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NOVEL ASPECTS OF CARDIAC ISCHEMIA/REPERFUSION INJURY IN TRANSLATIONAL DRUG DEVELOPMENT

PhD thesis

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List of abbreviations

ACC/AHA: American College of Cardiology/American Heart Association
ACEI: angiotensin-converting-enzyme inhibitors
AHA: American Heart Association
AMI: acute myocardial infarction
APD75: action potential duration at 75% of repolarization
ATC: Anatomical Therapeutic Chemical classification system
AUC: area under the curve
BARI: Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index
BSA: body surface area
CABG: coronary-artery bypass grafting
CI: Cardiac index
CiPa: Comprehensive in vitro Proarrhythmia Assay
CK: creatine kinase
CK-MB: creatine kinase myocardial band
CMRI: cardiac magnetic resonance imaging
CO: cardiac output
COX-2: cyclooxygenase-2
CS: clinical safety
EMA: European Medicines Agency
ESC: European Society of Cardiology
FDA: U.S. Food and Drug Administration
GLP: good laboratory practice
HF: heart failure
HFrEF: HF with reduced ejection fraction
hiPSC-CMs: human induced pluripotent stem cell derived cardiomyocytes
hiPSCs: human induced pluripotent stem cells
HR: heart rate
h-SOD: recombinant human superoxide dismutase
I/R: ischemia/reperfusion
ICD: Implantable cardioverter-defibrillator

ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

IND: Investigational New Drug Process

IPC: ischemic preconditioning

IPOST: ischemic postconditioning

IS: infarct size

LAD: left anterior descending coronary artery

LAVi: left atrial volume indexed to body surface area

LV: left ventricle

LVED mass: left ventricular end-diastolic mass

LVEDV: left ventricular end-diastolic volume

LVEF: left ventricular ejection fraction

LVESV: left ventricular end-systolic volume

LVESVI: left ventricular end-systolic volume index

LVSV: left ventricular stroke volume

MACE: Major adverse cardiac events

MAP: mean arterial pressure

MRA: mineralocorticoid receptor antagonists

N2BA:N2B: titin element A and B

α-HBDH: alpha-ahydroxybutyrate dehydrogenase

NDA: New Drug Application

NSAID: nonsteroidal anti-inflammatory drug

NT-proBNP: N-terminal pro-B type natriuretic peptide

post-MI HF: post-myocardial infarction heart failure

PK: pharmacokinetics

PPCI: Primary percutaneous coronary intervention

PTCA: Percutaneous transluminal coronary angioplasty

RIC: remote ischemic conditioning

RIPC: remote ischemic preconditioning

RTCA: Real Time Cell Analysis

RVED: Right ventricular end-diastolic mass

RVEDV: right ventricular end-diastolic volume

RVEF: right ventricular ejection fraction

RVESV: right ventricular end-systolic volume

RVSV: right ventricular stroke volume

sGC: soluble guanylate cyclase

sIPC: simulated ischemic preconditioning

sI/R: simulated ischemia/reperfusion

SPECT: Single-photon emission computed tomography

STEMI: ST-Elevation Myocardial Infarction

TnI: Troponin I

TnT: Troponin T

VF: ventricular fibrillation

VT: ventricular tachycardia

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1. Introduction

1.1. Major reasons for attrition in drug development

Drug development is a time consuming, complex, and expensive process that consists of preclinical and clinical phases (1-3). Attrition in drug development in later phases, such as phase 3 and phase 4, may lead to high sunk costs, increased patient risks and waste of time of scientists (4). In addition, approximately 197.000 deaths are attributed to adverse drug reactions in the European Union annually (5) and nearly 500 medicinal products were withdrawn from the market worldwide (6).

Failure of drug development may occur due to several reasons (Table 1.) in different phases (1, 7, 8).

Table 1. Reasons for failure of drug development

Failure of drug development may occur in preclinical, clinical and both the phases. GLP: good laboratory practice.

Preclinical phase
Lack of reliable screening methods for prediction of patient safety
Lack of reliable screening methods for prediction of drug efficacy in patients
Insufficient knowledge of underlying biological mechanisms of diseases
Inability to reproduce non-GLP preclinical studies
Use of inadequate animal models and animal models that may not recapitulate an entire disease
Differences in endpoints between the preclinical and clinical studies
Experiments are performed in young and healthy animals without comorbidities, comedications and risk factors which fail to mimic the “real-world” situation
Clinical phase
Failing to demonstrate safety and efficacy
Entering the clinical phase when preclinical data were inconsistent
Issues with dosing, timing and route of administration of therapies
Problems related to study design (eligibility criteria, patient recruitment, underpowered trials, lack of valid biomarkers and surrogate endpoints)
High heterogeneity of patient populations
Lack of funding for clinical trials
Both phases:
Inadequate collaboration among academic researchers, industry, and government – hiding the neutral or negative data

From the reasons detailed above, the leading causes of attrition in drug development programs are related to unexpected cardiotoxicity and failure of translation of preclinical efficacy (1, 9-11).

Some cardiotoxic effects may manifest only in the presence of cardiac diseases, e.g., in myocardial ischemia/reperfusion (I/R) conditions and/or in the presence of cardiovascular comorbidities and comedications. Since they remain undetected during preclinical and early clinical toxicology and safety studies, we termed this phenomenon "hidden cardiotoxicity" (12). Hidden cardiotoxicity fails to be detected, since the current International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines require the assessment of drug safety only in healthy animals and healthy human volunteers (13-15). In clinical trials, cardiotoxic adverse events often occur in patients with cardiac diseases and/or with cardiovascular comorbidities, e.g., hyperlipidemia, hyperglycemia, hypertension, aging or inflammatory diseases (16, 17).

High attrition rates in drug development and frequent clinical safety issues suggest that more sensitive methods that, e.g., model disease states are required for toxicity studies, including *in vivo*, *ex viv*o and *in vitro* models of myocardial I/R and/or comorbidities.

Another important aspect of attrition in drug development is related to failure of translating preclinical efficacy to clinical efficacy. To date, reperfusion therapy is the only available treatment option to reduce the risk of post-myocardial infarction heart failure (post-MI HF) (18). A plethora of cardioprotective therapies effective even in large animal models have been described. Since failure rate of translation of efficacy to clinical phases is still considerable, there is a need for bridging the translational gap between preclinical and clinical trials. Potential reasons of the failed translation of the preclinical data might be the use of suboptimal post-MI HF animal models with low clinical relevance (19).

Clinically relevant pig models with long-term follow up and serial cardiac magnetic resonance imaging (CMRI) of post-MI HF are prerequisites for final proof-of-concept studies before entering into clinical trials in drug and medical device development.

Therefore, this thesis will focus on two relevant aspects of failure of drug development in preclinical phase. The first aspect is the lack of reliable screening methods that include comorbidities such as cardiac I/R for the prediction of patient safety with particular focus on drug-induced cardiotoxicities. The second aspect is related to the need to characterize

a clinically relevant translational pig model, which is a prerequisite for final proof-of-concept studies before entering into clinical trials in drug and medical device development, with particular focus on post-MI HF.

1.2. Need for more sensitive screening methods for prediction of patient safety and drug cardiotoxicities

1.2.1. Drug development and its economic aspects

Low success rate is accompanied by high costs of drug development (1-3). The entire process lasts 8-12 years, with 1.3 billion USD estimated average cost per new approved compound (20-22). The likelihood that a drug that enters clinical trials will be approved was estimated to be about 12% (20). Therefore, early detection of toxicities reduces sunk costs, patient risks and enables companies to develop a higher number of marketable compounds (4). The phases of drug development, attrition rates, average duration, number of involved patients and cumulating of costs are shown in Figure 1.

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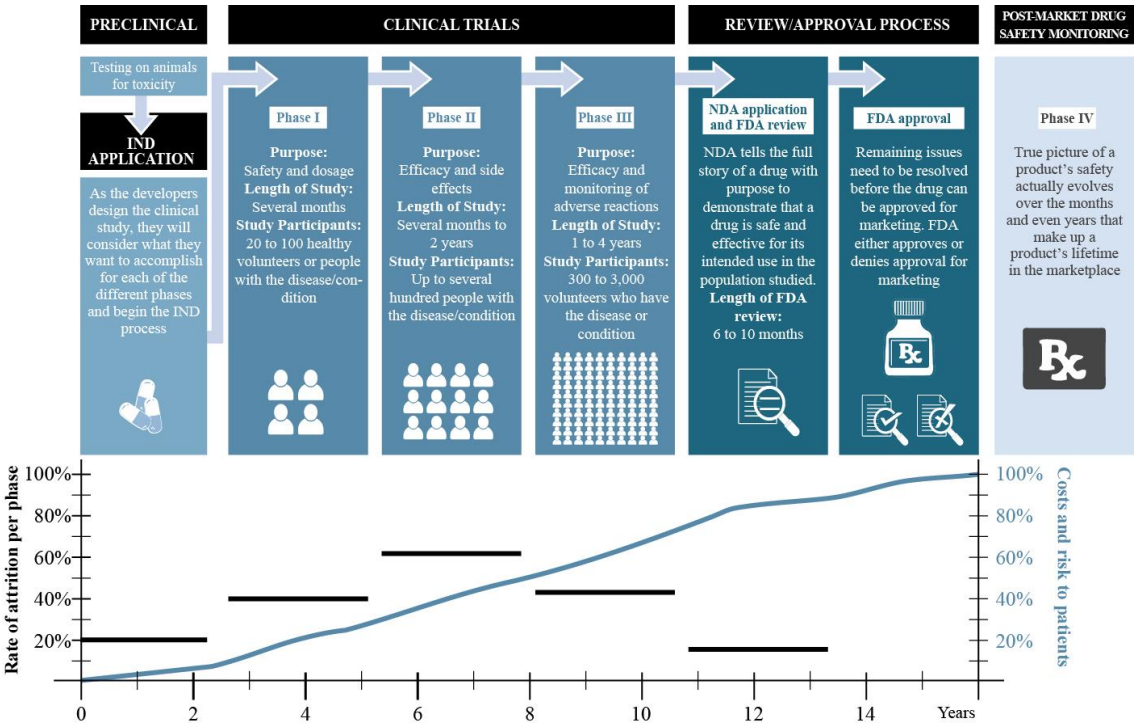

Figure 1. Preclinical and clinical phases of drug development with attrition rates, costs and risks to patients

Figure was prepared based on information and figures from references (9, 12, 23-26). Potential benefits of early, preclinical prediction of drug toxicities by establishing new methods may lead to potential savings of drug development cost and patient benefit by increase of patient safety. IND: Investigational New Drug Process; FDA: U.S. Food and Drug Administration; NDA: New Drug Application.

Since the drug-induced toxicities are among the leading causes attrition in drug development, we need better and more sensitive methods for their prediction to prevent loss of time, money and impact a patient's health, thus to increase productivity and safety of patients.

1.2.2. Significance of toxicity and clinical safety in attrition in drug development

Discontinuation of a drug development program may occur in preclinical, clinical phases and in post-market follow-up.

An analysis of drug candidates from 4 pharmaceutical companies showed that major sources of the attrition in drug development were related to preclinical toxicology and clinical safety reasons in period from 2000-2010 (27). Development of more than 20% of AstraZeneca's potential new medicines have been stopped for safety reasons at entry in preclinical GLP stage of toxicology testing (24).

In the past, pharmacokinetic and bioavailability issues played a significant role in failure of drug development. Since the 2000s, lack of efficacy and toxicity have become the leading causes, together accounting for about 60% of drug attritions (Figure 2) (9, 10).

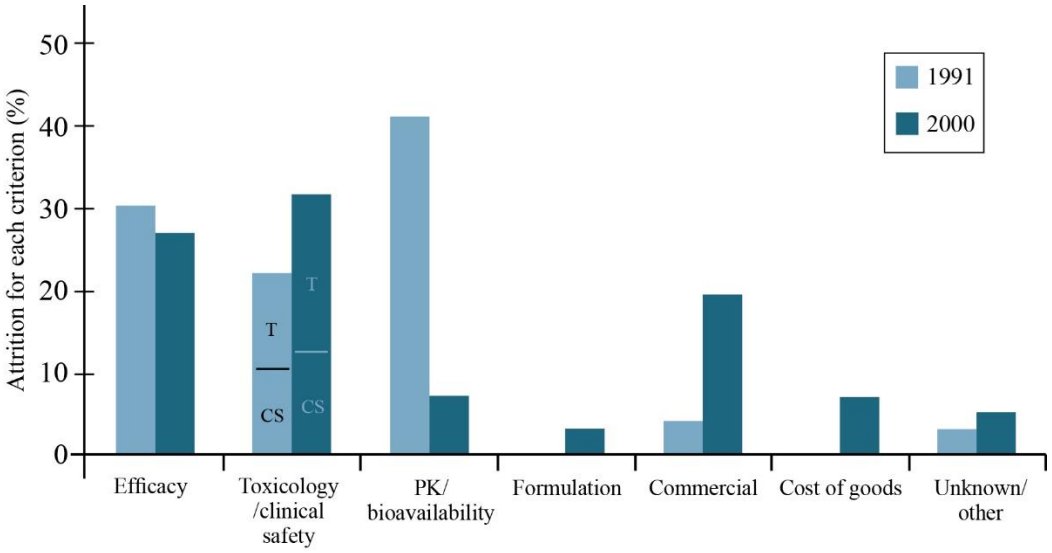

Figure 2. Reasons for attrition in drug development in different periods

Figure was adapted from reference (10) and (9). Reasons for attrition in drug development in 1991 and 2000. Safety reasons were the major causes of attrition in drug development. T: toxicology; CS: clinical safety; PK: pharmacokinetics.

An analysis of compounds entering first-in-man phase showed that the average success rate of development for all therapeutic areas was approximately 11% and the rate of failures was approximately 62% in Phase II and approximately 45% in Phase III trials (9). And finally, over 90% of the market withdrawals of drugs were related to drug toxicities (28).

Given that the rate of attrition in drug development due to clinical safety and toxicology issues is still high in last decades, more sensitive methods are needed to identify risk preclinically.

1.2.3. Cardiotoxicity as one of the major cause of drug withdrawal and its mechanisms

Causes of drug withdrawals were investigated in several publications. The percentage of occurrence of cardiotoxicities and hepatotoxicities, as the most frequents causes of toxicities, varied based on geographic area, examined time periods, and number of investigated drugs (Table 2).

Table 2. Proportions of cardiotoxicity and hepatotoxicity as the major causes of drugs withdrawals in different publications

Authors	Examined area	Examined period	Number of drugs	Withdrawal cause in %	
				Cardiotoxicity	Hepatotoxicity
Mcnaughton et al, 2014 (29)	European Union	2002-2011	19	47%	21%
P. La Rochelle et al, 2016 (30)	USA	1976-2010	34	44%	15%
Wilke et al, 2007 (31)	USA	1976-2005	28	28%	21%
Lexchin, 2014 (32)	Canada	1990-2009	19	37%	32%
Lexchin, 2005 (33)	Canada	1963-2005	41	20%	20%
Shah, 2006 (34)	Worldwide	1990-2006	38	45%	37%
Stevens and Baker, 2009 (35)	Worldwide	1975-2007	47	45%	32%
Onakpoya et al, 2016 (6)	Worldwide	1953-2013	462	14%	18%
Siramshetty et al, 2016 (36)	Worldwide	-	270	16%	21%

What can be clearly seen in Table 2 is that the rate of withdrawal due to cardiotoxicities reported in publications ranges between 14 and 47%, which highlights the relevance of studying the potential causes and mechanisms of cardiotoxicities from preclinical to post-approval phases of drug development.

Various mechanisms of cardiotoxicities were described as causes of drug withdrawals and drugs were prescribed for various indications. One of the most comprehensive summary of withdrawn drugs and mechanisms of toxicities were published by Onakpoya

et al. (6). An abbreviated summary of withdrawn drugs and mechanisms of cardiotoxicities are shown in Table 3.

Table 3. Ranking according to ATC (Anatomical Therapeutic Chemical ATC classification system) anatomical group of drugs withdrawn in Europe and/or USA from the market due to cardiotoxicities

Table was modified from reference (6) and reference (36). ?: drug has no ATC code but the ATC anatomical main group is evident.

Drug	ATC anatomical main group	ATC pharmacological subgroup	Mechanism of cardiotoxicity
amfepramone	A	antiobesity preparation	primary pulmonary hypertension, valve disorder
benfluorex	A	blood glucose lowering drugs	risk of heart valve disease
cisapride	A	propulsives	arrhytmias, QT-prolongation
dexfenfluramine	A	antiobesity preparation	pulmonary hypertension, heart valve disease
dolansetron	A	antiemetics and antinauseants	arrhythmias, QT-prolongation, torsade de pointes
domperidone (injectable)	A	propulsives	arrhythmias, cardiac arrest
fenfluramine	A	antiobesity preparation	heart valve disease and pulmonary hypertension, cardiac fibrosis
rosiglitazone	A	blood glucose lowering drugs	exacerbate congestive heart failure
sibutramine	A	antiobesity preparation	increased risk of cardiovascular and cerebrovascular events
tegaserod	A	drugs for constipation	increased risk of cardiovascular and cerebrovascular events
cloforex	A	antiobesity preparation	pulmonary hypertension
aprotinin	B	antifibrinolytics	heart failure, myocardial infarction
adesnosine phosphate	C	other cardiac preparations	cardiovascular?
bepridil	C	calcium channel blockers	ventricular arrhythmias, torsade de pointes
buflomedil	C	peripheral vasodilator	accelerated heart rate and cardiac arrest
dofetilide	C	antiarrhythmics, class I and III	QT-prolongation, drug interactions
encainide	C	antiarrhythmics, class I and III	increased death rates in patients who had asymptomatic heart rhythm abnormalities after a recent heart attack
naftidrofuryl oxalate	C	peripheral vasodilators	arrhythmias

Drug	ATC anatomical main group	ATC pharmacological subgroup	Mechanism of cardiotoxicity
prenylamine	C	vasodilators used in cardiac diseases	arrhythmias, QT-prolongation, multifocal ventricular tachycardia
probucol	C	lipid modifying agents	arrhythmias, QT-prolongation, torsade de pointes
terodiline	G	urologicals	arrhythmias, QT-prolongation
grepafloxacin	J	antiinfectives for systemic use	arrhythmias, QT-prolongation
sparfloxacin	J	antiinfectives for systemic use	arrhythmias, QT-prolongation
parecoxib	M	antiinflammatory and antirheumatic products, non-steroids	myocardial infarction, cerebrovascular accident
rofecoxib	M	antiinflammatory and antirheumatic products, non-steroids	increased risk of cardiovascular and cerebrovascular events
valdecoxib	M	antiinflammatory and antirheumatic products, non-steroids	myocardial infarction, cerebrovascular accident
droperidol	N	antipsychotics	arrhythmias, QT-prolongation
levacetylmethadol	N	drugs used in addictive disorders	torsade de pointes
pergolide	N	dopaminergic agents	risk for heart valve damage
propoxyphene	N	opioids	serious or even fatal heart rhythm abnormalities
sertindole	N	antipsychotics	arrhythmias, QT-prolongation, increased risk of sudden cardiac death
thioridazine	N	antipsychotics	arrhythmias
astemizole	R	antihistamines for systemic use	arrhytmias, QT-prolongation
clobutinol	R	cough suppressants	arrhythmias, QT-prolongation
orciprenalin (metaproterenol)	R	adrenergics, inhalants	palpitations and tachycardia
terfenadine	R	antihistamines for systemic use	arrhythmias, QT-prolongation
methylhexanamine (dimethylamylamine)	R?	used as nasal decongestant	heart attack, stroke, death
technetium (^{99}mtc) fanolesomab	V?	used as diagnostic radiopharmaceutical	life-threatening cardiopulmonary events including cardiac arrest, hypoxia, dyspnea and hypotension

Out of 38 substances from Table 3, 11 drugs (29%) were acting on alimentary tract and metabolism (anatomical main group A), 8 (21%) on cardiovascular system (anatomical main group C), 6 (16%) were used for nervous system (anatomical main group N), 5

(13%) for respiratory system (anatomical main group R), 3 (8%) for musculo-skeletal system (anatomical main group M), 2 (5%) drugs were from the group of anti-infectives for systemic use (anatomical main group J) and 1 drug (3%) was either in the group for blood and blood forming organs (anatomical main group B), or from group of drugs used in genito-urinary system (group G) from group of various compounds (group V). Focusing on the mechanism of cardiovascular toxicities, we can conclude that the use of the majority (58%) of compounds led to cardiac arrhythmias.

We can conclude that a considerable number of drugs were withdrawn from the market due to cardiotoxicities predominantly due to proarrhythmic events. This indicates a need for a better understanding of the mechanisms of toxicities and to further improve screening methods to reveal those toxicities.

1.2.4. Routine preclinical assessment of drug cardiotoxicities and guidelines

For screening of cardiotoxicities several widely used methods were developed. Cardiotoxicity screening is well regulated by the FDA and the European Medicines Agency (EMA) and harmonized by ICH guidelines (37). The ICH brings together the regulatory authorities and pharmaceutical industry to discuss scientific and technical aspects of drug registration with the mission to ensure that safe, effective and high quality medicines are developed and registered worldwide. Harmonization means publication of guidelines, with the current latest version, the ICH E6 (R2) Good Clinical Practice guideline. The ICH topics are divided into four categories with different topic codes: Q (Quality Guidelines), S (Safety Guidelines), E (Efficacy Guidelines) and M (Multidisciplinary Guidelines).

Preclinical *in vitro* and *in vivo* studies are described under the safety (S) topic which will be the focus of current thesis. The ICH safety topic is subdivided into 12 subtopics. The most relevant safety subtopics that are related to the focus of the current thesis are shown in Table 4 (37).

Table 4. Summary of the safety subtopics of the ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) guidelines that are related to the focus of current thesis

ICH summarizes safety guidelines to uncover potential risks like carcinogenicity, genotoxicity, reprotoxicity, testing strategy for assessing the QT interval prolongation and others.

ICH subtopic	The subtopic regulates
S3A and S3B	Toxicokinetics and Pharmacokinetics
S4	Duration of chronic toxicity testing
S6 (R1)	Preclinical safety evaluation of biotechnology-derived pharmaceuticals
S7A	Safety pharmacology studies
S7B	Describes a non-clinical testing strategy for assessing the potential of a test substance to delay ventricular repolarization
S9	Nonclinical evaluation for anticancer pharmaceuticals

Despite the detailed safety regulations, withdrawals due to drug cardiotoxicities are still occurring. This highlights the limitations of the current methods and the need for the development of novel assay systems to predict cardiotoxicity.

1.2.5. New approaches and limitations of preclinical assessment of drug cardiotoxicities

Detection of unsafe compounds in early stage of drug development by cardiotoxicity screening could reduce attrition rates, risk to patients and also money and time spent on drug development (4). Several new methodologies are trying to achieve these goals and to improve imperfections of currently available cardiotoxicity screening tests.

Adult somatic human cells reprogrammed into pluripotent stem cells (hiPSCs) and beating cardiomyocytes (hiPSC-CMs) can be used for drug cardiotoxicity screening in terms of arrhythmias, contractile dysfunction and structural abnormalities (38, 39). The hiPSC-CMs can be also used for estimation of drug effects on structural cardiotoxicity (4). In addition, serious limitations of the available *in vitro* blockade of Kv11.1 current (hERG current or IKr) in non-human cells or tissues can be avoided by use of recently proposed CiPa (Comprehensive *in vitro* Proarrhythmia Assay) assay. CiPa assay estimates proarrhythmic risk based on *in silico* reconstruction of human ventricular electric activity and the results are confirmed in human stem cell-derived cardiomyocytes

(hSC-CMs) (4). The hiPSC-CMs were also able to model diseases such as dilated cardiomyopathy (40), hypertrophic cardiomyopathy (41), chemotherapy-induced cardiomyopathy (42) and other diseases (for review see reference (43)). Despite the great advantages of use of human derived cells, this methodology has inherent limitations. Since the beating heart consists of diverse cell types, the lack of heterogeneity of cells may be an issue when stem cells are used for prediction of toxicities. Three-dimensional artificial tissues and mixing of different cell types may counter the limitations of cell-based assays (44, 45). The hiPSC-based monolayer and heart-on-a-chip platforms can be useful for cardiac functional assessment as well (46). *In vitro* repeated exposure toxicity testing of doxorubicin on hiPSC-CMs was performed to identify genomics biomarkers using microarrays and bioinformatics tools (47). Different cardiotoxic drugs altered the contractile properties of hiPS-CMs estimated by video microscopy-based motion field imaging (48). A biomarker, Rps6kb1 gene, that may predict cardiotoxicity was revealed by the use of Real Time Cell Analysis (RTCA) PCR Array technology in primary rat cardiomyocytes (49). Extracellular miRNAs were also explored as potential biomarkers for evaluation of cardiotoxicities (50).

To summarize, many different methodologies are under development for predicting drug-induced cardiotoxicities with particular focus on hiPSCs. Implementation of these methodologies will be time consuming and standardization of protocols are required. It is still challenging to overcome the major shortcoming of cell-based assays, the lack of heterogeneity of cells, that would be able to mimic the complexity of a cardiac tissue. Most of the aforementioned predictive screening methodologies are performed in "healthy" cells, tissues and organisms, completely ignoring the composition and complexity of a "real world" patient population that includes individuals with multiple comorbidities and comedications that may affect the outcome of the cardiotoxicity screening. E.g., a medium throughput cell-based hypercholesterolemia model was developed recently to mimic hypercholesterolemic conditions (51). Evaluation of drugs in hypercholesterolemic conditions may give valuable information and may predict cardiotoxicities in metabolic comorbid patients. Therefore, modeling of cardiovascular diseases and risk factors would be beneficial to incorporate into new guidelines, since many drug-induced toxicities occur in diseased patients and remain hidden during drug development.

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1.2.6. Definition of hidden cardiotoxicity

With the aim of differentiating overt toxicity from toxicity that can be triggered by disease states, the novel concept of hidden cardiotoxicity was introduced by our working group. Hidden cardiotoxicity is by definition a cardiotoxic effect that remain undetected during preclinical and clinical safety studies, but may become obvious in the presence of cardiac diseases, e.g., I/R injury, other diseases, or use of different drugs (12).

1.2.6.1. Mechanism of hidden cardiotoxicity, role of comorbidities

Hidden cardiotoxicity can manifest as ischemia-related lethal myocardial injury and/or as I/R-induced arrhythmias and/or as cardiac contractile dysfunction. Potential mechanisms of hidden cardiotoxicity (Figure 3) may include activation of cell death- or pro-arrhythmic processes during diseased states such as cardiac I/R. Drugs with hidden cardiotoxicities may also inhibit cardioprotective signaling pathways (as seen in, e.g., ischemic conditioning-induced protection). All the aforementioned mechanisms might be aggravated in the presence of comorbidities and comedications (12).

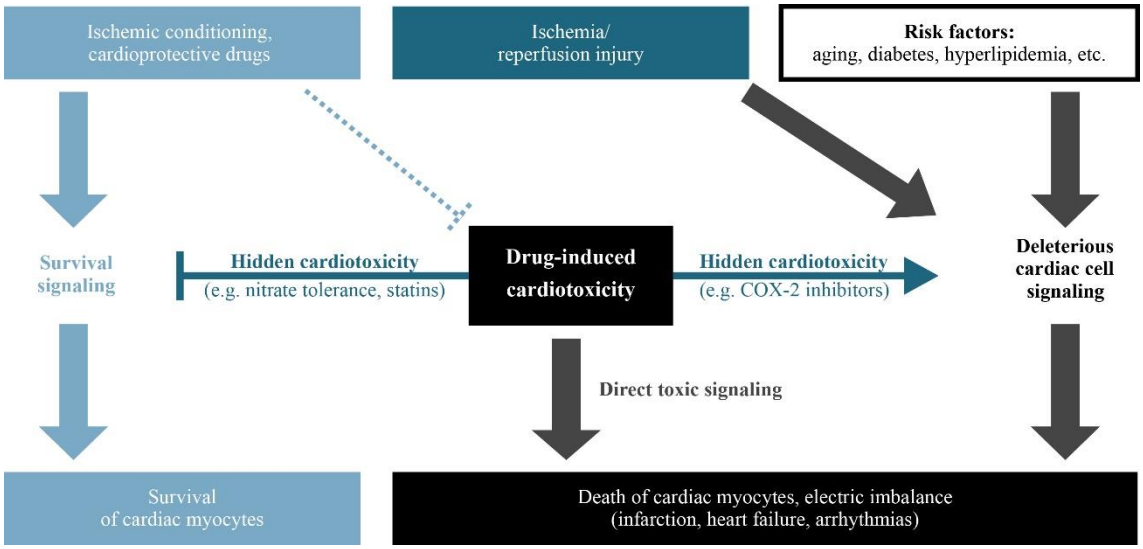

Figure 3. Mechanism of hidden cardiotoxicity

Figure was adapted from reference (12). Ischemia/reperfusion injury, cardiovascular risk factors and comedications may unmask the cardiotoxicity of drugs by inhibiting cell survival signaling or activating deleterious cardiac cell signaling

As per definition, drugs listed in Table 3 may have hidden cardiotoxic properties, since their toxicities were not revealed in preclinical and clinical safety screening. The withdrawal of rofecoxib due to safety concerns had high impact on the future of pharmaceutical industry. Rofecoxib and its toxicities will be discussed in detail below.

1.2.6.2. Hidden cardiotoxicity of rofecoxib

Rofecoxib, a nonsteroidal anti-inflammatory drug (NSAID) from the group of selective cyclooxygenase-2 (COX-2) inhibitors, was withdrawn from the market in 2004 due to an increased risk of cardiovascular adverse effects observed in the VIGOR and APPROVe trials (52, 53). A cumulative meta-analysis involving 42.174 patients concluded that rofecoxib should have been withdrawn several years earlier (54). Later, in another meta-analysis by Zhang et al, that included 116.094 participants, it was shown that the use of rofecoxib was associated with increased risk of arrhythmias (55). Later other details and mechanisms of cardiotoxicity of rofecoxib were revealed including inhibition of protection against I/R injury, prevention of production of epi-lipoxins, increase in blood pressure, inhibition of vascular remodeling (56). Since none of the aforementioned cardiotoxicities had been revealed in preclinical and clinical safety assessment of rofecoxib, according to our definition, rofecoxib had hidden cardiotoxic properties.

In addition, according to the scientific statement of the American Heart Association (AHA) the use of COX-2 inhibitors should be avoided in HF patients, since they may exacerbate underlying myocardial dysfunction (16). The explanation for this recommendation is that inhibition of prostaglandin synthesis leads to sodium- and water retention, increased systemic vascular resistance, and reduced response to diuretics (16). In a cohort study including 107.092 patients surviving their first hospitalization because of HF, the use of COX-2 inhibitors and other NSAIDs was associated with increased risk of death and cardiovascular morbidity (57).

From details above we can conclude that early detection of hidden cardiotoxicity of rofecoxib could have reduce a number of serious adverse events that appeared only in phase IV trials.

Since the hidden cardiotoxicity of an unknown number of drugs, including rofecoxib, remained unrevealed during preclinical safety assessment, there is a need to develop novel

methods to predict cardiotoxicities of drugs using *in vivo*, *ex vivo* and *in vitro* disease models such as I/R injury and to assess effect of drugs on intrinsic cardioprotection, e.g., elicited by ischemic preconditioning (IPC).

1.3. Use of translational large animal models in the development of therapies for post-myocardial infarction heart failure

1.3.1. Heart failure and post-myocardial infarction heart failure: significant burden for societies

European Society of Cardiology (ESC) defined HF as "a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral edema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress" (58). However, this definition should be updated, since significant portion of patients do not present signs and symptoms in early stages of HF and if using diuretic treatment. Also, the classification of HF is based on measurement of left ventricular ejection fraction (LVEF), elevated levels of N-terminal pro-B type natriuretic peptide (NT-proBNP) and presence of relevant structural heart disease and/or diastolic dysfunction (58), which is widely used in the literature but not directly included in the definition of HF.

HF affects 26 million patients worldwide and results in 1 million hospitalizations in both the USA and Europe each year (59). The prevalence of HF is 1–2% of the adult population in developed countries and is beyond 10% in patients >70 years of age (60-63). Worldwide distribution and prevalence of HF is shown in Figure 4.

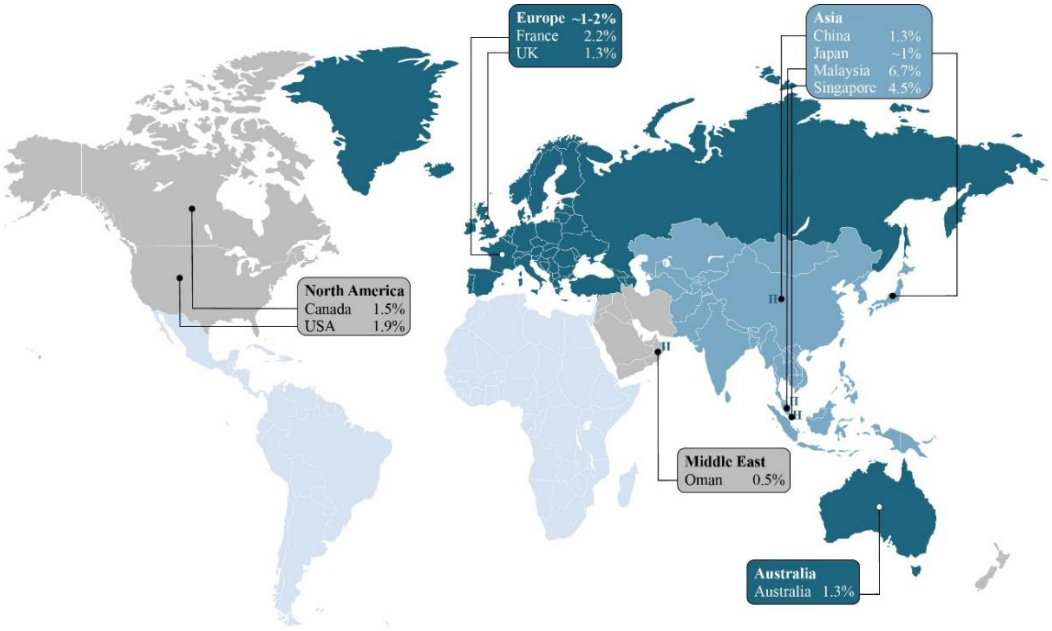

Figure 4. Worldwide distribution and prevalence of heart failure

Figure was adapted from web reference (64). Heart failure is a major public health issue affecting 26 million people worldwide.

About 14% of patients develop HF as a complication of acute myocardial infarction (AMI), hereinafter referred to as post-MI HF, and the cumulative HF rates are approximately 24% in patients surviving myocardial infarction at 1 year (65). In a review by Hellermann et al, the average in-hospital incidence of HF was 27.5% following myocardial infarction (66). Several mechanisms contribute to post-MI HF. Schematic progression of coronary artery disease to HF is shown in Figure 5 (67).

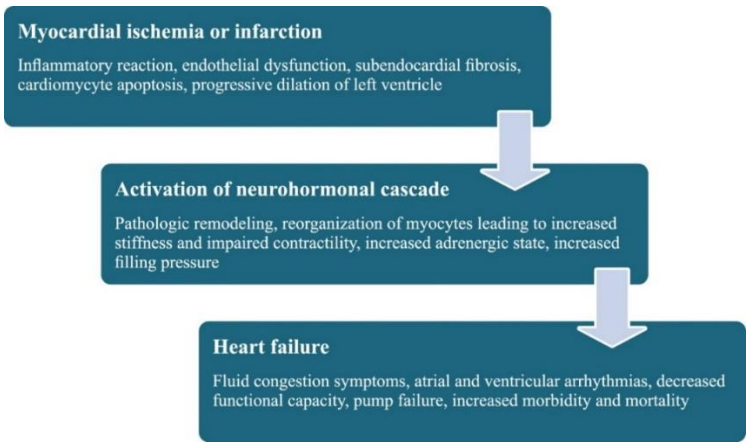

Figure 5. Progression of myocardial ischemia or infarction to heart failure

Figure was adapted from reference (67). Development of heart failure over time includes acute and late phases finally leading to decreased functional capacity.

HF is associated with worse outcome when fully developed and leads to increased morbidity and mortality. Despite the decreasing trend in mortality of AMI the incidence of HF has not decreased with implementation of novel therapeutic strategies (68). This fact highlights a strong need for development of new therapies to improve clinical outcome of post-MI HF (69).

1.3.2. Cardioprotection, current treatment and future perspectives in treatment of post-myocardial infarction heart failure

HF is one of the most powerful predictors of death in myocardial infarction patients (70). Prevention of onset of AMI would be the primary goal to avoid post-MI HF. If myocardial infarction occurs, reperfusion therapy is the only available treatment option to reduce infarct size (IS) and the risk of a subsequent HF (58, 71). Paradoxically, HF and other complications may arise from the detrimental effects of current standard treatment, i.e., reperfusion therapy, which is known as I/R injury (72, 73). Thus, there is still an unmet need for development of cardioprotective strategies (both pharmacological and non-pharmacological) beyond successful reperfusion to reduce I/R injury. I/R injury may manifest as a progressive irreversible tissue injury resulting in increase in myocardial IS. Since myocardial IS is strongly associated with all-cause mortality and hospitalization for HF within 1 year after primary percutaneous coronary intervention (PPCI) (74), cardioprotective strategies would be able to improve clinical outcomes in patients, preserve cardiac contractile function, reduce IS, therefore, the onset of HF (75).

More than 6400 articles on cardioprotection and prevention of HF were published since 1975 (76). Despite more than 3 decades of preclinical and clinical proof-of-concept studies that have shown that cardioprotection leads to IS reduction (Table 5), improve in clinical outcomes and translation of therapies into patients' benefit failed or were disappointing (76-78).

Table 5. Summary of most important clinical studies including more than 100 patients which have failed to demonstrate patients benefit of cardioprotective therapies after acute myocardial infarction

IS: infarct size; h-SOD: recombinant human superoxide dismutase; PTCA: Percutaneous transluminal coronary angioplasty; STEMI: ST-Elevation Myocardial Infarction; AMI: acute myocardial infarction; PPCI: Primary percutaneous coronary intervention; AUC: area under the curve; α-HBDH: alpha-ahydroxybutyrate dehydrogenase; CK: creatine kinase; LVEF: left ventricular ejection fraction; CMRI: cardiac magnetic resonance imaging; CK-MB: creatine kinase myocardial band; TnT: Troponin T; SPECT: Single-photon emission computed tomography; IPOST: ischemic postconditioning; LVESVI: left ventricular end-systolic volume index; TnI: Troponin I; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; HF: heart failure; RIC: remote ischemic conditioning

Study name/year of publication	Treatment	Number of patients	Outcome
SPRINT II/1993 (79)	Nifedipine usually within 3 hours of hospital admission	1006	No effect on IS, increased mortality
h-SOD with PTCA in Acute MI/1994 (80)	h-SOD before PTCA in STEMI patients	120	No improvement in left ventricular function
TAMI9/1994 (81)	Perfluorochemical emulsion (Fluosol) given with thrombolysis in AMI patients	430	No decrease IS or increase in left ventricular function
EMIP-FR/2000 (82)	Trimetazidine prior to thrombolysis followed by 48 h infusion	19 725	No difference in mortality at 35 days
ESCAMI/2001 (83)	Eniporide infusion prior to PPCI or after thrombolysis	2118	No difference in the IS (72 h AUC α-HBDH)
MAGIC/2002 (84)	Magnesium prior to reperfusion followed by 24 h infusion	6213	No difference in mortality at 30 days
CREATE-ECLA/2005 (85)	Glucose-insulin-potassium infusion for 24 h started after reperfusion in the majority of cases	20 201	No difference in mortality at 30 days
J-WIND-KTP/2007 (86)	Nicorandil bolus then 72 h infusion started after reperfusion	545	No difference in the IS (72 h AUC total CK) or 6 month LVEF
APEX-MI/2007 (87)	Pexelizumab bolus given prior to PPCI followed by infusion for 24 h	5745	No difference in all-cause death at 30 days
FIRE/2009 (88)	FX06 bolus given prior to PPCI and then repeated 10 min later	232	No difference in the IS by CMRI at 5 days or 4 months
HEBE-III/2010 (89)	Epoetin-alpha after (within 3 h) PPCI	529	No difference in the LVEF at 6 weeks. No difference in the IS (AUC CK-MB or TnT)
REVIVAL-3/2010 (90)	Epoetin-beta immediately after PPCI repeated 24 and 48 h later	138	No difference in LVEF at 6 months assessed by CMRI. No difference in the IS (5 days and 6 month CMRI)
REVEAL/2011 (91)	Epoetin-alpha immediately after PPCI repeated 24 and 48 h later	223	No difference in the IS by CMRI within 6 days and at 3 months
Atorvastatin pretreatment in STEMI/2011 (92)	Oral atorvastatin prior to PPCI and daily thereafter	173	No difference in the IS at 5–14 days using SPECT

Study name/year of publication	Treatment	Number of patients	Outcome
VIVIFY/2013 (93)	Ivabradine bolus over 30 s, followed by infusion over 8 h after PPCI	124	Lower LVEDV and LVESV, no change in LVEF
PROTECTION-AMI/2014 (94)	Delcasertib infusion before PPCI and continued for 2.5 h	1176	No difference in the IS measured by CK-MB AUC
CHILL-MI/2014 (95)	Hypothermia initiated before PCI and continued for 1 h after reperfusion	120	No effect on IS or LVEF, reduced death and HF at 45 days
NIAMI/2014 (96)	Sodium nitrite over 5 min before PPCI	229	No decrease in IS, no effect on LVEF
MITOCARE Study/2015 (97)	TRO40303 (n = 83) bolus injection prior to PPCI	163	No effect on IS or LVEF
CIRCUS/2015 (98)	Cyclosporine bolus injection before PPCI	970	No effect on deaths, HF or left ventricular remodeling
EMBRACE STEMI/2016 (99)	MTP-131 before PPCI for 1 h following reperfusion	297	No effect on IS by CK-MB AUC 0-72 h
CYCLE/2016 (100)	Cyclosporine A bolus before PPCI	410	No resolution of ST-segment, no effect on TnT at day 4 and remodeling at 6 months
EARLY-BAMI/2016 (101)	Metoprolol in bolus PPCI	683	No effect on IS measured by CMRI
MARIA/2017 (102)	Melatonin during PPCI	146	No effect on IS by CMRI, worsened LVEF evolution and LVEDV and LVESV
DANAMI-3–iPOST/2017 (103)	IPOST by 4x30 s angioplasty balloon inflation/deflation after PPCI	1234	Failed to reduce death from any cause and hospitalization for HF
NOMI/2018 (104)	Nitric oxide for inhalation starting before PPCI and for 4 h following PPCI	250	No effect on IS at 48-72h
CONDI-2/ERIC-PPCI/2019 (105)	RIC 4x5-min inflation/deflation of an automated cuff device before PPCI	5401	No improvement in cardiac death or hospitalization for HF at 12 months, no effect on IS by AUC 0–48 h

From the above Table 5 we can conclude that despite the still ongoing high interest to develop cardioprotective drug and intervention, besides the high number of failure to show efficacy there is no cardioprotective drug and intervention on the market.

In addition to Table 5, it is notable to mention that other drug/interventional therapies are being tested, but the results are not presented yet or the study was terminated or the number of involved patients was lower than 100. Such drug and interventional approaches are with adenosine (NCT00781404, promising primary results were published only (106)), Impella 2.5 (NCT01319760), sodium nitrite (107), exenatide (108), remote per-postconditioning (109), anti CD18 monoclonal antibody (110), desferoxamine (111), mangafodipir (112), sevoflurane (113) and insulin-like growth factor (114). In addition to AMI, translation of cardioprotection in settings of elective cardiac surgeries also failed.

The 2 most prominent phase III trials with neutral outcomes on efficacy of remote ischemic conditioningwere the ERICCA and RIPHeart trials (115, 116).

Although cardioprotective treatments do not show significant benefit as of now, evidence-based therapy and novel drugs are available for treatment of symptoms, prevention of rehospitalization and reduction in mortality.

According to the latest ESC guidelines on HF treatment, the evidence based therapy of patient with myocardial infarction include immediate administration of angiotensin-converting-enzyme inhibitors (ACEI), a beta-blocker and a mineralocorticoid receptor antagonists (MRA) after a myocardial infarction, especially when it is associated with left ventricular systolic dysfunction. This therapy is able to reduce the rate of hospitalization for HF and mortality. Implantable cardioverter-defibrillator (ICD) is recommended to prolong life in asymptomatic patients with LVEF <30% of ischemic origin who are ≥40 days after an AMI (58). In addition to above detailed conventional therapy, new pharmacological modalities have come in the focus of interest recently such as angiotensin receptor/neprilysin inhibitors (ARNIs) (58, 117-119), sodium-glucose co-transporter 2 inhibitors (120-122), soluble guanylate cyclase (sGC) stimulator (123) and inhibitor of the If channel (58, 124).

Although different treatment options are available for post-MI HF, the high incidence of mortality and rehospitalization shows that there is still an unmet need for development of new drugs to prevent rehospitalization, to decrease mortality and to improve symptoms and outcome in post-MI HF patients (68, 125).

1.3.3. How to improve translation of cardioprotective and post-myocardial infarction heart failure therapies?

As mentioned above, the leading causes of attrition in drug development programs are related to unexpected cardiotoxicity and failure of translation of preclinical efficacy (1, 9-11). Since failure rate of translation of efficacy to clinical phases is still considerable, there is a need for bridging the translational gap between preclinical and clinical trials. The way how to improve translation is a focus of great interest. According to ESC working group on cellular biology, improvement of the preclinical assessment of novel cardioprotective therapies is needed (126). Potential reasons for failed translation in terms

of efficacy of cardioprotective therapies, therapies of HF and potential solutions are shown in Table 6 (19, 126).

Table 6. Potential reasons for the failed translation of cardioprotective and post-MI HF therapies

Suggested opportunities for improving the likelihood of success of translation. MACE: Major adverse cardiac events; LV: left ventricle; CMRI: cardiac magnetic resonance imaging

Potential reason for failure of translation	Factors to consider	Potential solution
Inadequate study design	Infarct size is the primary endpoint Lack of randomization and blinding	Use clinically relevant endpoints (MACE, survival, LV remodeling, LV function with CMRI) Randomization and blinding
Inappropriate experimental model	Model that better represents the patients with myocardial infarction	Use closed-chest large animal models
Inappropriate experimental protocol	Duration of ischemia/reperfusion Route and timing of drug administration	Use clinically relevant parameters (ischemia time, long-term follow up), administer cardioprotective drug before or at onset of reperfusion
Confounding factors are not considered	Gender, age, comorbidities, comedications	Consider inclusion confounding factors in studies

In addition to Table 6, a guideline on the relevance of rigor and reproducibility in preclinical studies on cardioprotection recommends CMRI as a clinically relevant model for measurement of ventricular function in pigs (127).

Based on aforementioned observations, it can be concluded that for higher translational efficacy the use of large animal model, e.g., pigs, and clinically relevant protocols and study designs are needed. In the next chapter the relevance of the use of large animal models with clinically relevant endpoints and protocols will be emphasized.

1.3.4. Small and large animals as models of cardiovascular diseases including myocardial infarction and post-myocardial infarction heart failure and comparison to human characteristics

Beside the use of cell-based *in vitro* assays and *ex vivo* isolated perfused heart models of I/R injury to screen novel cardioprotective therapy, it is essential to demonstrate efficacy in small and large in vivo animal models. Small-sized rodents, particularly mice and rats, are extensively used in cardioprotection studies, mainly due to short gestation time, low costs and easier handling rather than clinical relevance. In addition, genome of mice and rats are well-characterized and easy to manipulate. Since some cardioprotective strategies effective in murine models showed their efficacy in small proof of concept clinical studies and since ICH guidelines require their use in drug development programs, the use of rat and mouse models remained essential in cardiovascular research (126).

Cardiovascular characteristics of murine and large animal models in comparison to humans are summarized in table (128-131).

Table 7. Cardiovascular characteristics of murine and large animal models and their comparison to humans

Table was adapted from reference (128) and amended by references (129-131). LAD: left anterior descending coronary artery; MHC: Myosin heavy chain; N2BA:N2B: titin element A and B; SERCA: sarco(endo)plasmic reticulum Ca2+-ATPase.

Parameter	Mouse	Rat	Pig/minipig	Sheep	Human
Body weight (kg)	0.02-0.05	0.2–0.5	80–100/ 25–50	50–80	50–86
Heart weight (g)	0.15–0.18	0.67 –0.99	148–435	188 ± 17	160–442
Heart rate (1/min)	310–840	250–493	50–116	60–120	72
Systolic Blood Pressure (mmHg)	113–160	84–184	135.8 ± 16.29	91–116	120
Diastolic Heart pressure (mmHg)	81-110	58-145	81.6 ± 14.10	102	80
Cardiac Output (mL/min)	13–15	16.11–61.4	3170 ± 450	1500 to 13200	3340–9790
Ejection Fraction (%)	59–94	87.15–95.1	52 ± 3	56.7 ±8.9	>55

Parameter	Mouse	Rat	Pig/minipig	Sheep	Human
Coronary anatomy	Left coronary artery branches into a large septal artery and LAD	Single or double coronary arteries	Right-coronary dominancy (66%) or balanced dominancy (33.5%)	Left coronary dominancy	Right-coronary dominancy
Localization of infarction in reperfused hearts	mid-myocardial, and sub-epicardial	mid-myocardial, and sub-epicardial	sub-endocardial	mid-myocardial	sub-endocardial
Coronary size (mm)	0.16	-	3.89 ± 0.15/ 1.5–2.0	3.8 ± 0. 2	3.70
MHC expression	Expression of α-MHC (>94–100%)	Expression of α-MHC (>94–100%)	Expression of β-MHC (~100%)	Expression of β-MHC (~100%)	Expression of β-MHC (>90–95%)
N2BA:N2B ratio	~0.05–0.25	~0.06–0.1	1–1.5	~0.35–0.4	~0.4–1.2
% of contribution of SERCA to calcium removal during relaxation	90–92%	90–92%	-	81.5%	76%
Multiple sampling of large volumes of tissues and biofluids	Not possible	Not possible	Possible	Possible	Possible

Based on the aforementioned data from the Table 7, we can conclude that large animals are better reflecting the human cardiovascular system. Among others, this indicates the need for use of large animals in cardiovascular research. According to the position paper of the ESC working group cellular biology of the heart the novel cardioprotective therapy should be investigated in all experimental I/R injury models including large animals (126).

Large animal studies that mimic the clinical reality in terms of observation time post MI, measurement of cardiovascular parameters by clinically relevant methods (with CMRI) are rare, partly due to ethical considerations but also due to high costs.

In cardiovascular research the use of pigs is more common than that of the sheep in Europe, this may be explained by that their breeding is more widespread. In addition, the scar formation after MI is very similar in humans and pigs. Collagen fibers are well organized in the core area of the infarction but have a random distribution in the outer areas in both humans and pigs (132).

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1.3.5. Comparison of different pig breeds and their use as models of post-myocardial infarction

Pig breeds that are used in biomedical and cardiovascular research belong to species of domestic pigs (*Sus scrofa*). Pigs in this species vary in size, appearance and genetic background (133, 134). According to their size, they can be classified in minipigs and large pigs. Major characteristics of minipigs and large pigs are shown in Table 8 (135).

Table 8. Major characteristics of pigs classified according to their size

Attributes	Minipigs	Large pigs
Handling	Easy due to size	Difficult due to size
Behavior	Tractable	Less tractable
Space requirements	Smaller	Larger
Fertility	Good	Good
Mature body weight	35-45 kg	Up to 220 kg
Multiple blood sampling	Feasible	Feasible
Test of human therapeutic devices	Feasible	Feasible
Requirement for food, pharmacological therapies, anesthetics	Lower	Higher

From the Table 8 we can conclude that minipigs and large swine differ first of all in physical attributes that makes minipigs easier to handle, perform interventions and keep in smaller stalls. In addition, smaller growth rate allows longer follow up periods with higher clinical relevance.

Although post-MI HF has been researched in pigs extensively, no study has been published with the aim of characterization and comparison the effect of MI on the outcome of post-MI HF in Landrace pigs and Göttingen minipigs.

In conclusion, clinically relevant pig models of post-MI HF are prerequisites for final proof-of-concept studies before entering clinical trials in most of the cardiovascular drug and medical device development projects. Therefore, a comprehensive characterization of the closed-chest post-MI HF models in Göttingen minipigs and Landrace pigs with the assessment of ventricular functions and anatomy using CMRI during long-term follow up may be useful for choosing the most fitting large animal models to study post-MI HF and to develop novel therapies.

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2. Objectives

2.1. Establishing a preclinical model with I/R to detect hidden cardiotoxicity of rofecoxib

Here we aimed to investigate that hidden cardiotoxicity of rofecoxib could have been detected in early preclinical phases in pathological conditions using cellular- (*in vitro*), and small animal models (*ex vivo* and *in vivo*) of acute I/R injury and to test the effect of rofecoxib on intrinsic cardioprotection elicited by IPC.

2.2. Characterization of post-MI HF pig model that resembles human pathology

We also aimed to characterize post-MI HF in Göttingen minipigs in comparison to Landrace pigs to show whether any of these models reflect post-MI HF parameters comparable to humans.

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3. Methods

Methods and other details are published in papers Brenner GB et al, Cells, 2020 (136) and Brenner GB, JoVe, 2021 (137) .

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4. Results

4.1. Chronic rofecoxib treatment increased acute mortality during cardiac ischemia/reperfusion

The rats were treated with rofecoxib for four weeks and then subjected to 30 min ischemia and 120 min reperfusion to investigate hidden cardiotoxicity of rofecoxib. Rofecoxib treatment increased the mortality rate as compared to the pooled data of other groups (OR=7.73, CI 95%=1.70–34.97 vs. I/R+vehicle + IPC+vehicle + IPC+rofecoxib; p<0.008; Figure 6). In the I/R+rofecoxib group, seven animals died due to irreversible ventricular fibrillation (VF) during the ischemic period and one animal died due to a sudden drop in blood pressure during reperfusion. In the I/R+vehicle group, only one animal died due to irreversible VF during the ischemic period. Animals died during the short I/R stimuli of IPC (six/each IPC group) were excluded from further evaluations and are not shown in Figures 7, 8, and 9. In the IPC+rofecoxib group, one animal died due sudden drop in blood pressure in the reperfusion period.

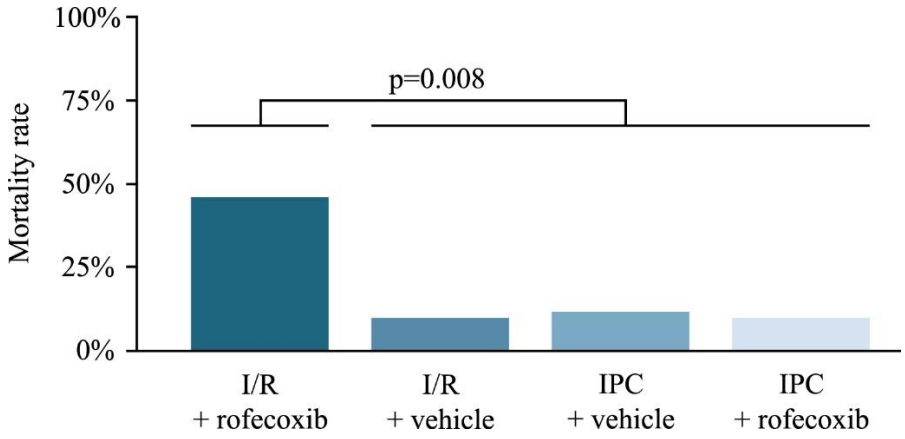

Figure 6. *Rofecoxib treatment increased the mortality rate in the ischemia/reperfusion (I/R) group in vivo when compared to the pooled data of other groups (OR = 7.73, CI 95% = 1.70–34.97, p < 0.008). IPC: ischemic preconditioning.*

4.2. Chronic rofecoxib treatment increased arrhythmia score in cardiac ischemia/reperfusion

The severity and duration of arrhythmias were evaluated by scoring 5 min intervals according to the Lambeth conventions during cardiac ischemia and early reperfusion. The

results are represented as an arrhythmia map in Figure 7, showing the type of most severe arrhythmias occurring during a given 5-min interval by a color scale.

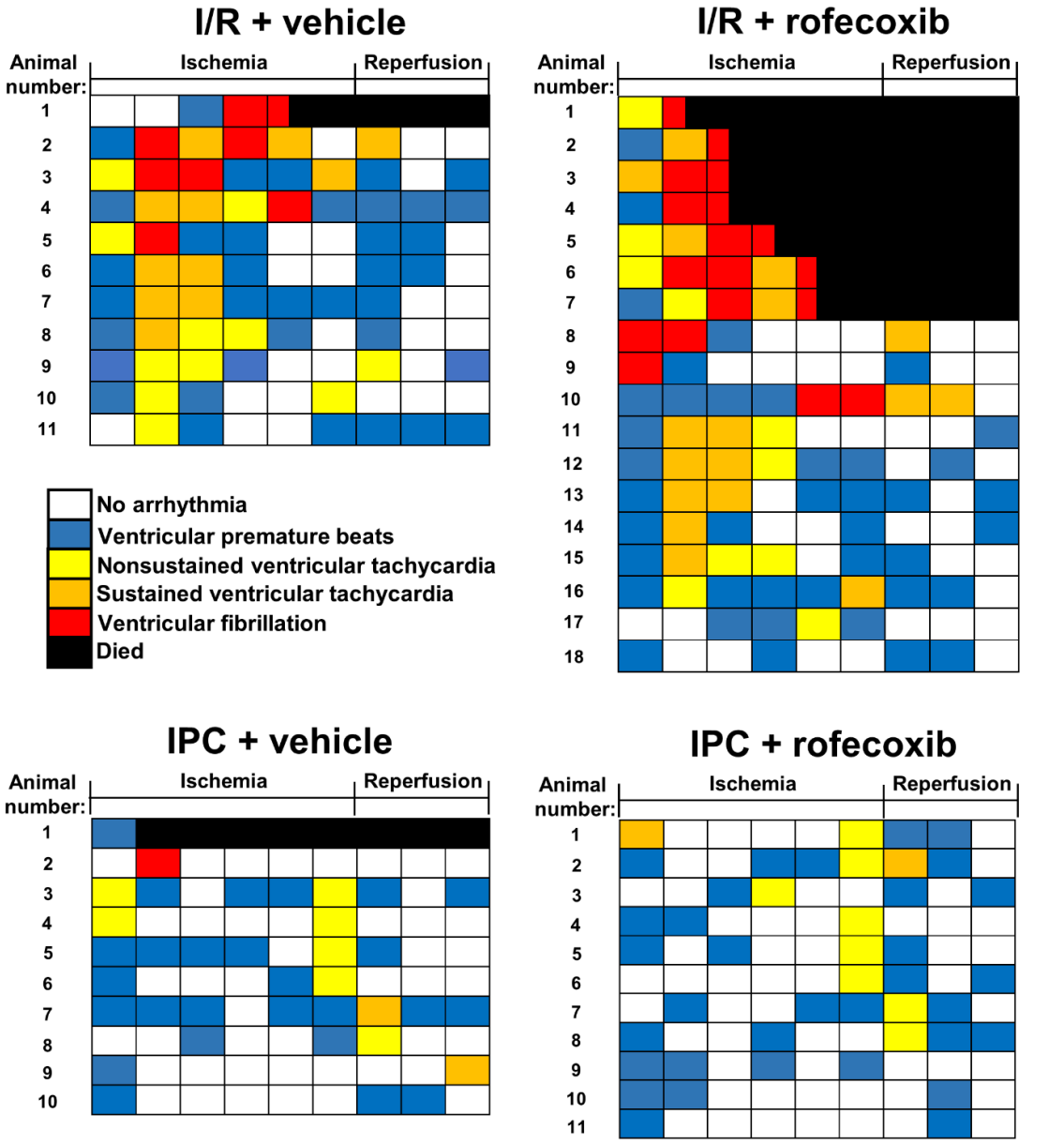

Figure 7. *Arrhythmia maps showing the arrhythmias in the order of severity during 30 min ischemia and at the first 15 min of reperfusion. Each row represents arrhythmias of each animal. The different color boxes show 5-min periods. The animals died during the IPC (ischemic preconditioning) are not shown. In the I/R+rofecoxib group animals 1–7 died due to ventricular fibrillation (red and black box).*

The peak arrhythmia scores were achieved in the I/R+vehicle groups after 10 min of ischemia (50th min of experiment) and, following that, they rapidly decreased (Figure 8). In contrast, in the I/R+rofecoxib group the initial increase runs parallel with the I/R+vehicle group, but, following that, the decline is much slower. We tested the statistical hypothesis that scores decrease in parallel by fitting the linear mixed regression model on the observations by excluding the data of the first period. The difference between slopes was highly significant ($p_{\text{Time x Group interaction}} = 0.00681$), thus suggesting a pronounced effect of rofecoxib on the recovery. Yet, such a difference does not exist between the estimated peak values at the end of 50th minute of experiment (p =0.66367). The initial increase of arrhythmia scores was not observed in the IPC groups.

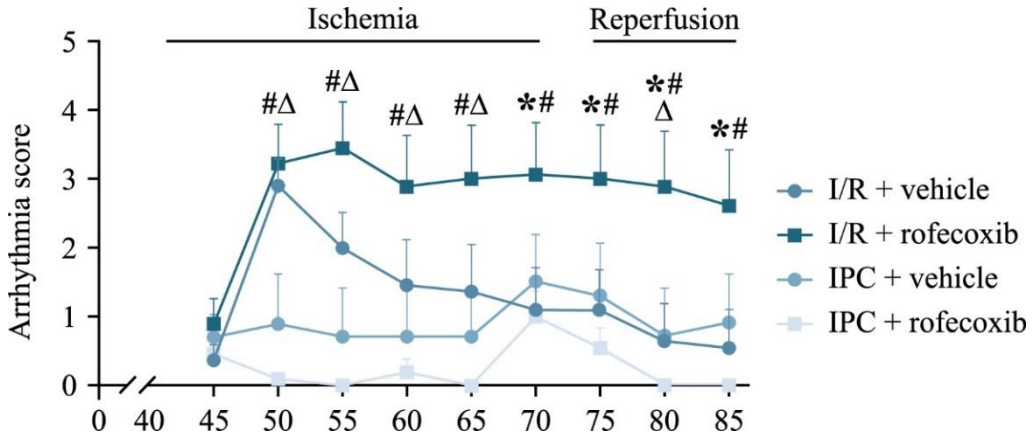

Figure 8. *Arrhythmia scores in 5-min intervals declined gradually starting from the 50th min in the I/R+vehicle (ischemia/reperfusion) group but remained elevated in the I/R + rofecoxib group (*p < 0.05 I/R + vehicle vs. I/R + rofecoxib). IPC (ischemic preconditioning) prevented initial increase of arrhythmia score (#p < 0.05 IPC + rofecoxib vs. I/R + rofecoxib, Δp < 0.05 IPC + vehicle vs. I/R + rofecoxib).*

4.3. Rofecoxib decreased infarct size and did not interfere with cardioprotection by ischemic preconditioning

We measured IS to explore the effect of rofecoxib on I/R injury and cardioprotection by IPC. Rofecoxib reduced IS (I/R + rofecoxib) as compared to the vehicle-treated (I/R + vehicle) group (Figure 7). IS was significantly smaller in the IPC+vehicle group as compared to I/R+vehicle. Chronic rofecoxib treatment did not affect IS-limiting effect of IPC in IPC+rofecoxib when compared to the IPC+vehicle group.

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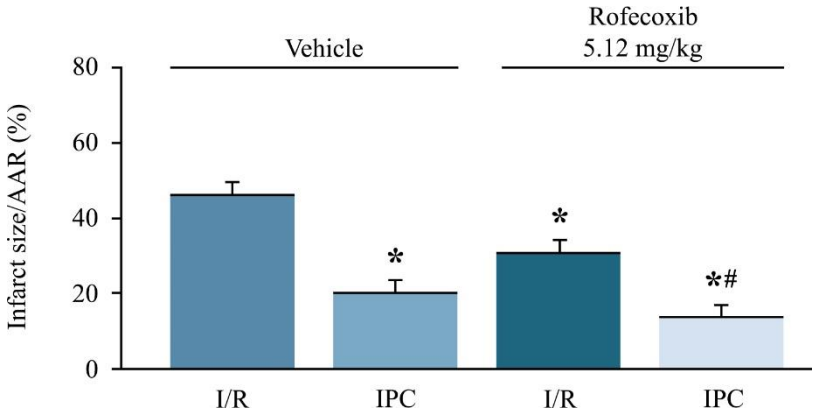

Figure 9. *Chronic rofecoxib treatment reduced infarct size in animals subjected to cardiac ischemia/reperfusion (I/R) and did not interfere with cardioprotection by ischemic preconditioning (IPC). (*p < 0.05 vs. I/R + vehicle, #p < 0.05 vs. I/R + rofecoxib). AAR: area at risk.*

No significant difference was observed between groups for the AAR expressed as a percentage of the left ventricle (I/R + vehicle: 51.1 ± 2.8%; IPC + vehicle: 41.6 ± 2.3%; I/R + rofecoxib: 44.8 ± 4.1%; IPC+rofecoxib 50.6 ± 4.9%).

4.4. Rofecoxib increased the action potential duration in rat isolated papillary muscles at the end of simulated ischemia/reperfusion and this effect was not observed ischemic preconditioning group

In a collaboration with the Department of Pharmacology and Pharmacotherapy from University of Szeged, *ex vivo* simulated ischemia/reperfusion (sI/R) and simulated ischemic preconditioning (sIPC) experiments were performed on isolated rat left ventricular papillary muscles in order to analyze the effect of rofecoxib on cardiac action potential parameters. Rofecoxib treatment did not change action potential duration at 75% of repolarization (APD75) (Figure 10 A and B) in normoxic conditions. As expected, the 30 min simulated ischemia significantly shortened APD75 (Figure 10 A) in all groups that were subjected to ischemia when compared to the respective normoxic groups. However, importantly, in the presence of sI/R rofecoxib increased APD75 (Figure 10 B) upon reperfusion following the 30 min simulated ischemia. In the sIPC group, these effects of rofecoxib on APD were not seen during reperfusion (Figure 10 B).

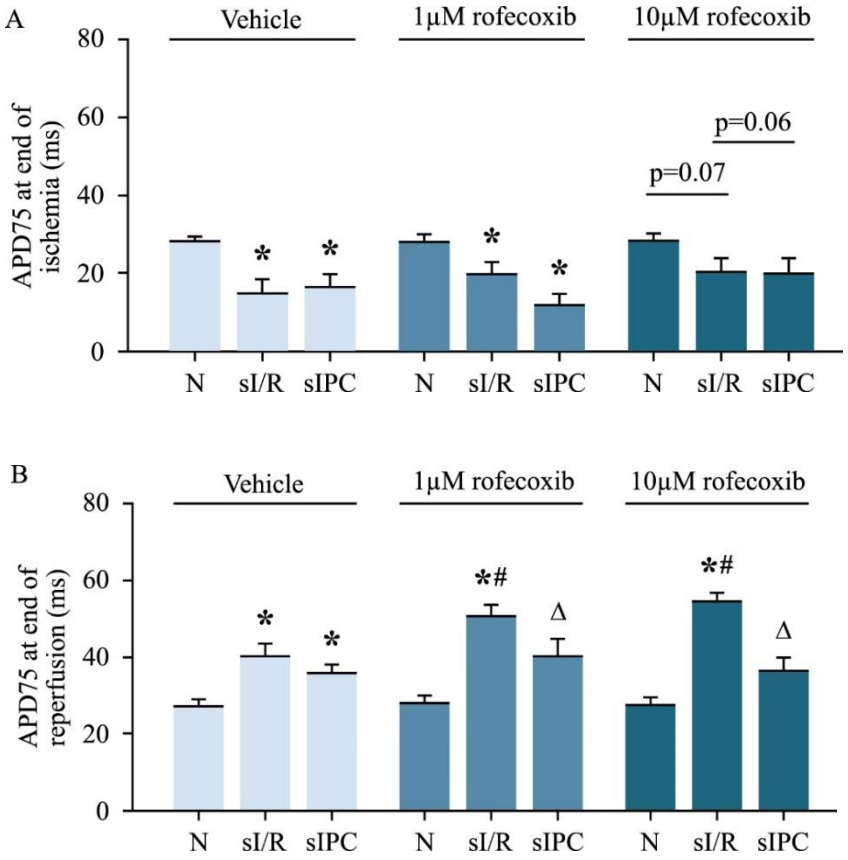

Figure 10. *(A) action potential duration at 75% of repolarization (APD75) decreased by the end of 30 min simulated ischemia in the simulated ischemia/reperfusion groups (sI/R) and simulated ischemic preconditioning groups (sIPC). (B) Rofecoxib increased the APD75 in adult rat isolated papillary muscles at the end of reperfusion and this effect was reversed by sIPC (*p<0.05 vs. corresponding normoxia group, #p<0.05 vs. sI/R +vehicle, Δp<0.05 vs. corresponding sI/R group).*

4.5. Rofecoxib treatment increased viability of isolated adult rat cardiac myocytes in normoxia and in simulated ischemia/reperfusion injury

In vitro sI/R experiments were performed in order to analyze the effect of rofecoxib on viability of isolated cardiac myocytes. sI/R caused significant cell death (Figure 11) as compared to normoxic control, which was reversed by rofecoxib treatment at 0.1, 0.3, 1, and 3 μM concentration, respectively, thereby supporting the *in vivo* data showing the IS reduction by rofecoxib (Figure 11).

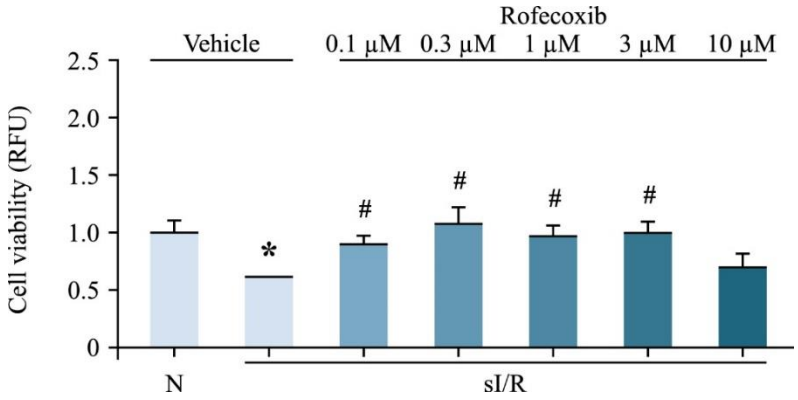

Figure 11. *Rofecoxib increased cell viability in isolated rat cardiac myocytes exposed to simulated ischemia/reperfusion (sI/R). Normoxia (N)+vehicle group was set to 1 relative fluorescence (RFU) arbitrary unit and all of the data were normalized to the averaged sI/R group (*p < 0.05 vs. Normoxia+vehicle, #p < 0.05 vs. sI/R+vehicle). RFU-arbitrary unit: Relative fluorescence unit.*

4.6. Mortality rate did not change between the two pig breeds

Out of 13 Göttingen minipigs subjected to myocardial infarction 2 animals died (15.4% mortality), 1 during the ischemic period due to irreversible ventricular tachycardia (VT) and 1 owing to asystole in reperfusion. In Göttingen minipigs 1 animal was successfully resuscitated during the cardiac ischemia. Mortality rate was 0% in Landrace pigs, 10 out of 10 animals survived, 2 of them required resuscitation due to VF in ischemic period. Mortality did not did not differ significantly between the two breeds.

4.7. Myocardial scar sizes were comparable between the two pig breeds

To measure the extent of cardiac scar as a consequence of AMI, CMRI was performed. Scar sizes and BARI scores (Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index) were comparable between the two breeds measured at the 2nd month of follow-up in Landrace pigs, and at the 3rd and 6th month in Göttingen minipigs (Figure 12 A and B). No differences were observed when scar sizes were related to the BARI scores in Landrace pigs at 2 months (0.55 ± 0.1) and in Göttingen minipigs at 3 months and 6 months respectively (0.75 ± 0.12 and 0.57 ± 0.08). The scars were

localized in the anterior, anteroseptal, septal, anteroapical and apical segments of the heart in both breeds. The lateral wall was affected only in Göttingen minipigs. Right ventricular infarction was negligible, affected only one animal out of eleven surviving Göttingen minipigs and one out of ten Landrace pigs (2.11 ± 2.11 vs. 0.97 ± 0.97).

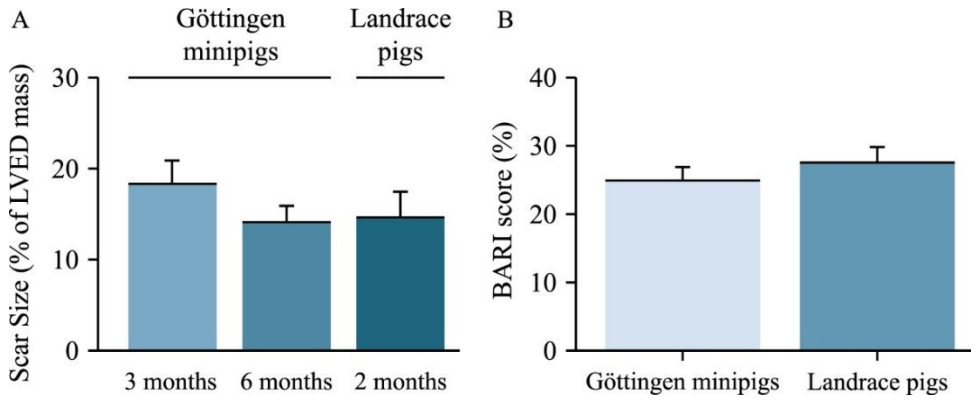

Figure 12. *(A) Left ventricular scar sizes in Göttingen minipigs and Landrace pigs measured by cardiac magnetic resonance imaging. Scar size is shown as a ratio of mass of infarction to the mass of left ventricle at end of diastole (LVED). (B) BARI (Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index) scores in Göttingen minipigs and Landrace pigs measured before coronary occlusion.*

4.8. Increase in left ventricular mass was more pronounced in Landrace pigs during follow-up

The cardiac growth rate was measured by CMRI. Left ventricular end-diastolic (LVED) mass in Göttingen minipigs increased only moderately (8%) at 6 months (Figure 13 A). In contrast, in Landrace pigs, LVED mass increased by almost 100% at 2 months (Figure 13 B).

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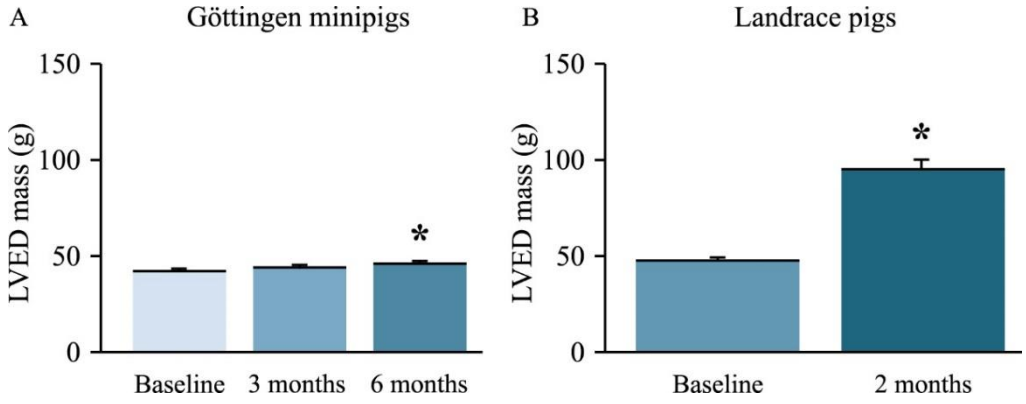

Figure 13. *(A) Left ventricular end diastolic (LVED) mass (g) of Göttingen minipigs and (B) Landrace pigs measured by cardiac magnetic resonance imaging (*p<0.05 vs. corresponding baseline).*

4.9. Left ventricular ejection fraction decreased only in Göttingen minipigs

LVEF, as the most widely used parameter of left ventricular systolic function, was measured by CMRI. MI resulted in a significant decrease in LVEF in minipigs at 3 months and 6 months (Figure 14 A). In Landrace pigs LVEF did not change after 2 months (Figure 14 B).

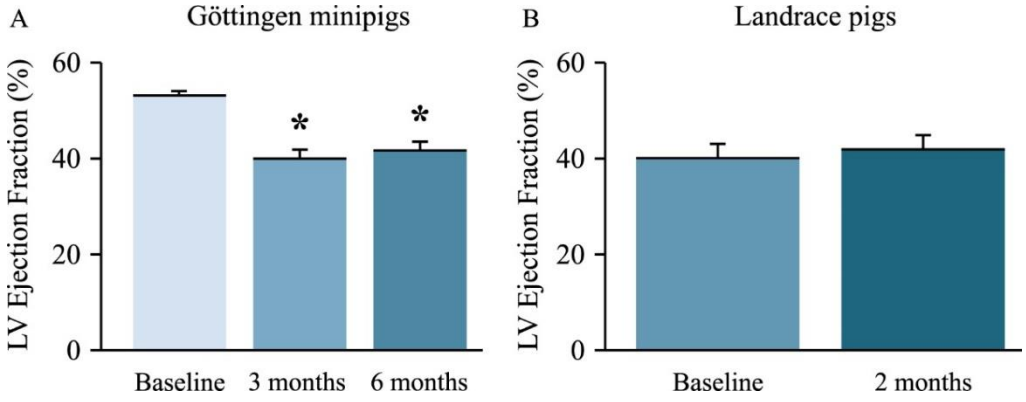

Figure 14. *(A) Left ventricular (LV) ejection fraction (%) of Göttingen minipigs and (B) Landrace pigs measured by cardiac magnetic resonance imaging (*p<0.05 vs. corresponding baseline).*

Post-infarction LVESV and LVEDV increased significantly in both breeds (Table 9). Left ventricular end-systolic volume (LVESV) increased by 69% and 80% in Göttingen minipigs after 3 and 6 months, respectively, and by 80% in Landrace pigs after 2 months. Left ventricular end-diastolic volume (LVEDV) showed a 28% increase after 3 months and a 42% increase after 6 months in Göttingen minipigs and an 82% increase in Landrace pigs after 2 months.

Left ventricular stroke volume (LVSV) of Landrace pigs increased by 85% in 2 months and LVSV of Göttingen minipigs did not increase significantly even at 6 months.

Table 9. *Left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and left ventricular stroke volume (LVSV) at the measured time points in Landrace pigs and Göttingen minipigs (*p<0.05 vs. corresponding baseline).*

Measured parameter	Göttingen minipigs			Landrace pigs	
	Baseline	3 months	6 months	Baseline	2 months
LVESV [ml]	25.77±1.73	43.65±4.53*	46.28±4.35*	54.59±2.00	98.26±8.60*
LVEDV [ml]	55.49±3.14	71.08±5.25*	78.81±5.46*	93.99±3.85	171.20±11.50*
LVSV [ml]	29.71±1.65	27.44±1.97	32.52±2.37	39.40±3.05	72.94±3.99*

4.10. Left atrial volume indexed to body surface area increased only in Göttingen minipigs, but both the breeds developed pulmonary edema following myocardial infarction

In order to further examine signs of HF, we performed measurement of the left atrial volume indexed to body surface area (LAVi). LAVi increased by 34% in Göttingen minipigs after 6 months (Figure 15 A) and did not change significantly in Landrace pigs after 2 months (Figure 15 B). Representative images show the tracing of the left atria (Figure 15 C-D). Moreover, the presence or absence of pulmonary edema was assessed by CMRI on the localizer images (Figure 15 E). Pulmonary edema was observed in both breeds as a result of cardiac decompensation. Ten out of eleven Göttingen minipigs and nine out of ten Landrace pigs showed signs of pulmonary edema.

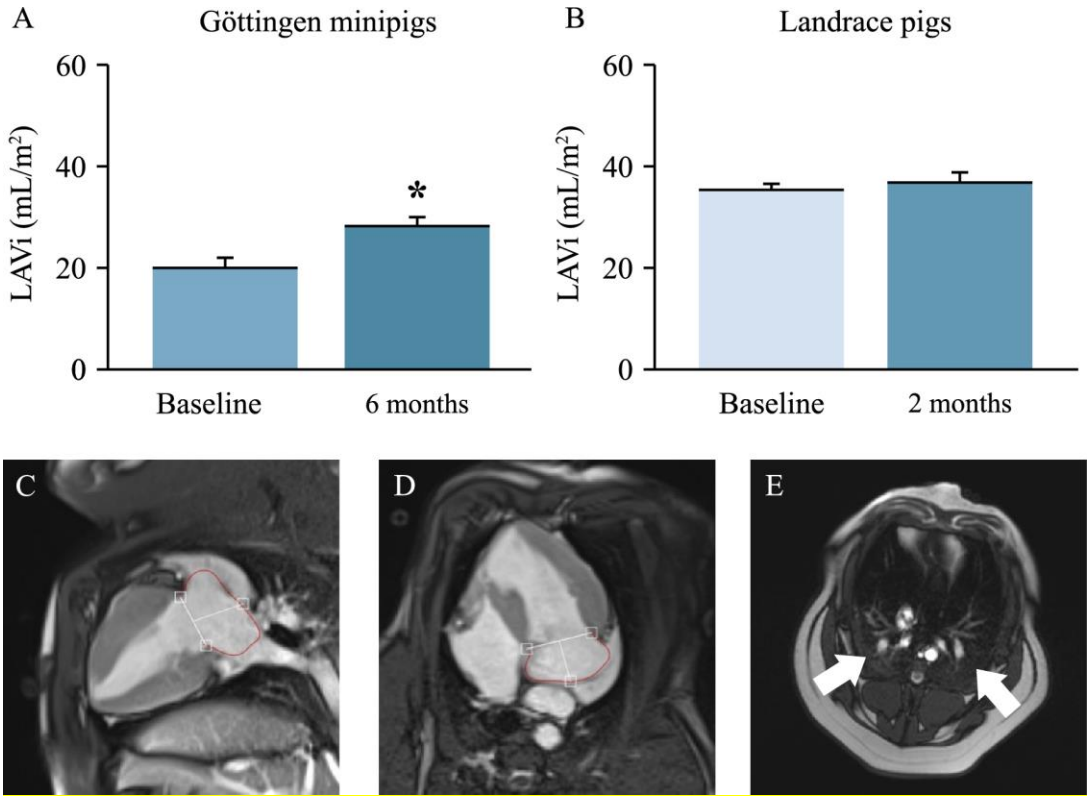

Figure 15. *(A) Left atrial volume indexed to body surface area (LAVi) in mL/m2 in Göttingen minipigs and (B) Landrace pigs measured by cardiac magnetic resonance imaging. Representative images of left atrial volumes, tracings were made on the two- (C) and four chamber (D) cine images. White arrows show the presence of pulmonary edema on the representative localizer image (E) (*p<0.05 vs. corresponding baseline).*

4.11. Increase in body weight was more pronounced in Landrace pigs during follow-up

In Göttingen minipigs body weight gain was only 8% after 3 months and 30% after 6 months (Figure 16A), whereas increased heart weight was accompanied by a nearly 100% increase in body weight in Landrace pigs at 2 months (Figure 16B).

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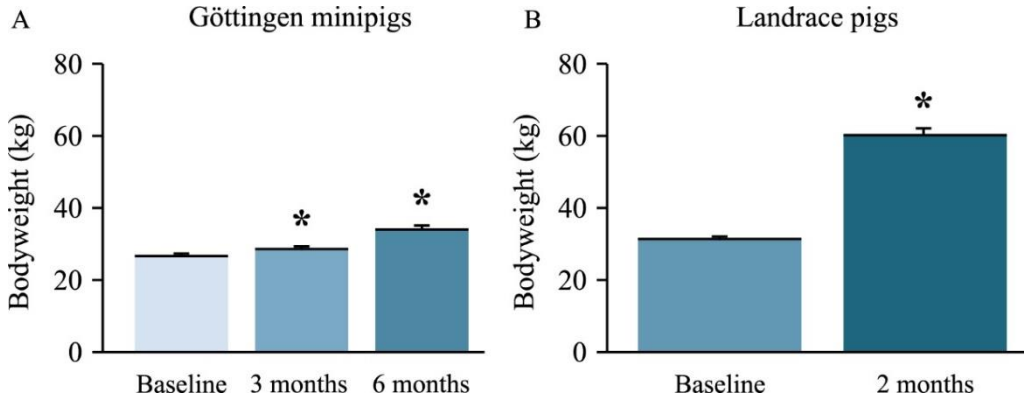

Figure 16. *(A) Bodyweights (kg) of Göttingen minipigs and (B) Landrace pigs (*p<0.05 vs. corresponding baseline).*

4.12. Trends in cardiac functional parameters differ between Göttingen minipigs and Landrace pigs

Coronary artery occlusion led to an almost significant decrease in mean arterial pressure (MAP) in Göttingen minipigs (57.9 ± 3.98 mmHg vs. 49.89 ± 1.24 mmHg) and decreased significantly in Landrace pigs (65.4 ± 5.97 mmHg vs. 45.47 ± 4.79* mmHg) in the early reperfusion phase as compared to the baseline (pre-infarction) values.

Cardiac index (CI) is a reliable indicator of cardiac performance which relates left ventricular cardiac output (CO) to body surface area (BSA). In Göttingen minipigs CI didn’t change at the measured timepoints (Figure 17 A), whereas in Landrace pigs a tendency to increase was detected in CI (Figure 17 B).

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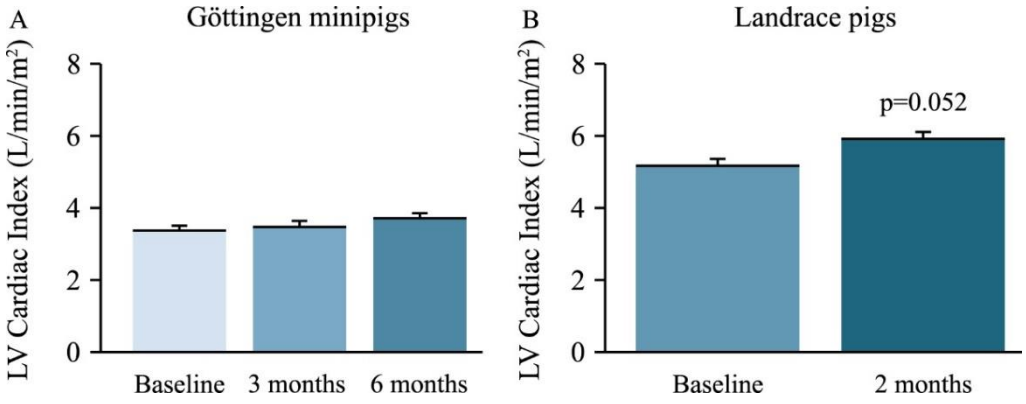

Figure 17. *(A) Left ventricular (LV) cardiac indices (L/min/m^2) of Göttingen minipigs and (B) Landrace pigs.*

Heart rate (HR) of Göttingen minipigs increased significantly at 3 (20%) and 6 months (22%) after MI compared to baseline values (Table 10). In contrast, the HR of Landrace pigs didn't change significantly during the follow-up period.

Table 10. *Heart rate (HR), cardiac output (CO) and body surface area (BSA) of Göttingen minipigs and Landrace pigs. *p<0.05 vs. corresponding baseline (repeated measures one-way ANOVA followed by Fisher's LSD test in Göttingen minipigs; paired t-test in Landrace pigs).*

Measured parameter	Göttingen minipigs			Landrace pigs	
	Baseline	3 months	6 months	Baseline	2 months
HR [L/min]	79.64±4.03	95.55±5.34*	97.00±4.46*	93.44±2.73	88.00±2.52
CO [L/min]	2.37±0.16	2.58±0.20	3.12±0.24*	3.65±0.25	6.41±0.39*
BSA [m^2]	0.70±0.01	0.73±0.01*	0.83±0.03*	0.70±0.01	1.08±0.03*

In Göttingen minipigs CO showed a significant, 32% increase only at 6 months of follow-up, whereas CO was increased by 76% in Landrace pigs after 2 months due to a significant increase in LVSV (Table 2). BSA increased significantly in both breeds at the measured time points (Table 2). BSA increased by 4% and 19% in Göttingen minipigs after 3 and 6 months, respectively, and by 54% in Landrace pigs after 2 months.

4.13. Increase in right ventricular morphofunctional parameters were observed in both Göttingen minipigs and Landrace pigs

MI affected not only left ventricular function but it also resulted in a significant increase of right ventricular ejection fraction (RVEF) in both breeds (Figure 18) measured by CMRI, despite the negligible right ventricular scar size.

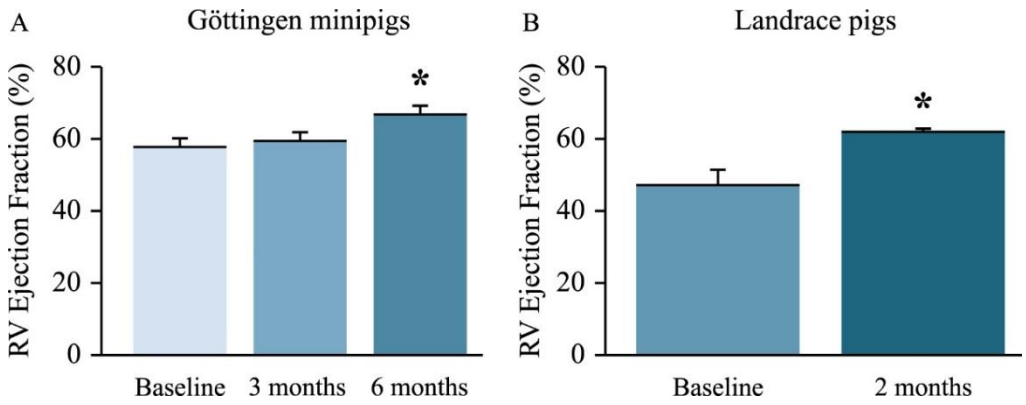

Figure 18. *Right ventricular (RV) ejection fractions (%) of Göttingen minipigs (**A**) and Landrace pigs (**B**) (*p<0.05 vs. corresponding baseline).*

RVED mass increased in Landrace pigs only (Table 11).

Table 11. *Right ventricular end-diastolic (RVED) mass, right ventricular end-systolic volume (RVESV), right ventricular end-diastolic volume (RVEDV) and right ventricular stroke volume (RVSV) in Göttingen minipigs and Landrace pigs (*p<0.05 vs. corresponding baseline).*

Measured parameter	Göttingen minipigs			Landrace pigs	
	Baseline	3 months	6 months	Baseline	2 months
RVED mass [g]	8.64±0.68	8.98±0.76	7.94±0.77	16.49±0.90	23.61±1.40*
RVESV [ml]	18.27±1.47	16.91±1.80	14.57±1.02	43.59±3.68	42.65±2.37
RVEDV [ml]	44.16±2.61	42.14±2.83	46.27±3.45	83.03±3.42	113.72±5.12*
RVSV [ml]	25.82±1.72	25.25±1.67	31.71±2.99*	39.44±3.52	71.06±3.38*

Right ventricular end-systolic volume (RVESV) did not change during follow up in any of the breeds. Right ventricular end-systolic volume (RVEDV) increased significantly by 37% only in Landrace pigs (Table 11). While right ventricular stroke volume (RVSV) in Göttingen minipigs increased significantly by 23% only after 6 months, in Landrace pigs a significant 80% increase in RVSV was observed at 2 months.

5. Discussion

In summary, as the leading causes of attrition in drug development programs are related to unexpected cardiotoxicity and failure of translation of preclinical efficacy (1, 9-11). this thesis focused on these two relevant aspects of failure of drug development in preclinical phase. The first aspect was the need of new reliable screening methods for prediction of drug-induced cardiotoxicity, highlighting hidden cardiotoxicities. Here, we demonstrated for the first time in the literature that rofecoxib increased acute mortality due to its proarrhythmic effect as indicated by increased APD75 during I/R. We also showed that rofecoxib did not interfere with the cardioprotective effect of IPC and that IPC was able to protect against rofecoxib-induced hidden cardiotoxicity. The second aspect was related to the need to characterize a clinically relevant translational pig model of post-MI HF, which is a prerequisite for final proof-of-concept studies before entering into clinical trials in drug and medical device development. We described a detailed protocol of acute MI and the evaluation of post-MI HF in a closed-chest model of adult Göttingen minipigs. In addition, this was the first characterization of post-MI HF in Göttingen minipigs in comparison to commonly used Landrace pigs, showing that the Göttingen minipig model reflects post-MI HF parameters comparable to humans.

We have shown that chronic rofecoxib treatment increased mortality following acute cardiac I/R in rats. This finding also is in line with the clinical data. Selective COX-2 inhibition with rofecoxib and celecoxib increased mortality at all doses in patients with prior myocardial infarction, however, the underlying mechanisms were not explained (138). Our findings suggest that the increased mortality due to rofecoxib treatment can be attributed to its proarrhythmic property that is masked in normoxic conditions, but can manifest during I/R. With the aim of further analyzing the hidden proarrhythmic effects of rofecoxib, we subjected left ventricular papillary muscles to sI/R conditions. APD was significantly prolonged by rofecoxib only in cardiac tissues subjected to sI/R. This finding broadly supports our in vivo data and highlights that myocardial ischemia is critical in induction of arrhythmias on top of treatment with drugs with hidden cardiotoxic properties (12). Mechanistically, increased spatial dispersion of repolarization between normoxic and ischemic myocardium promotes the development of ischemia-induced arrhythmias (139, 140) and rofecoxib may further exacerbate differences in APD

between normoxic and ischemic myocardium, further increasing the arrhythmia substrate in I/R (141).

Although here we did not assess the mechanism of rofecoxib-induced arrhythmias, suppression of COX-2-derived PGI2 and PGE2 synthesis and other "off target" effects of COX-2 inhibitors can be assumed (142). Supporting the theory of potential "off target" effects of rofecoxib, the risk for arrhythmia events was not increased with COX-2 inhibitors other than rofecoxib in a comprehensive meta-analysis of 114 randomized trials (55). However, subgroup analysis of patients with ischemic heart diseases was not performed in this meta-analysis, which may have revealed its cardiotoxicity even earlier. These results clearly show that the exclusion of patients with preexisting cardiovascular diseases from clinical trials and the lack of subgroup analyses on patients with underlying co-morbidities in clinical settings may lead to the loss of valuable safety information on drugs with potential hidden cardiotoxic effects (12, 143). In accordance with our preclinical data, previous clinical studies have demonstrated that myocardial ischemia is crucial in unmasking cardiotoxic effects of COX-2 inhibitors. Repeated administration of valdecoxib had no effect on QTc interval duration in healthy volunteers (144), and valdecoxib and parecoxib did not increase the risk for cardiac adverse events in patients recovering from major noncardiac surgical procedures (145). However, the use of valdecoxib and parecoxib in patients subjected to coronary-artery bypass grafting (CABG) was associated with an increased incidence of cardiovascular events (146). In the latter studies incidence of arrhythmias was not evaluated, however increased incidence of cardiovascular events further highlights the critical role of I/R as a provocative factor to exacerbate cardiotoxic effects of certain drugs. In addition, mortality was increased by celecoxib in a chronic post-MI HF in pigs due to left ventricular rupture and cardiac decompensation (147). In clinical settings, celecoxib use was associated with a dose-related increase in death from cardiovascular causes, myocardial infarction, stroke or HF (148). In contrast to this finding, low dose celecoxib treatment was noninferior in terms of cardiovascular safety as compared to naproxen and ibuprofen in patients at increased cardiovascular risk and osteoarthritis or rheumatoid arthritis (149). Based on these findings, it is plausible that hidden cardiotoxic effects may occur dose dependently. Since the hidden cardiotoxic effect of drugs may manifest not only as aggravation of I/R injury but also as attenuation of ischemic adaptation of the myocardium by ischemic

conditioning (12), here we also investigated the interaction of rofecoxib with IPC. We found that rofecoxib alone decreased IS and did not interfere with the protective effect of IPC. To further test if the IS limiting effect was due to direct cardio-cytoprotective effect or was an artefact due to the significantly less survival of animals with larger IS, we tested the cytoprotective effect of rofecoxib in cardiac myocytes subjected to sI/R and found an increased cell survival due to rofecoxib treatment. These results show that rofecoxib has a direct cardio-cytoprotective effect. IS-limiting effect was also shown in rats using another COX-2 inhibitor celecoxib. Furthermore, in this study mortality rate of celecoxib treatment was not reported, which can also alter outcomes of cardioprotection significantly (150). DFU, a compound structurally related to rofecoxib, led to a significant improvement in left ventricular end-diastolic pressure and LV systolic pressure and a reduction in IS after myocardial infarction in Lewis male rats (151). A neutral effect on IS was shown in different animal models of myocardial infarction with the use of various COX-2 inhibitors (152-155). In contrast, Inserte *et al*. showed that in transgenic mice constitutively expressing human COX-2 in cardiomyocytes functional recovery was improved, cell death was reduced after 40 min of *ex vivo* ischemia, and that pretreatment of mice with the COX-2 inhibitor DFU attenuated cardioprotection (156). In a recent publication inducible cardiac-specific COX-2 overexpression showed infarct-limiting effect in mice (157). Confirming our finding on direct cardioprotection by rofecoxib *in vivo*, here we also showed that rofecoxib increased cell survival in sI/R on isolated cardiomyocytes. These findings are in line with a previous report where cytoprotective effects of COX-2 inhibition was demonstrated in H9c2 cells and primary rat cardiomyocytes in a simulated hypoxia/reoxygenation (H/R) model, showing that a pretreatment with a COX-2 inhibitor NS-398 significantly attenuated H/R-induced cellular injury. The activation of RISK cardiomyocyte survival cascade following COX-2 inhibition was proposed (158).

Our results and other previous observations suggest that the presence and/or extent of cardioprotection by COX-2 inhibitors may vary due to both the nature of the applied inhibitor and the model species according to different anatomy, physiology, and pharmacokinetics, dosing, etc., and due to differences in surgery protocols (anesthetics, co-medications, chronic or acute cardiac ischemia etc.). Janus-like nature and central role of COX-2 inhibition on cardioprotection seems plausible. Here we found that rofecoxib

pretreatment did not affect the IS limiting effect of IPC. Confirming our results, parecoxib administered intravenously 15 min prior to IPC did not affect the IS limiting effect of IPC in male Wistar rats (159). Somewhat in contrast, the protective effect of late phase of ischemic conditioning was attributed to the increased expression and activity of COX-2 (160, 161). Similarly, Sato *et al.* showed upregulation of COX-2 expression in Harlan Sprague Dawley rats in the ischemic-reperfused cardiac region by late preconditioning but not by postconditioning and the use of celecoxib completely abrogated the infarct-sparing effect of the combination of the two interventions (162). In summary, it is unlikely that a class effect of COX-2 inhibitors regarding their influence on cardioprotection by ischemic conditioning exist. Moreover, it should be emphasized that in our present study the action potential-prolonging effect of rofecoxib in papillary muscle preparations subjected to sI/R was not observed when sIPC was applied. These results show that the hidden cardiotoxic effects of rofecoxib can be prevented by ischemic conditioning. Similarly, Maulik *et al*. showed that sIPC protected primary adult rat cardiomyocytes against the direct cardiotoxic effect of doxorubicin (163). However, there are no clinical data so far on the potential protective effect of IPC on drug-induced cardiotoxicity. The currently ongoing (or still unpublished) ERIC-ONC trial aimed to demonstrate whether remote ischemic preconditioning (RIPC) reduces subclinical myocardial injury due to anthracycline chemotherapy (164).

The most important clinically relevant finding of our work was that hidden cardiotoxicity of rofecoxib can be revealed by preclinical cardiotoxicity testing by using experimental I/R models. These results suggest that cardiac safety testing with simple preclinical models of I/R injury might reveal hidden cardiotoxicity of drugs under early stages of development.

As another leading cause of attrition in drug development programs beside unexpected cardiotoxicity is the failure of translation of preclinical efficacy [1, 9-11], here we aimed to characterize a clinically relevant translational pig model of post-MI HF, which is a prerequisite for final proof-of-concept studies before entering into clinical trials in drug and medical device development (126, 127, 165). In addition, this was the first characterization of post-MI HF in Göttingen minipigs in comparison to commonly used Landrace pigs, showing that the Göttingen minipig model reflects post-MI HF parameters comparable to humans.

Pig models resemble humans in anatomy, physiology and biochemical properties in particular in the field of myocardial infarction research as they develop trans-mural infarctions due to the lack of collateral perfusion (128). Therefore, pig models can serve as models for the analysis of cardioprotective therapies and their mechanisms (166-171). In order to analyze the scientific interest on post-MI HF in pigs we performed literature search on PubMed using the following search string: *(pig OR swine OR porcine OR sus-scrofa OR minipig OR mini-pig OR miniature-pig OR miniature-swine) AND (infarct* OR ischem* OR ischaem* OR reperfus*) AND (heart OR cardi* OR myocard*) AND (LAD OR left-anterior* OR LCX OR left-circumflex OR RCA) AND (heart-failure OR lvef OR ejection-fraction OR infarct-size OR infarction-size).* We found that pig models of cardiac ischemia/reperfusion are frequently used to study MI and post-MI HF, but only 17% (71 out of 425 articles) of studies involved minipigs and 7% (30 out of 425 articles) used Göttingen minipigs. Only about 1% (5 out of 425) of studies used Göttingen minipigs and clinically relevant protocols with long-term follow-up (1-9 months of reperfusion) and CMRI to analyze cardiac function. The small number of clinically relevant studies highlights the translational gap between basic research and clinical trials. Here we have found that despite the equal scar sizes, mortality rates and BARI scores in the two breeds, left ventricular dysfunction characterized by decreased LVEF was observed only in Göttingen minipigs. Here we observed a 15.4% acute mortality in Göttingen minipigs and no mortality in the follow-up period, the letter is comparable to that in clinical studies. Indeed, a patient-level meta-analysis of 10 randomized clinical trials found that the Kaplan-Meier estimated 1-year rate of all-cause mortality was as low as 2.2% following myocardial infarction (74). Scar sizes reported here are comparable to those in clinical trials. In clinical trials performed by Lonborg et al and Stone et al in patients surviving ST-elevation myocardial infarction the median scar sizes, measured as % of left ventricular myocardial mass were 9.5% and 17.9 % respectively (74, 172). Moreover, scar sizes in our present study are similar to those reported in previous publications in Göttingen minipigs (12-25%) (173-178) and in Landrace pigs (14-18%) (179-181). Our present finding on baseline LVEF in Landrace swine is in the range of data reported by others in large swine (182-184). These values in large swine are smaller as compared to healthy human LVEF reference ranges (58-61%) (185) and baseline (pre-infarction) values in Göttingen minipigs (55-73%) (174, 186, 187). Nevertheless, it is

worth noting that only the post-infarction data or delta changes of LVEF are reported in most publications (188-192). In accordance with the present results, previous studies of either post-MI HF induced by 45 to 90 min LAD occlusion followed by reperfusion or by permanent LAD occlusion have demonstrated either no reduction or modest reduction of LVEF in Landrace or Yorkshire swine after 4-6 weeks follow up as compared to baseline (pre-infarction) LVEF (193-195). However, Schuleri et al. compared morphofunctional parameters between Göttingen minipigs and Yorkshire swine and found that both breeds showed a decrease of LVEF 8 weeks after induction of MI by 120 to 150 min LAD occlusion-reperfusion; however, baseline LVEF values in Yorkshire swine were not reported (196). In other experiments in female Dalland Landrace pigs post-MI adverse remodeling was induced by 90 min LAD occlusion, however, LVEF was not reported after 4 weeks of follow-up (197). In contrast to our findings, in a study by de Jong et al., LVEF markedly decreased in Landrace pigs subjected to open chest occlusion of the LAD and by a 12 week follow up (198). This difference can be attributed to substantially longer cardiac ischemic period (150 min), which resulted in larger infarction size (23.4 ± 2.1% of LV), therefore, representing patients with severe myocardial infarction and long ischemic time. Elsewhere, 120-min closed-chest occlusion of left circumflex (LCX) coronary artery in German Landrace pigs led to a significant reduction in LVEF after eight weeks of reperfusion, suggesting that the different location of MI may also affect global left ventricular function (199). Although the LCX occlusion results in lower procedural mortality and thus is widely used in cardiovascular research as I/R model, in humans the culprit lesion is most frequently located in the LAD. Occlusion of the LAD in swine results in myocardial infarctions located in anteroapical, lateral and septal LV walls. The same localization and distribution is observed in humans when LAD is occluded which leads to more pronounced dysfunction and different compensatory changes as compared to when LCX is occluded (182). Thus, occlusion of LAD may be the best approach to mimic the clinical scenario which represents the largest patient population surviving AMI. Our present findings are consistent with others showing significant reduction in LVEF in post-MI HF in Göttingen minipigs after long-term follow up (174, 186, 187).

The reduction of LVEF in Göttingen minipigs following MI is consistent with clinical data showing cardiac dysfunction as a consequence of ventricular remodeling in patients

after AMI (200). In conclusion, Göttingen minipigs mimic the human conditions better, than the Landrace pigs, since pre-infarction LVEF, scar size, post-infarction LVEF, and mortality all are comparable to these parameters found in humans.

Here we observed only a slight increase in LVED mass after six months in Göttingen minipigs and an almost 100% increase in LVED masses in Landrace pigs after two months. Similar data were reported by Schuleri et al. in Yorkshire pigs, where a 40% increase in heart weight was observed after two months. In contrast, in other experiments of closed-chest post-MI HF in Göttingen minipigs no significant changes in left ventricular masses were observed (174, 186). Therefore, differences between the two breeds regarding LVEF can be attributed to an intensive cardiac growth rate in Landrace pigs and thus altered cardiac remodeling.

In clinical settings, beside LVEF, left ventricular volume provides valuable insight into long-term prognosis and mortality rate in post-MI patients (201). LVESV is the primary determinant of both early and late mortality in patients after AMI (202, 203). Here we have shown that ventricular volume assessed by CMRI increased significantly in both breeds. Post-MI remodeling induced a more pronounced increase in LVESV than in LVEDV in Göttingen minipigs, while both LVESV and LVEDV were increased by a similar rate in Landrace pigs. Consequently, left ventricular ejection fraction (LVEF) was significantly decreased at 3 and 6 months only in Göttingen minipigs but not in Landrace pigs after 2 months. These results must be interpreted with caution in Landrace pigs, where increased LVESV, LVEDV, and LVSV (calculated as the difference between the LVESV and LVEDV) are more likely related to an intensive increase in cardiac mass. Increased LVESV and LVEDV are consistent with clinical data of patients with post-MI HF (204-206). Moreover, adverse left ventricular remodeling was defined as an increase of 15% or more in the LVEDV in clinical studies (98, 207) and we found here a 28% increase after 3 months and a 42% increase after 6 months in LVEDV in Göttingen minipigs showing a clinically relevant adverse remodeling. In addition, here we have shown that LAVi increased only in Göttingen minipigs, but not in Landrace pigs. Increase of left atrial volume is an additional key structural alteration in the context of HF and is an independent predictor of death and HF hospitalization in patients surviving MI (208). Although the LAVi is not reported in the pig post-MI HF studies routinely, it may serve as a reliable parameter to further characterize the post-MI HF models.

Right ventricular function is rarely studied in post-MI HF models. Here we have found that right ventricular ejection fraction increased in both breeds. Although RV was practically not involved in myocardial necrosis, RVEF increased significantly in both breeds indicating RV volume overload and hence left ventricular dysfunction. Similarly, a clinical study enrolling 2008 patients with chronic systolic HF showed that 733 patients (37%) belonged to normal right ventricular function category with RVEF≥40% (209).

The most important finding of our work from the aspect of drug development is that the adult Göttingen minipig model with long-term follow-up mimics functional and morphological parameters of post-MI HF comparable to humans. Our present data also show that Landrace pigs are not suitable for the evaluation of post-MI HF mainly due to consequences of the rapid increase in body- and heart weight that does not allow long-term follow-up and interferes with post-MI HF pathology. In contrast, Landrace pigs might be suitable to assess the consequences of acute myocardial infarction. In addition, by choosing the proper endpoints and follow-up times, Landrace pigs may serve as models of cardiac diseases due to their lower price and easier accessibility. This present comprehensive characterization of the closed-chest infarction models in Landrace pigs and Göttingen minipigs will be useful for choosing the optimal large animal models to study post-MI HF and developing novel therapies against this pathology.

Despite our promising results, questions remain and further work is required to establish new methods to prevent high attrition rates in drug development.

In our study to predict hidden cardiotoxicity results strongly suggest that by supplementing current toxicity screening methodologies with a pathological condition, in this case I/R, useful information can be easily obtained about the behavior of a drug in diseased state. To mimic the “real world” patient population, further factors can be added to the test system, such as other diseases and medications. Differences of drug response and effect on action potentials across species have been also reported. Rats possesses minimal I_{Kr} as compared to humans. Thus, further research might be necessary to investigate the effect of I/R in clinically relevant hiPSC-CMs. However, at an organ level, differences in current densities within the ventricles may occur, suggesting that measurement of a single current may lead to misleading conclusions and further development may be required to better model the complexity of in vivo physiology (4).

In recent years, increasing interest was focused on miRNAs that may serve as reliable

biomarkers of diseases (210). A number of studies suggest that early changes in microRNA expression profiles might also predict cardiotoxicity (50, 211, 212). Thus, unbiased analysis of the raw small RNA sequencing data from samples gathered from subjects treated with hidden cardiotoxic drug and its bioinformatics evaluation may also give valuable information on effect of treatment on miRNA fingerprints. These investigations may also lead to finding of new toxicity biomarkers. According to Zhang et al, time-cumulative meta-analytic approach would have revealed cardiotoxicity of rofecoxib earlier (55), suggesting that quicker detection of adverse drug reactions and improved early warning systems are needed in the field of pharmacovigilance.

Regarding the need to characterize a more robust clinically relevant translational pig model of post-MI HF we can conclude that our approach and model answers a number of issues: relevance of long-term follow up and the use of clinically relevant endpoints. Therefore, we took a step toward bridging the translational gap between basic science and clinical efficacy. However, in the future investigations it might be mandatory to validate cardioprotective and symptomatic/causative therapies of post-MI HF in aged swine with comorbidities and comedications using clinically relevant endpoints, protocols and multicenter preclinical approach (19, 143).

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6. Conclusions

6.1. Hidden cardiotoxicity of rofecoxib can be revealed by experimental I/R models

Here we demonstrated for the first time in the literature that rofecoxib increased acute mortality due to its proarrhythmic effect as indicated by increased APD75 during I/R. We also showed that rofecoxib did not interfere with the cardioprotective effect of IPC and that IPC was able to protect against rofecoxib-induced hidden cardiotoxicity. Therefore, our data highlights the value of testing drug candidates for hidden cardiotoxicity in disease models such as I/R injury.

6.2. Göttingen minipig model is superior to the Landrace pig in long-term follow-up post-MI HF studies

We have shown here that the adult Göttingen minipig model with long-term follow-up mimics functional and morphological parameters of post-MI HF comparable to humans. Our data also show that Landrace pigs are not suitable for the evaluation of post-MI HF mainly due to consequences of the rapid increase in body and heart weight that does not allow long-term follow-up and interferes with post-MI HF pathology. Landrace pigs might be suitable to assess the consequences of acute myocardial infarction. Our comprehensive characterization of the closed-chest infarction models in Landrace and Göttingen minipigs will be useful for choosing the optimal large animal models to study post-MI HF and developing novel therapies against this pathology.

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7. Summary

Drug development is a time-consuming, complex and expensive process that consists of preclinical and clinical phases (1-3). Attrition in drug development in later phases, such as phase 3 and phase 4, may lead to high sunk costs, increased patient risks and waste of time of scientists (4). As the leading causes of attrition in drug development programs are related to unexpected cardiotoxicity and failure of translation of preclinical efficacy, this thesis focused on these two relevant aspects of failure of drug development in preclinical phase. The first aspect was the need of new reliable screening methods for prediction of drug-induced cardiotoxicitiy, highlighting hidden cardiotoxicities. Here, we demonstrated for the first time in the literature that rofecoxib increased acute mortality due to its proarrhythmic effect as indicated by increased APD75 during I/R. We also showed that rofecoxib did not interfere with the cardioprotective effect of IPC and that IPC was able to protect against rofecoxib-induced hidden cardiotoxicity. The second investigated aspect was related to the need to characterize a clinically relevant translational pig model of post-MI HF, which is a prerequisite for final proof-of-concept studies before entering clinical trials in drug and medical device development. Here we described a detailed protocol of acute MI and the evaluation of post-MI HF in a closed-chest model of adult Göttingen minipigs. In addition, this was the first characterization of post-MI HF in Göttingen minipigs in comparison to commonly used Landrace pigs, showing that the Göttingen minipig model reflects post-MI HF parameters comparable to humans.

In summary, this thesis highlighted the value of testing drug candidates for hidden cardiotoxicity in disease models such as I/R injury. In addition, we have shown that Göttingen minipig model is superior to the Landrace pig for following up the development of post-MI HF.

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9. Bibliography of the candidate's publications

9.1. Own publications involved in the current thesis

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9.2. Own publications not involved in the current thesis

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