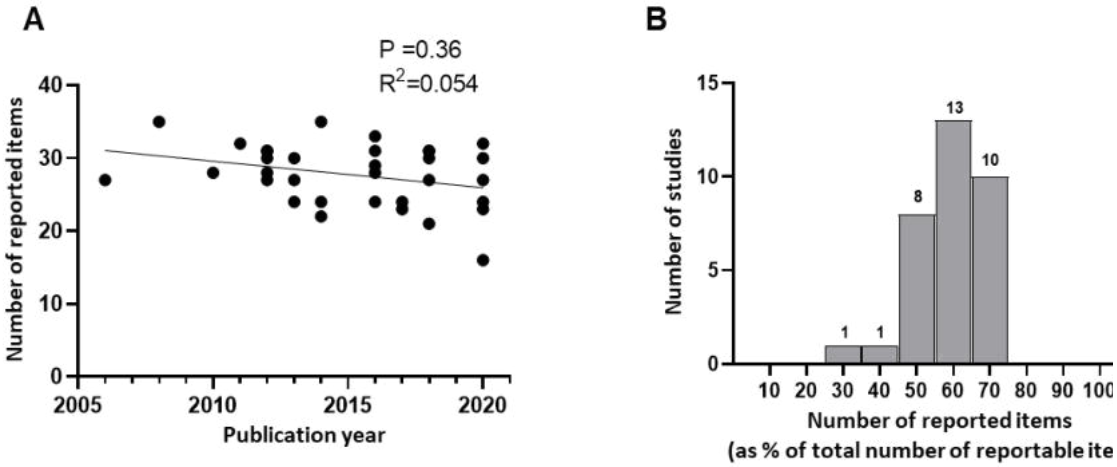

Figure 9. A: Number of reported items per study correlated to publication year (non-parametric Spearman correlation), each dot represents a single study included into the systematic review. **B:** Histogram on the distribution of studies with different numbers of reported items, calculated as a percentage of the total number of reportable, number of studies are represented as numbers marked on the corresponding columns. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

Reporting frequencies on each parameter are shown in Figure 8, resulting in a notable lack of reporting on animal housing; use of quality control measures, e.g., anesthetic reflex surveillance, AAR/LV data; and measuring other consequences of myocardial I/R-injury such as arrhythmias or MVO. We also measured the distribution of studies with

different levels of reported data items (shown in Supplementary Figure 9B), demonstrating that only 30% of the included studies reported 60%-70%, but none of them reported more than 70% of the investigated study parameters. These data suggest that the number of reported items in the majority of the reviewed studies is insufficient or inadequate for full evaluation and reproduction.

The number of reported data items of 44 was achieved in the Budapest, Szeged and Amsterdam study centres, resulting in 92% of the scored data items. As no prospective sample size calculation was done in the three study centres, no clear adherence to the ARRIVE guidelines could be stated, resulting in the loss of 2 points out of 48. In the Budapest and Szeged study centres no blood gas analysis and no cardiac necroenzyme measurement was performed, whereas in the Amsterdam study centre no IPC positive control group was used, and no MVO measurement was conducted."

4.1.5. Meta-analysis shows an overall IS-limiting effect independently from the number of reported items and showed no significant publication bias in in vivo rat studies on cardioprotective effects of limb RIPC

"To be able to compare the IS-limiting effect of RIPC in the current in vivo studies to findings of previous publications, we conducted a meta-analysis on the reviewed studies. In addition, we assessed the relation between the number of reported data items and effect size using meta-regression. Furthermore, since we could not identify differences between the methodological parameters of our neutral in vivo studies and that of the studies in the literature, the question was raised whether there may be studies with smaller IS-limiting or neutral outcomes regarding limb RIPC withheld from publication. To assess the possibility of this phenomenon, we conducted a publication bias assessment.

From the 33 studies included in the systematic review, only 22 were included in the meta-analysis, as the remaining 11 articles did not describe exact IS/AAR values as a mean ± SEM or SD, or did not give exact information on group sizes (Figure 5.), necessary for meta-analysis. Of these 22 articles, data on 23 controlled comparisons of RIPC in rat models of acute myocardial I/R-injury without any comorbidity or comedication were extracted, including a total of 189 animals in the control groups, and 188 animals in the RIPC groups. In case of studies, where the effect of different anesthetics on RIPC efficacy was investigated, only the groups with the reference anesthetic was included.

Heterogeneity of the studies was found to be significant (I2 = 75.51% and τ2 = 42.87; p < 0.001). RIPC reduced IS/AAR by 21.28% (95% CI 18.07 - 24.49) compared to control group (df=22; p<0.00001), as summarized in Figure 10A. By re-performing the analysis using NMD, similar results were obtained, as heterogeneity was observed to be significant (I2 = 49.21% and τ2 = 74.15; p < 0.001), and the overall effect was 34.68 favouring RIPC towards control (95% CI 29.37 – 39.99).

We investigated the impact of the number of reported data items on the outcomes by performing meta-regression using the number of reported items as the independent variable, and mean difference as the dependent variable, and found no significant relationship between them (estimated meta-regression coefficient: -0.666 [95% CI: -1.395 - 0.063]; p=0.07).

Publication bias was assessed by visual interpretation of the funnel plot (Figure 10B), suggesting that small studies with small or no cardioprotective efficacy of RIPC may be underrepresented among published results, however, the Egger's regression test showed no significant publication bias (p=0.07), and the non-parametric trim-and-fill method did not indicate missing studies which would compensate the asymmetry of the funnel plot. The funnel plot using NMD, and the result of the Egger's test (p=0.07) was similar to the analysis using MD. In this case the trim-and-fill analysis indicated three missing studies to compensate for the asymmetry of the funnel."

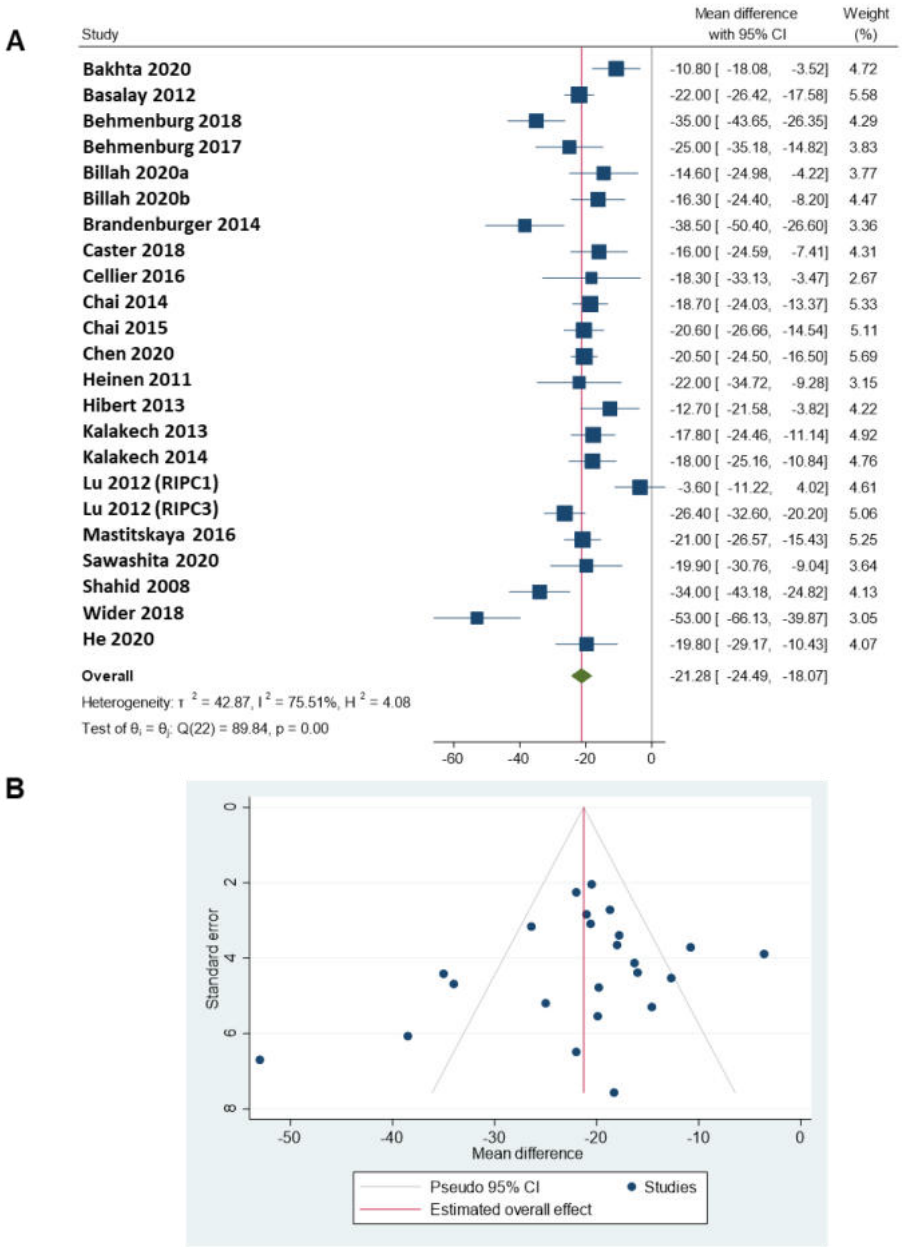

Figure 10. A: Forest plot of the meta-analysis on cardioprotective efficacy (defined as a reduction in IS/AAR%) of RIPC in *in vivo* rat models of acute myocardial I/R-injury, using random-effects DerSimonian-Laird method. A total of 22 controlled comparisons were made, with a total of 194 and 195 animals included in the control and RIPC groups, respectively. **B:** Funnel plot for assessment of publication bias. The red line represents the estimated overall mean effect size, and the grey lines represent the pseudo 95% CI accordingly. Publication bias was assessed visually, followed by Egger's regression test, and non-parametric trim-and-fill analysis. *Figure was adapted without modifications from: Nabil V. Sayour et al., Cardiovasc Res, 2023* [40].

4.2. Results for Study no. 2

4.2.1. Echocardiographic characterization of pressure-overload-induced cardiac dysfunction in rats

"To identify novel pharmacological targets for HF with a high possibility to be a candidate for drug repurposing, we aimed to investigate GPCR expression in pressure-overload-induced cardiac dysfunction vs. normal rat hearts. To this end, we used a widely established rat model by performing TAC surgery and assessed cardiac function and morphology by terminal echocardiography (Figure 11.). TAC surgery significantly reduced ejection fraction, cardiac output and fractional shortening compared to SHAM surgery at 15-18 weeks following operation. These findings were paralleled by a significant enlargement in cardiac dimensions, i.e., an increase in end-systolic and end-diastolic volumes and a significantly decreased stroke volume in the TAC group compared to the SHAM. Morphological analyses showed a significant increase in anterior, posterior and relative wall thicknesses of the heart, resulting in a significantly higher calculated left ventricular mass in the TAC vs. SHAM group (Figure 11.). All in all, TAC surgery caused a severe phenotype of left ventricular pressure-overload-induced systolic dysfunction and cardiac hypertrophy, as expected. After terminal echocardiography, cardiac samples were obtained from the left ventricles of both TAC vs. SHAM animals, and comparative transcriptional analyses were performed (Figure 12/A.)."

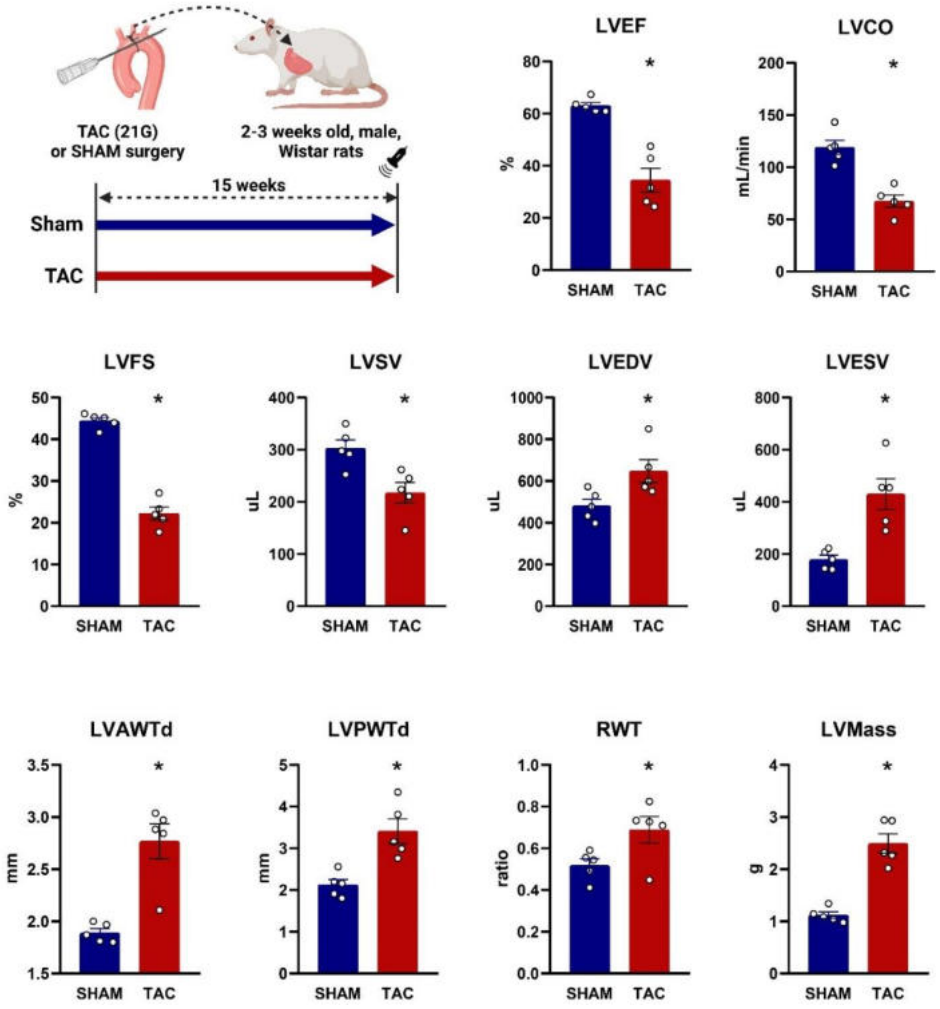

Figure 11.: Echocardiographic parameters 15 weeks after transverse aortic constriction or sham surgery in rats. Left upper panel represents the experimental design. **TAC**: transverse aortic constriction; **LVEF**: left ventricular ejection fraction; **LVCO**: left ventricular cardiac output; **LVFS**: left ventricular fractional shortening; **LVSV**: left ventricular stroke volume; **LVEDV**: left ventricular end-diastolic volume; **LVESV**: left ventricular end-systolic volume; **LVAWTd**: left ventricular anterior wall thickness in diastole; **LVPWTd**: left ventricular posterior wall thickness in diastole; **RWT**: relative wall thickness; **LVMass**: calculated left ventricular mass. Measurements were performed on n=5 individual animals for both the SHAM and the TAC groups. *: $p<0.05$, unpaired Student's *t*-test, shown as mean ± SEM. *Figure was adapted without modifications from: Nabil V. Sayour et al., IJMS, 2023* [41].

4.2.2. Bulk RNA sequencing identified 69, and ddPCR identified 27 cardiac GPCR genes to be differentially expressed in TAC vs. SHAM rat hearts

*"After the completion of in vivo disease modelling, unbiased whole transcriptome analysis on the cardiac samples of both TAC and SHAM hearts was performed by bulk RNA sequencing, resulting in a total of 5864 genes to be significantly differently expressed in TAC vs. SHAM hearts, after correcting for multiple comparisons (*Supplementary table S1 in the original article shows all significantly differentially expressed genes found by bulk RNA sequencing [41]*). Of note, several genes characteristic to the failing heart was found to be significantly differentially expressed in TAC vs. SHAM hearts, supporting echocardiographic findings [e.g. nppa (FC: 4.216, p<0.001), nppb (FC: 1.799, p<0.001), atp2a2 (FC: 0.898, p<0.001), myh7 (FC: 1.424, p<0.001), and several collagene genes].*

Results of the bulk RNA sequencing were further filtered for a total of 288 GPCR genes (Figure 12/A.). Of these, 69 GPCR genes were found to be significantly differently expressed in TAC vs. SHAM hearts, 53 of which were up-regulated, and 16 were down-regulated (Figure 12/B., left panel; Supplementary table S2 in the original article shows all significantly differentially expressed GPCR genes measured by bulk RNA sequencing [41]*).*

*Parallel to the bulk RNA sequencing, we investigated the gene expression of a total of 288 GPCR genes by using ddPCR (Figure 12/A.). Of these GPCR genes, ddPCR found 27 genes to be significantly differentially expressed in TAC vs. SHAM hearts, 20 of which were up-regulated, and 7 of which were down-regulated (*Figure 12/B., right panel; Supplementary table S3 in the original article shows all significantly differentially expressed GPCR genes measured by ddPCR [41]*). After the completion of the transcriptomic analyses, we aimed to compare the cardiac GPCR gene expression profiles measured by bulk RNA sequencing and ddPCR."*

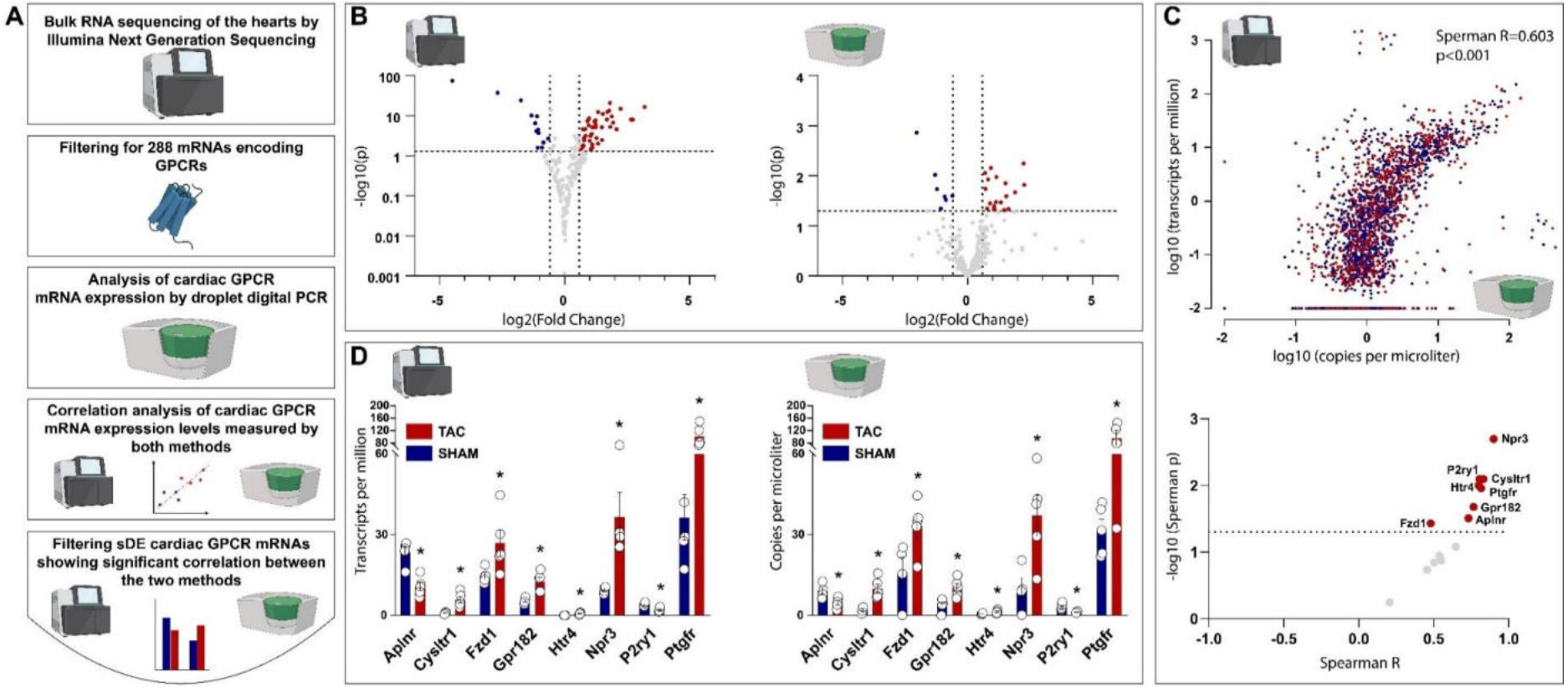
A
Bulk RNA sequencing of the hearts by Illumina Next Generation Sequencing
Filtering for 288 mRNAs encoding GPCRs
Analysis of cardiac GPCR mRNA expression by droplet digital PCR
Correlation analysis of cardiac GPCR mRNA expression levels measured by both methods
Filtering sDE cardiac GPCR mRNAs showing significant correlation between the two methods
B
-log10(p)
log2(Fold Change)
C
Sperman R=0.603
p<0.001
log10 (transcripts per million)
log10 (copies per microliter)
-log10 (Sperman p)
Spearman R
Npr3
P2ry1
Htr4
Cysltr1
Ptgfr
Gpr182
Aplnr
Fzd1
D
Transcripts per million
Copies per microliter
TAC
SHAM
Aplnr
Cysltr1
Fzd1
Gpr182
Htr4
Npr3
P2ry1
Ptgfr

Figure 12.: Transcriptional characterization of G-protein coupled receptors in TAC vs. SHAM rat hearts by bulk RNA sequencing and droplet digital PCR. (**Panel A**) represents the workflow for transcriptional characterization of cardiac G-protein coupled receptors (GPCRs), followed by the comparison of results obtained from bulk RNA sequencing and droplet digital PCR (ddPCR), and by the selecting of GPCRs that were found by both methods to be significantly differentially expressed in a significant correlation. (**Panel B**) volcano plots showing down- and up-regulation of cardiac GPCR expression measured by bulk RNA sequencing (left figure) and by ddPCR (right figure). (**Panel C**) upper figure represents correlation between bulk RNA sequencing (Y axis) and ddPCR (X axis) values for gene expression, each dot represents one GPCR gene measured in one sample; lower figure represents GPCR genes that were found to be significantly differentially expressed (sDE) in TAC vs. SHAM rat hearts identified by both methods, only eight of which showed significant correlation between the two methods. (**Panel D**) box plots showing those eight GPCR genes that were found to be sDE in TAC vs. SHAM rat hearts identified by both methods in significant correlation between the two methods. For each GPCR gene, measurements were obtained from n=5 individual rat heart from both the SHAM and the TAC groups. For bulk RNA sequencing, *: $p<0.05$ vs. SHAM, Wald test; for ddPCR, *: $p<0.05$ vs. SHAM, Student's unpaired *t*-test, shown as mean ± SEM. *Figure was adapted without modifications from: Nabil V. Sayour et al., IJMS, 2023* [41].

4.2.3. Comparative analysis of cardiac GPCR gene expression profiles measured by bulk RNA sequencing and ddPCR shows significant correlation

"To compare how GPCR gene expression profiles may differ between bulk RNA sequencing and ddPCR, we performed a correlation between the TPM values (obtained from bulk RNA sequencing data) and the copies/µL values (obtained from ddPCR data) of all the 288 cardiac GPCR genes investigated from both the TAC and SHAM animals. The overall results of both methods showed significant correlation (Figure 12/C., upper figure, Spearman R=0.603, P<0.001).

*To avoid sources of selection bias for identifying possible GPCR targets in HF, we aimed to focus on GPCR genes that were found to be significantly differentially expressed in TAC vs. SHAM hearts both by bulk RNA sequencing, and by ddPCR, and show a significant correlation between the two methods (Figure 12/A.). 14 cardiac GPCR genes were identified by both methods to be significantly differentially expressed with similar direction of expression changes (up-, or down-regulated) in TAC vs. SHAM hearts, 8 of which genes showed a significant correlation (*Figure 12/C., lower figure; Supplementary Figure S1 in the original article shows correlation analyses for each significantly differentially expressed GPCRs identified by the two methods [41]*). Individual values for gene expression levels of these cardiac GPCR genes are shown on Figure 12/D. (left figure for bulk RNA sequencing, right figure for ddPCR)."*

4.2.4. Filtering for novel GPCR targets identifies prostaglandin F2α receptor to be a potential GPCR target with relevant clinical translatability in heart failure

"Of the 8 cardiac GPCR genes that were identified to be significantly differentially expressed in TAC vs. SHAM hearts by both methods and a significant correlation, we sought for genes that (i) can be targeted by commercially available small molecules, and (ii) have not been described yet in the context of HF.

Previous investigations have demonstrated that inhibition of Npr3 improves HF after myocardial infarction in mice [87], and that Htr4 was involved in the pathogenesis of ischemic HF in rats [88]. However, to the best of our knowledge, Ptgfr was demonstrated to be involved in atherosclerosis and blood pressure regulation [89], bleomycin-induced pulmonary fibrosis [90], as well as in the collagen synthesis of cardiac fibroblasts [91], but Ptgfr has never been investigated in the pathogenesis of systolic dysfunction so far.

All in all, Ptgfr was chosen to be the candidate for further analyses, as this GPCR gene (i) showed a significant increase in TAC vs. SHAM hearts both by bulk RNA sequencing and ddPCR, with a significant correlation between the two methods (Spear-man R=0.817, P=0.011), (ii) has a commercially available antagonist (AL-8810), and (iii) has not been described yet in the context of HF.

Of note, Cysltr1 was also found to fit to most of the above-stated criteria [as (i) the expression profile of Cysltr1 also significantly correlates between bulk RNA sequencing and ddPCR, and (ii) Cysltr1 has already approved antagonists that are currently indicated in maintenance treatment of asthma], however, Cysltr1 was previously brought in touch with HF [92]*."*

4.2.5. Ptgfr is expressed by cardiac fibroblasts and cadiomyocytes

"First we aimed to characterize which cell types of the cardiac tissue expresses Ptgfr. To this end, we performed RNAScope in situ hybridization analyses on the cardiac tissue of mice. We sought for co-localization of Ptgfr mRNA with markers of cardiomyocytes (RYR2), endothelial cells (CD31), fibroblast cells (VIM), smooth muscle cells (TAGLN), or macrophages (CD68). We found that Ptgfr gene was primarily expressed on RYR2+ cardiomyocytes and VIM+ fibroblast cells (Figure 13/A. and 13/B., respectively, Supplementary figure S2 in the original article shows representative RNA Scope images of Ptgfr expression in CD31+, TAGLN+ and CD68+ cardiac cells [41]*). Our findings showed similar results to that of the Tabula Muris open dataset for single cell transcriptomic data* [93]*."*

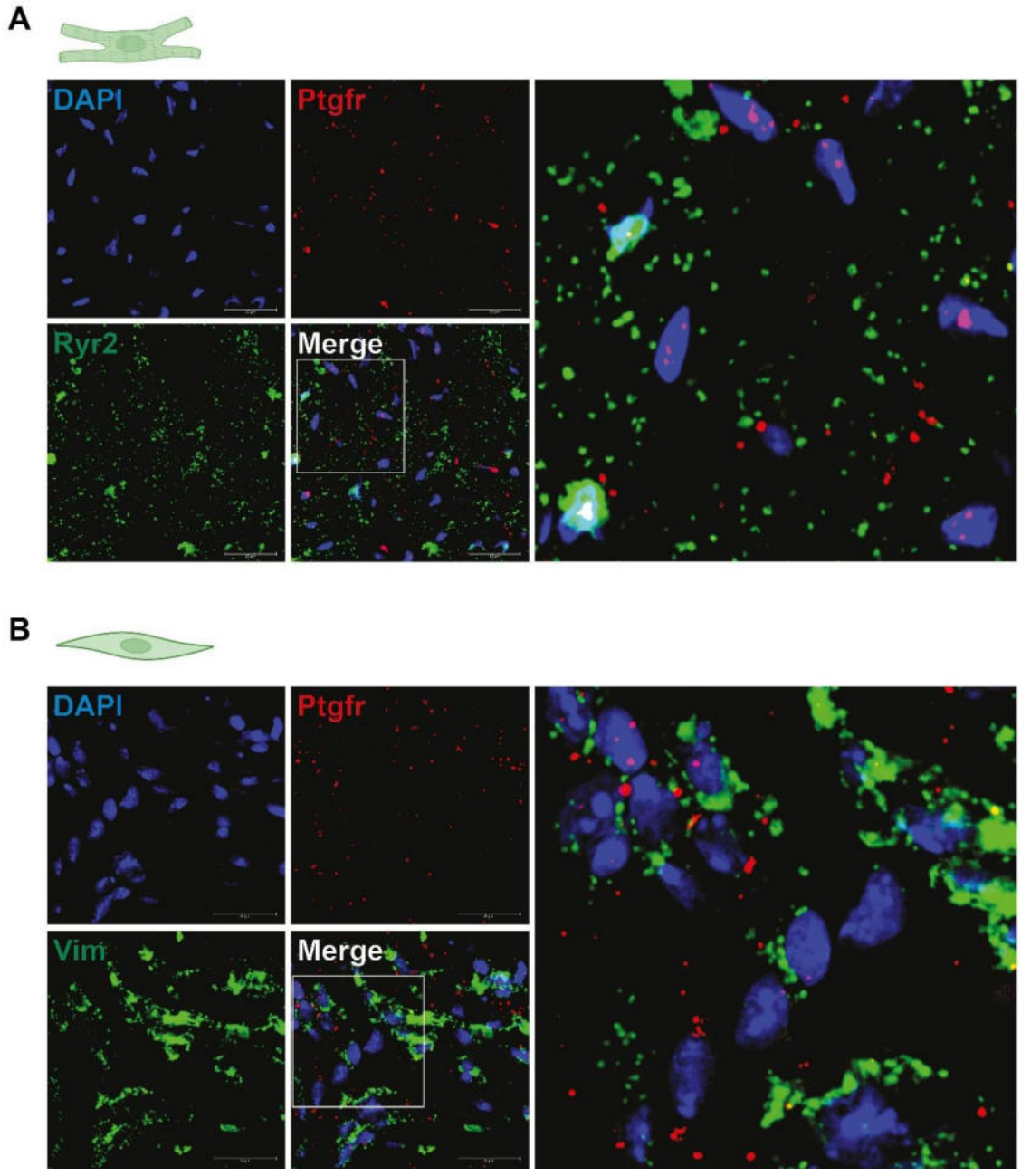

Figure 13.: Prostaglandin $F_{2\alpha}$ receptor expression profile in murine hearts. (**A**) and (**B**) demonstrated representative RNA-Scope images of murine hearts showing *Ptgfr* expression on cardiomyocytes ($Ryr2^+$ cells) and cardiac fibroblasts (Vim^+ cells), respectively, with no technical replication or quantification. *Figure was adapted without modifications from: Nabil V. Sayour et al., IJMS, 2023* [41].

4.2.6. Ptgfr inhibition by AL-8810 reverts angiotensin-II induced hypertrophy of neonatal rat cardiomyocytes.

"Cardiomyocytes were identified to express Ptgfr in murines, as shown above. As the TAC model of HF is characterized by pressure overload-induced cardiac hypertrophy, we aimed to test the effect of Ptgfr-inhibition in an in vitro model that recapitulates these characteristics of TAC model. To this end, we used an in vitro model of ANG-II-induced hypertrophy of neonatal rat cardiomyocytes, as described previously [94] *(Figure 14/A.). In this model, we tested the effect of AL-8810, a highly selective Ptgfr inhibitor of lower (1 μM) or higher (10 μM) concentrations. These concentrations were selected based on previous publications using AL-8810 on in vitro cell cultures* [95–98].

We found that ANG-II treatment resulted in a significant, ~1.25-fold increase in cell surface area of NRCM cells compared to the vehicle treated group (Figure 14/B. and 14/C.). Cardiomyocyte hypertrophy could be reverted by the 10 μM, but not the 1 μM concentrations of AL-8810 treatment (Figure 14/B. and 14/C.). This is the first demonstration of the anti-hypertrophic effect of Ptgfr-inhibition on cardiomyocytes."

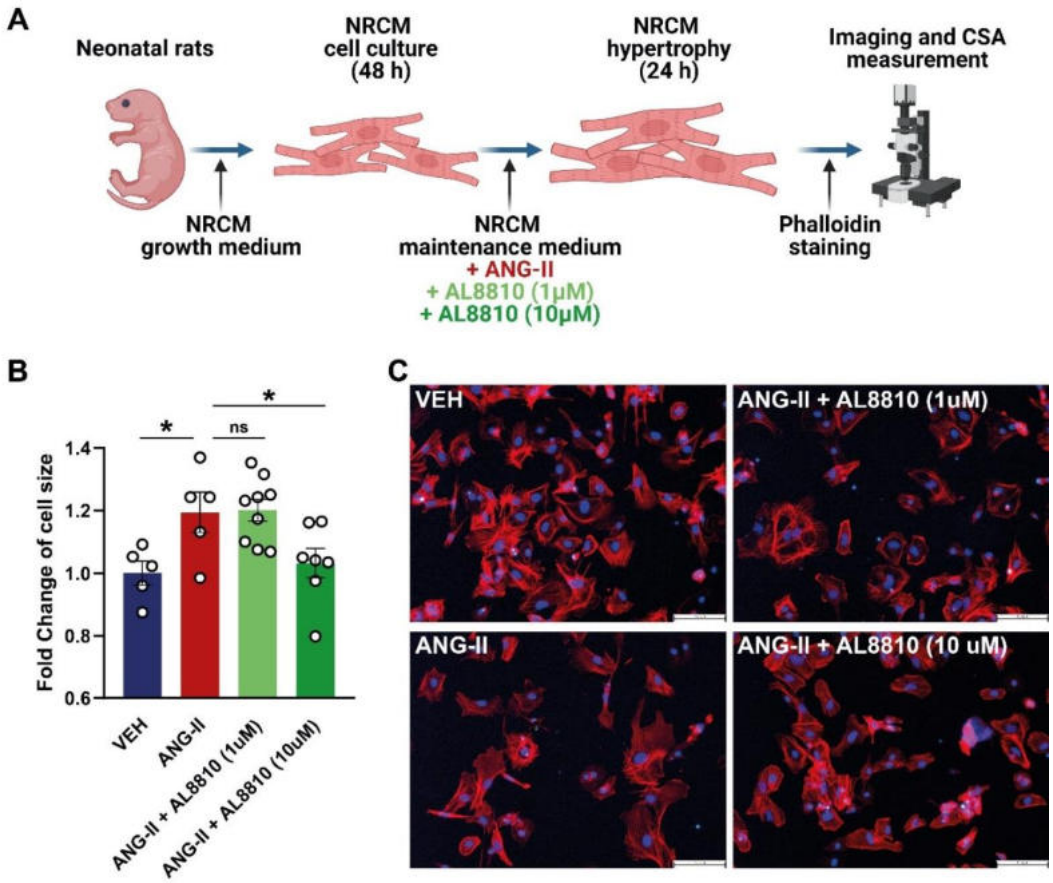

Figure 14.: Effect of Prostaglandin $F_{2\alpha}$ receptor inhibition on *in vitro* angiotensin-II-induced cardiomyocyte hypertrophy. (**A**) represents the experimental workflow of neonatal rat cardiomyocyte (NRCM) cell culturing, induction of hypertrophy by angiotensin-II (ANG-II), treatment with prostaglandin $F_{2\alpha}$ receptor (*Ptgfr*) antagonist AL8810 in lower (1 μM) and higher (10 μM) doses, and measurement of cell surface area (CSA) after phalloidin staining. (**B**) shows significant increase in CSA of NRCM cells by ANG-II, which could be reverted by 10 μM of AL8810; biological replicates of n=5-9/group, *: $p<0.05$ vs. ANG-II, One-way ANOVA, Dunett's post hoc test, shown as mean ± SEM. (**C**) shows representative images of phalloidin staining for each treatment groups. *Figure was adapted without modifications from: Nabil V. Sayour et al., IJMS, 2023* [41].

5. DISCUSSION

5.1. Cardioprotective efficacy of RIPC in myocardial I/R-injury

In study no. 1, we performed in vivo rat experiments in three laboratories to assess the cardioprotective efficacy of RIPC in myocardial I/R-injury in an individually designed, randomized and blinded manner. In addition, in two of the study centres, IPC was used as a "positive control" for setting the level of maximal cardioprotection achievable in those labs. In addition, we tested the cardioprotective efficacy of different RIPC protocols by using different numbers of limb I/R cycles, different methods to exert limb I/R, different numbers of limbs, and different durations of myocardial ischemia.

Unexpectedly, for the first time in the literature, we demonstrated no cardioprotective effects of limb RIPC, as we found (i) no decrease in myocardial IS, (ii) no decrease in the size of microvascular obstruction, and (iii) no change in duration and occurrence of reperfusion arrhythmias in neither of the study sites. Of note, IPC used as a positive control, was cardioprotective. As our experimental results were in contrast to those in the literature, we hypothesized that some of the key methods (e.g. in performing the RIPC procedure) in our in vivo study differs from the previously published studies. To explore this, we performed a systematic review of similar RIPC studies in rats.

We analysed parameters ranging from animal husbandry characteristics to preoperative or intraoperative procedures, including quality control measures, as these parameters are pertinent for pre-clinical reproducibility. Overall, we found that the majority of the included studies reported 50-60% of the data items, and when reported, methods are heterogeneous. Of note, the methodological settings in our in vivo experiments were within the boundaries of the published literature.

Although data items for the "interventions" regarding myocardial I/R-injury and limb RIPC are well-reported, perioperative measures ranging from details in anaesthetic regimes to monitoring vital parameters. For instance, it has been well evidenced that certain types of anesthetic protocols influence the cardioprotective efficacy of ischemic conditioning. A key pre-clinical study by Behmenburg et al. demonstrated that propofol anesthesia diminishes cardioprotection by RIPC in vivo [78], a factor that has been brought in context with the neutral results of trials assessing cardioprotective efficacy of RIPC in patients undergoing CABG [99]. On the other hand, volatile anesthetics (e.g. isoflurane) or ketamine/xylazine anesthesia were also demonstrated to be cardioprotective per se [100101], and thus, should be used with caution in studies assessing

cardioprotective efficacy of pharmacological or non-pharmacological approaches. Pre-clinical recommendations suggest that pentobarbital should be the anesthetic of first-choice in pre-clinical studies of cardioprotection [102], and accordingly, in the vast majority of the reviewed RIPC studies, and in our in vivo experiments pentobarbital was used. Nevertheless, regarding pentobarbital, doses, administration sites, and administration frequency for maintenance are reported in a highly heterogeneous manner in the reviewed studies.

Another key perioperative parameter for such studies is ventilation, including tidal volumes, respiratory rates, and the use of oxygen supplementation, as all of these parameters influence cellular and systemic homeostasis, and thus, could influence the cardioprotective efficacy of RIPC [103]. Bromage and colleagues have described a pattern (but not a piece of evidence) that remote ischemic conditioning may be more efficacious in animals ventilated with oxygen supplementation [104], however, in our systematic review, we found that more than one-third of the included studies reported no parameters on respiratory approaches.

Monitoring and maintaining basic vital parameters, such as body temperature, cardiac electrical activity by ECG, and blood pressure, are also of paramount importance in pre-clinical studies. These are not only needed for reproducibility, but also for describing potential causes of death or potential need for exclusion of an animal from the analysis part of the study. For instance, the absence of ST-segment elevation after LAD occlusion may indicate an unsuccessful induction of the myocardial ischemia, and thus such animals should be excluded from the analyses. Nevertheless, less than 50% of the included studies reported on the use of ECG, and less than 60% of the included studies reported information about the ascertainment of signs of myocardial ischemia (including ST-segment elevation). In addition, more than 75% of the included studies lack description of predefined exclusion criteria.

Beside the perioperative methods, certain parameters that should be reported according to the ARRIVE criteria, including randomization, blinding and report on mortality were also evaluated by our systematic review of RIPC studies in rats. We found that ~40% of the included studies did not report on randomization, and ~70% did not report on blinded evaluation of the results. These findings suggest that reliability and reproducibility of most of the studies are of serious concern.

One quality criteria specific to studies assessing cardioprotection in myocardia I/R-injury is the reporting on AAR/LV, as this parameter provides information on the consistency of the location of coronary ligation. Nevertheless, only 70% of the included studies gave information of this

data, and when reported, it ranged between 18-57% indicating a very high heterogeneity between studies. These findings also question the reliability and reproducibility of RIPC studies in rats, in general.

Of note, the cardioprotection community has recognised that poor reporting quality contributes to the gap between pre-clinical and clinical results, and reacted by establishing pre-clinical guidelines to improve reporting rigor and reproducibility [102]. Nevertheless, as assessed in our systematic review, the number of reported data items did not change over time, despite that pre-clinical guidelines and consensus documents were published regularly.

It should be emphasized, that reporting quality does not reflect the study quality, and does not influence the effect size, as evidenced by our non-significant meta-regression results. On the other hand, however, we found a tendency (but no statistical significance) for publication bias analyses towards positive results, meaning that there may be some chance that studies with smaller group sizes have larger effect sizes (i.e. greater IS-reduction by RIPC) than studies with larger group sizes. Although, statistically, it only means that smaller studies tendentially influence the outcome of the meta-analysis, it may also translate to the notion that studies with "near-neutral" or "neutral" results (i.e. studies in which IS-reduction was smaller, or could not be achieved by RIPC) are withheld from publication.

Based on the above findings, we emphasize the importance of publishing studies with neutral results, as they can provide vital information that helps refining hypotheses and experimental designs when translated into the clinical setting. Building a "trustable" body of pre-clinical literature by strictly adhering to quality control standards and by transparently reporting methods and results, irrespective of the outcomes, is pivotal for inter-laboratory reproducibility, and thus, for a successful translation.

5.2. Identifying novel GPCR targets in heart failure

In study no. 2, we performed a systematic screening for novel cardiac GPCR targets of HF by using the gold standard RNAseq, as well as ddPCR. As a result, we found several cardiac GPCR mRNAs that were significantly differentially expressed in failing vs. healthy rat hearts. From these, we aimed to further characterize those GPCRs that (i) were identified by both screening methods, with a significant correlation between the two methods, (ii) have commercially available modifiers, and (iii) have not been described in the context of HF previously. We have found that Ptgfr matched the above criteria, and demonstrated the this GPCR is primarily expressed in cardiac muscle- and fibroblast cells. In line with our pre-clinical results on rats,

single-nucleus transcriptomic data of human failing hearts (available through the Broad Institute's Single Cell Portal under project ID SCP1303) has also identified a significantly increased level of *Ptgfr* expression compared to healthy hearts [105]. Finally, in an in vitro model, we have demonstrated that inhibition of Ptgfr prevented cardiomyocyte hypertrophy, a condition that is present in certain forms of HF.

We aimed to screen for GPCRs, as these receptors represent the largest family of targets for already approved drugs, and thus, are relevant candidates to look for possibilities of drug repositioning for a novel indication in HF. From a pharmacological point of view, GPCR targeting agents, mostly exogenous small molecules, continued to expand over the past decades. Based on a 2021 report, of the 826 human GPCRs, around 350 non-olfactory GPCRs are considered druggable, of which 165 are validated drug targets, further enhancing the possible utility of drug repositioning for HF [106]. Importantly, GPCRs are deeply involved in the physiological and pharmacological mechanisms of the cardiovascular system, and a remarkable number of cardiovascular drugs already target GPCRs (e.g. ß-adrenoceptor blockers, angiotensin receptor blockers) [39].

Our approach was to assess the differential expression of GPCRs in failing vs. healthy rat hearts on the level of mRNAs. Although measurements of target molecule expression on the level of proteins (e.g. by western blots or proteomics) are generally accepted to be more translatable, in the case of GPCRs it is largely unsuccessful with multiple underlying reasons. For instance, specific, sensitive, and high-affinity antibodies to mark GPCRs are seldom, if ever given. Secondly, GPCRs are non-soluble integral membrane proteins with relatively low expression levels. Thirdly, diversity in GPCR protein conformations further complicates their detection on the protein level [107]. Therefore, in the current study, differential expression of genes was measured to screen for novel targetable GPCRs in the heart.

Bulk RNA sequencing is considered to be an unbiased, highly sensitive, and high-throughput method, and thus, it became a gold standard choice for target screening based on differential gene expression profiling [108]. Nevertheless, RNA sequencing requires an extensive bioinformatics background to gain good-quality data, and to perform reliable statistics. On the other hand, ddPCR is a highly precise, absolute quantitative method for analyzing gene expression that does not require bioinformatic background to obtain comparable transcriptomic data [109]. Although ddPCR may not be feasible to analyze the whole transcriptome, when the

transcriptional profiling is restricted to a family of targets, e.g. to GPCRs or tyrosine kinases, ddPCR provides unbiased data.

In the current study, the differential gene expression levels of a total of 288 non-olfactory GPCRs were measured in TAC-induced failing hearts vs. sham-operated healthy hearts of rats. Of these, RNAseq identified 69, whereas ddPCR identified 27 GPCRs to be significantly differentially expressed in the failing vs. healthy cardiac tissues. As the current study was not designed to validate the accuracy and sensitivity of one measurement method over the other, only those GPCRs were selected that were identified by both methods with strongly correlated expression levels ascertained by the two methods.

As a result of the above screening process, a total of 8 cardiac GPCRs were identified, the majority of which have already been studied in previous pre-clinical or clinical investigations. This fact further supports the validity of the currently used target screening approach. Of these GPCRs, we selected *Ptgfr* to characterize further, as this receptor has never been brought directly in context with HF.

Previous studies have demonstrated that the endogenous ligand of *Ptgfr*, prostaglandin F2α (PGF2α), is mainly produced by fibroblasts in the cardiac tissue, and that myocardial ischemia leads to an increased production of PGF2α, which enhances tissue fibrosis [110]. Another in vitro study has demonstrated that PGF2α leads to cardiomyocyte hypertrophy with an intensity similar to phenylephrine or endothelin-1. This finding was paralleled by a significant increase in A-type natriuretic peptide production of neonatal rat cardiomyocytes, and was specific only to PGF2α amongst all other prostanoids investigated in the study [111]. A study led by Mallat and colleagues demonstrated that the level of 8-iso-PGF2α in the pericardial fluid is significantly increased in patients with HF, strengthening the human relevance of the PGF2α-Ptgfr axis in this disease [112].

Overall, in the current study, we demonstrated that expression of Ptgfr shows a significantly different expression in TAC vs. SHAM rat hearts, and that inhibition of this GPCR prevents Ang-II-induced cardiomyocyte hypertrophy in vitro. This finding is in alignment with the possible beneficial effect of Ptgfr inhibition for certain pathomechanistic pathways of HF, as suggested by previous publications.

On the other hand, with the currently established target screening approach, we have identified *Cysltr1* as a potential pharmacological target in HF. This receptor is a suitable target for drug repositioning, as its antagonists (montelukast and zafirlukast) are currently indicated for the

maintenance treatment of asthma. Moreover, observational evidence showed that, among asthmatic patients, users of montelukast have a significantly decreased risk for cardiovascular events compared to non-users [113,114]. In addition, a recent pre-clinical study showed that montelukast improves cardiac function dose-dependently [92]. Overall, these findings serve as a basis for further investigation of the potential beneficial effect of *Cysltr1* antagonism in cardiovascular diseases, more specifically, in HF.

As for other GPCRs identified by our target screening approach to be potentially brought in relationship with HF, recent pre-clinical studies have explored that targeting them in HF models improves outcomes. For instance, the natriuretic peptide clearance receptor *Npr3*, a GPCR involved in the clearance of natriuretic peptides, has been shown to be expressed in a significantly higher number in failing human and mouse hearts, vs. normal hearts [115,116], and the inhibition of this receptor was protective in animal models of HF [87]. Another example is the apelin receptor *Aplnr*, a GPCR that is linked with many cardio-metabolic diseases, has been demonstrated to be protective in TAC- and in myocardial infarction-induced HF when activated [117]. On the other hand, endothelin receptors (*Ednra, Ednrb*) are among the few GPCR targets that have been tested for HF in the clinical reality. However, unfortunately, inhibition of these receptors by bosentan failed to improve outcomes in severely ill HF patients [118].

Overall, we emphasize the use of a combination of screening modalities to identify novel pharmacological targets for HF. In addition, we suggest to screen for such molecular targets that have a pharmacological modifier (agonist or antagonist) already available for clinical use for another indication, and thus, that could be repurposed for a new indication in HF. Such strategies would certainly decrease (i) the time and resources used for drug development starting from drug discovery to clinical testing, and thus, (ii) the risk for unsuccessful translation from bench to bedside.

6. CONCLUSIONS

As AMI and HF are still two of the leading causes of morbidity and mortality worldwide, novel cardioprotective approaches – as an adjunct to the already existing ones – are needed to improve outcomes for patients' benefit. Basic research studies, including in vivo and in vitro models, are invaluable for identifying and testing new pathophysiological pathways or new pharmacological or non-pharmacological approaches in the field of cardiology. Nevertheless, the success of translating pre-clinical results into clinical practice is very low, emphasizing the need for improving pre-clinical (and clinical) testing.

As stated in the objectives for this doctoral work, here we aimed to establish pre-clinical approaches that contribute to an improved reproducibility and translatability of studies investigating cardioprotection in acute myocardial ischemia/reperfusion (I/R)-injury, as well as in chronic HF.

In one study, we investigated the cardioprotective efficacy of remote ischemic preconditioning (RIPC) in a rat model of acute myocardial I/R-injury. RIPC is a well-known cardioprotective therapy for acute myocardial I/R-injury that is described to robustly decrease myocardial infarct size (IS) in the pre-clinical setting, nevertheless, it was found to be ineffective in the clinical reality. To this end, we carried out our experiments in an individually designed, blinded, and randomized fashion in three study centres in Hungary and the Netherlands. Our methods were not different from those reported in previously published studies. For the first time, we describe that limb RIPC in this model was ineffective in decreasing myocardial IS, microvascular obstruction, and I/R-related arrhythmias in all three laboratories. This result was in discrepancy with our meta-analysis of similar in vivo rat studies showing a robust 21.28% absolute reduction in IS by RIPC. We found that this discrepancy may be due to the insufficiently reported methodological details and design parameters in the majority of studies, and the high heterogeneity in a number of experimental settings, as identified by the current systematic review. In addition, publications reporting on conditions when RIPC did not work (other than the well-known confounders of comorbidities and comedications), or on methodological details that are crucial for RIPC to be cardioprotective are lacking. Together, these factors hinder reproducibility, which is necessary for successful translation.

In another set of experiments, we aimed to identify and test novel pathomechanistic targets for HF using a rat model of pressure-overload-induced cardiac dysfunction. Here we demonstrated for the first time that prostaglandin F2α receptor (*Ptgfr*), a cardiac G-protein-coupled receptor

(GPCR) is a potential pharmacological target in HF showing a significantly increased expression in failing vs. healthy rat hearts. *Ptgfr* was identified as a result of a systematic screening approach for cardiac GPCR genes to be differentially expressed in the two groups using bulk deep RNA sequencing, as well as droplet digital polymerase chain reaction, a method that – to the best of our knowledge – was used for the first time for target screening purposes. In line with the literature, we demonstrated that *Ptgfr* was expressed in cardiac muscle cells and fibroblasts. We also showed that inhibition of *Ptgfr* by AL-8810 prevented *in vitro* cardiomyocyte hypertrophy induced by Ang-II. Moreover, with this screening approach, cysteinyl leukotriene receptor 1 (*Cysltr1*) was also identified as a significantly differentially expressed GPCR in failing vs. healthy rat hearts. As *Cysltr1* has already available antagonists (e.g. montelukast or zafirlukast) indicated for stable asthma, and observational evidence showed that users of these drugs have decreased cardiovascular events compared to non-users, montelukast and zafirlukast have a potential to be repurposed for a novel indication in HF, and might be tested in a phase-III clinical trial as an adjunct to the current HF therapy.

In conclusion, to successfully translate pre-clinical results into clinical outcomes, reporting rigor, reproducibility, and the publication of studies with neutral results are the cornerstones in pre-clinical investigations. Otherwise, hypotheses for a possible clinical investigation will rely on biased evidence, leading to clinical outcomes contradicting pre-clinical results. On the other hand, the implementation of a new drug into clinical practice (from discovery to approval) is of very low success rate, again despite promising pre-clinical results. One potential method to overcome obligatory molecular characterization, pre-clinical studies, and clinical safety profiling could be drug repositioning. Therefore, screening for and pre-clinically testing molecular targets of drugs that are potentially repositionable for a novel indication could contribute to the success of translation, as at least the pre-Phase-II investigations have already passed.

7. SUMMARY

In this doctoral work, we aimed to analyse cardioprotective approaches for acute myocardial ischemia/reperfusion-injury and for pressure-overload-induced chronic heart failure. To this end (i) we tested the cardioprotective efficacy of limb remote ischemic conditioning, an already known cardioprotective method for acute myocardial ischemia/reperfusion-injury, and (ii) identified and tested novel, possible cardioprotective pathways for chronic heart failure using small animal models of disease, in two separate studies. Overall, these studies were the first (i) to demonstrate the absence of cardioprotective efficacy of limb remote ischemic conditioning in a rat model of acute myocardial ischemia/reperfusion-injury, (ii) to identify possible publication bias towards positive results of published studies testing the cardioprotective efficacy of limb remote ischemic conditioning, (iii) to use droplet digital PCR-based screening for pharmacologically targetable G-protein-coupled receptors in chronic heart failure – and in any disease in general, and (iv) to show antihypertrophic efficacy of prostaglandin-F2α receptor blockade, potentially targetable in heart failure.

In conclusion, based on the above studies, to improve the success of translation of potential cardioprotective approaches for acute myocardial ischemia/reperfusion-injury and for chronic heart failure from pre-clinical testing into clinical reality, it is suggested to publish well-reported, methodologically reproducible studies, irrespective of the outcome, as without such studies, clinically translatable cardioprotective interventions could not be identified. In addition, we emphasize that screening for molecular targets that have already available pharmacological modifiers, i.e. that can be subjects for drug repositioning, could also improve the translational success rate.

Overall, we believe that our studies and results contribute to the improvement of translation from bench to bedside in the field of cardioprotection.

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