Multi-Omics Profiling of Myocardial Stunning and Necrosis across Experimental and Clinical Settings

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To My People

“Where there is ruin, there is hope for a treasure.”

— Rumi

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ABSTRACT

The myocardial response to ischemia–reperfusion (I/R) injury ranges from reversible post-ischemic contractile dysfunction, termed myocardial stunning, to irreversible myocardial necrosis. Understanding the mechanisms that determine these divergent outcomes is fundamental to the study of endogenous cardioprotection. Ischemic preconditioning (IPC) increases myocardial tolerance to I/R injury and shifts the post-ischemic response toward more reversible outcomes; however, the molecular mechanisms underlying this protective effect remain incompletely understood. This thesis investigates these mechanisms using an experimental *in vivo* rat model in combination with plasma proteomic analyses of blood samples from patients. By integrating phosphoproteomics, proteomics, transcriptomics, and DNA methylation analyses, the thesis aims to characterize the molecular programs associated with IPC-induced cardioprotection.

Study I: IPC reduced infarct size while shifting post-ischemic outcomes toward reversible myocardial stunning following ischemia–reperfusion. Phosphoproteomic profiling demonstrated extensive regulation of phosphorylation sites in proteins related to the sarcomere, Z-disc, cytoskeleton, and actin-binding structures. These findings suggest that IPC induces rapid phosphorylation-dependent contractile changes during ischemic stress.

Study II: Quantitative myocardial proteomics further characterized time-resolved molecular responses to IPC. Early adaptive pathways associated with endocytosis and Fc gamma receptor-mediated phagocytosis were upregulated, whereas later inflammatory, complement, and coagulation cascades were downregulated compared with non-preconditioned myocardium. These findings indicate that IPC may affect inflammatory remodeling after reperfusion.

Study III: Transcriptomic analyses demonstrated that IPC is accompanied by reprogramming of the cardiac gene-expression landscape together with epigenetic changes. IPC was associated with reduced global DNA methyltransferase activity and selective promoter methylation changes in genes involved in inflammation, stress signaling, and DNA repair, consistent with reduced activation of maladaptive pathways. These findings are also consistent with an effect of IPC on post-ischemic inflammation and remodeling.

Study IV: In a clinical study, temporal plasma proteomics using proximity extension assays distinguished Takotsubo syndrome from ST-elevation myocardial infarction in women. Distinct subacute inflammatory and stress-response signatures were identified in patients with ST-elevation myocardial infarction versus Takotsubo syndrome.

Conclusion: Collectively, these studies demonstrate that IPC induces phosphoproteomic, proteomic, transcriptomic, and epigenetic changes that may play a role in IPC-induced myocardial resilience to ischemic injury. The findings are consistent with the concept of myocardial stunning as a biologically regulated and potentially adaptive state.

Keywords: Ischemic preconditioning; Myocardial stunning; Ischemia–reperfusion injury; Cardioprotection; Phosphoproteomics; Proteomics; Transcriptomics; Takotsubo syndrome

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SAMMANFATTNING PÅ SVENSKA

Myokardiets svar på ischemi-reperfusionsskada spänner över ett spektrum från reversibel post-ischemisk kontraktil dysfunktion, så kallad myokardiell stunning, till irreversibel myokardnekros. Att klarlägga de mekanismer som avgör myokardiets post-ischemiska förlopp är centralt för förståelsen av endogen kardioprotektion. Ischemisk prekonditionering (IPC) kan förskjuta post-ischemiska mekanismer mot ökad myokardiell tolerans och mer reversibla utfall, men den molekylära grunden för denna effekt är fortfarande ofullständigt känd. Denna avhandling undersöker den molekylära grunden för dessa svar med hjälp av en experimentell *in vivo*-råttmodell tillsammans med en kompletterande klinisk plasmaproteomisk analys. Genom att integrera fosfoproteomiska, proteomiska, transkriptomiska, och DNA-metyleringsrelaterade analyser syftar avhandlingen till att definiera molekylära program associerade med IPC-inducerad kardioprotektion.

Studie I: IPC minskade infarktstorleken samtidigt som graden av reversibel myokardiell stunning ökade efter ischemi–reperfusion. Fosfoproteomisk profilering påvisade omfattande reglering av fosforyleringsställen i proteiner kopplade till sarkomeren, Z-disken och aktinbindande strukturer. Dessa fynd tyder på att IPC inducerar snabb fosforyleringsbaserad strukturell och kontraktil omprogrammering som kan bidra till att stabilisera sarkomerer och cytoskelettet under ischemisk stress.

Studie II: Kvantitativ myokardiell proteomik karakteriserade de tidsberoende molekylära svaren på IPC. Tidigt sågs uppreglering av endocytosrelaterade signalvägar, medan senare inflammatoriska processer samt komplement- och koagulationskaskader nedreglerades jämfört med icke-prekonditionerat myokardium. Dessa resultat indikerar att IPC orsakar molekylära anpassningar som skulle kunna dämpa maladaptiv inflammatorisk remodellering efter reperfusion**.**

Studie III: Transkriptomiska analyser visade att IPC omprogrammerar hjärtats genuttryck och åtföljs av epigenetiska förändringar. IPC var associerad med minskad global aktivitet av DNA-metyltransferaser samt selektiva förändringar i promotormetylering i gener involverade i inflammation, stressignalering och DNA-reparation.

Studie IV: I en klinisk studie visade plasmaproteomik med proximity extension assay tidsberoende skillnader mellan Takotsubo-syndrom och ST-höjningsinfarkt hos kvinnor. Distinkta subakuta inflammatoriska och stressrelaterade proteomiska signaturer identifierades, vilket belyser molekylära skillnader mellan övergående myokardiell dysfunktion vid Takotsubo och irreversibel hjärtcellsdöd vid hjärtinfarkt.

Slutsats: Sammantaget talar dessa studier för att ischemisk prekonditionering är associerad med fosfoproteomiska, proteomiska, transkriptomiska och epigenetiska förändringar som kan bidra till att öka myokardiets motståndskraft mot ischemisk skada. Resultaten är också förenliga med att myokardiell stunning kan uppfattas som ett reglerat och potentiellt adaptivt tillstånd.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Elmahdy A, Shekka Espinosa A, **Kakaei Y**, et al. Ischemic preconditioning affects phosphosites and accentuates myocardial stunning while reducing infarction size in rats. ***Front Cardiovasc Med.*** **2024;11:1376367.**

II. **Kakaei Y**, Hussain S, Elmahdy A, et al. Comparison of the proteomic landscape in experimental ischemia–reperfusion with versus without ischemic preconditioning. ***Sci Rep.*** **2025;15:11836.**

III. Khan AW, Hussain S, Elmahdy A, **Kakaei Y**, et al. Ischemic preconditioning regulates cardiac transcriptome via DNA methylation conferring cardioprotection from ischemia–reperfusion injury. ***Eur Heart J Open.*** **2025;5:oeaf124**.

IV. **Kakaei Y**, Kalani M, Jha S, et al. Temporal plasma proteomic differences between Takotsubo syndrome and ST-elevation myocardial infarction identified by proximity extension assay. ***In manuscript***

CONTENTS

ABBREVIATIONS

ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
CA2	Carbonic Anhydrase 2
ChIP	Chromatin Immunoprecipitation
DDA	Data-Dependent Acquisition
DNMT	DNA Methyltransferase
ECG	Electrocardiogram
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Extracellular Vesicle
FAC	Fractional Area Change
FASP	Filter-Aided Sample Preparation
FDR	False Discovery Rate
FS	Fractional Shortening
GLS	Global Longitudinal Strain
GO	Gene Ontology
I/R	Ischemia–Reperfusion
IPC	Ischemic Preconditioning
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAD	Left Anterior Descending (artery)
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
LOD	Limit of Detection
LVEF	Left Ventricular Ejection Fraction

MAOA	Monoamine Oxidase A
MI	Myocardial Infarction
mPTP	Mitochondrial Permeability Transition Pore
NIPC	Non-Ischemic Preconditioning
NSTEMI	Non-ST-Elevation Myocardial Infarction
PEA	Proximity Extension Assay
SEM	Standard Error of the Mean
STAMI	Stunning in Takotsubo versus Acute Myocardial Infarction
STEMI	ST-Elevation Myocardial Infarction
TMT	Tandem Mass Tag
TS	Takotsubo Syndrome
TTC	2,3,5-Triphenyltetrazolium Chloride
WMSI	Wall Motion Score Index

DEFINITIONS IN SHORT

Ischemia–Reperfusion (I/R) Injury	Tissue injury that occurs following restoration of blood flow after a period of ischemia. I/R injury is characterized by oxidative stress, calcium overload, mitochondrial dysfunction, inflammation, and cell death.
Ischemic Preconditioning (IPC)	A cardioprotective phenomenon in which brief, non-lethal episodes of ischemia render the myocardium more resistant to subsequent prolonged ischemic injury.
Myocardial Necrosis	Irreversible cardiomyocyte death, typically caused by prolonged ischemia and associated with membrane disruption, inflammatory activation, and structural myocardial damage.
Multi-Omics	An integrative analytical framework that combines multiple high-dimensional molecular datasets, including phosphoproteomics, proteomics, transcriptomics, and epigenetic analyses, to characterize coordinated biological responses.
Myocardial Stunning	Reversible post-ischemic contractile dysfunction that persists after reperfusion despite restoration of adequate perfusion and the absence of irreversible necrosis.

1 INTRODUCTION

1.1 ACUTE ISCHEMIC HEART DISEASE: EPIDEMIOLOGY AND CLINICAL HETEROGENEITY

Acute ischemic heart disease remains one of the leading causes of mortality worldwide and continues to impose a substantial burden on healthcare systems through high rates of hospitalization, recurrent cardiovascular events, and long-term disability (1, 2). Despite considerable advances in prevention, pharmacological therapy, and revascularization strategies, clinical outcomes after acute ischemic events remain highly variable (3, 4). Whereas some patients recover with limited long-term impairment, others progress toward adverse ventricular remodeling, arrhythmias, and chronic heart failure (5).

This variability indicates that restoration of coronary perfusion, although central to treatment, does not by itself fully determine myocardial outcome or long-term prognosis (6). Rather, the response to ischemic stress reflects a complex interplay of biological processes involving metabolic adaptation, ion homeostasis, mitochondrial integrity, inflammatory signaling, and tissue repair (7). These responses influence not only the degree of cellular injury during ischemia but also the capacity of the myocardium to recover after reperfusion.

Importantly, the biological heterogeneity of acute myocardial injury cannot be fully explained by coronary anatomy alone. Acute myocardial injury may occur in the absence of an obstructive epicardial culprit lesion, and clinically similar presentations may arise from distinct upstream pathophysiological mechanisms (8). These observations support a broader mechanistic framework in which irreversible structural injury and reversible functional suppression may represent partially distinct biological responses to acute cardiac stress.

1.2 CLINICAL SPECTRUM OF ACUTE ISCHEMIC MYOCARDIAL INJURY

1.2.1 DEFINITION AND CLINICAL PRESENTATIONS

Acute ischemic heart disease encompasses clinical conditions characterized by an abrupt mismatch between myocardial oxygen supply and demand, resulting in acute myocardial ischemia and, in some cases, myocardial injury (9). In most cases, this imbalance arises from impaired coronary perfusion due to atherosclerotic plaque rupture or erosion with superimposed thrombosis, but it may also result from vasospasm, coronary microvascular dysfunction, or other mechanisms that reduce oxygen delivery relative to demand (3).

Clinically, acute ischemic myocardial injury most commonly presents within the spectrum of acute coronary syndromes (ACS), including ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI), and unstable angina (10). The diagnosis of acute myocardial infarction is based on a rise and/or fall in cardiac troponins together with evidence of myocardial ischemia, such as compatible symptoms, electrocardiographic abnormalities, or imaging findings (9). However, these clinical definitions do not fully capture the mechanistic diversity underlying acute myocardial injury. Similar clinical presentations may reflect markedly different combinations of reversible dysfunction, irreversible myocyte loss, inflammatory activation, and recovery processes after ischemia–reperfusion (I/R) (7).

1.3 PATHOPHYSIOLOGY OF ACUTE MYOCARDIAL ISCHEMIA

1.3.1 MYOCARDIAL ISCHEMIA

Myocardial ischemia develops when coronary blood flow becomes insufficient to meet the metabolic demands of the myocardium (7). Because the heart has high and continuous energy requirements, even brief reductions in oxygen delivery can rapidly disrupt cellular homeostasis (11). Under physiological conditions, cardiomyocytes rely predominantly on mitochondrial oxidative phosphorylation to generate adenosine triphosphate (ATP), which is required to maintain contractile activity, ionic gradients, and cellular integrity (12).

During ischemia, oxygen deprivation forces a metabolic shift from aerobic ATP production toward anaerobic glycolysis (13). This transition leads to progressive ATP depletion and accumulation of metabolic by-products such as lactate, resulting in intracellular acidosis (14). Declining ATP levels impair the function of energy-dependent ion transporters, including Na^+/K^+-ATPase and Ca^{2+}-ATPases, thereby promoting ionic disequilibrium and intracellular sodium accumulation (15). As intracellular sodium rises, the sodium-calcium exchanger may operate in reverse mode, driving calcium influx and contributing to intracellular calcium overload, a key mediator of ischemic dysfunction and injury (**Figure 1**).

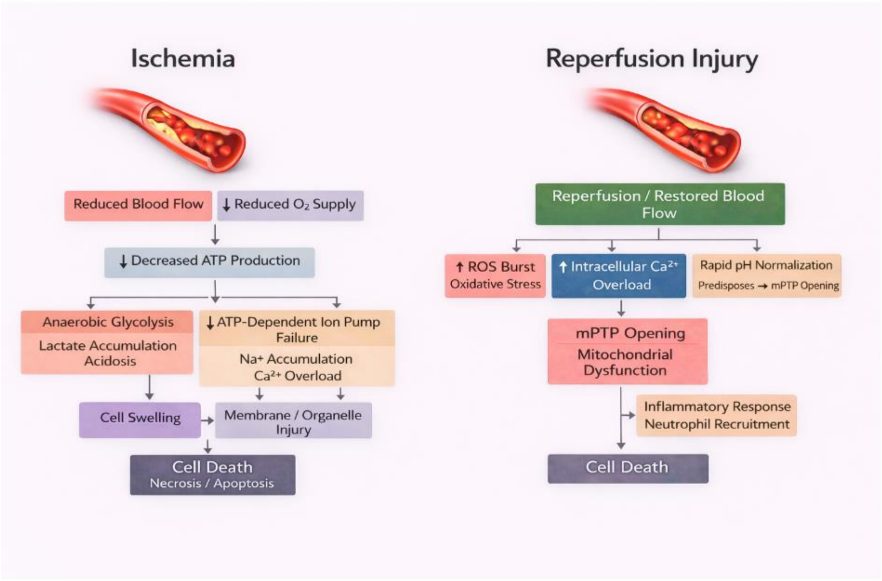


Figure 1**. Schematic overview of myocardial ischemic and reperfusion injury.* *During ischemia, reduced coronary blood flow and oxygen supply impair ATP production, promoting anaerobic glycolysis, intracellular acidosis, ion pump failure, cellular swelling, and membrane/organelle injury. With increasing severity or duration, these processes may progress to irreversible cell death. Upon reperfusion, restoration of blood flow can salvage viable myocardium but also triggers additional injury pathways, including reactive oxygen species (ROS) generation, intracellular Ca^{2+} overload, rapid pH normalization, mitochondrial permeability transition pore (mPTP) opening, mitochondrial dysfunction, and inflammatory activation, which may further contribute to myocardial injury and cell death.* ***Created with BioRender.com.

Elevated cytosolic calcium disrupts excitation-contraction coupling, impairs sarcomeric performance, and activates calcium-dependent proteases and phospholipases that further compromise cellular structure and function (16). In parallel, ischemia induces mitochondrial dysfunction and metabolic disturbances that sensitize cardiomyocytes to subsequent oxidative stress during reperfusion (17). The duration and severity of ischemia are therefore major determinants of outcome: short-lived ischemia may result predominantly in transient contractile dysfunction without irreversible structural injury, whereas prolonged ischemia ultimately leads to loss of membrane integrity and cell death (18).

1.3.2 ISCHEMIA–REPERFUSION INJURY

Although restoration of coronary blood flow is essential for myocardial salvage, reperfusion can paradoxically trigger additional injury pathways, a phenomenon known as ischemia–reperfusion injury (17). The abrupt reintroduction of oxygen and metabolic substrates initiates a series of oxidative, ionic, inflammatory, and microvascular events that may amplify tissue damage beyond that incurred during ischemia alone (13).

A central mechanism in reperfusion injury involves mitochondrial dysfunction. During ischemia, mitochondria become functionally compromised. Upon reperfusion, rapid re-oxygenation is associated with an early burst of reactive oxygen species, which can promote opening of the mitochondrial permeability transition pore (mPTP), resulting in further mitochondrial depolarization, impaired ATP generation, and activation of cell death pathways (17). Reperfusion is also accompanied by rapid normalization of intracellular pH, which can intensify ionic fluxes and promote calcium loading, thereby potentiating necrotic and apoptotic signaling (19).

At the same time, reperfusion activates innate immune responses. Damaged cardiomyocytes release danger-associated molecular patterns , which initiate inflammatory signaling cascades and recruit neutrophils, macrophages, and other immune mediators to the injured myocardium (20). Complement activation, cytokine release, endothelial dysfunction, platelet aggregation, and leukocyte plugging may all contribute to further tissue injury and microvascular impairment. These processes can culminate in the no-reflow phenomenon, in which tissue-level perfusion remains inadequate despite restoration of epicardial coronary patency (21). Thus, successful reopening of an epicardial vessel does not necessarily equate to effective reperfusion at the myocardial tissue level (**Figure 1**).

Taken together, ischemia and reperfusion constitute a dynamic and interconnected injury process in which reperfusion may either salvage viable tissue or exacerbate ongoing damage. The balance between injurious pathways and endogenous protective mechanisms during this period is a major determinant of functional recovery and structural outcome.

1.4 REVERSIBLE AND IRREVERSIBLE MYOCARDIAL INJURY

Acute myocardial ischemia does not invariably result in uniform structural damage. Rather, myocardial responses exist along a continuum ranging from fully reversible functional impairment to irreversible myocyte death (14). Distinguishing these outcomes is fundamental to prognosis, mechanistic understanding, and therapeutic strategy, as it separates recoverable dysfunction from permanent structural remodeling.

1.4.1 IRREVERSIBLE INJURY: MYOCARDIAL NECROSIS

When ischemia exceeds the threshold of cellular tolerance, cardiomyocytes undergo irreversible injury. Sustained ATP depletion, persistent calcium overload, oxidative stress, and mitochondrial failure progressively compromise sarcolemma integrity and cellular viability (16). Loss of membrane integrity, a defining feature of necrotic cell death, permits leakage of intracellular proteins such as cardiac troponins into the circulation. In this context, troponin release serves as a key biochemical correlate to cardiomyocyte injury and, in the setting of membrane disruption, is consistent with irreversible structural damage (9).

Necrosis initiates a robust inflammatory response. Cellular debris activates innate immune pathways and recruits neutrophils and macrophages to the injured region. Although these responses are essential for clearance of dead tissue and subsequent repair, they can also contribute to secondary injury, extracellular matrix remodeling, and expansion of myocardial dysfunction (20). Over time, necrotic myocardium is replaced by fibrotic scar tissue, which preserves wall integrity but lacks contractile capacity and predisposes to adverse ventricular remodeling, systolic impairment, and arrhythmogenesis (22). Irreversible injury therefore has enduring structural and functional consequences that extend well beyond the acute ischemic event.

1.4.2 REVERSIBLE INJURY: MYOCARDIAL STUNNING

In contrast, shorter or less severe ischemic insults may preserve cellular viability while producing marked contractile dysfunction. Myocardial stunning was classically defined as prolonged but reversible post-ischemic systolic dysfunction that persists after restoration of coronary blood flow in the absence of overt infarction or necrosis (23). Central to this concept are preserved viability, absence of substantial irreversible injury, and delayed but eventual functional recovery.

In stunned myocardium, widespread cell death does not occur, and membrane integrity is largely maintained. Cardiac biomarkers may be absent, minimal, or transiently elevated depending on the clinical context, but the predominant abnormality lies in reversible impairment of excitation-contraction coupling, dysregulated calcium handling, and altered contractile protein function (9, 16). Oxidative stress, transient calcium overload, and post-translational modification of contractile and cytoskeletal proteins have all been implicated in the pathogenesis of myocardial stunning (16). Functional recovery generally occurs over hours to days as metabolic, redox, and ionic homeostasis are progressively restored (23) (**Figure 2**).

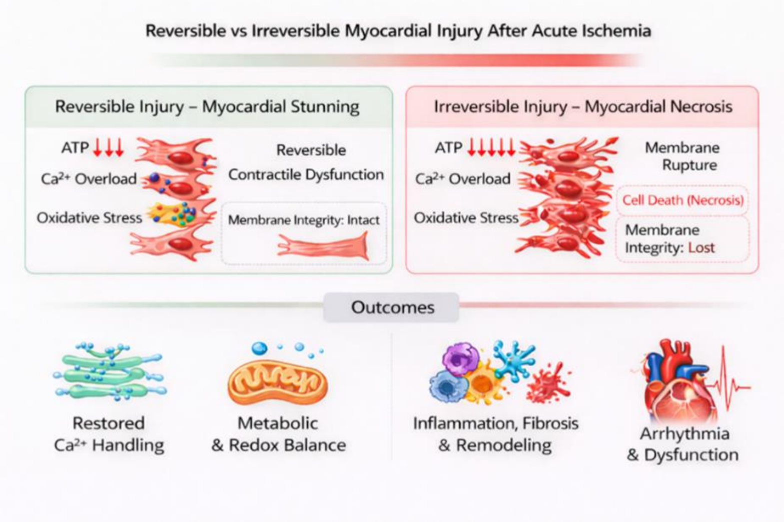


***Figure 2*. Conceptual framework of reversible and irreversible post-ischemic myocardial outcomes after acute ischemia.**
Acute ischemic stress may give rise to a continuum of myocardial responses, ranging

*from reversible post-ischemic contractile dysfunction (myocardial stunning) to irreversible structural injury with myocyte necrosis. In myocardial stunning, ATP depletion, calcium overload, and oxidative stress impair contractile function despite preserved membrane integrity and cellular viability, allowing recovery. In contrast, more severe or prolonged ischemia leads to loss of membrane integrity, necrotic cell death, and subsequent inflammatory and remodeling responses that may contribute to persistent dysfunction and arrhythmogenesis. The left side of the figure highlights recovery-associated processes in viable stunned myocardium, whereas the right side illustrates irreversible myocardial injury***, Created with ChatGPT*.**

Importantly, myocardial stunning and necrosis are not necessarily mutually exclusive. Following a single ischemic insult, different myocardial regions may exhibit different fates, with irreversible necrosis in some areas and reversible dysfunction in adjacent viable myocardium (7). This coexistence highlights that myocardial outcome after ischemia is spatially and biologically heterogeneous rather than uniform.

Traditionally, myocardial stunning has been regarded as a residual consequence of ischemic injury, proportional to insult severity. However, observations from cardioprotective models challenge a strictly linear interpretation. In some experimental settings, interventions that reduce irreversible injury, such as ischemic preconditioning (IPC), have also been associated with greater transient post-ischemic contractile dysfunction (24). This apparent dissociation between structural preservation and functional suppression suggests that reversible dysfunction and irreversible injury may not simply represent different points along a single severity continuum but may instead be influenced by partially distinct biological processes.

These observations have contributed to the hypothesis that myocardial stunning may, at least in part, represent a regulated and potentially adaptive response to metabolic stress rather than merely incomplete injury. In this framework, transient suppression of contractile activity may reduce ATP consumption during periods of limited energetic reserve, thereby supporting maintenance of cellular homeostasis and viability (12). From this perspective, stunning may be interpreted not only as a consequence of stress or injury but also as a state that may accompany endogenous cardioprotective signaling. This possibility is particularly relevant in the context of IPC, in which structural protection and transient functional suppression may coexist (**Figure 3**).

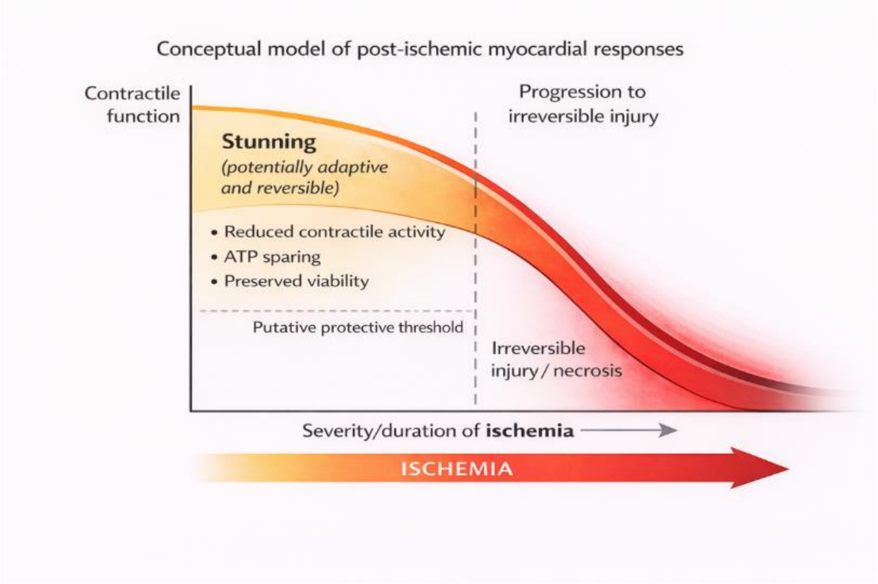


Figure 3. Conceptual model of post-ischemic myocardial responses across increasing ischemic severity and duration.
The schematic illustrates a conceptual relationship between contractile function and the severity/duration of ischemia, highlighting a transition from reversible post-ischemic dysfunction (myocardial stunning) to irreversible injury with necrosis. In the earlier phase, myocardial stunning is depicted as a potentially adaptive and reversible state characterized by reduced contractile activity, relative ATP sparing, and preserved viability. With increasing ischemic burden, this response may progress beyond a putative protective threshold toward irreversible structural injury, loss of viability, and necrosis. The figure is intended as a schematic representation of how transient functional suppression and irreversible injury may relate to one another during ischemic stress, rather than as a strict deterministic model. ***Created with BioRender.com.***

1.5 ISCHEMIC PRECONDITIONING: AN ENDOGENOUS CARDIOPROTECTIVE MECHANISM

IPC is one of the most extensively studied endogenous cardioprotective phenomena in cardiovascular biology (25). IPC refers to the observation that brief, non-lethal episodes of ischemia render the myocardium more resistant to a subsequent prolonged ischemic insult (25). This protective effect has been demonstrated reproducibly across multiple experimental species and is classically characterized by a reduction in infarct size following sustained coronary occlusion and reperfusion. Compared with non-preconditioned myocardium, preconditioned tissue exhibits greater tolerance to ischemic stress, delayed onset of irreversible injury, and improved cellular survival (7).

IPC may be induced directly in the myocardium (classical IPC), but protection can also be triggered remotely through brief ischemia in distant tissues, such as a limb, a phenomenon known as remote ischemic preconditioning (RIPC). In this setting, neural and/or humoral signals are thought to transmit protective information to the heart. The effects of IPC are also temporally biphasic. An early "first window" develops rapidly and wanes within a few hours, whereas a delayed "second window" emerges after approximately 12–24 hours and can persist for 48–72 hours, reflecting transcription-dependent reinforcement of stress-resistance pathways (26).

Mechanistically, IPC activates an interconnected network of kinases, second messengers, and mitochondrial effectors that converge on pathways regulating redox balance, calcium homeostasis, cell survival signaling, and mitochondrial permeability transition pore opening (7, 27, 28). Through these pathways, IPC reduces susceptibility to lethal reperfusion injury and limits irreversible tissue loss. However, the functional consequences of IPC extend beyond infarct limitation alone. In selected experimental settings, IPC has paradoxically been associated with greater transient post-ischemic contractile dysfunction despite improved structural preservation and maintained viability (24). This observation raises the possibility that transient contractile suppression may accompany, rather than oppose, cardioprotection.

This apparent paradox is of particular relevance to this thesis. If IPC reduces irreversible injury while accentuating reversible dysfunction, then post-ischemic contractile depression cannot be interpreted solely as a marker of greater tissue damage. Instead, it may reflect molecular adaptations that favor survival over immediate restoration of mechanical performance. Understanding these programs is therefore essential for clarifying the relationship between myocardial stunning and cardioprotection.

1.6 CLINICAL CORRELATES OF DIVERGENT MYOCARDIAL INJURY PHENOTYPES

1.6.1 TAKOTSUBO SYNDROME: DEFINITION, DIAGNOSTIC HALLMARKS, AND PUTATIVE DRIVERS

Takotsubo syndrome (TS), also referred to as stress-induced cardiomyopathy, is an acute heart failure syndrome characterized by transient left ventricular systolic dysfunction with regional wall-motion abnormalities (i.e. myocardial stunning) that typically extend beyond the territory of a single epicardial coronary artery (29). It commonly presents with acute chest pain and/or dyspnea, electrocardiographic abnormalities, and elevated cardiac biomarkers, often in a pattern that mimics acute myocardial infarction (30). Diagnostic frameworks emphasize the absence of an obstructive culprit coronary lesion or acute plaque rupture sufficient to explain the extent of ventricular dysfunction, together with exclusion of important alternative diagnoses such as myocarditis and pheochromocytoma (31).

Although ventricular systolic function often recovers from days to weeks, TS is not a benign condition and may be complicated by cardiogenic shock, arrhythmias, thromboembolism, and acute heart failure (31). Mechanistically, TS is most strongly linked to acute sympathetic activation and catecholamine excess, which can induce myocardial stunning through perturbation of β-adrenergic signaling, coronary microvascular dysfunction and/or vasospasm, and downstream metabolic, oxidative, and calcium-handling disturbances within cardiomyocytes(30, 31). Susceptibility is influenced by host-related factors including autonomic regulation, endothelial function, and post-menopausal status, consistent with the marked predominance of TS in women (29).

TS is particularly relevant in the context of this thesis because it represents a clinical phenotype dominated by reversible systolic dysfunction rather than overt irreversible necrosis. While some degree of biomarker release may occur, the dominant feature is transient myocardial dysfunction with subsequent recovery. As such, TS provides a clinically important counterpart to infarction-related myocardial injury and offers a framework for the biology of reversible myocardial dysfunction in humans.

1.6.2 ST-ELEVATION MYOCARDIAL INFARCTION: CLINICAL DEFINITION, DIAGNOSTIC FEATURES, AND PATHOPHYSIOLOGY

ST-elevation myocardial infarction (STEMI) is a form of acute coronary syndrome most commonly caused by acute atherothrombotic occlusion of an epicardial coronary artery after plaque rupture or plaque erosion, leading to prolonged myocardial ischemia and irreversible cardiomyocyte necrosis if coronary blood flow is not rapidly restored. The diagnosis of STEMI is based on the integration of clinical presentation, electrocardiographic findings, and biochemical evidence of acute myocardial injury, particularly a rise and/or fall in cardiac troponin concentrations with at least one value above the 99th percentile upper reference limit in an appropriate ischemic context. In clinical practice, STEMI typically presents with acute chest pain, chest pressure, or dyspnea, together with persistent ST-segment elevation in anatomically contiguous leads and, in many cases, angiographic identification of an obstructive culprit lesion requiring urgent reperfusion therapy (32).

At the mechanistic level, STEMI is primarily driven by abrupt cessation of coronary perfusion, which results in oxygen deprivation, impaired oxidative metabolism, ATP depletion, ionic disequilibrium, loss of membrane integrity, and activation of necrotic and regulated cell-death pathways. When ischemia is sustained, myocardial injury progresses in a transmural wavefront from the subendocardium toward the epicardium, ultimately culminating in irreversible infarction. Although early reperfusion is the cornerstone of myocardial salvage, restoration of blood flow may itself provoke additional injury through oxidative stress, calcium overload, endothelial and microvascular dysfunction, and inflammatory activation, processes collectively referred to as ischemia–reperfusion injury (33).

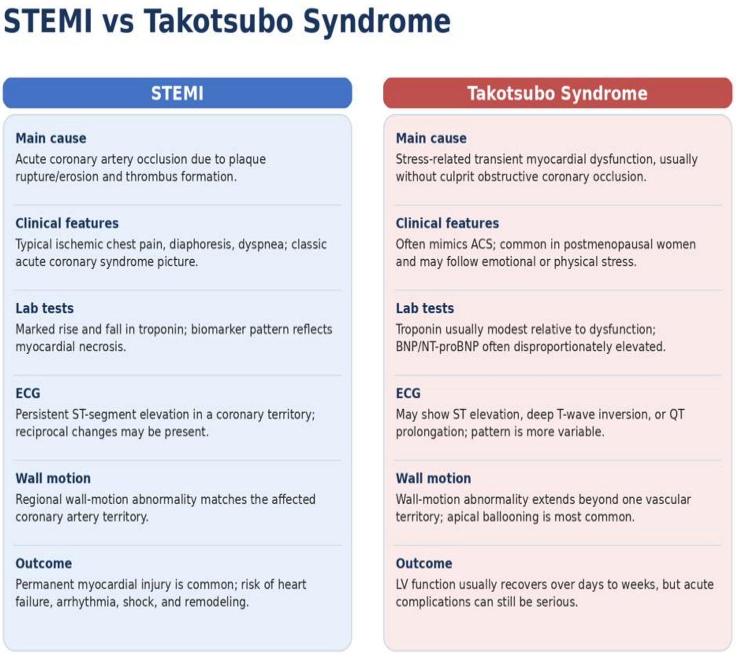
Despite substantial advances in primary percutaneous coronary intervention, antithrombotic therapy, and systems of care, STEMI remains associated with considerable morbidity and mortality worldwide (34). Major early complications include cardiogenic shock, malignant ventricular arrhythmia, acute heart failure, and mechanical complications such as papillary muscle rupture, ventricular septal rupture, and free-wall rupture (32). From an epidemiological perspective, the incidence of STEMI has declined in several high-income settings over recent decades, likely reflecting improved prevention, risk-factor control, and changes in population-level cardiovascular care; however, ischemic heart disease remains a major global contributor to death and disability, and important socioeconomic disparities in STEMI incidence and outcomes persist. Established risk factors include cigarette smoking, hypertension, diabetes mellitus, dyslipidemia, obesity, and older age, together with other determinants of atherosclerotic cardiovascular disease (35).

STEMI differs from NSTEMI in that it more commonly reflects complete and persistent coronary occlusion with a larger area of jeopardized myocardium, typically manifested by ST-segment elevation on the electrocardiogram. By contrast, NSTEMI more often occurs in the setting of subtotal, intermittent, or distal coronary obstruction and is usually associated with predominantly subendocardial ischemic injury. Both conditions are characterized by cardiomyocyte necrosis and elevated cardiac troponin concentrations, but STEMI generally requires immediate reperfusion because of the greater urgency of restoring coronary blood flow and limiting infarct size (36).

Within the context of this thesis, STEMI is the most relevant clinical phenotype of myocardial stunning co-existing with irreversible ischemic myocardial injury and permanent structural loss of cardiomyocytes. While the experimental studies are based on an I/R model, patient-derived STEMI data provides a clinically relevant context for interpreting molecular signatures associated with acute ischemic necrosis and its downstream consequences.

1.6.3 ST-ELEVATION MYOCARDIAL INFARCTION VERSUS TAKOTSUBO SYNDROME: CONTRASTING MYOCARDIAL INJURY TRAJECTORIES

Acute myocardial injury may present with a similar clinical picture across distinct disease entities, including chest pain, electrocardiographic abnormalities, and elevated cardiac troponins, thereby necessitating urgent diagnostic evaluation (10). Beneath this shared presentation, however, lie biologically divergent myocardial injury trajectories (**Figure 4**).



STEMI vs Takotsubo Syndrome

	STEMI	Takotsubo Syndrome
Main cause	Acute coronary artery occlusion due to plaque rupture/erosion and thrombus formation.	Stress-related transient myocardial dysfunction, usually without culprit obstructive coronary occlusion.
Clinical features	Typical ischemic chest pain, diaphoresis, dyspnea; classic acute coronary syndrome picture.	Often mimics ACS; common in postmenopausal women and may follow emotional or physical stress.
Lab tests	Marked rise and fall in troponin; biomarker pattern reflects myocardial necrosis.	Troponin usually modest relative to dysfunction; BNP/NT-proBNP often disproportionately elevated.
ECG	Persistent ST-segment elevation in a coronary territory; reciprocal changes may be present.	May show ST elevation, deep T-wave inversion, or QT prolongation; pattern is more variable.
Wall motion	Regional wall-motion abnormality matches the affected coronary artery territory.	Wall-motion abnormality extends beyond one vascular territory; apical ballooning is most common.
Outcome	Permanent myocardial injury is common; risk of heart failure, arrhythmia, shock, and remodeling.	LV function usually recovers over days to weeks, but acute complications can still be serious.

Figure 4. Comparison of ST-segment elevation myocardial infarction (STEMI) and Takotsubo syndrome. This schematic summarizes the main differences between STEMI and Takotsubo syndrome in terms of cause, clinical features, laboratory findings, electrocardiographic characteristics, wall-motion pattern, and outcome. STEMI is typically caused by acute coronary artery occlusion due to plaque rupture or erosion with thrombus formation, resulting in myocardial necrosis and regional wall-motion abnormality corresponding to the affected coronary territory. In contrast, Takotsubo syndrome is a stress-related transient myocardial dysfunction that usually occurs without culprit obstructive coronary occlusion and is characterized by wall-motion abnormalities extending beyond a single vascular territory, often with subsequent recovery of left ventricular function over days to weeks. STEMI = ST-segment elevation myocardial infarction; ACS = acute coronary syndrome; BNP = B-type natriuretic peptide; NT-proBNP = N-terminal pro-B-type natriuretic peptide; ECG = electrocardiogram; QT = interval from the beginning of the Q wave to the end of the T wave on the electrocardiogram; LV = left ventricle/left ventricular. Created with BioRender.com.

In the context of this thesis, comparison of STEMI and TS is relevant because these syndromes represent contrasting myocardial injury states, predominantly irreversible ischemic injury versus largely reversible myocardial dysfunction. This distinction is important because acute ventricular impairment does not necessarily reflect the extent of irreversible cardiomyocyte loss but may instead arise from biologically distinct processes with different implications for tissue recovery, remodeling, and circulating molecular profiles.

These contrasting clinical entities provide a human context for interpreting the experimental distinction between reversible dysfunction and irreversible injury, along with their divergent circulating molecular signatures.

1.7 CENTRAL CLINICAL AND BIOLOGICAL CHALLENGE

The observations above converge on a central question in contemporary cardiovascular medicine: what determines myocardial fate following acute ischemic stress? Despite standardized reperfusion strategies and evidence-based care pathways, outcomes remain variable, ranging from irreversible necrosis with adverse remodeling to predominantly reversible dysfunction with recovery (32) . Despite extensive investigation of ischemia–reperfusion injury, the molecular programs that determine whether myocardium progresses toward irreversible necrosis or reversible functional suppression remain incompletely understood.

Although myocardial stunning has traditionally been conceptualized as a milder manifestation along a continuum of ischemic injury, observations from cardioprotective models indicate that functional suppression and structural preservation may not always align proportionally (24). These findings support the interpretation that reversible dysfunction may represent a biologically distinct response characterized by functional suppression in the absence of extensive irreversible tissue injury, rather than simply a milder form of damage. In this framework, transient suppression of contractile activity may reduce ATP consumption during metabolic stress, prioritize cellular homeostasis, and support cardiomyocyte viability when energetic reserve is limited (12). From this perspective, myocardial stunning may be understood not merely as a byproduct of injury, but as a potentially adaptive response that may accompany cardioprotective signaling.

This framework raises testable mechanistic questions: Which molecular pathways determine whether cardiomyocytes proceed toward irreversible death or functional recovery? Are these trajectories governed predominantly by protein abundance, post-translational modification, and signaling network dynamics, or by transcriptional reprogramming over time? And critically, do these divergent myocardial responses generate systemic signatures that can be detected using circulating biomarkers?

Addressing these questions is essential for mechanistic stratification of acute myocardial injury and for moving beyond a purely anatomical model of coronary obstruction. A temporal, systems-level approach integrating transcriptomic, proteomic, phosphoproteomic, and circulating proteomic readouts offers a direct route to define pathway-level responses, distinguish adaptive from injurious programs, and may identify biomarker signatures that discriminate reversible dysfunction from irreversible tissue loss.

1.8 MULTI-OMICS APPROACHES TO DECIPHER MYOCARDIAL INJURY AND RECOVERY

Despite decades of research, many mechanistic insights into ischemia–reperfusion injury have arisen from reductionist studies of individual pathways (37). Multi-omics approaches extend beyond such reductionist designs by enabling parallel characterization of molecular changes across complementary regulatory layers. In the setting of myocardial injury, this provides an opportunity to relate transcriptional responses to protein abundance, post-translational signaling, and circulating biomarker profiles across time (38) .

Traditional clinical assessment and imaging remain essential but provide only indirect readouts of the molecular processes governing injury and recovery. High-throughput omics technologies enable comprehensive profiling across molecular layers, including transcripts, proteins, and post-translational modifications, thereby offering a framework to characterize temporal molecular patterns, and identify biomarkers linked to myocardial outcome (39).

1.8.1 TRANSCRIPTOMICS

The transcriptome reflects dynamic regulation of gene expression in response to physiological and pathological stimuli. RNA sequencing enables quantitative analysis of thousands of transcripts simultaneously and can reveal coordinated activation or repression of biological programs during ischemia and reperfusion (40). Transcriptomic studies have identified induction of inflammatory pathways, stress-response signaling, metabolic reprogramming, and cell-survival processes in the injured heart (41) . However, transcript levels do not necessarily predict protein abundance or activity, as they are influenced by post-transcriptional regulation, translational efficiency, and protein turnover. Transcriptomics therefore provides insight primarily into regulatory direction and biological potential rather than direct functional execution.

1.8.2 PROTEOMICS

Whereas transcriptomics reflects gene regulation, the proteome represents the functional molecular machinery through which phenotype is expressed. Proteomic analyses quantify changes in protein abundance, pathway composition, and molecular organization, and have identified alterations in contractile proteins, metabolic enzymes, inflammatory mediators, and extracellular matrix-related proteins during ischemic injury and repair (42). Because proteins directly mediate cellular function, proteomics can provide a closer approximation of phenotype than transcriptomics alone. Nonetheless, total protein abundance does not capture all functionally relevant changes, and detection may be limited by dynamic range, technical variability, and incomplete coverage of low-abundance proteins.

1.8.3 PHOSPHOPROTEOMICS

Post-translational modifications add a rapid and highly dynamic regulatory layer to myocardial biology, with phosphorylation playing a central role in excitation-contraction coupling, calcium handling, stress signaling, cytoskeletal regulation, and mitochondrial function. Phosphoproteomics enables large-scale analysis of phosphorylation across thousands of sites and thereby provides a means to infer signaling network activity during ischemia–reperfusion (43). Because phosphorylation changes occur within seconds to minutes, they are particularly relevant to acute conditions, where they are more likely to reflect biologically important mechanisms of early myocardial dysfunction than transcriptional or translational changes, which generally require longer time to emerge. This level is particularly relevant to reversible dysfunction, where transient modulation of protein activity may have major consequences for contractile performance even in the absence of overt structural loss or major changes in total protein abundance.

1.8.4 CIRCULATING PROTEOMICS

Whereas tissue-based omics provides mechanistic insight into myocardial biology, circulating proteomics offers translational relevance by capturing systemic responses associated with cardiac injury. Plasma proteomic profiling can identify patterns related to inflammation, stress signaling, coagulation, tissue remodeling, and neurohumoral activation, and may help distinguish clinically overlapping syndromes with different underlying biology (44). At the same time, plasma proteins reflect contributions from multiple tissues and immune compartments, which complicates cardiac attribution and underscores the importance of careful interpretation, temporal sampling, and clinical context.

1.8.5 INTEGRATIVE MULTI-OMICS

Each omics layer provides complementary information. Transcriptomics captures regulatory activation, proteomics reflects molecular execution, phosphoproteomics resolves rapid signaling control, and circulating proteomics links tissue-level biology to clinical phenotype. Integration across these layers provides a more complete framework for reconstructing coordinated molecular programs rather than isolated pathway snapshots (45). Such an approach is particularly valuable in the study of acute myocardial injury, where relatively subtle differences in regulation may determine whether the myocardium follows a trajectory of recovery or irreversible damage.

At the same time, omics datasets require rigorous statistical processing, pathway-level interpretation, and biological validation. Temporal sampling is especially important, since molecular programs evolve rapidly after ischemic stress and the distinction between adaptive signaling and injury-associated bystander effects may change over time. Nevertheless, multi-omics offers a powerful framework for moving from descriptive characterization toward mechanistic stratification of myocardial injury and recovery.

Within this framework, the present thesis integrates complementary phosphoproteomic, proteomic, transcriptomic, and circulating proteomic approaches to investigate the molecular determinants of reversible versus irreversible myocardial injury

2 AIMS AND OBJECTIVES

The overall aim of this thesis is to elucidate how IPC modifies myocardial responses to I/R injury, with a particular focus on the relationship between reversible myocardial stunning and irreversible necrosis. The thesis seeks to define the molecular mechanisms underlying IPC-induced cardioprotection through integrated phosphoproteomic, proteomic, transcriptomic, and epigenetic analyses, and to extend these findings to the clinical setting through temporal plasma proteomics.

This thesis comprises four studies, each designed to address a specific aim:

I. To investigate how ischemic preconditioning influences infarct development and myocardial stunning following ischemia–reperfusion injury in rats, and to characterize associated alterations in myocardial phosphoproteomic signaling.

II. To define temporal myocardial proteomic changes induced by ischemic preconditioning and to identify pathways associated with adaptive responses and those linked to inflammatory and necrotic processes.

III. To determine how ischemic preconditioning reprograms the cardiac transcriptome and to examine the role of DNA methylation in the epigenetic regulation of gene expression linked to myocardial stunning and necrosis.

IV. To characterize temporal plasma proteomic signatures distinguishing Takotsubo syndrome from ST-elevation myocardial infarction and to identify systemic molecular patterns associated with reversible and irreversible myocardial injury.

3 METHODS

3.1 ANIMALS, SURGICAL PROCEDURES, AND EXPERIMENTAL GROUPING

3.1.1 ANIMAL MODEL SELECTION

To investigate acute ischemic myocardial phenotypes with high mechanistic and temporal resolution, a controlled and reproducible *in vivo* model was required, permitting precise regulation of ischemia duration, reperfusion timing, and regional tissue sampling (46).

Rats were selected as the experimental animal for this study based on their extensive validation in myocardial I/R research and their physiological robustness under surgical manipulation (47). These attributes allow the implementation of tailored ischemia protocols with a level of experimental control sufficient to support systematic model optimization. Accordingly, a substantial component of this work was dedicated to the design and validation of a rat model capable of delineating duration-dependent transitions between reversible myocardial dysfunction and irreversible injury.

Given the strong dependence of myocardial outcome on ischemia duration (48), pilot experiments were incorporated into the study design to calibrate ischemic exposure parameters and to establish a reproducible framework for distinguishing non-lethal from lethal ischemic phenotypes prior to the main experimental investigations.

3.1.2 ANIMALS, ETHICS, AND HOUSING CONDITIONS

All experiments included in this thesis were conducted in male Sprague–Dawley rats. Animals were six to eight weeks old and weighed 250–350 g at the time of surgery. Rats were sourced from Janvier Labs (Le Genest-Saint-Isle, France) and allowed to acclimatize for one week prior to experimental procedures at the Laboratory for Experimental Biomedicine, University of Gothenburg, Sweden. Only male rats were used to reduce biological variability related to sex-specific hormonal influences on I/R injury. Consequently, the potential impact of sex differences on myocardial stunning and ischemic preconditioning was not addressed in this thesis and warrants investigation in future studies. Throughout both the acclimation and experimental periods, rats were maintained under controlled environmental conditions with a 12-hour light/dark cycle and a regulated ambient temperature of 21 °C. Animals had *ad libitum* access to standard laboratory chow and water. All experimental procedures were designed to minimize animal suffering and perioperative mortality.

All studies were conducted in accordance with the ARRIVE guidelines and complied with Swedish national legislation governing animal research. Experimental protocols were approved by the Gothenburg Animal Ethics Committee (Dnr. 5.8.18-13778/2020 with amendments Dnr 5.8.18-13352/2021 and Dnr 5.8.18-11014/2023).

3.1.3 SURGICAL PROCEDURE FOR MYOCARDIAL ISCHEMIA–REPERFUSION

Rats were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (5 mg/kg). Following loss of the pedal withdrawal reflex, animals were endotracheally intubated and mechanically ventilated using a small-animal ventilator. Ventilation parameters were adjusted to maintain physiological gas exchange, and end-tidal CO_2 was continuously monitored and maintained between 5–6%.

To ensure a stable depth of surgical anesthesia throughout the procedure, a continuous intravenous infusion of ketamine and xylazine (0.125 mg/mL and 3 mg/mL, respectively), diluted in Ringer’s solution, was administered via cannulation of the lateral tail vein. Body temperature was maintained at approximately 37 °C using a thermostatically controlled heating pad.

Following left thoracotomy at the fourth intercostal space, the mediastinum was gently opened to expose the heart. Myocardial ischemia was induced by temporary ligation of the left anterior descending (LAD) coronary artery using a 6-0 polypropylene suture placed 3–4 mm distal to the origin of the LAD. Successful coronary occlusion was confirmed by regional myocardial blanching, characteristic electrocardiographic changes, and the immediate development of regional wall motion abnormalities assessed by echocardiography.

After the predefined ischemic duration, reperfusion was achieved by carefully releasing the ligature, with restoration of coronary blood flow verified visually.

The chest was closed in layers, negative intrathoracic pressure was restored, and animals were allowed to recover under close monitoring. Postoperative care was provided in accordance with institutional guidelines, and animals were returned to their cages once fully awake and breathing spontaneously.

3.1.4 ISCHEMIA–REPERFUSION MODEL AND EXPERIMENTAL DESIGN

Following surgical preparation as described above, myocardial I/R experiments were performed using transient occlusion of the LAD coronary artery, with ischemia duration predefined according to the experimental design.

Animals were randomly assigned to sham-operated controls, I/R without preconditioning, or I/R preceded by IPC. Within I/R groups, ischemia durations were selected according to the experimental endpoint to induce either reversible or irreversible ischemic injury.

Functional assessments and tissue sampling were performed at standardized time points following reperfusion. Molecular analyses were conducted on myocardial tissue harvested from regions corresponding to functional impairment, allowing direct integration of regional myocardial function with molecular responses (**Figure 5**). This experimental design provided a reproducible platform for investigating acute ischemic myocardial phenotypes while minimizing variability related to ischemic severity, post-ischemic timing, and tissue sampling.

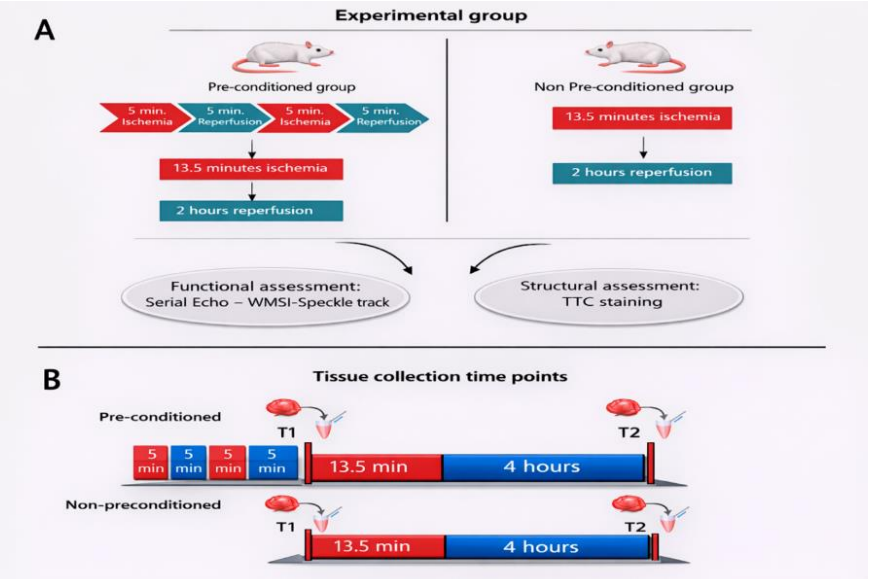


Figure 5**. **Overview of the experimental design for ischemic preconditioning and non-preconditioned myocardial ischemia-reperfusion protocols.
*(**A**) Functional and structural assessment protocol. Rats were assigned to either a preconditioned group, in which two cycles of 5 min ischemia followed by 5 min reperfusion were applied before the index ischemic insult, or a non-preconditioned group without IPC. Both groups were subjected to 13.5 min LAD occlusion followed by 2 h reperfusion. Functional outcome was assessed by serial echocardiography, including wall motion score index (WMSI) and speckle tracking, and structural injury was evaluated by TTC staining.*
*(**B**) Tissue collection protocol for molecular analyses. Preconditioned and non-preconditioned groups underwent the same index ischemic insult, followed by 4 h reperfusion. Myocardial tissue was collected at two predefined time points: T1, representing an early time point in both groups, and T2, after 4 h reperfusion. At the 4 h reperfusion time point, the preconditioned group predominantly exhibited myocardial stunning, whereas the non-preconditioned group showed infarction.*
***Created with Biorender.com**.*

3.1.5 ISCHEMIC PRECONDITIONING PROTOCOL

IPC was implemented using two consecutive cycles of transient LAD occlusion, each comprising 5 minutes of ischemia followed by 5 minutes of reperfusion, applied immediately prior to the index ischemic episode (25). Coronary occlusion and subsequent reperfusion during IPC cycles were verified using the criteria described above. Following completion of the IPC protocol, animals proceeded directly to the index LAD occlusion according to their assigned experimental group.

3.2 FUNCTIONAL ASSESSMENT AND TISSUE COLLECTION

3.2.1 ECHOCARDIOGRAPHY AND WALL MOTION ANALYSIS

Transthoracic echocardiography was performed to assess regional and global myocardial function at baseline and at predefined time points following reperfusion, with late follow-up at 48 h. In subsets of experiments used for functional characterization, echocardiography was also performed at later time points (e.g., 24–48 h) to document recovery after IPC. Imaging was conducted using a high-resolution ultrasound system equipped with a 15–30 MHz linear array transducer. To ensure reproducible imaging conditions, animals were positioned supine on a temperature-controlled platform, and core body temperature was maintained at approximately 37 °C throughout the procedure.

Parasternal long-axis views were acquired to visualize the left ventricular cavity, mitral valve, and aortic valve, followed by parasternal short-axis views obtained approximately 6 mm below the mitral valve. Image acquisition was performed using ECG-gated high–frame-rate imaging, and cine loops comprising more than 1,000 frames were recorded for subsequent offline analysis.

In a subset of experiments, regional myocardial function was evaluated using the wall motion score index (WMSI). The left ventricle was divided into 17 segments, and each segment was assigned a score based on visual wall motion assessment (1 = normal contraction, 2 = hypokinesia, 3 = akinesia). Analyses focused on segment 12, corresponding to the anterolateral/anterior free wall supplied by the LAD coronary artery and exposed to ischemia during coronary occlusion. Segment 12 was assessed at baseline and at 4 h and 48 h following reperfusion. Reversible myocardial stunning was operationally defined as the presence of akinesia at 4 h with recovery of wall motion by 48 h (**Figure 6**).

To minimize observer bias, all echocardiographic analyses were performed independently by two experienced investigators blinded to experimental group allocation, and discrepancies were resolved by consensus.

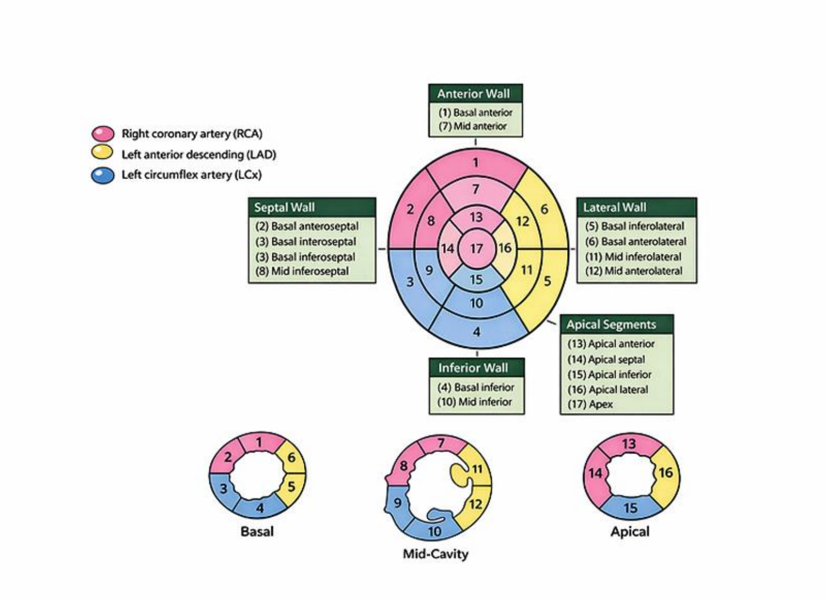


Figure 6. Schematic representation of the 17-segment left ventricular model and corresponding coronary artery territories.
The left ventricle is divided into basal, mid-cavity, and apical levels according to the standardized 17-segment model. Segments are grouped into anterior, septal, lateral, inferior, and apical regions, and are color-coded to indicate their predominant coronary arterial supply: right coronary artery, left anterior descending artery , and left circumflex artery . The lower panels illustrate the short-axis arrangement of basal, mid-cavity, and apical segments. ***Created with ChatGPT****.*

3.2.2 SPECKLE-TRACKING STRAIN ANALYSIS

To obtain a sensitive and quantitative assessment of myocardial deformation, two-dimensional speckle-tracking echocardiography was performed (49). This analysis was conducted in selected experimental groups based on prior WMSI assessments.

Both parasternal long-axis and mid-ventricular short-axis views were analyzed. The left ventricle was divided into six segments, and the endocardial border was manually traced using 48 sampling points to enable automated tracking of myocardial motion throughout the cardiac cycle. The ischemic region was consistently identified as the anterior mid-segment in the long-axis view and the anterior free-wall segment in the short-axis view.

Regional myocardial deformation in the ischemic segment was quantified using longitudinal, radial, and circumferential peak strain (%), as well as time-to-peak strain (ms).

Measurements were obtained at baseline and repeated at 4 h and 48 h following reperfusion to characterize temporal changes in myocardial deformation and functional recovery.

In addition to regional strain analysis, global functional parameters, including left ventricular ejection fraction (LVEF), fractional shortening (FS), fractional area change (FAC), cardiac output, and global longitudinal strain (GLS) were calculated. All strain analyses were performed by investigators blinded to treatment group and ischemia duration.

3.2.3 INFARCT SIZE ASSESSMENT

Myocardial infarct size was quantified using Evans blue/triphenyl tetrazolium chloride (TTC) double staining in a subset of experiments designed to assess irreversible myocardial injury, with or without IPC.

After 2 h of reperfusion, the LAD was re-ligated at the original ligation site, and 5% Evans blue dye was injected via the lateral tail vein to delineate the non-ischemic myocardium. After 1 min, hearts were rapidly excised, rinsed in cold saline, and sectioned transversely into five slices of approximately 2 mm thickness from apex to base. Tissue sections were incubated in 1% TTC solution for 10 min at 37 °C in the dark to differentiate viable from infarcted myocardium, followed by fixation in 10% formalin.

Following staining, sections were weighed, mounted on glass slides, and scanned. Infarct size was quantified as the percentage of infarcted myocardium relative to the area at risk, defined as the myocardial region not stained by Evans blue. All measurements were performed independently by two investigators blinded to experimental group allocation, and values were averaged for analysis.

3.2.4 TISSUE COLLECTION AND REGIONAL MYOCARDIAL SAMPLING

At predefined reperfusion time points, animals were deeply anesthetized and hearts were rapidly excised to minimize post-mortem ischemic effects. Hearts were immediately rinsed in ice-cold saline to remove residual blood and processed without delay.

For molecular analyses, the left ventricle was dissected, and regional myocardial sampling was guided by the anatomical distribution of the LAD. The ischemic region, corresponding to the anterior and anterolateral left ventricular free wall, was delineated using the position of the coronary ligature together with intraoperative anatomical landmarks and functional localization by echocardiography. This approach ensured that molecular profiling was performed on myocardium directly exposed to the ischemic insult and corresponding to the functionally affected territory.

When required, non-ischemic myocardial tissue was collected from remote regions of the left ventricle for comparative analyses. All tissue samples were snap-frozen in liquid nitrogen immediately after dissection and stored at −80 °C until further processing.

To preserve regional specificity and minimize biological variability, tissue sampling was performed using a standardized protocol by the same experienced operator. This targeted regional sampling strategy was chosen to avoid whole-ventricle averaging and to enable direct integration of phosphoproteomic, proteomic, and transcriptomic findings with functional outcomes.

In selected experiments, tissue was collected immediately following IPC (or no preconditioning) prior to the index ischemia (T1) and/or at 4 h post-surgery (T2), depending on the study-specific experimental design.

3.3 MOLECULAR AND COMPUTATIONAL ANALYSES

3.3.1 PHOSPHOPROTEOMIC ANALYSIS

Phosphoproteomic profiling was carried out on myocardial tissue collected from the LAD-perfused region of the left ventricle that was functionally affected. The samples were homogenized in lysis buffer containing 2% sodium dodecyl sulfate and 50 mM triethylammonium bicarbonate using a FastPrep®-24 instrument (five cycles, 40 s each, at 6.5 m/s). Following homogenization, the lysates were centrifuged at 8,000 × g for 20 minutes, and protein concentrations were subsequently measured using a bicinchoninic acid (BCA) assay.

Protein aliquots (200 µg) were first reduced with 100 mM dithiothreitol (DTT) for 60 minutes and subsequently processed using a modified filter-aided sample preparation (FASP) protocol, including alkylation with 10 mM methyl methanethiosulfonate. Proteolytic digestion was carried out with sequencing-grade trypsin at an enzyme-to-protein ratio of 1:100, initially overnight at 37 °C, followed by an additional 4-hour incubation. For phosphoproteomic analysis, peptides underwent a third round of trypsin digestion.

Isobaric labeling was performed using tandem mass tag (TMTpro) 16plex reagents. Labeled samples were pooled and concentrated; sodium deoxycholate was removed by acidification with 10% trifluoroacetic acid, and peptides were purified by C18-based desalting.

Phosphopeptides were enriched using sequential phosphopeptide enrichment, first with High-Select titanium dioxide followed by High-Select iron–nitrilotriacetic acid. The pooled phosphopeptides were fractionated into 13 fractions by increasing acetonitrile concentration (7% to 50%) using a high-pH reversed-phase fractionation kit.

Enriched phosphopeptides were analyzed by high-resolution nano–liquid chromatography–tandem mass spectrometry (nano-LC–MS/MS) using an Orbitrap Lumos™ Tribrid™ mass spectrometer interfaced with an Easy-nLC1200 system operated in data-dependent acquisition (DDA) mode.

3.3.2 BIOINFORMATIC ANALYSIS

Raw phosphoproteomic data were processed using Proteome Discoverer (version 2.4), with database searching performed using Mascot (version 2.5.1) against the Rattus norvegicus UniProt proteome. Phosphopeptide identification was validated using Percolator, and TMTpro 16plex-based quantification was performed using reporter ion intensities normalized to total peptide abundance, with identifications filtered at a 1% false discovery rate (FDR).

Downstream analyses were conducted using Perseus (version 1.6.12.0). Phosphopeptides were required to have at least three valid values per group and were $\log_2$-transformed prior to analysis. Differential phosphorylation between groups was assessed using Welch’s t-test ($P < 0.05$ and fold change $\geq$

1.2). Functional enrichment analysis of differentially regulated phosphoproteins was performed using ShinyGO (version 0.76) with FDR correction.

3.4 GLOBAL PROTEOMIC ANALYSIS

3.4.1 PROTEOMIC SAMPLE PREPARATION AND LC–MS/MS

Global proteomic profiling was performed on ischemic left ventricular myocardium using a standardized mass spectrometry–based workflow. Frozen tissue samples were homogenized under denaturing conditions to ensure efficient protein solubilization, followed by centrifugation to remove insoluble material. Protein concentrations were determined using the BCA assay to allow equal protein input across samples. Aliquots containing 30 µg of protein were incubated for 60 min with 100 mM DTT prior to modified FASP processing, including alkylation with 10 mM methyl methanethiosulfonate in digestion buffer for 30 min.

Proteolytic digestion was performed using sequencing-grade trypsin overnight at 37 °C, followed by an additional 4 h digestion. The resulting peptide mixtures were desalted using C18 solid-phase extraction cartridges and dried under vacuum prior to mass spectrometric analysis.

Protein abundance was determined using TMTpro 16-plex isobaric labeling. Peptide samples were initially separated into 70 fractions by basic reversed-phase chromatography, then pooled into 23 concatenated fractions before analysis by nano-LC–MS/MS

Peptide separation was carried out by nano–liquid chromatography coupled to tandem mass spectrometry using LC–MS^3 analysis on an Orbitrap Lumos™ Tribrid™ mass spectrometer coupled with an Easy-nLC1200 system operated in DDA mode.

3.4.2 BIOINFORMATIC ANALYSIS

Raw proteomic data were processed using Proteome Discoverer (version 2.4), with database searching performed using Mascot (version 2.5.1) against the Rattus norvegicus UniProt reference proteome. Protein quantification was based on TMTpro reporter ion intensities normalized to total peptide abundance, and identifications were filtered using a 1% FDR.

Differential protein abundance was assessed using Perseus (version 1.x, if applicable), requiring a minimum of three valid values per group. Proteins displaying a ≥20% change and a P-value <0.05 (Welch's t-test; paired for within-sample time comparisons where applicable) were considered significantly altered between groups. Functional enrichment analyses were performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, and protein–protein interaction networks were explored using Search Tool for the Retrieval of Interacting Genes/Proteins . Results were visualized using volcano plots and enrichment-based plots.

3.4.3 WESTERN BLOT ANALYSIS

To validate selected targets identified in the proteomic analyses, western blot analysis was performed using rat left ventricular tissue. Samples (collected and preserved as described above) were homogenized using a TissueLyser in ice-cold radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitor cocktails. Lysates were clarified by centrifugation, and protein concentrations were determined using the Bradford protein assay.

Equal amounts of protein were mixed with Laemmli sample buffer containing β-mercaptoethanol and heat-denatured at 95 °C for 5 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Criterion TGX 4–15% gradient gels and transferred onto nitrocellulose membranes. Uniform protein loading and transfer efficiency were verified using stain-free imaging.

Membranes were blocked for 1 hour at room temperature with EveryBlot blocking buffer, followed by overnight incubation at 4 °C with primary antibodies against carbonic anhydrase 2 (CA2; AB124687-1001, Abcam) and monoamine oxidase A (MAOA; AB126751-1001, Abcam), each diluted 1:1000 in the same buffer. After washing steps, membranes were exposed to a horseradish peroxidase–conjugated anti-rabbit IgG secondary antibody (12–348, Sigma-Aldrich). Protein bands were subsequently visualized using enhanced chemiluminescence.

3.5 TRANSCRIPTOMIC AND EPIGENETIC ANALYSIS

3.5.1 RNA EXTRACTION, RNA SEQUENCING, AND DNA METHYLATION ANALYSES

Frozen left ventricular myocardial tissue from the ischemic region was used for transcriptomic and epigenetic profiling. Tissue samples were homogenized under liquid nitrogen, and total RNA was extracted using a phenol–chloroform–based method (TRIzol reagent) followed by column purification to ensure high RNA purity. RNA concentration was measured spectrophotometrically, and RNA integrity was assessed by electrophoretic analysis. Only samples meeting predefined quality and integrity criteria were included in downstream analyses.

Genome-wide transcriptomic profiling was conducted using RNA sequencing. Ribosomal RNA was removed with the NEBNext® Ribo-Zero Magnetic Kit (New England Biolabs), and sequencing libraries were generated using the NEBNext® Ultra™ Directional RNA Library Preparation Kit for Illumina®. Libraries were sequenced on an Illumina NovaSeq 6000 system with paired-end 150 bp reads. Two control samples showing high levels of residual ribosomal RNA were excluded from subsequent analyses.

To investigate epigenetic regulation of transcriptional responses, DNA methylation–related analyses were conducted in parallel using genomic DNA isolated from the same myocardial regions. Global DNA methyltransferase (DNMT) activity was measured using a colorimetric enzymatic assay that quantifies methyl group transfer to a universal DNA substrate. DNMT activity was calculated according to the manufacturer’s protocol and normalized to total protein input.

Promoter-specific DNA methylation was assessed using an enrichment-based approach. Genomic DNA was purified and subjected to methylated DNA capture using a methyl-CpG–binding domain–based enrichment system. Enriched methylated DNA fractions were quantified by quantitative polymerase chain reaction using promoter-specific primers targeting selected genes. Candidate genes were chosen based on differential expression, CpG density within promoter regions, and biological relevance to inflammation, stress response, DNA repair, and cardioprotection. Promoter methylation levels were normalized to input DNA for each sample.

Chromatin immunoprecipitation (ChIP) assays targeting Dnmt1 and enzyme-linked immunosorbent assay (ELISA) quantification of Jun and Parp14 were performed as described in the corresponding transcriptomics/epigenetics study.

3.5.2 TRANSCRIPTOMIC BIOINFORMATIC ANALYSIS

Raw RNA sequencing data were processed using established bioinformatic pipelines. Adapter trimming and quality filtering of raw paired-end reads were performed using Skewer (version x.x). High-quality reads were aligned to the Rattus norvegicus reference genome (rn7 assembly) using the Spliced Transcripts Alignment to a Reference aligner.

Aligned reads were assigned to genomic features based on Ensembl annotations (release 108), and gene-level read counts were generated using FeatureCounts (Subread package). Samples exhibiting excessive ribosomal RNA content were excluded from downstream analyses.

Normalization and differential gene expression analyses were performed using the DESeq2 package within the Partek® Flow software environment. Genes with low expression levels (average read count < 10 across samples) were filtered prior to statistical analysis.

Exploratory data analysis, including principal component analysis, was conducted to assess sample clustering and overall variance between experimental groups and time points. Visualization of transcriptomic data was performed using volcano plots, heatmaps, and box-and-whisker plots generated within Partek Flow.

3.6 CLINICAL PLASMA PROTEOMIC ANALYSIS

3.6.1 STUDY DESIGN AND POPULATION

To complement the myocardial tissue–based mechanistic analyses described above, circulating plasma proteomics was performed in a prospective clinical cohort of women with TS or STEMI enrolled in the Stunning in Takotsubo versus Acute Myocardial Infarction (STAMI) study (NCT04448639) at Sahlgrenska University Hospital. The study was conducted in accordance with the Declaration of Helsinki and approved by the Swedish Ethical Review Authority (registration number 2022-01003-02). Written informed consent was obtained from all participants prior to inclusion.

Patients with TS were diagnosed according to contemporary European Society of Cardiology (ESC) criteria (30), including transient left ventricular dysfunction in the absence of obstructive coronary artery disease. STEMI was diagnosed based on guideline-defined criteria, including clinical presentation, electrocardiographic changes, and angiographic confirmation of acute coronary occlusion (3). The study included 38 women with TS and 34 women with STEMI.

3.6.2 BLOOD SAMPLING AND PLASMA PROCESSING

Venous blood samples were collected longitudinally following hospitalization to capture dynamic systemic responses. Samples were scheduled for collection on days 0, 1, 2, 3, 7, 14, and 30 after admission. Predefined sampling windows were used to define the acute phase (day 0–1), subacute phase (day 2–3), and convalescent phase (day 14–30). To preserve temporal resolution while accommodating missing samples, the acute phase used day 0 (or day 1 if day 0 was unavailable), the subacute phase used day 2 (or day 3 if day 2 was unavailable), and the convalescent phase used day 30 (or day 14 if day 30 was unavailable). Within each window, the exact sampling day was retained and included as a covariate in statistical models (**Figure 7**).

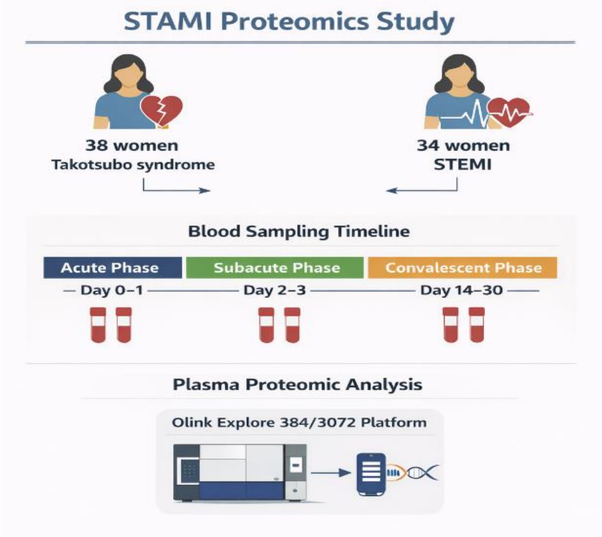


Figure 7. Overview of the clinical design of the STAMI plasma proteomics study. *Women with Takotsubo syndrome (TS; n = 38) and ST-elevation myocardial infarction (STEMI; n = 34) were enrolled in the prospective Stunning in Takotsubo versus Acute Myocardial Infarction (STAMI) study. Serial blood sampling was performed after symptom onset, and plasma proteomic analyses were organized into predefined analytical windows representing the acute (day 0–1), subacute (day 2–3), and convalescent (day 14–30) phases. Plasma protein abundance was quantified using the Olink Explore 384/3072 platform, enabling longitudinal comparison of circulating proteomic signatures between TS and STEMI across distinct clinical phases.* ***Created with Biorender.com****.*

Blood was drawn into ethylenediaminetetraacetic acid–coated tubes and processed under standardized conditions. Samples were kept at 4 °C and centrifuged at 2,000 × g for 10 min within 30 min of collection. Plasma was aliquoted to avoid repeated freeze–thaw cycles and stored at −80 °C until analysis. All samples underwent a single freeze–thaw cycle prior to proteomic profiling.

3.6.3 PROXIMITY EXTENSION ASSAY–BASED PROTEOMIC PROFILING

Plasma protein abundance was quantified using the Olink Explore 384/3072 platform (Olink Proteomics, Uppsala, Sweden), an antibody-based multiplex proximity extension assay (PEA) technology enabling simultaneous measurement of a large panel of predefined proteins.

The PEA technology relies on pairs of target-specific antibodies, each conjugated to a unique oligonucleotide. Upon dual binding of both antibodies to the same protein molecule, the attached oligonucleotides are brought into close proximity, allowing hybridization and subsequent enzymatic extension to form a unique DNA reporter sequence. This DNA barcode is amplified and quantified by next-generation sequencing. Because signal generation requires dual antibody recognition, the method provides high specificity, while DNA-based amplification enables sensitive detection of low-abundance proteins in small plasma volumes (50).

Internal assay controls were included to monitor incubation, extension, and detection efficiency. Raw sequencing data were processed using the manufacturer's standardized computational pipeline, including quality control, inter-plate normalization, and intensity scaling. Protein abundance was reported as normalized protein expression values on a $\log_2$ scale, allowing relative quantification across samples. Proteins with a high proportion of values below the limit of detection (LOD) were handled according to Olink guidelines; analytes with excessive missingness were excluded prior to analysis, and values below the LOD were retained as reported by the platform. The final dataset included 1,072 proteins at each time point.

3.7 STATISTICAL ANALYSIS

All statistical analyses described below apply to animal, molecular, and clinical experiments unless otherwise specified. Data was presented as mean ± standard error of the mean (SEM), except for WMSI which was presented as median (Q1, Q3).

For animal functional and molecular experiments, comparisons between two groups were conducted using an unpaired two-tailed Student's t-test, whereas comparisons involving multiple groups or repeated measurements were analyzed using one-way or mixed-design ANOVA with Bonferroni correction.

Proteomic and phosphoproteomic data were log 2-transformed prior to analysis. Differential abundance was assessed using Welch's t-test, with a significance threshold of $P < 0.05$ and 1% FDR where applicable.

RNA sequencing data were analyzed using a negative binomial model implemented in DESeq2, with multiple-testing correction using the FDR approach. Differential gene expression was defined as FDR < 0.05 and absolute fold change ≥ 2.

Clinical plasma proteomic differences between TS and STEMI were assessed using linear regression models adjusted for age and exact sampling day within each predefined time window. Multiple testing correction was performed using the Benjamini–Hochberg FDR procedure, with FDR < 0.05 considered statistically significant.

All analyses were performed using GraphPad Prism, SPSS, R, Perseus, and Partek Flow as appropriate.

4 RESULTS

4.1 ESTABLISHING THE CORE PHENOTYPE

4.1.1 DEFINING THE FUNCTIONAL PHENOTYPE OF REVERSIBLE POST-ISCHEMIC DYSFUNCTION

A central objective of **Study I** was to establish an *in vivo* experimental framework capable of distinguishing reversible post-ischemic contractile dysfunction (myocardial stunning) from irreversible structural injury (necrosis or infarction) under controlled I/R conditions. To achieve this, a rat LAD occlusion–reperfusion model with graded durations of index ischemia was employed.

In this model, myocardial stunning was defined as the presence of segmental akinesia during early reperfusion (4 h) followed by resolution of akinesia at late follow-up (48 h), assessed by serial echocardiographic evaluation of the ischemic myocardial segment. This operational definition enabled systematic assessment of the relationship between ischemia duration and functional recovery.

In non-preconditioned (NIPC) animals, the transition from preserved myocardial function to overt regional dysfunction occurred within a relatively narrow ischemic time window. Across the ischemia durations tested, no interval consistently produced reversible akinesia in NIPC animals. When akinesia was observed at 4 h after reperfusion, it most frequently persisted at 48 h, indicating sustained contractile impairment in the absence of preconditioning.

In contrast, IPC markedly altered this ischemia–outcome relationship. Following IPC, ischemia durations that were associated with persistent dysfunction in NIPC hearts more frequently resulted in reversible contractile impairment. In particular, ischemia durations of 13.5–15 minutes induced reversible akinesia in the majority of IPC animals, whereas reversible dysfunction was rarely observed in NIPC animals exposed to similar ischemic durations. Other indices of global and regional cardiac function, including LVEF, FS, FAC, GLS, and regional strain parameters, showed significant deterioration at 4 h after reperfusion in both groups. At 48 h, these parameters recovered substantially in IPC animals, whereas NIPC animals remained persistently impaired.

Collectively, these observations indicate that IPC increases the likelihood that early post-ischemic contractile dysfunction follows a reversible trajectory.

4.1.2 DISSOCIATION BETWEEN EARLY FUNCTIONAL DEPRESSION AND IRREVERSIBLE STRUCTURAL INJURY

Because an increased incidence of early akinesia could theoretically reflect more severe myocardial injury, it was essential to determine whether the shift toward reversible dysfunction occurred at the expense of increased structural damage.

To address this, infarct size was quantified under a matched 13.5-minute ischemia protocol using Evans blue and TTC staining. IPC animals demonstrated a markedly smaller infarct size compared with NIPC controls (1.8 ± 1.1% vs 12.8 ± 3.3% of the area at risk; **Figure 8**).

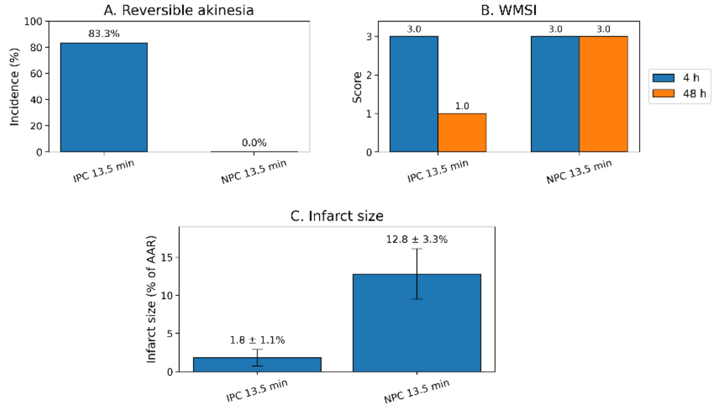


Figure 8. IPC increases reversible post-ischemic dysfunction while reducing infarct size after 13.5 min ischemia.

*(**A**) Incidence of reversible akinesia in rats subjected to 13.5 min LAD occlusion followed by reperfusion, with (IPC) or without (NPC) prior ischemic preconditioning. Reversible akinesia was defined as akinesia at 4 h with recovery by 48 h.*

*(**B**) Segmental wall motion score index (WMSI) in the ischemic segment at 4 h and 48 h after reperfusion. IPC animals showed recovery of wall motion by 48 h, whereas NIPC animals exhibited persistent dysfunction.*

*(**C**) Infarct size quantified by Evans blue/TTC staining after 13.5 min ischemia and 2 h*

reperfusion, expressed as percentage of area at risk. IPC was associated with markedly reduced infarct size compared with NIPC. Data are presented as mean ± SEM in panel C. Together, these findings demonstrate dissociation between early functional depression and irreversible structural injury under conditions of ischemic preconditioning. ***Created with GraphPad Prism.***

These findings indicate that the enhanced propensity for reversible dysfunction observed in IPC hearts did not occur at the cost of increased myocardial necrosis. Taken together, the results demonstrate that under the present experimental conditions early contractile dysfunction and irreversible structural injury can be experimentally dissociated *in vivo*. After IPC, early functional impairment was more likely to follow a reversible course while infarct size was simultaneously reduced.

4.1.3 IPC-INDUCED PRE-ISCHEMIC PHOSPHOREGULATION OF THE CONTRACTILE APPARATUS

To investigate potential mechanisms underlying the altered functional trajectory observed after IPC, **Study I** examined rapid post-translational remodeling with a focus on protein phosphorylation.

Phosphoproteomic profiling was performed immediately after the IPC stimulus (or sham) and prior to the index ischemia, thereby capturing the molecular state induced by preconditioning in the absence of confounding effects from prolonged ischemia, reperfusion injury, or secondary structural remodeling. Phosphoproteomic analysis identified 9,103 quantified phosphopeptides, of which 786 phosphopeptides were differentially regulated between IPC and NIPC groups according to predefined statistical criteria. Among these, a total of 412 phosphopeptides showed increased abundance, whereas 374 were decreased, indicating that IPC induced bidirectional remodeling of the myocardial phosphoproteome rather than uniform activation or suppression.

Functional enrichment analyses demonstrated a pronounced structural focus of IPC-associated phosphoregulation within the contractile architecture of cardiomyocytes. Z-disc represented the most significantly enriched cellular compartment, with additional enrichment observed in the I-band, sarcomere, actin filament bundles, myofibrils, and contractile fibers.

At the molecular-function level, actin filament binding emerged as the most strongly enriched functional category, followed by actin binding and related cytoskeletal interaction processes. These findings indicate that IPC preferentially affects phosphorylation of proteins involved in the sarcomeric and cytoskeletal machinery responsible for myocardial contraction.

4.2 PROTEOMIC SIGNATURES OF IPC ACROSS PRE-ISCHEMIC AND EARLY REPERFUSION PHASES

4.2.1 IPC REMODELS THE MYOCARDIAL PROTEOME IN A TEMPORALLY STRUCTURED MANNER: FROM A PRE-ISCHEMIC PRIMING STATE TO ALTERED EARLY-REPERFUSION BIOLOGY

Building on the functional dissociation established in **Study I**, **Study II** examined whether this divergence was accompanied by time-dependent remodeling of the myocardial proteome. In **Study I**, IPC promoted recovery after a 13.5-minute LAD ischemic insult, whereas NIPC hearts more frequently progressed toward persistent dysfunction and infarction.

Using the same 13.5-minute LAD I/R paradigm, left ventricular tissue was collected at two predefined time points: T1, immediately after IPC (or sham) and prior to index ischemia; or T2, 4 hours after reperfusion. The 4-hour time point corresponds to the phase of maximal contractile depression in IPC hearts, as identified in **Study I.**

This design therefore distinguishes an IPC-induced pre-ischemic molecular state (T1) from early reperfusion biology (T2), enabling temporal interpretation of proteomic changes.

4.2.2 EARLY PRE-ISCHEMIC PHASE (T1): IPC ESTABLISHES A TRAFFICKING- AND PROCESSING-ENRICHED PROTEOMIC CONFIGURATION

At T1, 3,804 proteins were quantified, of which 117 proteins differed significantly between IPC and NIPC (95 upregulated and 22 downregulated in IPC; $P < 0.05$ and $\geq 20\%$ change).

This early IPC-associated proteomic profile did not resemble canonical injury- or necrosis-associated signatures. Instead, enrichment analyses indicated a proteomic configuration dominated by cellular trafficking and intracellular processing pathways. Within GO Biological Process, the most prominent enrichment was positive regulation of endocytosis (11.63-fold enrichment), involving proteins such as *PPT1, CDC42, PPP3CC, BIN1,* and *FMR1*.

In KEGG pathway analysis, Fc gamma receptor-mediated phagocytosis showed significant enrichment (13.29-fold enrichment), driven by proteins including *BIN1, WASF2, CDC42,* and *ARF6.*

Proteins downregulated at T1 did not cluster into coherent biological pathways, suggesting that IPC at this stage primarily induces selective activation of trafficking-related processes rather than suppression of injury-related pathways.

Because these changes were detected prior to index ischemia, they most plausibly represent an IPC-induced pre-ischemic molecular configuration rather than secondary consequences of ischemic injury or reperfusion-associated inflammation.

4.2.3 EARLY REPERFUSION PHASE (T2): IPC IS ASSOCIATED WITH ATTENUATION OF REMODELING-, IMMUNE-, AND COAGULATION-RELATED PROTEOMIC SIGNATURES

At 4 hours after reperfusion (T2), proteomic differences between IPC and NIPC shifted toward pathways commonly associated with early I/R responses.

In the IPC versus NIPC comparison at T2, 173 proteins were significantly altered, with 43 proteins upregulated and 130 proteins downregulated in IPC relative to NIPC.

Proteins reduced in IPC were strongly enriched in pathways related to tissue remodeling, including fibrinolysis (28.25-fold enrichment), humoral immune response (15.89-fold enrichment), and blood coagulation (15.69-fold enrichment).

At the pathway level, the most prominent enrichment among proteins reduced in IPC was the complement and coagulation cascade (19.94-fold enrichment), involving proteins such as *CPB2, C2, C4A, FGG, SERPING1, C1QA/B, SERPINC1, FGA, F13A1,* and *PROS1.*

These results indicate that IPC is associated with relative attenuation of complement-, coagulation-, and remodeling-related proteomic signatures during early reperfusion, compared with NIPC hearts**.** However, whether these proteomic differences are causally involved in protection or instead represent downstream consequences of reduced injury remains unclear.

Complement and coagulation proteins may partially reflect circulating plasma components and interpretations are therefore framed as relative between-group differences rather than assuming exclusive myocardial cellular origin (**Figure 9**).

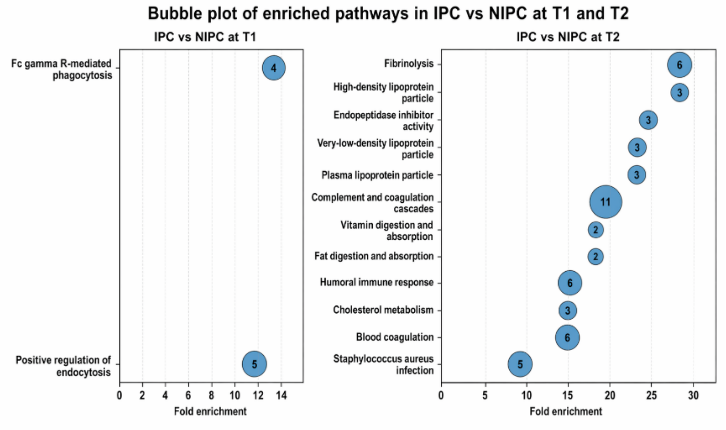


Figure 9. Bubble plot of enriched pathways in IPC versus NIPC at T1 and T2. *The plot shows significantly enriched biological pathways identified in ischemic preconditioned myocardium (IPC) compared with non-preconditioned myocardium (NIPC) at two time*

points, T1 and T2. Each bubble represents one enriched pathway and is positioned according to its fold enrichment value on the x-axis. Bubble size is proportional to the number of proteins associated with each pathway based on the proteins reported in the Results section, with larger bubbles indicating pathways containing more identified proteins. The number displayed within each bubble indicates the exact protein count for that pathway. ***Created with GraphPad Prism.***

4.2.4 TIME-DEPENDENT REMODELING WITHIN GROUPS

Within-group temporal analyses demonstrated that reperfusion induces substantial proteomic remodeling regardless of preconditioning status.

In NIPC hearts, 459 proteins changed significantly between T1 and T2 (229 upregulated and 230 downregulated), with strong enrichment for complement and coagulation cascades (13.43-fold enrichment), fibrinolysis (16.3-fold enrichment), and humoral immune response (11.2-fold enrichment).

In IPC hearts, 103 proteins differed significantly between T1 and T2, with enrichment of pathways related to leukocyte migration (60.89-fold enrichment), complement activation, and immune-response-associated processes.

These findings indicate that early reperfusion is accompanied by activation of immune- and complement-related pathways in both groups, although the magnitude and composition of these responses differ between IPC and NIPC hearts. Again, the extent to which these proteomic differences are causally involved in protection versus represent downstream consequences of reduced injury remains unclear.

4.2.5. TARGETED VALIDATION SUPPORTS PROTEOMIC DIRECTIONALITY

To validate the proteomic dataset using an independent method, two proteins that differed significantly between IPC and NIPC at T1 were selected for orthogonal validation by Western blot analysis: monoamine oxidase A and carbonic anhydrase 2 (CA2).

Consistent with the proteomic data, Western blot analysis demonstrated lower *MAOA* expression and higher *CA2* abundance in IPC compared with NIPC (Student's t-test, $P < 0.05$), supporting cross-platform agreement between mass spectrometry and immunoblotting results.

4.3 TEMPORAL TRANSCRIPTOMIC AND EPIGENETIC REMODELING FOLLOWING ISCHEMIC PRECONDITIONING

4.3.1 IPC IS ASSOCIATED WITH TIME-STRUCTURED TRANSCRIPTOMIC REMODELING LINKED TO GENE-SELECTIVE DNA METHYLATION CONTROL

Study III extended the mechanistic investigation upstream from protein-level remodeling described in **Study II** to transcriptional and epigenetic regulation. Using the same *in vivo* 13.5-minute LAD I/R model, we examined whether IPC was associated with reshaping of the cardiac transcriptome in association with DNA methylation-dependent regulatory mechanisms.

Like in **Study II**, myocardial tissue was collected at two predefined time points: T1, immediately after IPC or sham and prior to index ischemia, and T2, 4 hours after reperfusion.

4.3.2 IPC INDUCES TEMPORALLY STRUCTURED TRANSCRIPTOMIC REMODELING

RNA sequencing demonstrated that IPC modulates myocardial gene expression at both phases, although with distinct magnitude and breadth.

At T1, IPC induced a relatively focused transcriptional response. A total of 53 genes were differentially expressed compared with NIPC hearts, including 46 upregulated and 7 downregulated genes, using a false discovery rate threshold of less than 0.05 and a fold change of at least two.

At T2, transcriptomic divergence between IPC and NIPC hearts increased substantially. In this comparison, 166 genes were differentially expressed, including 55 upregulated and 111 downregulated genes in IPC relative to NIPC hearts.

Several IPC-regulated transcripts were associated with pathways involved in inflammatory regulation, stress responses, DNA repair, and transcriptional control. Among these were *Nfkbia, Gadd45b, Jun, Parp14,* and *Cebpd*, several of which ranked among the most significantly altered candidates across both time points.

Gene set enrichment analysis indicated coordinated regulation of pathways related to immune signaling, stress responses, apoptosis, metabolic adaptation, and inflammatory regulation. These findings suggest that IPC induces a structured transcriptional program rather than indiscriminate activation of gene expression.

4.3.3 IPC IS ASSOCIATED WITH REDUCED GLOBAL DNMT ACTIVITY AND GENE-SELECTIVE PROMOTER METHYLATION

A central observation was that global DNMT activity was significantly reduced in IPC hearts at both T1 and T2, indicating sustained suppression of methylation activity across the early reperfusion window (**Figure 10**).

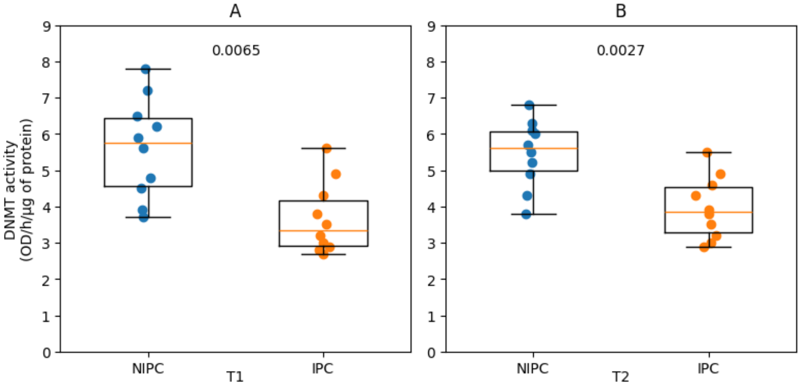


Figure 10. DNA methyltransferase (DNMT) activity in non-preconditioned (NIPC) and preconditioned (IPC) myocardium.
(A) At T1, IPC is associated with reduced DNMT activity compared to NIPC.
(B) At T2, DNMT activity remains decreased in IPC. ***Created with GraphPad Prism.***

Despite this global reduction in DNMT activity, promoter-level analyses demonstrated that IPC does not induce uniform hypomethylation across the genome. Instead, IPC was associated with a dynamic and gene-selective pattern of DNA methylation.

During the early phase (T1), IPC was associated with promoter hypomethylation of several adaptive genes, including *Cebpd, Nfkbia, Gadd45b, Jun,* and *Aplod1*. Reduced promoter methylation at these loci was accompanied by increased mRNA expression, consistent with epigenetic depression of genes involved in stress adaptation, inflammatory regulation, DNA repair, and transcriptional responses.

During the reperfusion phase (T2), despite reduced global DNMT activity, several genes demonstrated increased promoter methylation in IPC hearts accompanied by reduced gene expression. Examples included *Tmem200c, Fgfr4,* and *Stk32c*. In contrast, *Parp14*, which was upregulated in IPC hearts at T2, showed promoter hypomethylation consistent with transcriptional activation.

Together, these findings suggest that IPC is associated with locus-specific and phase-dependent redistribution of DNA methylation, allowing activation of protective gene programs while constraining selected pathways during early reperfusion.

4.3.4 PROMOTER-LEVEL MECHANISTIC SUPPORT: DYNAMIC MODULATION OF *DNMT1* OCCUPANCY

To further investigate the mechanistic basis of these epigenetic changes, ChIP assays were performed to assess *Dnmt1* binding at gene promoters. During the early phase (T1), IPC significantly reduced *Dnmt1* occupancy at the *Jun* promoter, corresponding with reduced promoter methylation and increased *Jun* expression. At T2, IPC reduced *Dnmt1* binding at the *Parp14* promoter, which was accompanied by increased *Parp14* mRNA expression and protein abundance. ELISA analysis confirmed increased *Jun* and *Parp14* protein levels at the corresponding time points. These findings provide promoter-level evidence that IPC dynamically modulates *Dnmt1* promoter binding and are consistent with epigenetic regulation of gene expression during early reperfusion.

4.4 TRANSLATIONAL PLASMA PROTEOMIC DIVERGENCE BETWEEN TAKOTSUBO SYNDROME AND REPERFUSED STEMI

4.4.1 TEMPORAL PLASMA PROTEOMIC DIVERGENCE BETWEEN TAKOTSUBO SYNDROME AND REPERFUSED STEMI

Study IV extended the thesis into a clinical setting by comparing two syndromes with overlapping acute clinical presentations but distinct myocardial injury phenotypes: TS, characterized by transient left ventricular dysfunction without obstructive coronary artery disease sufficient to explain the phenotype, and timely reperfused STEMI, representing ischemic necrotic injury (**Figure 11**).

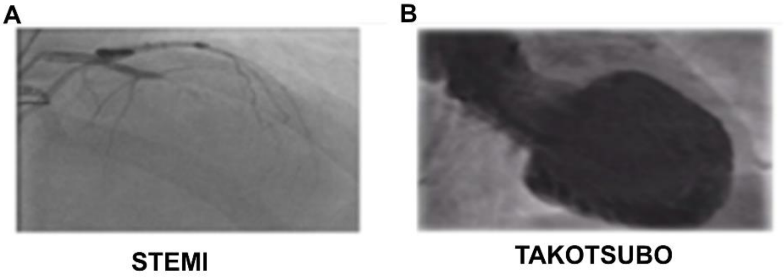


Figure 11. Representative angiographic images of STEMI and Takotsubo cardiomyopathy.
(A) Coronary angiography image from a patient with ST-elevation myocardial infarction (STEMI), showing obstructive coronary artery disease.
(B) Left ventriculography image from a patient with Takotsubo cardiomyopathy, demonstrating the characteristic apical ballooning.

Plasma proteomic profiling was performed using PEA technology (Olink platform) in women enrolled in the STAMI study. Plasma samples were analyzed across predefined temporal windows reflecting different phases of disease evolution, including the acute phase (0–1 days after symptom onset), a subacute phase (2–3 days after symptom onset), and the convalescent phase (~30 days after symptom onset).

A defining feature of the results was their temporal structure. In the acute phase, only seven proteins met the FDR threshold for differential abundance between TS and STEMI, indicating limited early proteomic separation despite the distinct pathophysiological mechanisms underlying transient dysfunction and necrotic injury. In contrast, proteomic divergence increased markedly during the subacute phase. A total of 236 proteins were differentially abundant, of which 227 were increased in TS and nine were increased in STEMI.

By the convalescent phase, between-group differences diminished substantially, with minimal differential protein abundance observed between the two conditions. This reduction in proteomic separation is consistent with partial convergence of circulating protein profiles during recovery. Taken together, these observations revealed a three-phase temporal pattern characterized by minimal separation at presentation, maximal divergence during the early subacute phase, and attenuation of differences during convalescence.

4.4.2 PATHWAY ENRICHMENT ANALYSIS OF SUBACUTE TS-ASSOCIATED PROTEINS

Pathway enrichment analysis was performed for proteins that were differentially abundant during the subacute phase and stratified according to the direction of change between TS and STEMI.

Among proteins increased in TS, over-representation analysis demonstrated enrichment of pathways related to cytokine and interleukin signaling, innate immune activation, and receptor tyrosine kinase-mediated signal transduction. These pathways collectively indicate coordinated activation of inflammatory and stress-related signaling processes reflected in the circulating proteome.

Platelet and hemostatic pathways were also enriched among proteins increased in TS. However, interpretation of these pathways requires caution because patients with STEMI routinely receive dual antiplatelet therapy, whereas patients with TS typically do not. Accordingly, relative enrichment of platelet-related pathways in TS may partly reflect treatment-related suppression of platelet-associated proteins in STEMI rather than disease-specific activation in TS.

Notably, enrichment of cytokine signaling, innate immune activation, and receptor tyrosine kinase pathways remained a prominent feature of the TS-associated proteomic profile, indicating that the subacute signal was not explained solely by platelet- or treatment-related differences between groups. Proteins increased in STEMI during the subacute phase were few in number and did not generate a coherent pathway-level enrichment pattern.

4.4.3 TEMPORAL INTERPRETATION OF PLASMA PROTEOMIC DIVERGENCE

The temporal distribution of proteomic differences between TS and STEMI suggests that disease-specific circulating protein signatures are most evident during the evolving phase following the initial clinical presentation rather than at the moment of symptom onset.

At presentation, shared systemic responses associated with acute cardiac injury, including sympathetic activation and inflammatory signaling, may contribute to the relative similarity of plasma proteomic profiles between the two conditions. By contrast, during the subacute interval the circulating proteome reflects more distinct biological processes, including immune activation and stress-response signaling pathways that are more prominently represented in TS.

During convalescence, the reduction in proteomic differences between groups suggests partial normalization of circulating molecular signals as acute processes resolve.

5 DISCUSSION

5.1 REFRAMING MYOCARDIAL STUNNING: FROM PASSIVE DYSFUNCTION TO REGULATED CARDIOPROTECTION

Myocardial stunning has traditionally been defined as a transient impairment of contractile function that persists after restoration of coronary blood flow following I/R injury (51). In its classical interpretation, this phenomenon has largely been seen as a consequence of reperfusion-associated metabolic disturbance, oxidative stress, and impaired calcium handling, all of which disrupt the contractile apparatus during early reperfusion (13, 23). The findings presented in this thesis support a broader interpretation in which, particularly in the context of IPC, reversible contractile dysfunction may reflect a regulated biological state associated with cardioprotection.

Across the experimental studies, IPC appeared to shift the post-ischemic myocardial response away from irreversible injury and toward reversible dysfunction. In the *in vivo* rat model, this was reflected by a greater tendency for early post-ischemic contractile depression to be followed by functional recovery, together with a reduction in infarct size. These observations suggest that reversible dysfunction should not be regarded as evidence of incomplete protection but may instead represent transiently reduced contractile performance despite preserved myocardial viability. This interpretation is consistent with concepts derived from the myocardial hibernation literature, in which reduced contractile activity has been proposed as a means of restoring metabolic balance and maintaining viability under stress (52).

The omics findings further strengthen this framework. Phosphoproteomic analyses showed that IPC was associated with altered phosphorylation patterns in proteins involved in sarcomeric organization and contractile regulation, indicating that post-ischemic dysfunction in the preconditioned heart may arise from controlled changes within the contractile apparatus rather than from passive injury alone. Complementary proteomic and transcriptomic findings pointed to concurrent alterations in inflammatory signaling, cellular stress responses, repair-related processes, and epigenetic regulation, consistent with a broader adaptive response to ischemic stress.

Taken together, these findings support the idea that myocardial stunning may represent not only a pathological consequence of ischemic stress, but also, under specific conditions, an adaptive response during early reperfusion (53). Within this framework, a transient reduction in mechanical work may reduce energetic demand at a time when mitochondrial recovery and cellular homeostasis remain incomplete. Such a state may provide the myocardium with a temporary protective window during which cellular integrity can be preserved despite ongoing functional impairment. Although the present data do not establish a direct causal mechanism, they support the interpretation that reversible post-ischemic dysfunction may form part of an IPC-associated cardioprotective response linked to a lower likelihood of irreversible myocardial necrosis.

5.2 MULTILAYER REGULATION OF POST-ISCHEMIC TRAJECTORY

A central theme emerging from this thesis is that IPC reshapes myocardial responses to I/R injury across multiple biological layers. These layers span rapid post-translational modification, tissue-level proteomic remodeling, and upstream transcriptional and epigenetic regulation, which together influence the trajectory of myocardial recovery.

At the post-translational level, phosphoproteomic analyses demonstrated that IPC induces extensive remodeling of phosphorylation within the myocardial proteome before the ischemic insult. The most strongly enriched protein categories were related to structural components of the contractile apparatus, including sarcomeric proteins and Z-disc-associated complexes. These observations suggest that IPC establishes a pre-ischemic phosphorylation landscape capable of modulating mechanical performance during subsequent ischemia-reperfusion. Such rapid post-translational modulation has previously been implicated as a central mechanism of ischemic conditioning, enabling cardiomyocytes to adapt quickly to metabolic stress during reperfusion (27, 28). In this framework, phosphoproteomic remodeling appears to represent an early priming layer, whereas the proteomic and transcriptomic findings capture how this regulatory state is translated into the post-ischemic response.

At the tissue proteome level, temporal profiling revealed structured remodeling during early reperfusion. Compared with non-preconditioned hearts, IPC hearts showed relative attenuation of complement-, coagulation-, and remodeling-related protein signatures. Importantly, reperfusion-associated biological processes remained active even in hearts that ultimately recovered function. The key distinction between trajectories therefore appears to lie not in the simple presence or absence of these injury-associated pathways, but in their relative weighting and temporal coordination during early reperfusion. This interpretation is consistent with previous work showing that cardioprotective interventions often modifies inflammatory and remodeling pathways rather than completely suppressing them (20).

Transcriptomic and epigenetic analyses extended this framework further upstream. IPC induced coordinated changes in gene expression accompanied by dynamic regulation of DNA methylation. Reduced global DNMT activity was associated with selective promoter hypomethylation of genes involved in stress adaptation, inflammatory regulation, and DNA repair (51). At the same time, promoter hypermethylation of selected genes was observed despite reduced global DNMT activity, suggesting locus-specific redistribution of methylation rather than uniform epigenetic suppression. These findings support growing evidence that epigenetic mechanisms contribute to the regulation of myocardial responses to ischemic stress (52, 53).

Promoter-level analyses provided additional support for this model by demonstrating dynamic modulation of *Dnmt1* occupancy at specific gene promoters. Reduced *Dnmt1* binding at the *Jun* and *Parp14* promoters was associated with promoter hypomethylation, increased gene expression, and elevated protein levels. Such targeted regulation suggests that IPC influences transcriptional programs through selective epigenetic remodeling rather than through global suppression of DNA methylation.

In addition to these intracellular and tissue-level regulatory layers, the coordinated responses observed after IPC may also involve intercellular signaling mechanisms. In this context, extracellular vesicles have increasingly been implicated in the literature as mediators of ischemic conditioning and cardioprotective communication. Although extracellular vesicles were not examined in the present thesis, they represent a plausible pathway through which IPC-associated signals could be propagated between myocardial and extra-myocardial compartments, thereby contributing to the coordination of inflammatory, survival, and repair-related responses (54).

Collectively, the multi-omics observations presented in this thesis indicate that cardioprotection emerges from coordinated regulatory processes operating across multiple biological layers. Rapid sarcomeric phosphoregulation, temporal reweighting of tissue-level proteomic programs, and upstream epigenetic control of transcriptional responses appear to converge in shaping the myocardial trajectory following ischemia-reperfusion (**Table 1-2**).

Table 1. *Integrated multi-omics summary of the early myocardial response immediately after ischemic preconditioning and before index ischemia (T1).*

Biological theme	**Transcriptomics / DNA methylation**	**Proteomics**	**Phosphoproteomics**	**Integrated interpretation**
Epigenetic and transcriptional priming	**Early protective gene reprogramming; ↓ DNA methyltransferase activity**	**No dominant epigenetic protein signal**	**No major epigenetic phosphosite signal**	**IPC primarily acts at gene-regulatory level**
Inflammation control and stress adaptation	**Protective stress-response genes upregulated**	**Adaptive immune trafficking (not injury signaling)**	**Not a dominant phosphosite theme**	**Controlled pre-stress state (not full suppression)**
Endocytosis, membrane trafficking, phagocytosis	**Not a leading transcriptomic signal**	**Enrichment of endocytosis/phagocytosis proteins**	**Cytoskeletal & membrane remodeling present**	**Preparation for adaptive trafficking before ischemia**
Contractile apparatus and structure	**Not a dominant transcriptomic signal**	**No major contractile protein abundance change**	**Strong sarcomeric & cytoskeletal phosphoregulation**	**Post-translational tuning of contractile machinery**
Metabolic / vascular resilience	**Signals consistent with stress & vascular resilience**	**Limited metabolic protein changes**	**No dominant metabolic phosphosite pathway**	**Supportive role, not main coordinated program**
Functional implication	**Protective pre-ischemic profile**	**Adaptive handling rather than damage remodeling**	**Favors reversible stunning**	**Primed state favoring protection over necrosis**

Table 2. *Integrated multi-omics summary of the post ischemic myocardial response after reperfusion (T2).*

Biological theme	Transcriptomics / DNA methylation	Proteomics	Phosphoproteomics	Integrated interpretation
Epigenetic control of late injury response	**Protective programs persisted, while maladaptive genes were suppressed.**	**No dominant epigenetic protein cluster.**	**Not assessed at T2.**	**IPC still acts as a gene-regulatory filter during reperfusion.**
Inflammation and immune response	**Inflammatory pathways appeared regulated rather than unchecked.**	**Less injury-associated inflammatory burden than NIPC, but not a silent response.**	**Not assessed at T2.**	**IPC reshapes inflammation toward a controlled reparative profile.**
Complement, coagulation, and fibrinolysis	**Compatible with attenuation of injury-amplifying cascades.**	**These pathways increased after I/R overall, but were less pronounced in IPC than NIPC.**	**Not assessed at T2.**	**IPC limits excess coagulation/complement activation associated with necrotic injury.**
Tissue remodeling and extracellular injury response	**Late maladaptive injury responses were reduced.**	**Remodeling-related proteins were downregulated versus NIPC.**	**Not assessed at T2.**	**The stunned IPC myocardium appears less committed to destructive remodeling.**
Repair and survival signaling	**Repair/survival signals remained evident at the transcript level.**	**Direct proteomic support was limited.**	**Not assessed at T2.**	**Late protection still appears to be driven mainly by gene-expression programs.**
Phenotypic endpoint	**Profile is consistent with reversible stunning rather than necrosis.**	**IPC hearts showed less inflammatory and remodeling burden than NIPC hearts.**	**T1 phosphosite findings provide a plausible upstream mechanism, but no direct T2 data are available.**	**Across studies, stunning appears as an actively regulated protective state, not passive consequence**

5.3 TEMPORAL STRUCTURE AS AN ORGANIZING PRINCIPLE OF CARDIOPROTECTION

An important implication of this thesis is that cardioprotection cannot be understood fully from single end points such as infarct size reduction or functional recovery alone (7, 55, 56). Across the experimental studies, IPC was associated with a different pattern of myocardial response over time rather than only less injury. This was evident across both functional and molecular levels, including contractile performance, phosphoproteomics, proteomics, transcriptomics, and epigenetic analysis.

This distinction was particularly relevant in the setting of post-ischemic dysfunction. An early reduction in contractile performance may reflect a reversible trajectory, whereas a similar phenotype at a later stage may instead indicate progression toward irreversible injury (55, 56). Consistent with this, proteomic and transcriptomic analyses suggested that the preconditioned myocardium did not merely show a weaker injury response, but a different sequence of biological events, with early signatures of stress adaptation and later divergence in pathways related to inflammation, remodeling, and injury progression. The phosphoproteomic findings extend this interpretation further by showing that IPC-associated phosphorylation changes were already present before the sustained ischemic insult, supporting the concept of molecular priming before the full post-ischemic phenotype became apparent.

A similar time-dependent pattern was also observed in the clinical study. Plasma proteomic profiling showed limited separation between TS and reperfused STEMI at presentation, whereas clearer differences emerged during the subacute phase, particularly in inflammatory and stress-response pathways, before diminishing again during convalescence. Taken together, these findings indicate that the interpretation of cardioprotection depends not only on which phenotypes are observed, but also on when they emerge. This has important implications for both experimental design and therapeutic translation, since assessment at a single time point may obscure meaningful distinctions between delayed recovery, reversible dysfunction, and progression to irreversible damage.

5.4 BIOLOGICAL ACTIVITY WITHIN REVERSIBLE DYSFUNCTION STATES

The translational findings extend a central theme of this thesis by showing that distinct forms of acute myocardial dysfunction are associated with different downstream biological response patterns. In this context, TS provides a clinically relevant contrast to reperfused STEMI: both may present with acute ventricular dysfunction yet they differ fundamentally in their underlying injury profile, with TS characterized by largely reversible dysfunction and reperfused STEMI defined by substantial ischemic necrotic injury.

In the clinical proteomic study, the clearest molecular separation between TS and reperfused STEMI emerged during the subacute phase rather than at presentation. TS was characterized by a systemic proteomic signature enriched for cytokine and interleukin signaling, innate immune pathways, and receptor tyrosine kinase-mediated signaling, whereas acute and convalescent profiles were considerably more similar. These findings indicate that proteomic distinction between the two syndromes is not captured most clearly at the time of initial presentation, but during the evolving phase of recovery, when their dominant biological response patterns become more clearly differentiated.

This interpretation is consistent with growing evidence that the transient disorder of ventricular mechanics in TS is accompanied by sustained biological activity beyond the initial acute phase. Earlier studies have demonstrated myocardial and systemic inflammatory activation in TS, including macrophage infiltration, altered monocyte profiles, and persistent cytokine elevation, consistent with the view that immune signaling forms part of the syndrome rather than representing a secondary epiphenomenon (57, 58). In the present study, the persistence of inflammatory and stress-response pathway enrichment after exclusion of platelet- and coagulation-related processes further supports the interpretation that immune- and stress-associated signaling are integral features of the subacute TS response.

At the same time, these findings should not be interpreted as indicating an absence of biological activity in reperfused STEMI. Rather, they suggest that, within the circulating proteomic space examined here, TS displays a more distinctive subacute inflammatory and stress-associated signature. The significance of this observation lies in showing that reversible dysfunction and necrotic injury are not distinguished solely by the extent of structural damage, but also by the character of the biological programs that unfold during recovery.

Biological sex may also influence the interpretation of these findings. Experimental and clinical studies suggest that sex can affect susceptibility to ischemia-reperfusion injury and may modify responses to cardioprotective conditioning, although these effects are not consistent across models (59, 60). This issue is particularly relevant here because the experimental studies were performed in male rats, whereas the clinical proteomic study was restricted to women to reduce sex-related proteomic confounding and because TS predominantly affects women. The present findings therefore support the biological relevance of the identified trajectories, but do not establish whether the same molecular and functional patterns are preserved across sexes.

Within the broader framework of this thesis, these observations provide a clinical parallel to the experimental findings by showing that recovery-prone dysfunction may remain biologically active and cannot be understood solely in terms of reversibility. Instead, its significance depends on the nature and timing of the associated molecular response.

6 LIMITATIONS

This thesis has several limitations. First, the experimental studies were conducted in male rats. Although this provided a controlled setting for examining post-ischemic trajectories of reversible dysfunction and irreversible injury, it limits direct translational relevance to human disease and does not account for sex-related biological variation. Second, although multiple omics approaches were applied across the experimental studies, the thesis provides an integrated biological interpretation rather than a fully causal systems-level account of the underlying mechanisms.

Third, the molecular findings are primarily associative, and the temporal sequence between many molecular changes and functional or injury-related phenotypes cannot be fully established in these analyses. This limits causal interpretation, since some observed phosphorylation events, protein alterations, and epigenetic changes may represent downstream consequences rather than upstream drivers of injury or protection. The causal roles of specific phosphorylation events, protein alterations, and epigenetic changes therefore require further targeted validation. Fourth, the clinical study was limited to circulating proteomics in women with Takotsubo syndrome and STEMI, which restricts both generalizability and tissue-specific interpretation

7 CLOSING REMARKS FUTURE PERSPECTIVES

7.1 STUDY I

Study I establishes the experimental models used in three of the studies in this thesis and showed that IPC is associated with reduced irreversible myocardial injury together with an increased propensity for reversible post-ischemic dysfunction followed by functional recovery. The reproducible recovery of segmental contractile impairment after IPC suggests that preconditioning shifts the threshold at which ischemic stress progresses from reversible dysfunction to irreversible infarction. In this context, IPC appears to shift the myocardial response away from irreversible necrosis and toward a state of transient but recoverable dysfunction, thereby extending the window for myocardial salvage.

These functional findings are supported by the molecular data. Phosphoproteomic analysis of IPC-treated myocardium showed early phosphorylation changes in proteins linked to the sarcomere, Z-disc, and actin-binding structures. While the exact functional effects of these modifications are not yet fully understood, their presence in key structural regions suggests that IPC may help stabilize the contractile apparatus before ischemia. Taken together, these results suggest that myocardial stunning is not simply a byproduct of ischemic stress, but may instead represent a regulated and potentially adaptive response associated with cardioprotection.

7.2 STUDY II

Study II demonstrates that the cardioprotective effects of IPC are accompanied by temporally structured remodeling of the myocardial proteome. Before index ischemia, IPC established a proteomic configuration enriched in trafficking- and processing-related pathways, suggesting that the myocardium enters the ischemic insult in a biologically primed rather than unchanged state. During early reperfusion, IPC was associated with relative attenuation of complement-, coagulation-, and remodeling-related proteomic signatures compared with non-preconditioned myocardium.

7.3 STUDY III

Study III extends the mechanistic framework of this thesis upstream to transcriptional and epigenetic regulation by demonstrating that IPC is associated with structured remodeling of the cardiac transcriptome together with selective regulation of DNA methylation. IPC was associated with reduced global DNA methyltransferase activity, yet this reduction was not accompanied by uniform hypomethylation. Instead, promoter-level analyses revealed a dynamic and gene-selective redistribution of methylation linked to genes involved in stress adaptation, inflammatory regulation, DNA repair, and transcriptional control. Although the broader causal hierarchy linking these epigenetic events to functional myocardial recovery remains to be fully clarified, the findings support the concept that IPC engages selective epigenetic mechanisms as part of its protective program.

7.4 STUDY IV

Study IV extends the central concepts of this thesis to a clinical setting by demonstrating that Takotsubo syndrome and ST-elevation myocardial infarction, despite overlapping acute clinical presentations, are associated with distinct temporal plasma proteomic trajectories. Whereas early molecular separation between the two conditions was limited, a more pronounced divergence emerged during the subacute phase, during which proteins related to cytokine signaling, innate immune activation, and receptor tyrosine kinase-mediated pathways were enriched in Takotsubo syndrome.

7.5 GENERAL CONCLUSION

Overall, this thesis suggests that ischemic preconditioning may not simply reduce myocardial injury; it may also actively shift the myocardial response toward a protected phenotype characterized by transient contractile suppression rather than irreversible structural damage. Across the experimental studies, IPC was associated with reduced infarct size, a greater tendency toward reversible post-ischemic dysfunction, rapid phosphoregulatory changes in sarcomeric and cytoskeletal proteins, and later proteomic and transcriptomic signatures consistent with attenuated inflammatory and remodeling responses. At the same time, the thesis does not establish that all observed molecular changes are causally upstream of protection, and some may instead reflect downstream consequences of reduced injury. Nevertheless, the overall pattern across phosphoproteomic, proteomic, transcriptomic, and epigenetic analyses is consistent with IPC inducing intra-cellular changes that shifts the post-ischemic myocardium away from necrosis and toward reversible dysfunction and recovery.

8. FUTURE DIRECTIONS

8.1 MECHANISTIC VALIDATION OF IPC-ASSOCIATED MOLECULAR SIGNATURES

An important next step will be to determine which molecular changes identified across the experimental studies are causally involved in cardioprotection and recovery, rather than simply associated with IPC exposure. In particular, candidate phosphorylation events, proteins, and epigenetic alterations should be prioritized for targeted validation using perturbation-based approaches. Such studies would help distinguish molecular correlates of protection from mechanisms that actively direct the myocardium toward reversible dysfunction and away from irreversible injury.

A major priority will also be to clarify how signaling events observed after IPC but before ischemia are translated into functional recovery during reperfusion. Extending sampling into additional early and intermediate reperfusion time points, while integrating phosphoproteomic, proteomic, transcriptomic, and epigenetic readouts, may help define the temporal sequence through which IPC establishes a recovery-compatible myocardial trajectory.

8.2 EXTRACELLULAR VESICLE-MEDIATED SIGNALING IN CARDIOPROTECTION

The findings of this thesis raise the possibility that part of the protective program induced by IPC may be mediated through extracellular vesicle-associated signaling. Future studies should therefore investigate whether extracellular vesicles (EVs) released before ischemia or during reperfusion carry proteins, phosphoregulated molecules, or regulatory nucleic acids that contribute to myocardial adaptation. Profiling EV cargo across the relevant experimental phases may help identify transferable signals linked to reversible dysfunction and functional recovery.

Such work could also provide insight into whether IPC-induced cardioprotection is mediated exclusively within the myocardium or is partly propagated through intercellular communication between cardiac, vascular, and immune compartments. Integrating EV profiling with tissue-based omics may therefore help establish a broader system-level understanding of protective signaling.

8.3 CELL CULTURE AND REDUCTIONIST EXPERIMENTAL MODELS

To strengthen mechanistic interpretation, future work should complement *in vivo* findings with reductionist experimental systems. Cardiomyocyte cultures, co-culture systems involving endothelial or immune cells, and engineered heart tissue models may provide tractable platforms for testing whether candidate signaling pathways directly influence contractile recovery, calcium handling, inflammatory activation, or cellular resistance to ischemia–reperfusion injury. These systems would also enable more controlled perturbation of selected pathways than is possible in whole-animal models.

In particular, applying EV fractions, candidate proteins, or pathway-specific interventions to cell-based models could help determine whether the molecular signatures identified in this thesis are functionally active drivers of protection or secondary reflections of a broader biological response. Such experiments would serve as an important bridge between descriptive multi-omics observations and causal mechanistic understanding.

8.4 SEX DIFFERENCES AND COMORBIDITY-ORIENTED VALIDATION

Because the experimental studies were performed in male rats, future research should determine whether the molecular and functional signatures identified in this thesis are preserved in females. This will be particularly important given the known influence of sex on cardiovascular biology and the clinical relevance of sex differences in syndromes characterized by reversible myocardial dysfunction.

It will also be important to examine whether the protective trajectories observed after IPC remain conserved in the presence of metabolic and cardiovascular comorbidities, such as diabetes, obesity, aging, or hypertension. Extending the current framework to such models would substantially improve translational relevance and may also reveal whether comorbidity alters the balance between reversible dysfunction and irreversible injury.

8.5 TRANSLATIONAL AND BIOMARKER-ORIENTED CLINICAL STUDIES

A central translational priority will be to determine whether the circulating molecular signatures identified in the clinical study can be reproduced in larger and more heterogeneous patient cohorts. Validation across broader populations, including both sexes and patients with different comorbidity profiles, will be necessary to establish the robustness and generalizability of the observed proteomic trajectories.

Beyond syndrome discrimination, future work should assess whether selected circulating proteins or protein panels have diagnostic, prognostic, or mechanistic value. In particular, biomarker-oriented studies should test whether temporal plasma or EV-associated signatures can predict ventricular recovery, persistence of dysfunction, recurrence, or adverse remodeling. Combining circulating omics with serial imaging and clinical outcomes may help identify biomarkers that reflect not only disease category, but also biological trajectory and recovery potential.

A further translational step will be to investigate whether patient-derived plasma or EV fractions exert functional effects in experimental cell systems. Exposure of cardiomyocytes, endothelial cells, or immune cells to circulating material collected at different clinical phases may help clarify whether the identified signatures are passive biomarkers or active contributors to disease evolution and recovery. In this way, future studies may bridge mechanistic insight, biomarker development, and clinical applicability.

USE OF GENERATIVE AI

Generative AI tools, including ChatGPT, were used to assist with grammar correction, language refinement, and the preparation of selected figures.

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