Cardiovascular Impacts of Stress and a Western Diet: Synergistic Effects of Lifestyle Risk Factors on Infarct Tolerance and Cardioprotection

Makayla Nicholas

BBiomedSc

School of Medical Science

Griffith University

Submitted in fulfilment of the requirements of the degree of

Master of Medical Research

January 2022

Griffith UNIVERSITY

I. Abstract

Introduction: Ischemic heart disease (IHD) is one of the leading causes of death from a non-communicable disease globally. Multimorbidity - the co-occurrence of two or more chronic conditions - is becoming highly prevalent in patients suffering IHD. Recently, mood and metabolic disorders have been found to share mechanistic pathways in disease progression, with mood disorders such as major depressive disorder (MDD) and chronic stress (CS), and metabolic disorders such as obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome (MetS) contributing to cardiovascular disease (CVD) risk and development, and worsening cardiovascular outcomes in IHD sufferers. The intertwining pathological mechanisms of how these metabolic and mood disorders promote cardiovascular disease (perhaps synergistically) is not well known and is currently under investigation. Emerging research suggests that subclinical levels of CS and consumption of a Western diet promote pathological impairments in cardiac infarct tolerance and may diminish the heart's innate protective signalling pathways. We further investigated these claims and examined the impacts of CS exposure and/or WD consumption on cardiometabolic risk factor development, mood impairment, and cardiac responses to classical pre-conditioning stimuli.

Methods: 64 male C57BL/6J 8-week-old mice were randomly allocated to one of four groups (control diet, CD; Western diet, WD; control diet + restraint stress, CD + RS; or a Western diet + restraint stress, WD + RS). Mice were fed either a CD (calorie content: 14% fat, 59% carbohydrates, and 19% protein) or a WD (57% carbohydrates, 32%, fat, and 11% from protein) for 17 weeks. At week 15, mice in RS groups were exposed to 14 days of two-hour daily RS in a clear Perspex restraint device. Behavioural analyses (open field test and sucrose preference test) were undertaken at baseline (week 0) and post-intervention (week 17) to determine if these interventions had an impact on markers of mood. Fasted circulating glucose was measured at baseline and post-intervention, with serum samples taken at these

times for subsequent assessment of fasted insulin, triglycerides, and cholesterol. After post-intervention behavioural assessment and blood collection, mice were anaesthetised and euthanised via heart excision. Serum samples were taken from the thoracic cavity immediately after heart removal for assessment of circulating markers. These hearts were used for immediate Langendorff perfusion experiments. Hearts were divided into two groups: one group that underwent ischemia and reperfusion (I/R), and one that was subjected to an ischaemic pre-conditioning (IPC) protocol (3x cycles of 5 minutes ischemia/5 minutes reperfusion) before exposure to I/R. Myocardial ventricular tissue was isolated and underwent analysis via ELISA and Western Blot assessment. Efflux of the two cell damage markers, lactate dehydrogenase (LDH) and cardiac troponin, was assessed.

Results and conclusions: The WD resulted in significant weight gain and development of insulin resistance, without changes in the lipid profile. RS alone did not have an impact on metabolic parameters. Mood disturbances were found in the mice exposed to RS, however the WD did not improve or worsen behaviour (in the absence or presence of RS). No differences were found in intrinsic cardiac function between the control or the intervention groups. The protective efficacy of IPC was reduced in hearts from WD *vs*. control diet animals. However, WD hearts were nonetheless able to be pre-conditioned, with improvements found in % recovery of left ventricular developed pressure (LVDP) and absolute recovery of end-diastolic pressure (EDP). Regardless of diet, chronic RS impaired or eliminated reperfusion function protective effects of IPC, an inhibitory effect associated with reduced AKT expression and a trend to increased GSK-3β phosphorylation (significant in WD+RS hearts). Thus, both a WD and chronic stress are detrimental to cardioprotection and kinase signalling, with these adverse effects of stress more pronounced than those of diet.

II. Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signed ……. Date 30/01/2022

III. Table of Contents

IV. List of Figures and Tables

Ordered as they appear in the thesis.

V. List of Abbreviations and Terms

ACE	Angiotensin converting enzyme
AGE	Advanced glycation end products
AMI	Acute myocardial infarction
AMPK	Adenosine activated monophosphate-activated protein kinase
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
CAD	Coronary artery disease
CMS	Chronic mild stress
CPMS	Chronic predictable mild stress
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CRS	Chronic restraint stress
CS	Chronic Stress
CVD	Cardiovascular disease
CVS	Cardiovascular system
EDP	End-diastolic pressure
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
GLUT4	Glucose transporter type 4
GPCR	G-protein coupled receptor
GSK-3β	Glycogen synthase kinase-3β
HDL	High density lipoprotein

HFD	High fat diet
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPA	Hypothalamic-pituitary-adrenal
I/R	Ischemia/reperfusion
ICAM-1	Intercellular adhesion molecule-1
IHD	Ischemic heart disease
IL-1	Interleukin-1
IL-6	Interleukin-6
IPC	Ischemic pre-conditioning
K_{ATP}	ATP sensitive potassium channel
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LVDP	Left ventricular developed pressure
MDD	Major depressive disorder
MDD-AF	Major depressive disorder with atypical features
MetS	Metabolic syndrome
MI	Myocardial infarction
mPTP	Mitochondrial permeability transition pore
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OFT	Open field test
PAI-1	Plasminogen activator inhibitor-1
PI3K	Phosphoinositide 3-kinase
PINE	Psycho-immune neuroendocrine
PKC	Protein kinase C
RAAS	Renin-angiotensin-aldosterone system

RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
RS	Restraint stress
SNS	Sympathetic nervous system
SPT	Sucrose preference test
STAT3	Signal transducer and activator of transcription 3
T2DM	Type 2 diabetes mellitus
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion protein 1
VLDL	Very low-density lipoprotein
WD	Western diet
WHO	World Health Organisation

VI. Acknowledgements

I would like to thank Associate Professor Joss Du Toit, Associate Professor John Headrick, and Associate Professor Jason Peart for their continual guidance and support throughout this project. Thank you for giving me this opportunity to undertake the Master of Medical Research program. Thank you, Joss, for the late nights and weekends you spent drafting my thesis. I am extremely grateful.

I would also like to thank Diabetes Australia for the grant that funded most of this project. I am very thankful for this rare opportunity.

I would also like to thank Brock Lyon, who conducted the animal study (including the behavioural tests and blood collection) alongside me.

Thank you to Tessa Helman for her assistance, support, and guidance during the animal study, for analysing the open field test results, and for taking the time to teach me lab protocols essential for the completion of this thesis.

I would also like to thank Joshua Ingles for dedicating his time and skills to performing the LDH assays during the Langendorff heart perfusion experiments, which were essential to this project.

I would also like to thank Trissha Ybanez for her time and assistance with cryo-homogenising and fractioning the heart tissue required for Western immunoblotting.

I want to express gratitude to Lauren Went for teaching me how to analyse Western blots and for her guidance on this topic.

I would also like to thank my fellow laboratory members, Kyle Hatton-Jones, Saba Naghipour, and Kai Robertson for sacrificing their time during the Langendorff perfusion experiments to ensure these heart perfusions and the tissue and serum collection ran smoothly.

1.0 Literature Review

1.1 Incidence of Metabolic and Mood Disorders and its Impact on CVD

Non-communicable diseases such as cardiovascular disease (CVD), metabolic syndrome (MetS), and mood disorders are the primary cause of premature death. Worldwide, 126 million people suffer from ischemic heart disease (IHD), with 9 million deaths resulting from this condition per year[1]. In 2021, one quarter of the population in high income countries had obesity[2]. MetS is estimated to impact a quarter of the global population, although it is difficult to determine its prevalence due to differing diagnostic criteria[3]. We are also currently facing a mental health epidemic in the developed world. 1 in 16 Australians are currently suffering from a depressive disorder, while 1 in 7 are suffering from anxiety[4]. More than half of the mental health diagnoses are made before an individual is 25 years old[5]. It is becoming increasingly common that patients experiencing long-term psychological stress or depression may also be overweight or obese, and that these psychological and metabolic disorders may act together to cause or worsen disease, such as IHD[6,7].

1.2 Multimorbidity in Contemporary Society

Multimorbidity is the co-occurrence of two or more chronic conditions, where no individual disease or disorder is given precedence over another in a person[8,9]. People with multimorbidities are difficult to treat and place a great burden on the healthcare system, with the incidence of multimorbidity increasing in older adults[9]. Those suffering multiple non-communicable chronic diseases typically pay more in healthcare than what each disease would cost individually[10]. Multimorbidity has become the 'new normal' in IHD, with ~2/3 of acute myocardial infarction (AMI) sufferers having at least two comorbid conditions[11]. Recent findings suggests that metabolic and mood disorders may act synergistically as they share mechanistic pathways and neuroendocrine and metabolic sequelae[12]. However, current research evidence is contradictory. How metabolic and mood disorders interact to promote CVD is poorly understood and should therefore be investigated. The CVD promoting effects

of and interactions between metabolic (eg. MetS) and mood disorders (depression, chronic stress) are considered below: these are two of the most common and strongly interlinked comorbidities in CVD[13,14].

1.3. Impacts of Dietary Obesity and Metabolic Disorder on CVD

1.3.1 Metabolic Syndrome

It is estimated that by 2025, 75% of Australian females and 83% of males aged 20 years and over will be overweight or obese[15]. Currently, 35% of Australians over the age of 18 have been diagnosed with metabolic syndrome[16]. Globally, 3% of children (aged 6-12 years old) and 5% of adolescents (aged 13-18 years old) suffered from MetS in 2020[17]. Metabolic syndrome (also known as syndrome X) encompasses a constellation of cardiovascular and diabetic risk factors including hyperglycaemia, high blood pressure (BP), dyslipidaemia, and abdominal obesity – all which typically manifest due to an imbalance in energy expenditure vs. intake, a sedentary lifestyle and/or genetics[3,18-20]. There are differing definitions for MetS - e.g., WHO requires type 2 diabetes mellitus (T2DM), insulin resistance or impaired glucose tolerance along with two other previously mentioned criteria (which can also include microalbuminuria). The Adult Treatment Panel III (most commonly used for MetS diagnosis) requires any three of the four formerly specified CVD risk factors be met for a positive diagnosis[3,21,22]. The pathological effects of MetS risk factors overlap, with one factor of MetS manifesting and promoting development of another and vis versa[23]. However, obesity typically develops first, followed by dyslipidaemia, insulin resistance, then hypertension. Primary treatment for MetS involves lifestyle changes, including daily physical exercise, weight loss, and a healthier diet (e.g., lower caloric diet consisting of lower levels of sugar and saturated fat, along with high fibre). However certain medications targeted at treating specific risk factors

such as statins and angiotensin converting enzyme (ACE) inhibitors are prescribed to some patients[20].

1.3.2 Mechanistic changes in MetS and its relation to CVD.

1.3.2.1 IHD and MetS:

The pathological cardiovascular impacts of obesity are thought to be due to the influences of the other MetS risk factors, with individuals who are not metabolically healthy found to have an increased risk of developing CVD - including IHD - irrespective of BMI category[18,24]. The pathological effects of obesity are thought to be caused by the insulin resistance promoted by obesity, rather than obesity itself[12]. However, overweight or obese individuals have a higher CVD risk compared to those of healthy weight, regardless of metabolic health[12,25,26]. IHD is a hallmark of MetS and obesity, with MetS increasing the risk of developing IHD threefold[27]. Each risk factor of the metabolic syndrome is individually linked to increased risk of CVDs. Both MI and IHD risk is increased through pathological changes in the endothelium, nervous system, and immune system. These mechanisms and their role in atherogenesis and IHD will be discussed.

Visceral obesity and insulin resistance are associated with endothelial dysfunction - and therefore atherosclerosis. These changes are due to elevated non-esterified fatty acids, impaired suppression of lipolysis in adipocytes, decreased adiponectin levels, systemic and local inflammation, increased e-selectin, leptin resistance, and abnormal leptin and ghrelin levels[28-32]. Acute increase in non-esterified fatty acids in obesity results in hyperinsulinemia by decreasing glucose uptake in tissues, while chronic increases act to promote lipotoxicity and pancreatic β-cell apoptosis, which can lead to cellular dysfunction and death, and result in T2DM[33-36]. A decrease in amino acids in the bloodstream occurs in the presence of chronically elevated non-esterified fatty acids, resulting in an inhibition in nitric oxide production due to

decreased circulating L-arginine which, along with endothelial nitric oxide synthase (eNOS), produces the vasodilator, nitric oxide[23,33].

Higher levels of activated hepatic lipase and cholesteryl ester transfer protein have been found in individuals with MetS, particularly in those with visceral obesity and insulin resistance. High levels of small dense low-density lipoprotein (LDL) particles in these syndromes are lipolysed by these proteins resulting in smaller, denser LDL particles. These particles can easily pass through the endothelial lining of the blood vessel wall, causing damage resulting in atherosclerosis, and are more susceptible to modification (i.e., by glycation or oxidation). In MetS, macrophages uptake more oxidised or acetylated small dense LDL primarily via scavenger receptors (e.g., CD-36 and SR-A receptors) compared to the LDL receptor, thereby causing foam cell formation[28,37,38].

Systemic and local adipocyte inflammation can occur in obesity and MetS due to release of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), VCAM-1, fibrinogen, and P-selectin. Release of these inflammatory markers increases risk of CVD and IHD occurrence. These inflammatory responses can promote progression to insulin resistance, atherosclerotic plaque formation and rupture, and MetS development in obese individuals[23,28,39,40]. IL-6 and TNF-α are released from adipocytes and induce the secretion of the inflammatory marker, CRP, in the liver, which has been seen to increase the CVD risk of males with MetS when elevated[39,41]. IL-6 has also been linked to increased coagulation and dyslipidaemia and plays an important role in linking endothelial dysfunction in visceral obesity to oxidative stress[39]. Atherosclerotic plaque formation may also be promoted by the reduction of adiponectin in obese and insulin resistant individuals since the adipokine has anti-inflammatory and anti-atherogenic properties[29,42,43].

Ghrelin has been found to be lower in individuals with obesity, insulin resistance and T2DM and exists in two forms: acylated ghrelin and unacylated ghrelin. Obese individuals

with MetS have a higher circulating acetylated:deacetylated ghrelin ratio despite having lower levels of unacetylated and total ghrelin compared to non-obese individuals with MetS[44,45]. Ghrelin is regarded as the 'hunger hormone,' being released as a signal to eat food, and may play a role in adipocyte production, lipid accumulation, glucose metabolism and insulin regulation[46-48]. Under normal conditions, ghrelin has also been shown to play a role in protecting the heart against myocardial ischemia/reperfusion (I/R) injury due to the high density of ghrelin receptors in the aorta and myocardium[49,50]. The involvement of ghrelin in protection against I/R has not been fully determined but could be linked to PKC activation[51]. Ghrelin is thought to play a role in atherogenesis in pathological states, such as MetS. Low circulating ghrelin levels potentially results in lower levels of nitric oxide production, impaired suppression of pro-inflammatory cytokine production (Il-6, TNFα), and increased mononuclear cell binding (ICAM-1, VCAM), thereby increasing risk of IHD development[52-54].

Hyperleptinemia is often seen in obese individuals with MetS[55-58]. The adipokine, leptin, reduces food intake and increases energy expenditure, reflecting an individual's adipose tissue storage[55]. Independent of insulin resistance, the occurrence of hyperleptinemia and leptin resistance in obese individuals may increase CVD risk by promoting atherogenesis. Potential mechanisms through which leptin promotes atherogenesis include promoting thrombosis, endothelial wall calcification, reduced arterial compliance, stimulating smooth muscle proliferation, hypertrophy, and migration, and increasing oxidative stress - therefore leading to arterial wall injury[28,59,60,61,62,63]. Obese individuals also exhibit leptin resistance in the hypothalamus, modifying the effect high leptin levels normally have on energy expenditure pathways and eating behaviour, eventually leading to the promotion of insulin insensitivity and the progression of obesity into MetS in these patients[59,64,65]. Leptin resistance therefore promotes hyperleptinemia and overindulgence of food.

1.3.2.2 Promotion of hypertension and sympathetic dysfunction in MetS, and its relation to CVD risk:

Hypertension is one of the key factors that increase IHD risk in MetS patients and is caused by a number of factors, including raised insulin levels, increased sympathetic nervous system activity, and renin-angiotensin-aldosterone system (RAAS) overactivity (increasing angiotensin II production). Patients with MetS and hypertension have a significantly higher risk of a CVD incidence, independent of conventional cardiovascular risk factors, with the risk of CVD increasing as the number of MetS risk factors increases[66]. In MetS, RAAS activity is increased due to increased IL-6 and TNF-α levels, enhanced renin production via SNS overactivation or via the macula densa sensing reduced levels of sodium in the circulation. This results in an increase in angiotensin II production, reducing plasma potassium levels and increasing plasma sodium levels, consequently leading to increased blood pressure due to water retention. Increased RAAS activity also promotes sympathetic nervous system (SNS) activation, which creates a positive feedback loop as the SNS stimulates renin release, further stimulating RAAS activity, which further promotes SNS activity. SNS overactivity can cause hypertension in individuals with MetS through systemic vasoconstriction, increasing vascular resistance through arteriole remodelling, and increasing sodium reabsorption from the renal tubule. SNS overactivation drives an increased release of noradrenaline from the adrenal glands resulting in vasoconstriction of arterioles, inducing increased blood pressure. Cardiac output is increased because of the direct effect of increased circulating noradrenaline on the heart. The increase in circulating non-esterified fatty acids seen in obesity is partially due to increased plasma noradrenaline, which promotes lipolysis in white adipose tissue.

Hypothalamic-pituitary-adrenal (HPA) dysregulation occurs in individuals with MetS, with increased cortisol levels – a risk factor for IHD - associated with hypertension, dyslipidaemia, and T2DM[67-70]. Other factors influencing the development of endothelial

dysfunction and associated high blood pressure include low adiponectin, increased levels of leptin and insulin, increased cytokines (TNF, IL-6), and leptin and insulin resistance[71]. Insulin promotes fluid retention via increasing sodium and water reabsorption in the proximal tubule. Since individuals with insulin resistance have higher levels of insulin than normal, this effect is exacerbated in MetS and untreated obese individuals with insulin resistance. Insulin also promotes endothelin-1 and its vasoconstrictive actions on the arterial wall, along with smooth muscle hypertrophy - increasing arterial wall thickness. However, it is important to note that while many papers have found an association between hypertension and insulin resistance, some have suggested that insulin resistance plays a role in sustaining hypertension, rather than being a cause of it[14]. Long-term elevated circulating leptin levels primarily increase blood pressure through promoting vasoconstriction through chronic adrenergic activation[72]. While reduced levels of ghrelin in the circulation (of which has vasodilatory effects) have also been found in those with hypertension.

1.3.3 Cardiac Remodelling, Heart Failure, and Tolerance to I/R in MetS:

MetS promotes cardiac remodelling and contractile dysfunction, leading to an increased risk of heart failure, atrial arrythmias, and cardiac arrest. Myocardial morphological changes arise in MetS due to numerous interacting factors, including hypertension, hyperlipidaemia, elevated levels of advanced glycation end products (AGEs), oxidative stress, cellular apoptosis, and impaired cardiovascular compliance[28,73,74]. Hyperglycaemia causes extracellular matrix modifications (leading to cardiac fibrosis) and impaired calcium handling. While insulin resistance and hyperinsulinemia stimulate an increase in circulating AGEs, oxidative stress, SNS dysregulation, and growth-promoting function, resulting in increased myocardial mass and reduced arterial compliance[75]. Glucose transport into myocytes is reduced due to impaired glucose transporter type 4 (GLUT4) translocation and reduced GLUT4 in insulin resistance[76].

LV dysfunction may be promoted in individuals with insulin resistant MetS due to impaired insulin signalling of AKT and FOXO1[77-79]. Fibrosis, reduced angiogenesis, apoptosis, ROS production and impaired mitochondrial function are promoted due to diminished insulin signalling in the heart[80-83]. Due to decreased glucose transport into the myocyte, FFAs are the primary source for energy production in insulin resistant individuals, impairing the heart's ability to adapt to increased energy demand. Due to the myocardial cellular changes that occur during insulin resistance (previously mentioned), cardiomyopathy and heart failure are promoted[84-86]. Decreased myocyte autophagy in patients with MetS stimulates cardiac apoptosis, with apoptotic markers present in early diabetic cardiomyopathy[87]. MetS has been found to be independently associated with worsening of diastolic dysfunction across several studies, with participants presenting with reduced early diastolic velocity and a higher early mitral inflow velocity to mitral annular early diastolic velocity (E/E') ratios - indicating impaired relaxation[14,88]. Development of diastolic dysfunction precedes systolic dysfunction in diabetic cardiomyopathy, with suggestions of myocardial apoptosis being a key factor in promoting the progression from diastolic to systolic dysfunction in this disease state[87].

It has been suggested that obesity is the likely driving force for several cardiac structural changes in MetS, including increased end diastolic volume, narrowing of aortic valves, reduced ejection fraction, and increased left ventricle and interventricular septum mass[89-91]. Heart damage may occur due to the accumulation of lipids in the myocardium, with cardiac lipotoxicity an established cause of left ventricular dilated cardiomyopathy in T2DM and obesity[92]. Obesity and cardiac hyperlipidaemia impair cardiac electrophysiological activity, likely through increased cardiac fibrosis impeding the electrical signal conduction between cardiac myocytes in these patients, leading to cardiac arrythmia[93-95]. Cardiac fibrosis in MetS patients may also be due to RAAS overactivation, oxidative stress, and increased AGEs.

Cardiac remodelling occurs at sub-clinical levels of obesity in Wistar rats fed a WD for 6 weeks[96]. The WD fed rats displayed diastolic dysfunction, biventricular cardiac remodelling, and larger subcutaneous and visceral adipose tissue deposits despite no significant weight gain. While these high-fat diet fed rats maintained their body weight, they presented with metabolic dysfunction, exhibiting hyperglycaemia, insulin resistance, and glucose intolerance[96]. Gonçalves, et al. [96] found increased levels of galectin-3 in non-obese WD fed animals, which is believed to play a role in promoting fibrosis, inflammation, and cardiac hypertrophy[96]. Increased collagen deposition, and cardiac remodelling mediators including TNF-α, IL-6, and chemotactic and proliferative agents were present, contributing to diastolic dysfunction[96]. These mice were also insulin resistant, which has been suggested to be the first indicator of myocardial changes in the heart, due to the impact insulin resistance has on endothelial dysfunction and fibrosis deposition[75]. Left ventricular diastolic dysfunction was evident in the animals with this pre-diabetic state[88].

In obesity/MetS, insulin resistance appears to the be primary mechanism through which myocardial ischemia/reperfusion (I/R) tolerance is impaired, typically through impairment of the reperfusion injury salvage kinases (RISK) pathway. Cardioprotective AKT and GSK-3β have been reported to display altered expression and phosphorylation of these proteins in obese, insulin resistant mice[97,98]. Isolated obesity has been found to be protective against I/R injury (the obesity paradox), but these effects are abolished in obese, insulin insensitive phenotypes[99].

Overall, dietary obesity, insulin resistance, and MetS have been found to have a significant impact on cardiovascular function, morphology, tolerance to I/R exposure, and heart failure progression. In addition to profoundly influencing risk of cardiovascular disease, these conditions may also impair the heart's ability to withstand disease associated damage and respond to potential protective interventions. These will be discussed later.

1.4 Stress, Major Depressive Disorder, and Ischemic Heart Disease

1.4.1 Major Depressive Disorder, Stress and CVD Risk

Chronic stress (CS) and associated mood disorders may be key determinants of CVD risk and outcomes. There is a strong association between stress, major depressive disorder (MDD), and CVD development. Individuals who have MDD, regardless of presence of pre-existing CVD, have an increased risk of suffering from IHD[100,101]. CS induced MDD has been reported to promote cardiac hypertrophy, hypertension, diastolic dysfunction, fibrosis, inflammation, and cardiac nitrosative and oxidative stress markers[102,103]. The INTERHEART study investigated the effect of CS on the cardiovascular system (CVS). This study found that chronic external stressors - including financial stress, stress at work or home, depression, and a sense of lack of control - increased the risk of AMIs to a quantitatively similar extent as typical risk factors (smoking, hypertension, hyperlipidaemia, and diabetes)[102]. Financial stress in particular was associated with increased IHD risk. The Jackson Heart study found that participants who experienced moderate to high financial stress were at increased risk of CHD compared to those that did not experience financial hardship, and that financial stress and CHD may be related through psychological/emotion-based pathways. The data also suggested that aside from financial hardship being a risk factor for IHD, it is also a significant risk factor for hypertension, diabetes, and depressive symptoms[104]. This again highlights the potential interactions among metabolic and mood disorders in the context of CVD.

1.4.2 Mechanisms Linking MDD to Cardiovascular Pathology/Abnormalities

1.4.2.1 Cardiac Cellular Abnormalities in MDD/CS

As discussed above, there is ample support for detrimental impacts of CS and MDD on the heart. However, the mechanistic basis of these outcomes remains to be defined, though several candidates are implicated. MDD and CS appear to impair all major cellular components of the

heart. Cardiac myocyte abnormalities include changes in the mitochondria, sarcoplasmic reticulum, and cell nuclei, along with cellular oedema, myofibrillar damage, and impaired myocyte contractility[105-107]. In addition, nitrosative and oxidative stress markers have been shown to be involved in the worsening of myocardial infarction due to CS exposure[103]. Decreased intracellular Ca^{2+} levels have been observed, which is thought to reflect decreased Ca^{2+} loading in the sarcoplasmic reticulum, likely contributing to cardiomyocyte contractile dysfunction observed in models of MDD[107]. Myocardial contractile dysfunction is also related to abnormal rates of myocyte apoptosis, as cardiomyocytes are not renewable[106]. A significant increase in myocyte apoptosis is observed in models of MDD induced by chronic mild stress (CMS), correlating with increased levels of pro-apoptotic Bax mRNA and protein expression, and decreased levels of anti-apoptotic Bcl-2 expression[106,108,109]. Interestingly, normotensive rats exposed to stress have a higher risk of post-ischemic arrythmias due to contractile dysfunction, while stressed hypertensive rats had improved contractile function and decreased risk of post-ischemic arrythmias. Additionally, myocardial ultrastructure was greatly changed in these normotensive rats post-CS, however hypertensive rodents exposed to stress did not display a great change in ultrastructure compared to non-stressed hypertensive rats[110]. This suggests that hypertension causes adaptive changes in the heart, resulting in it being able to better withstand the impacts of CS on cardiac tissue compared to normotensive rats.

1.4.2.2 Systemic dysregulation in CS/MDD and the promotion of CVD and I/R intolerance

Myocardial tolerance to ischemia has been found to be impaired under CS conditions[103,111,112]. Larger post-ischemic infarcts, worsened left ventricular developed pressure (LVDP) recovery, and arrythmias have been found post-I/R in studies on mice[110,113]. Sympathetic over-activity, vagal under-activity, HPA axis abnormalities, and changes in immune function and inflammation are four inter-connected systems that are altered with CMS

exposure and are thought to possibly cause dysregulation of cardioprotective pathways, resulting in the heart being more susceptible to I/R injury and infarction[13] (Figure 1). The HPA axis and sympathetic nerve activity are both involved in the innate stress response and are over-stimulated under conditions of CS[114]. Chronic stimulation of the HPA axis and sympatho-adrenomedullary pathway promotes development of IHD, metabolic dysfunction, and mental health problems[115,116]. CMS has also been shown to reduce heart rate variability and increase cardiovascular reactivity and heart rate as a result of increased sympathetic vs. decreased parasympathetic tone[117-119]. The sympathetic nervous system (SNS) releases catecholamines at the neuromuscular junction for a prolonged period under these conditions, which increases the risk of arrythmias. Long-term activation of the SNS in CS and MDD can increase reactive oxygen species and activate apoptotic pathways[114,120]. As previously discussed, excessive SNS activity will also drive hypertension and cardiac hypertrophy, impairing function and ischaemic tolerance[121]. Pro-death processes are also promoted, including altered Bcl-2 protein expression and increased NADPH oxidase activity, excessive nitric oxide production, and reactive nitric oxide species[122,123]. These are promoted by increased stimulation of β-adrenergic receptors, which interact with immune-inflammatory function to exacerbate these responses further[124,125].

Dysregulation of the immune-inflammatory response includes increased release of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) in models of CMS, promoting HPA axis overactivation and depressive behaviours, including social isolation, anhedonia, altered cognition, and drowsiness. Multiple studies confirm the presence of chronic low-grade inflammation in MDD, which is considered an important mechanism in promoting its development[126-128]. Interestingly, direct immunoinflammatory challenge (eg. LPS) induces a series of behavioural outcomes - termed 'sickness behaviour' - that mimic MDD and anxiety-like symptoms. These symptoms are diminished after anti-depressive treatment[127,129]. Inflammation will in turn impact the cardiovascular and other systems[130]. In addition, increased

release of macrophages in CS has been found to promote IHD and worsen myocardial ischemic recovery[131].

The HPA axis regulates the body's natural stress response and is overactivated during CS. Under normal conditions, the anterior hypothalamic release of corticotropin-releasing hormone (CRH) and pituitary secretion of the adrenocorticotropic hormone stimulate secretion of glucocorticoids (including cortisol) by the adrenal cortex. When cortisol levels rise in the bloodstream, this causes inactivation of the HPA axis through a negative feedback system, resulting in normal resolution[13,132]. Note that the stress response evolved as a short-term biological adaptation to acute environmental threats (eg. predation), enhancing physiological, metabolic, and behavioural preparedness[133]. However, with 'chronic' stress this normal feedback control response is impaired, resulting in abnormally prolonged increases in cortisol and downstream adrenal catecholamines[13]. This occurs as the negative feedback loop is disrupted by the progressive downregulation of the glucocorticoid receptors in response to CS, resulting in a continual release of CRH[132,134]. As stress can be defined as a combination of the type of external stimulus (both actual and perceived) combined with an individual's internal perception of that stimulus and behavioural outcomes, the threshold of where the stress becomes pathological varies with each individual[135,136]. Over-activation of the HPA axis in MDD and CS has been well researched and is thought to have both direct and indirect effects on the heart's structure (remodelling), function, and tolerance to ischemia.

Excess cortisol during HPA axis overactivation promotes inflammation, despite cortisol being an anti-inflammatory mediator under healthy conditions. Due to glucocorticoid receptor downregulation in CS, cortisol is unable to assert its anti-inflammatory effect on these receptors. The inflammatory mediators: cytokine IL-6, eicosanoids, platelet activating factor and serotonin appear particularly important in HPA axis activation under conditions of CS [13,137]. These inflammatory mediators are risk factors for metabolic dysregulation and IHD

development[138,139]. Glucocorticoids influence cytokine expression, increase cardiac output, elevate blood pressure, and promote CVS sensitivity to catecholamines (adrenaline, noradrenaline) through increasing arteriole β-adrenergic receptor binding affinity[13,140]. The RAAS system is upregulated by these catecholamines, resulting in a prothrombotic state and endothelial dysfunction. RAAS activation in this state in turn promotes activation of pro-inflammatory cytokines and adhesion of leukocytes – encouraging endothelial dysfunction. Furthermore, angiotensin II promotes oxidative stress, hypertension, shear stress, and atherosclerosis[115].

Overstimulation of the HPA axis under CS conditions promotes hyperglycaemia (via insulin inhibition and stimulation of lipolysis) and further advances insulin resistance via cortisol promotion of angiotensin II activity. The insulin resistance then further exacerbates hyperglycaemia and impairs myocardial ischemic tolerance[13,141-143]. Atherosclerotic plaque formation is promoted through HPA axis overactivation, through cortisol induced AMPK inhibition in heart tissue and activation in the liver, resulting in promotion of VLDL formation, in addition to cardiac lipotoxicity, oxidative stress, and insulin resistance[115]. Stimulation of the HPA axis also reduces secretion of gonadal hormones (limiting the protective cardiovascular effects of gonadocorticoids) and decreases production of IGF-1 and growth hormone, (which have a role in protecting myocardium from I/R injury and reduce CVD risk)[140,144,145]. It is clear that CS and MDD share mechanistic pathways in disease pathology with obesity, T2DM, and MetS. Chronic activation of these pathways further promotes mood and metabolic disease, while increasing CVD risk.

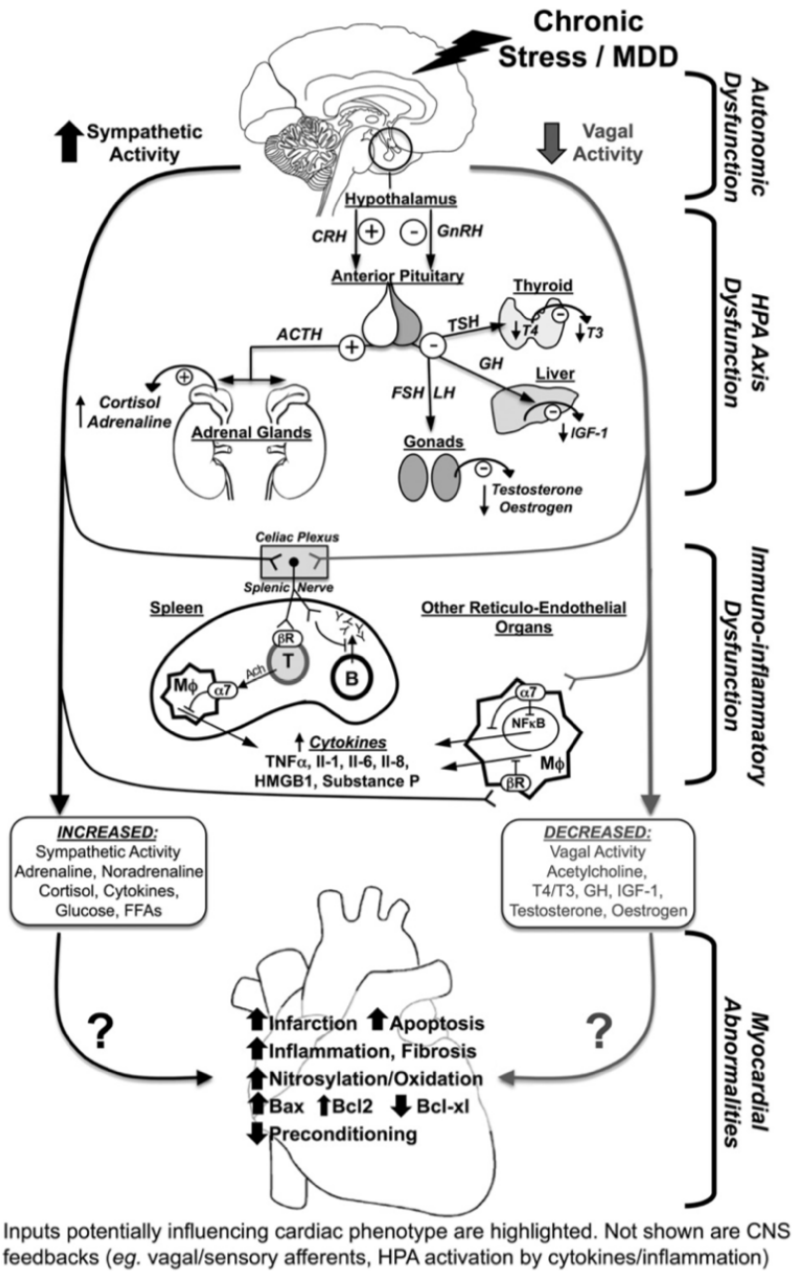

Figure 1: Potential signalling pathways between four inter-related systems impacted in MDD that govern cardiovascular stress phenotype[13].

1.5 Integration of System-Wide Mechanisms - the PINE Network:

The development of and relationships between major chronic diseases such as MDD, T2DM, MetS, and CVD can only be adequately understood from a holistic 'systems biology' perspective. Mood and metabolic disorders are prevalent in modern society, and frequently

comorbid with shared risk factors, co-occurrence, and bi-directional influences on disease outcomes pointing to more than mere association. These chronic conditions (together with associated CVD) may share important pathogenic mechanisms spanning regulatory networks and organ systems[7,116]. Obesity and MDD are strongly interlinked conditions. Weight loss is associated with the more prevalent or typical form of MDD; however, it is thought that obesity can promote the onset of typical MDD. The atypical MDD subtype - major depressive disorder with atypical features (MDD-AF) – specifically promotes an obese phenotype[7]. In an attempt to better understand not only MDD development but its relationship with other chronic diseases, an inter-related regulatory network of pathways has been proposed, including the varied paths already discussed above. This psycho-immune-neuroendocrine (PINE) network includes the CNS, immune, autonomic, gut, and endocrine networks. The PINE network provides a holistic systems biology view of disease development, and is proposed as existing in 3 states: the PINE physiome - considered the healthy state, the PINE pre-diseasome - the reversible unstable pre-disease state induced by exposure to CS (for example), and the PINE pathome - the stable but potentially irreversible disease state, resulting in MDD[146]. Transition from the physiome state to the pathome state is critical in disease development and may be heralded by the release of biomarkers or other changes specific to elements of this network[7,147]. In the PINE network model, it is proposed that MDD arises over time with exposure to different forms of CS, which causes dysregulation of normal PINE network pathways. As outlined in a recent paper, this may involve shifts from a healthy dominance of physiological negative feedback loops to a growing dominance of pathological positive feedbacks, pushing the entire system into a disease state[146,148]. Thus, MDD results when CS drives the system beyond a threshold over time marking the transition to the PINE pathome state[146].

Two proposed models for the development of typical MDD in the presence of pre-existing obesity have been forwarded[7]. The first suggests that CS and MDD share the same

metabolic, inflammatory, neuroendocrine, and gut-related pathways as obesity, resulting in pre-existing obesity promoting the pathways involved in MDD pathogenesis and thus influencing its development. The second proposed model (Figure 2) suggests that the psychosocial consequences of obesity, in particular body image perception, results in CS which causes exaggerated dysfunction of the PINE network into the PINE pathome state. This second model involves impairment of inflammatory pathways, the autonomic nervous system, levels of leptin and ghrelin (of which result in energy imbalance), and microbial imbalance[7]. While providing a systems biology perspective on MDD, metabolic disruption, and other disease development, how these psychological and physiological disorders interact specifically to impair cardioprotective responses and increase CVD risk factors is yet to be established. In particular, how highly prevalent sub-clinical stress and metabolic disturbance (e.g., WD) interact to influence the heart is unknown and a topic worthy of investigation.

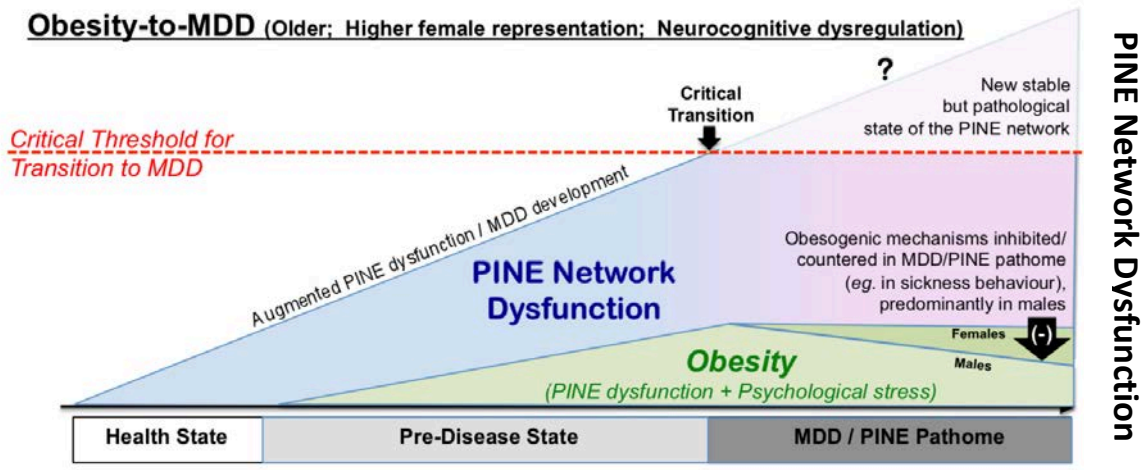

Figure 2: Proposed mechanisms of how obesity may cause MDD through PINE Network Dysfunction[7]

1.6 Subclinical Levels of Stress and a Western Diet – Unappreciated CVD Risks

Conflicting evidence surrounds the possible synergistic effects of CS and a WD on cardiometabolic outcomes and whether CS exacerbates the metabolic effects of a high fat diet. There is some evidence to suggest that stress-dependent reduction in feeding and thus body weight may attenuate cardiometabolic risk outcomes[6,149,150]. CS has also been found to both worsen and ameliorate metabolic dysfunction, in particular T2DM and insulin sensitivity[151,152]. Research has been conducted investigating the combined impact of a high fat diet (HFD) and chronic social/psychological stress in mice on the cardiovascular system[153,154]. Findings indicate that the combination of stress with a HFD results in systolic-diastolic dysfunction, interstitial collagen deposits, cardiomyocyte apoptosis, remodelling of coronary arterioles, and inflamed myocardium, without histological hallmarks of hypertrophy present[153]. One study suggested that the HFD may have been protective against common anxiety and depression symptoms induced by stress in mice, however another study in female rats found that the combination of a cafeteria diet and chronic stress showed higher anxiety levels and depressive behaviours[154,155]. Psychological stress was also suggested to act synergistically with obesity to alter both the levels of the neurotrophin brain-derived neurotrophic factor (BDNF) and function of its TrkB receptor, promoting a highly oxidative environment in the heart, together with left ventricle dysfunction and myocyte apoptosis[153]. However, little research has been conducted into the combined effects of sub-clinical, apparently benign levels of stress and a WD on the CVS. While a moderately elevated caloric intake and mild levels of stress may be relatively benign individually, emerging evidence indicates that when combined, they may have additive or synergistic impacts on cardiovascular health and infarct tolerance. Du Toit, et al. [12] recently investigated the impact of these commonly co-existing risk factors on myocardial infarct tolerance, metabolic homeostasis, and CVD risk. Levels of stress and a WD that did not independently impact the heart were discovered to synergistically worsen metabolic

homeostasis and mood, along with cardiac ischaemic tolerance. There was evidence of emerging depressive and pre-diabetic states in these mice, independent of weight gain, while ischemic intolerance was found to be independent of post-ischemic apoptosis and expression of pro-death proteins[12]. These findings are significant as they highlight the importance of investigating the combined effects of what may appear to be relatively benign levels of stress and a WD on different organ systems, in particular the heart, with these conditions widespread in modern societies, and multimorbidity increasingly the new normal in IHD.

1.7 Ischemia/reperfusion injury

In Australia, 440 people are hospitalised per day due to IHD[156]. Myocardial ischemic events, cardiac arrest, and heart surgery all lead to myocardial I/R injury, including arrythmias and cardiomyocyte death. Ischemia is classified as a period in which oxygen supply doesn't meet demand due to reduced or no blood flow to an area – resulting in ATP depletion and cell death[157]. Reperfusion is essential to prevent necrosis and irreversible tissue damage. However, it has been found to also cause injury to the ischemic tissue at its commencement due to pathological opening of the mitochondrial permeability transition pore (mPTP) at the beginning of reperfusion. Opening of this pore at reperfusion has been deemed one of the primary mechanisms through which cell death occurs during an episode of I/R, however the cardioprotective signalling processes that occur during both ischemia and reperfusion determine if the mPTP is opened[158-161]. Cardioprotective interventions that inhibit mPTP opening during reperfusion to reduce cell death have been thoroughly investigated in the past several decades.

1.8 Ischaemic Pre-conditioning

Protective interventions to reduce damage to the heart in IHD and with surgical ischemia (e.g., percutaneous coronary intervention, transplantation) - clinical cardioprotection - remains an elusive goal in cardiology. Most research and trials to date focus on so-called 'conditioning' responses. Pre-conditioning is an experimentally effective cardioprotective procedure that may be utilised to decrease myocardial susceptibility to I/R and reduce myocardial infarct size[162-164]. Pre-conditioning exerts its protective effect through the activation of pro-survival kinases in the PI3K/AKT RISK pathway via a pre-ischaemic stimulus. As a generalised overview of the pre-conditioning response (figure 3), release of G-protein coupled receptor (GPCR) agonists are stimulated via periodic non-injurious ischemia (thus 'conditioning'), which in turn inhibits pro-death or injury pathways (eg. GSK-3β signalling) and activates the pro-survival RISK pathway. This pro-survival pathway then acts on the mitochondrial permeability transition pore (mPTP) and ATP sensitive potassium (K_{ATP}) channels on the mitochondria, preserving their function and limiting the activation of death paths. Survival kinase AKT (also known as protein kinase B) is activated in the RISK pathway via the accumulation of adenosine, bradykinin etc, phosphorylating eNOS (among other targets), which in turn activates the PKG pathway (which targets the mitochondrial K_{ATP} channel and Na^+ proton exchanger), inhibits Bax, Bad and GSK-3β, and activates Bcl-2 and p70s6K (decreasing cell apoptosis), thus inhibiting opening of the mPTP and activation of cell death processes[165]. When activated, PKG may inhibit the opening of the mPTP through upregulating PKC activity (activating ERK 1/2 which then inhibits the activity of GSK-3β) and inducing the formation of NO. Activated PKG may also limit Ca^{2+} overload via interacting with phospholamban on the sarcoplasmic reticulum to limit Ca^{2+} uptake[165]. PKA activates eNOS increasing NO synthesis, reduces lactate dehydrogenase release, and increases cAMP phosphorylation[166]. A certain number of cycles of non-injurious ischemia/reperfusion are

required to exert a protective effect in the heart (due to GPCR agonists having an additive effect during these cycles). But too many or too few cycles (or cycles that are too short/long) can result in a diminished cardioprotective effect[167-170].

While highly effective in experimental models, ischemic conditioning has not translated well clinically and multiple trials have yielded disappointing outcomes, certainly in comparison to profound outcomes in animal models[165,171]. While few investigate the basis of this bench-to-bed disconnect, evidence suggests that common comorbid conditions in patients may impair RISK related signalling and interventions that target these pathways. These cardioprotective stimuli are rendered ineffective in the presence of pathological conditions, including obesity, T2DM, and depression[99,172,173]

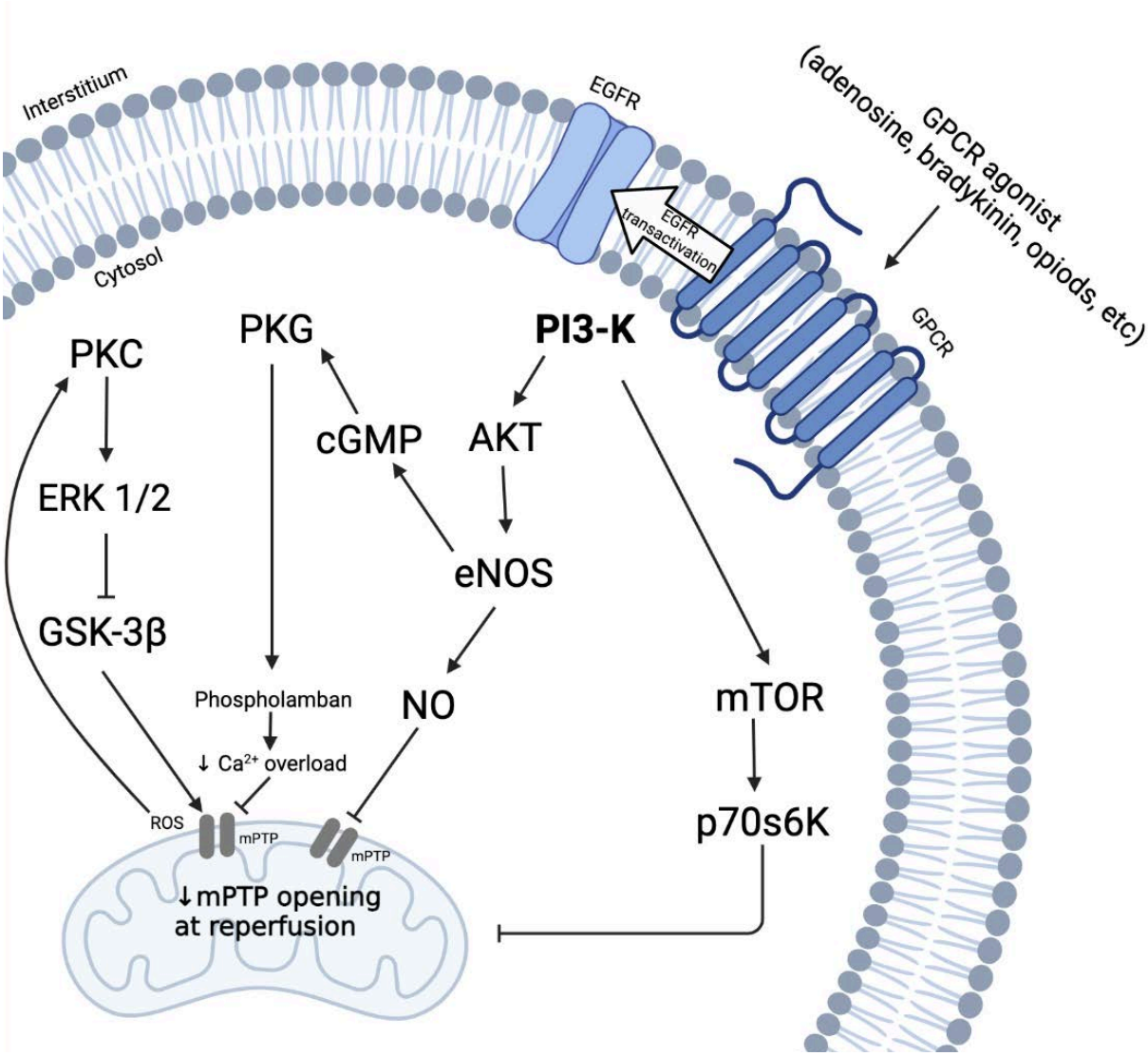

Figure 3: Generalised overview of cardioprotective signalling in response to conditioning stimuli.

1.8.1 Pre-conditioning responses in MetS

Limited research has been conducted on myocardial conditioning responses and MetS components, with much of the current research conducted having focussed on ischemic post-conditioning responses rather than pre-conditioning[174,175]. While much remains to be determined, one potential mechanism by which MetS abolishes post-conditioning responses is thought to involve impaired GSK-3β, as seen in mice with MetS (resulting in increased incidence of mPTP opening), impairment of phosphorylated AMPK to total AMPK ratio and

phosphorylated levels of ERK 1/2 and p70S6K1[174,175]. As post-conditioning shares similar pathways as pre-conditioning, it is suggested that these impairments may be present in the pre-conditioning pathway in MetS individuals.

Regarding obesity, smaller infarct size and higher levels of active AKT and eNOS, and inactive GSK-3β have been found in diet-induced obese rats compared to control, indicating improved cardioprotective response to ischemia and reperfusion[99,176]. Nonetheless, obese hearts could not be conditioned via ischemic pre-conditioning (IPC)[176]. Others investigating the efficacy of post-conditioning in an obese phenotype report decreased levels of phosphorylated AKT, p70S6K1, AMPK, ERK 1/2 and eNOS and failed attempt to reduce infarct size, supporting the findings that the obese myocardium cannot be conditioned[175].

Hyperlipidaemia has been found to independently diminish cardioprotective pre-conditioning stimuli and thereby worsen infarct tolerance. Reduction of ecto-5-nucleotidase activity (which plays a role in the production of adenosine in the IPC response), activation of metalloproteinases, and increased cardiac nitrosative stress (causing reduced nitric oxide bioavailability) have been suggested to contribute to this impaired protective response in hyperlipidaemia[157,177-179].

Numerous studies have reported diminished cardioprotective efficacy of IPC in T2DM, with diabetic hearts presenting with arrythmia, Ca^{2+} handling abnormalities, K^{+} efflux, increased Na^{+} accumulation, and increased infarct size when compared to control hearts[180-182]. IPC has also been shown to worsen myocardial ischemic tolerance compared to non-IPC diabetic hearts[182]. However, some findings suggest that increasing the intensity of the IPC stimulus in the diabetic myocardium may in fact result in reduced myocardial susceptibility to ischemia in T2DM hearts. This has been suggested to be linked to AKT phosphorylation levels[183]. Impaired humoral signalling has been proposed to be a potential cause of diminished cardioprotective efficacy of remote ischemic pre-conditioning in early T2DM, with depletion

of exosomes in the serum in T2DM mice found to be cytotoxic, worsening cardiac infarct size[184]. Insulin resistance, whether co-morbid with another MetS risk factor or an isolated phenotype, impacts cardiac ischemic tolerance and ischemic pre-conditioning, with reports of disrupted mitochondrial K^+ channel activation in this disease state[185]. This is consistent with IPC in T2DM findings, and highlights the importance of insulin insensitivity, suggesting that a pre-diabetic phenotype is just as susceptible to impaired myocardial protective mechanisms against I/R. How these cardioprotective processes and outcomes are influenced by commonly comorbid mood disorders, such as MDD, remains to determined.

1.8.2 Effects of CS and MDD on Cardioprotection

While there is growing evidence for worsened outcomes from IHD and ischemia with CS/MDD, much less is known about its effects on cardioprotection. How pre- and post-conditioning affect the heart in depressed individuals is currently not well researched. One study has provided evidence that depression diminishes the cardioprotective effect of post-conditioning in a rat model of depression, and this diminished capacity is likely due to impaired AKT and STAT3 activation at reperfusion[173]. However, this is currently the only study investigating the impact of CMS induced depression on myocardial pre-conditioning, and further studies investigating how both pre- and post-conditioning are affected by MDD and CS are needed to corroborate the findings of this single study and better investigate underlying mechanisms.

2.0 Aims and Hypotheses

The aim of this study is to investigate the individual and combined effects of a WD and low-level CS on cardiometabolic risk factors, mood, myocardial ischaemic tolerance, and the myocardial response to a classical pre-conditioning stimulus.

It is hypothesised that:

1. A WD will worsen cardiometabolic risk factors and myocardial ischemic tolerance.
2. CS will worsen cardiometabolic risk factors and mood.
3. The WD and chronic low-level stress will act synergistically to worsen cardiometabolic risk factors, mood, and myocardial ischemic tolerance.
4. The combination of a WD and exposure to mild stress will diminish the cardioprotective effects of a classical pre-conditioning stimulus.

3.0 Methods

3.1 Animal Model:

A total of 64 male C57Bl/6J 8-week-old mice were obtained from the Animal Resources Centre (ARC, Perth, Western Australia) in two batches of 32 mice per batch, one month apart. C57Bl/6J mice were chosen as they are a well-established mouse model for studies in both cardiovascular and mood disorders. Mice were randomly allocated into four experimental groups, with 4 mice per cage. These four groups were: 1) Control diet (CD), 2) WD, 3) CD + restraint stress (RS - 2-hour daily restraint per day for 14 days), and 4) WD + RS. Green Line GM500 cages separately ventilated in DGM racks (Techniplast S.p.A, Varese, Italy) were used to house the mice. Two Living World Eco Glass bottles (PETstock, Victoria, Australia) were attached to the hopper of each cage. Mice had *ad libitum* access to food and water and received daily standard care. Housing conditions for all mice included a 12-hour day cycle (lights on at 7am), in a (21±1°C) temperature and (40±2%) humidity-controlled room. Mice were weighed daily from day three of habituation for three months, then weekly from then on. Daily weighing resumed during the stress protocol for the mice in the CD + RS and WD + RS groups, and the post-intervention behavioural week for the CD and WD groups. Tail marking using a black sharpie (Officeworks, Gold Coast, Australia) was used to mark each mouse in a cage in order to differentiate between them. Mouse one had one line drawn proximal on the tail; mouse two had two lines drawn near the proximal of the tail; mouse three had three proximal lines; and mouse four had one line drawn distal on the tail. The animal model was approved by the Griffith University Animal Ethics Committee (MSC/02/20/AEC) and the study was undertaken within their guidelines, along with the *Australian code of practice for the care and use of animals for scientific purposes* to reduce the possibility of the mice undergoing unnecessary and preventable suffering.

Habituation

Mice were habituated for two weeks before baseline behavioural tests and experimental procedures commenced. During the first seven days of habituation, a Techniplast red mouse house (Techniplast S.p.A, Varese, Italy) was placed in each cage to reduce fighting between mice and was removed on day 8 of habituation for the remainder of the study. During the first two days of habituation, each animal cage was opened for five minutes, and the handlers' hand was placed into the cage in attempt to familiarise the mice with the handlers' voices and smell before daily handling commenced. Daily handling began on the third day of habituation, in which the mice were taken out of their cage (by allowing them to crawl onto the handler's hand to be picked up) for tail marking and weighing[186].

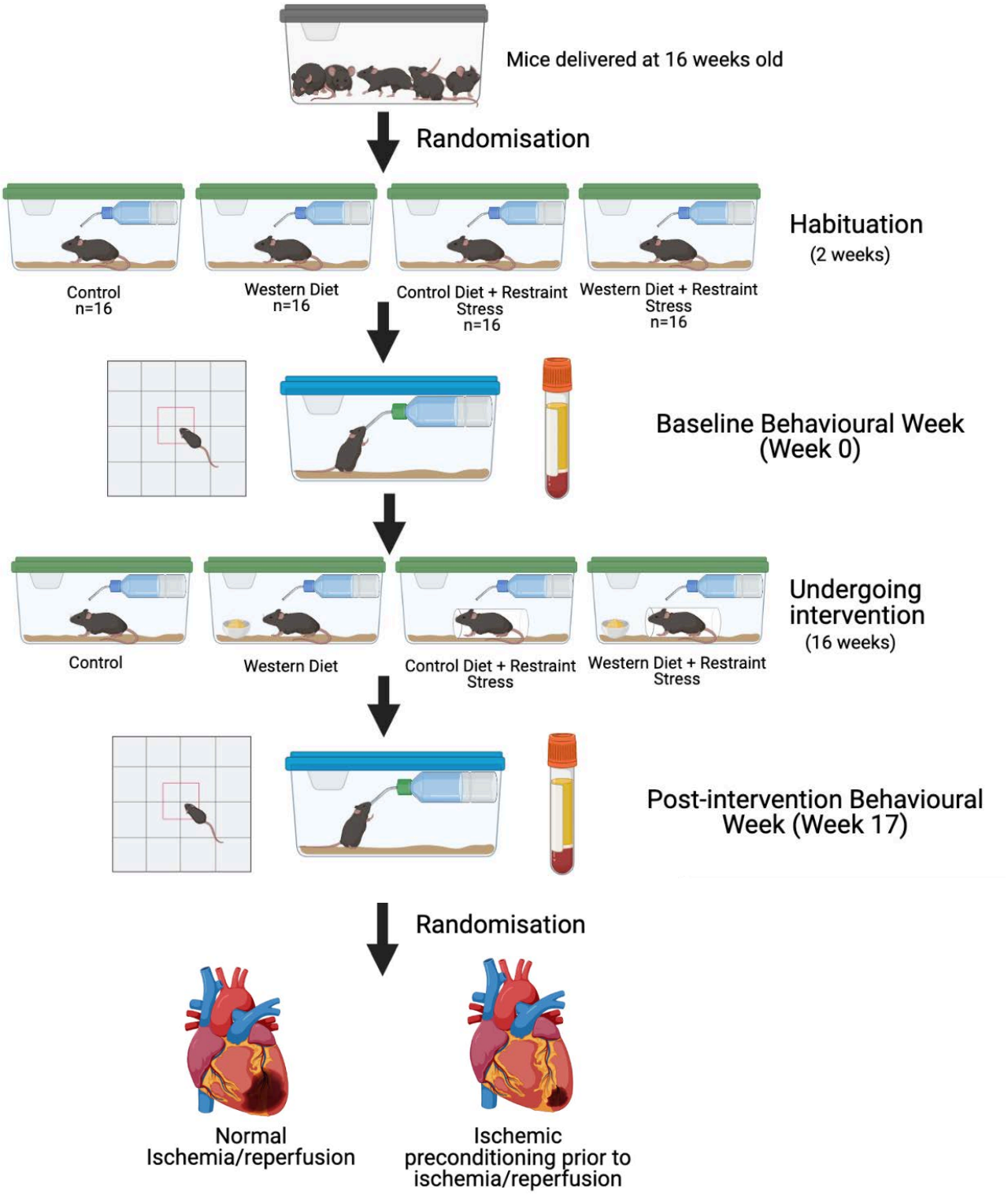

Figure 4: Timeline of the Entire Study.

3.2 Dietary Intervention:

CD and CD + RS animals were fed a pre-made standard laboratory chow diet (Irradiated Rat and Mouse Cubes, Specialty Feeds, Glen Forrest, Western Australia). The macronutrient content of the standard laboratory diet was 14% fat, 59% carbohydrates, and 19% protein (Specialty Feeds, Glen Forrest, Western Australia). The WD and WD + RS mice were fed a Western diet which derives 57% of its calories from carbohydrates, 32% from fat, and 11% from protein[12]. Mice were subjected to the WD for 16 weeks as this time induces obesity without the presence of overt T2DM. Mice had ad libitum access to food. Each WD group cage had approximately 40-50g of food put in the cage each day. Mice were fed fresh food between 10:30am and 12:30pm each day for the duration of the experiment, and their daily food consumption was recorded.

Western diet composition.

In a bowl or container, 250g solidified edible animal fat and oil (Supafry, Goodman Fielder Consumer Foods, Sydney, Australia) was melted and mixed with 280g white sugar (Coles Group, Victoria, Australia), 4 x 397g tins sweetened condensed milk (Coles Group, Victoria, Australia), and 1.6kg powdered standard laboratory chow (control diet) (Irradiated Rat and Mouse Cubes, Specialty Feeds, Glen Forrest, Western Australia) until a dough-like consistency was obtained. Mixture was rolled into balls, wrapped into baking paper and stored in an airtight container in the refrigerator (4°C). Fresh food was made fresh approximately every 10 days.

3.3 Chronic predictable mild stress protocol:

The chronic predictable mild stress (CPMS) protocol was introduced for the final 14 days of the study, which included the day the post-intervention open field test (OFT) was conducted[187]. The animals were subjected to the CPMS after the OFT to avoid it interfering/impacting with

the OFT results. Mice were exposed to the CPMS by confining them to ventilated clear Perspex restraint devices (3cm diameter and 10cm in length) for 2 hours daily (9:00am – 11:00am). These restraint devices were then individually placed in green line GM500 ventilated cage, which were then placed in a separate DGM rack to the one that the housing cages were in. These mice were shielded from the other mice in the animal facility by taping paper over the front of the cages. Four cages of mice were placed per row in the DGM rack (each row was one cage of mice next to each other).

3.4 Behavioural Tests:

Behavioural assessments were performed at baseline (prior to week 1 of study) and at post-intervention (week 17). Mice first underwent an open field test (OFT) before being subjected to the sucrose preference test (SPT). A recovery period of 24 hours was allowed between each behavioural test to prevent the possible psychological effects of the OFT from impacting the SPT outcomes.

3.4.1 Open Field Test (OFT) Procedure:

OFTs were conducted on the first day of the behavioural tests in a separate behavioural room to the animal housing room. These tests were conducted between 09:40am and 2pm. The test required an 80cm^2 Perspex area with 30cm high walls. The floor of the arena was marked in the centre with a 40cm^2 centre square and a peripheral area which encompassed the space between the walls of the arena and the centre square. Mice were habituated for a minimum of 30 minutes in the room prior to commencing the OFT. The OFT lasted 30 minutes, with the first 5 minutes of the video used as habituation, and the subsequent 5 minutes analysed to obtain the OFT results. Videos were analysed by EthoVision XT 14 (Nodulus Information Technology, Wageningen, Netherlands) which assessed the following behavioural parameters:

wall seeking behaviour, wall seeking duration (seconds), and distance travelled (cm). Filming begun prior to mice being placed in the centre square at the beginning of the test. Mice were left alone in the room for the duration to the test to avoid confounding factors. A 30-minute timer was then started once all persons had exited the room and the time each mouse went into the OFT was recorded. At the end of 30 minutes, filming was stopped, and the mice were taken out of the OFT boxes and placed in their housing cages. The number of times a mouse defecated and/or urinated in the box was also recorded. Before commencement of the test on the next animal, the floor and walls of the OFT boxes were sprayed and wiped twice with ethanol and paper towels. The boxes were left to air dry for five minutes before the next OFT began[188].

3.4.2 Sucrose Preference Test (SPT):

The SPT was performed in the same IVC rack and room the mice were housed in. Clean Green Line GM500 IVC cages were used for the SPT, with one mouse per cage. Part of the bedding from the original housing cage of each mouse was placed into their SPT cage in an attempt to reduce potential stress of moving mice to a new clean cage. Mice were habituated to the SPT bottles from the first day of habituation, two weeks before the baseline behavioural tests were conducted, and were used for the remainder of the study as normal water bottles. Mice had ad libitum access to water, 1% sucrose solution and food (standard chow for baseline tests, and either standard chow or a WD for post-intervention tests). These water and 1% sucrose solution bottles were placed in the cage hopper that holds the animal chow. Baseline water and sucrose solution consumption values were recorded for each bottle, and liquid consumed was measured at 24 and 48 hour intervals. During the first 24 hours, sucrose solution was placed on the right side of the cage, which was then swapped to the left side of the cage in the final 24 hours to reduce side preference bias. Mice were then returned to their housing cage at the conclusion of the SPT[189].

3.5 Fasted blood collection:

A recovery period of 24 hours was allowed between the SPT and blood collection to prevent psychological and physiological effects of the SPT impacting the blood sample values, along with the need to fast the mice for the tail tip bleeds. Mice were fasted 4 hours before blood sampling was initiated via tail tip bleeds. Blood collection was performed in a separate room to where the animals were housed (surgery room) between 8am and 11:30am. 80% ethanol was wiped over the tail to reduce risk of infection before Numit 5% cream (Ego pharmaceuticals, Melbourne, Victoria) was applied to the tail and left to exert its effect for 5 minutes while the tail sat under a warm lamp infrared light. Numit cream was wiped off the tail and a small portion was cut from the tail tip of the mice via a scalpel. Blood glucose levels were measured with an Accu-Check Performa glucose monitor and recorded. Blood for serum analysis was collected via massaging the tail to increase blood flow and placed into a 1.5mL Eppendorf tube. Once enough blood had been collected to yield approximately 100-150ul of serum, the mouse's tail was wiped with 80% ethanol and slight pressure was applied to the tail tip to reduce bleeding. Mice awaiting blood collection were kept outside the procedure room which was thoroughly cleaned with 80% ethanol before the next mouse was brought in. Blood samples were placed on ice for 30 minutes to an hour before being spun in a centrifuge at 2000 RPM for 10 minutes. Serum was separated using 100µL pipette and stored at -80°C.

3.6 Cardiometabolic risk profile:

3.6.1 Body weight:

Body weight was measured daily using an A&D Weighing GX-2000 precision scale (A&D Australasia Pty. Ltd, Adelaide, South Australia) from day three of habituation for four and a half months, then weekly for the rest of the study. Daily weighing resumed once the animals

were subjected to the stress protocol commenced for RS and WD + RS groups, or the post-intervention behavioural week began for the C and WD cages.

3.6.2 Circulating metabolic and inflammatory marker assessment:

Baseline and post-intervention fasted serum was collected via tail bleeds at weeks 0 and 17, as detailed above. Non-fasted blood was collected at animal sacrifice via a syringe from the thoracic cavity. Fasted serum samples were analysed for insulin, triglycerides, and cholesterol using enzyme-linked immunosorbent assays (ELISAs) following manufacturer's instructions (Ultra-Sensitive Mouse Insulin ELISA Kit, Crystal Chem, Illinois, USA; Triglyceride Quantification Colorimetric/Fluorometric Kit, BioVision, California, USA; QuickDetect™ Total cholesterol (Mouse) ELISA Kit, Biovision, California, USA). Non-fasted blood was analysed for leptin, ghrelin, noradrenaline, and melatonin via ELISAs following manufactures' instructions (Mouse Leptin, LEP ELISA kit, Cusabio, Houston, Texas), (Mouse ghrelin (GHRL) ELISA Kit, Cusabio, Houston, Texas), (Norepinephrine ELISA Kit #KA3836, Abnova, Taipei, Taiwan), (Melatonin ELISA Kit #E4630, Milpitas, California). Homeostatic model assessment of insulin resistance (HOMA-IR) values were calculated for each mouse via multiplying their glucose (mg/dL) and insulin (µU/ml) values together, then dividing this value by 405.

3.7 Animal Sacrifice:

Animals were transported to a separate laboratory for animal sacrifice and Langendorff perfusion experiments. Transport occurred an hour before sacrifice to acclimatise mice to the laboratory. A 60 mg kg^{-1} intraperitoneal injection of sodium pentobarbital (lethabarb) was given to the mice to anesthetise them. They were then weighed, and non-fasted blood glucose was measured via tail tipping and collecting a glucometer reading.

The pedal withdrawal reflex test was undertaken to assess level of anaesthesia before proceeding to pin down the mouse's limbs and excising the heart. Blood was collected from the thoracic cavity promptly after heart removal via a syringe, placed on ice for 1 hour, then spun at 2000 RPM for 10 minutes. Serum was immediately frozen at -80°C. Immediately following heart excision, the brain was removed and sectioned with surgical scissors and tweezers, followed closely by the liver, pancreas, white adipose tissue, colon, and faeces.

3.8 Langendorff Heart Perfusion model:

A Langendorff heart perfusion model was used to assess cardiac function and tolerance to ischemia/reperfusion. This perfusion method has been successful in producing myocardial cell death and contractile function loss and is an accurate method of cardiovascular function assessment[190,191].

After heart excision, hearts were quickly placed onto a Krebs buffer ice block, then placed into the Langendorff perfusion machine via cannulating the aorta to perfuse the coronary circulation. Modified Krebs-Henseleit buffer, gassed with 95% O_2-5% CO_2 was circulated through the coronary system. This buffer contained 119mM NaCl, 11mM glucose, 22mM $NaHCO_3$, 4.7mM KCl, 1.2mM $MgCl_2$, 1.2mM KH_2PO_4, 1.2mM EDTA, and 2.5mM $CaCl_2$, and was kept at a temperature of 37°C. A polyvinyl chloride film balloon was filled with fluid and placed into the left ventricle via making an incision in the atria. Ventricular function was assessed by inflating the balloon to an end-diastolic pressure (EDP) of 5mmHg. The hearts were then placed into a jacketed bath of perfusate at 37°C. A thermal probe connected to a Physitemp TH-8 digital thermometer (Physitemp Instruments Inc., Clifton, NJ, USA) was used to monitor the temperature of the perfusate throughout the perfusions. An ultrasonic flow probe was placed proximal to the aortic cannula and connected to a T206 flowmeter (Transonic Systems Inc., Ithaca, NY, USA) to measure coronary flow. Systolic

pressure, coronary flow, heart rate, EDP, +d*P*/d*t* and -d*P*/d*t* were processed by a four-channel MacLab system (AD instruments Pty Ltd, Castle Hill, Australia) connected to an Apple iMac. Coronary effluent was collected at 5, 10, 15, and 45 minutes of reperfusion, measured in a 200mL measuring cylinder and snap frozen. Coronary effluent was used to measure troponin at 5 and 10 minutes via ELISA following manufacturer's instructions (ultra-sensitive mouse cardiac troponin-I ELISA, Life Diagnostics, Pennsylvania, USA). Before Langendorff perfusions, hearts were randomly divided into two groups: one in which hearts were exposed to IPC and one in which they were not exposed to IPC.

Non-IPC protocol:

Baseline function of each heart was monitored over 10 minutes during stabilisation. This allowed hearts with abnormal baseline function (low or high left LVDP and/or coronary flow) to be excluded[190]. Hearts were then subjected to 35 minutes of pacing using a SD9 stimulator (Grass Instruments, Quincy, MA, ISA) at 420 beats min^{-1} to assess baseline function before being exposed to 25 minutes of normothermic global ischemia by turning off aortic perfusion to stop flow of perfusate to the heart. Following ischemia, 45 minutes of aerobic reperfusion was started by restarting aortic flow of perfusate. Function was assessed at 5 minute intervals during the 45 minutes reperfusion.

IPC Protocol:

The protocol for baseline function and pacing was the same as for the non-IPC studies. However, after pacing and assessment of basal function, these hearts were exposed to three cycles of 5 minutes ischemia/5 minutes reperfusion as a pre-conditioning stimulus before undergoing 25 minutes of normothermic global ischemia and 45 minutes of aerobic reperfusion.

3.9 Lactate Dehydrogenase (LDH) Assay Protocol:

LDH release into coronary effluent was measured at 5, 10 and 15 minutes of reperfusion as a marker of cell death. Before the coronary effluent was snap frozen, a sample was taken for LDH measurement. LDH solution and BSA were used as reference values for the LDH assay. LDH solution, BSA, and coronary effluent were pipetted into individual tubes filled with NADH working solution and pyruvate, and scanned at 340nm on a Tecan Sunrise Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland) heated to 37°C.

3.10 Tissue Fractionation:

Tissue fractionation of the left ventricle was conducted via cryo-homogenisation. The left ventricle was put into a mortar filled with liquid nitrogen and homogenised with a pestle. The heart tissue was divided into RNA, Western blotting, and ELISA protein in a 1:2:1 ratio respectively. Homogenisation solution (Maxwell® RSC simply RNA Tissue Kit, Promega, Wisconsin, United States) was mixed with a protein sample designated for RNA sequencing and analysis, and snap frozen. Lysis buffer (97% cell lysis buffer, Bio-Plex® Cell Lysis Kit #171304011, Bio-Rad, Australia; 2% Bimake Phosphatase Inhibitor Cocktail, Sapphire Biosciences, NSW, Australia; 1% Bimake Protease Inhibitor Cocktail (EDTA-Free, 100X in DMSO), Sapphire Biosciences, NSW, Australia) was vortexed with protein allocated for analysis via ELISA kits. A bicinchoninic acid (BCA) sample was made and both the BCA sample and the ELISA protein were snap frozen and stored at -80°C. Homogenate for Western blotting was mixed with mitochondrial isolation buffer (70mM sucrose, 190mM mannitol, 20mM HEPES, 0.2mM EDTA, 1mM PMFS, 10µM leupeptin, 2mM benzamidine, 5µM, 1mM NaO). A sample of this was taken for whole cell lysate, and put into a tube with lysis buffer and stored at -80°C. The remainder of the homogenate was spun in a centrifuge at 600g for 10

minutes at 4°C, from which the nuclear fraction was extracted from the pellet and mixed with lysis buffer. The supernatant was centrifuged again with lysis buffer at 10 000g for 30 minutes at 4°C to obtain the mitochondrial fraction from the pellet. The supernatant was extracted and spun in an ultracentrifuge at 100 000g for 90 minutes at 4°C. The supernatant was extracted and put into a tube for the cytosolic fraction, and the pellet was stored with lysis buffer for the membrane fraction. A sample of the nuclear, mitochondrial, cytosolic, and membrane fractions were taken to make BCA samples for protein quantification. Samples were stored at -80°C.

3.11 BCA Protein Quantification:

Following cryo-homogenisation of heart tissue, BCA protein assays were performed on the cytosol, membrane, and ELISA homogenate following the manufacturer's instructions using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Massachusetts, United States) to determine the amount of protein in each sample. Absorbance was measured at 540nm on a Tecan Sunrise Microplate Reader. BCA protein assays allowed the determination of the amount of protein in each sample, which was then used to calculate the amount of sample and kinexus buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM β-glycerophosphate, 20 mM NaPP) on Microsoft Office Excel (Microsoft Corporation, Washington, USA) required in each aliquot. Aliquots were then made with 17.5µg/µL protein with kinexus buffer for Western immunoblots, and 44ug protein with kinexus for ELISAs and stored at -80°C.

3.12 Western Immunoblotting:

10% acrylamide gels were hand cast each morning. Samples were thawed, mixed with loading dye, and placed onto a heat block for 5 minutes at 100°C. 5µL of protein ladder (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, ThermoFisher Scientific, Waltham,

Massachusetts) was loaded into the second well of each gel, and 17.5µL of protein was loaded into wells 4-15. Gels were placed into an electrophoresis tank with running buffer and ran for 10 minutes at 50V, then 2 hours at 100V. Transfer of proteins was achieved via the BioRad Transblot Turbo Machine. Transfer buffer was placed in the cassette, with blotting and polyvinylidene difluoride fluorescent membrane paper. Gels were cut and rolled onto membrane paper. Blotting paper was then rolled on top of the gel. Transfer of proteins to membrane occurred at 20V for 20 minutes. Transferred proteins on membrane were agitated in 1x TBS for 5 minutes before blocking in Odyssey blocking buffer (LI-COR Biosciences, Nebraska, USA) for 1 hour 30 minutes at room temperature. After blocking, membranes were then incubated at 4°C with gentle agitation for 16-18 hours in the primary antibody (AKT 1:1000 dilution (9272, Cell Signaling); pAKT 1:1000 (4060, Cell Signaling), ERK 1:1000, pERK 1:1000 (9101, Cell Signaling); GSK-3β 1:500 (9315, Cell Signaling); pGSK-3β 1:500 (9336, Cell Signaling); GAPDH 1:50,000 (sc-32233, Santa Cruz). After incubation, the membrane was washed four times in 1x TBST (5 minutes each) and once in 1x TBS (5 minutes) before incubation at room temperature for 1 hour in the dark with the secondary antibodies (IRDye® 680RD goat anti-rabbit 1:30,000 (925-68071, LI-COR) and IRDye® 680RD donkey anti-mouse 1:30,000 (925-68072, LI-COR)). The PVDF membrane was then washed again four times in 1x TBST (5 minutes each) and once in 1x TBS (5 minutes) before being left to dry on Kim Wipes in the dark for 15 minutes. Proteins were then imaged on a Licor Odyssey Infrared Imaging System (Millenium Science, Mulgrave, Australia) and normalised to GAPDH during analysis.

3.13 Inflammatory Markers

Cytokine analysis was undertaken on blood serum (IL-6) and heart tissue (IL-6, TNF-α) via ELISA following manufacturer's instructions (IL-6 (mouse) AlphaLISA Detection Kit, 500

Assay Points, PerkinElmer, Waltham, Massachusetts), (TNF-α (mouse) AlphaLISA Detection Kit, 500 Assay Points, PerkinElmer, Waltham, Massachusetts).

3.14 Statistical analysis

Statistical analysis was conducted on GraphPad Prism, Version 9. A two-way ANOVA was used to compare pre-intervention and post-intervention, as well as non-IPC vs IPC hearts. A one-way ANOVA was used to analyse the four groups at one timepoint. The Holm-Šídák post-hoc test was used for all ANOVAs, with an alpha value of 0.05.

4.0 Results

Summary of Key Findings:

Cardiometabolic profile of animals subjected to the WD or chronic restraint stress (CRS) or their combination: Body weight and food consumption was increased in the WD fed groups from week 12. Once the RS protocol was introduced both RS groups showed a decrease in body weight, consistent with biological stress. Changes in fasted insulin, glucose, and HOMA-IR values between groups were also seen, while no differences were found in blood lipid levels.

Behavioural changes induced by the WD or CRS or their combination: Sucrose preference was decreased in the WD fed groups, while wall seeking behaviour and distance travelled in the OFT was increased.

Cardiac function and post-ischaemic outcome of animals subjected to the WD or CRS or their combination: ***Non-IPC hearts*** **-** There were no significant differences in LVDP recovery (%) between the four groups after reperfusion. ***IPC hearts*** - LVDP recovery (%) was significantly higher and EDP reduced in the CD and WD IPC groups.

4.1. Weekly Animal Body Weight and Food Intake

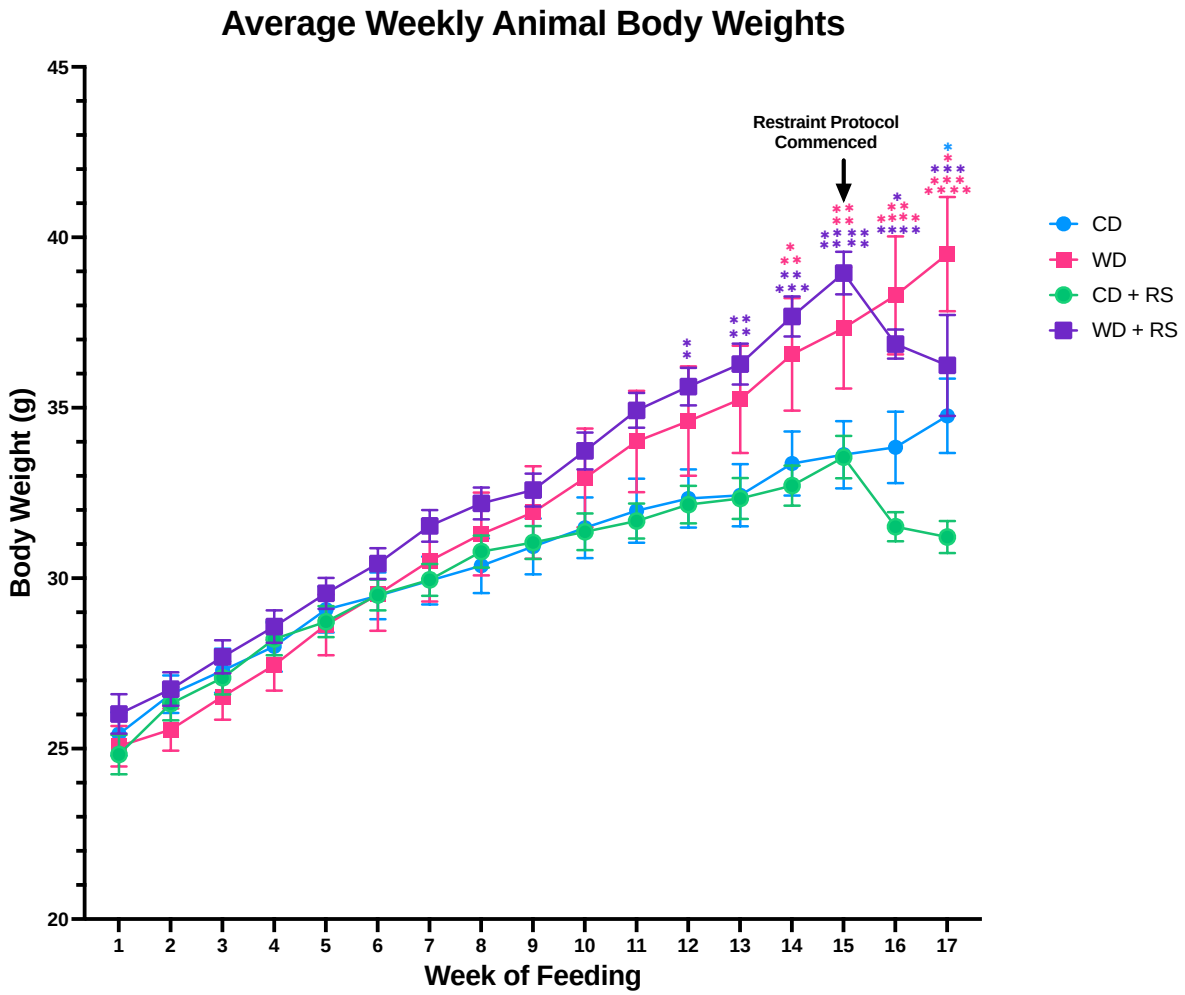

Figure 5: Average Weekly Body Weights Over the 17 Week Study Period. *Week 12*, *p < 0.05 vs CD, *p < 0.05 vs CD+RS. *Week 13*, **p < 0.01 vs CD, **p < 0.01 vs CD+RS. *Week 14*, **p < 0.01 vs CD, ***p < 0.001 vs CD+RS, **p < 0.01 vs CD+RS, *p < 0.05 vs CD. *Week 15*, ****p < 0.0001 vs CD, ****p < 0.0001 vs CD+RS, **p < 0.01 vs CD+RS, **p < 0.01 vs CD. *Week 16*, ****p < 0.0001 vs CD+RS, ****p < 0.0001 vs CD+RS, **p < 0.01 vs CD, *p < 0.05 vs CD. *Week 17*, ****p < 0.0001 vs CD+RS, ***p < 0.001 vs CD, ***p < 0.001 vs CD+RS, *p < 0.05 vs WD+RS, *p < 0.05 CD+RS. Data presented as mean±SEM. n = 16.

The WD caused significant body weight gains compared to the CD, with the diet resulting in significant differences in body weight from week 12 of the study onwards ($p < 0.05$) (figure 5). Introduction of the RS protocol in week 15 resulted in a significant loss in body weight in both the WD and CD groups without a recovery in weight by the end of the study.

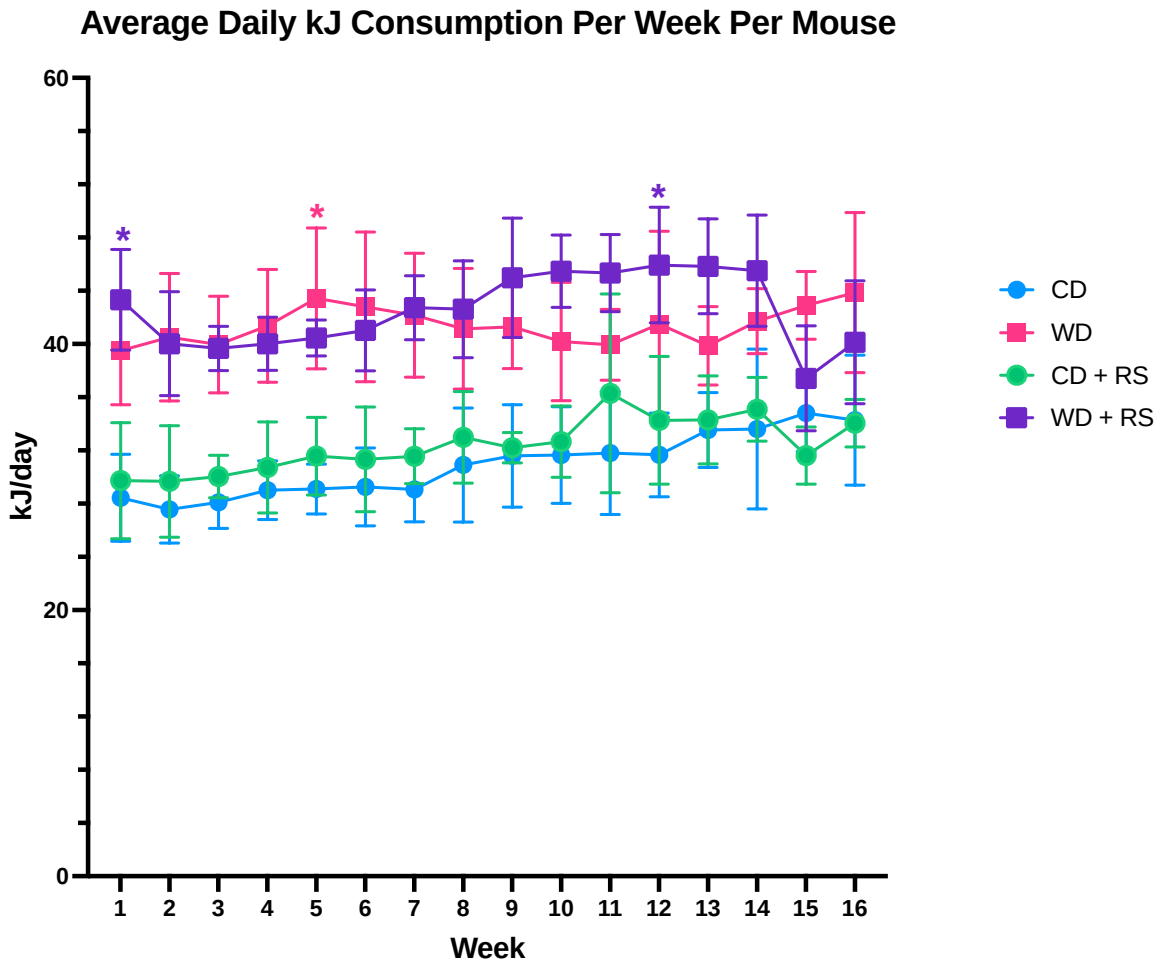

Figure 6: Average Daily kJ Intake Per Week Per Mouse. *Week 1*, *p < 0.05 vs CD. *Week 5*, *p < 0.05 vs CD. *Week 12*, *p < 0.05 vs CD. Results presented as mean±SEM. n = 16.

The average daily kilojoule (kJ) intake per mouse for each week is presented in figure 6. The WD + RS mice had a significantly higher ($p < 0.05$) daily energy consumption in weeks 1 and 12 compared to the CD group, as did the WD group in week 5 ($p < 0.05$) when compared to CD mice. An insignificant drop in kJ consumption was seen in both RS groups at the commencement of restraint stress in week 15, which correlates with a decrease in body weight. However, while body weight did not recover, caloric intake began increasing in week 16.

4.2 Behavioural Tests

Sucrose Preference Test:

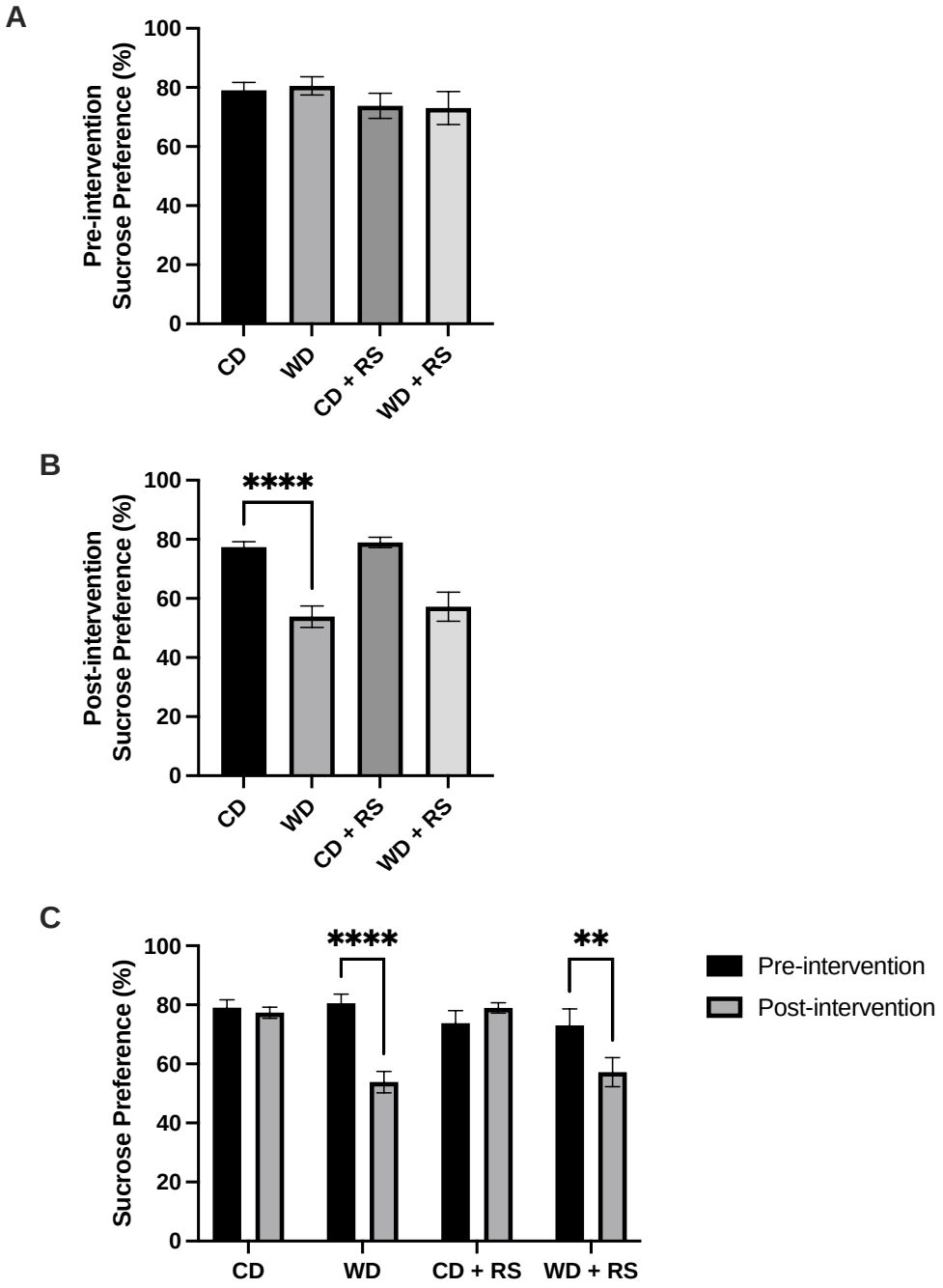

Figure 7: Sucrose Preference Test. A) Pre-intervention Sucrose Preference Test. **B)** Post-intervention Sucrose Preference Test. ****p < 0.0001. **C)** Pre-intervention vs Post-intervention Sucrose Preference Test. **p < 0.01, ****p < 0.0001. Data presented as mean ± SEM. n = 16. Pre-intervention week started at week 0. Post-intervention week commenced at week 17 of study.

There were no significant differences in sucrose preference between groups pre-intervention (before diet and stress introduction) as determined by the sucrose preference test (SPT) (figure

7). Post-intervention, the WD group had significantly lower sucrose consumption than the CD group. When comparing pre- vs post-intervention sucrose preference (figure 7C), both WD ($p < 0.001$) and WD + RS ($p < 0.01$) groups had significantly reduced sucrose consumption at the post-intervention test compared to pre-intervention.

Open Field Test Outcomes:

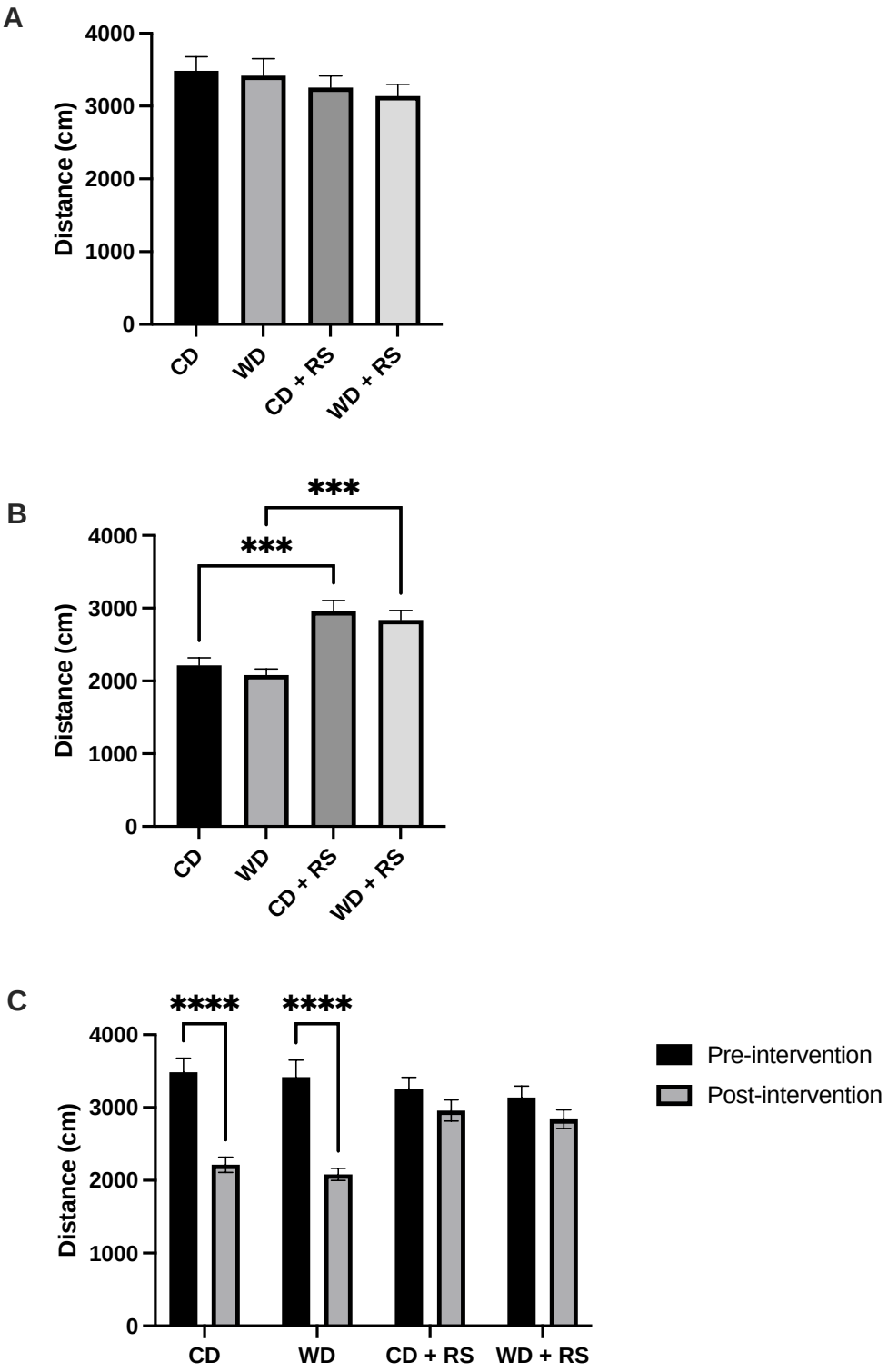

Figure 8: Distance Travelled in the Open Field Test. A) Distance travelled pre-intervention. **B)** Distance travelled post-intervention. ***p < 0.001. **C)** Distance travelled in pre- vs post-intervention. ****p < 0.0001. Results presented at mean±SEM. n = 16. Pre-intervention week started at week 0. Post-intervention week commenced at week 17 of study.

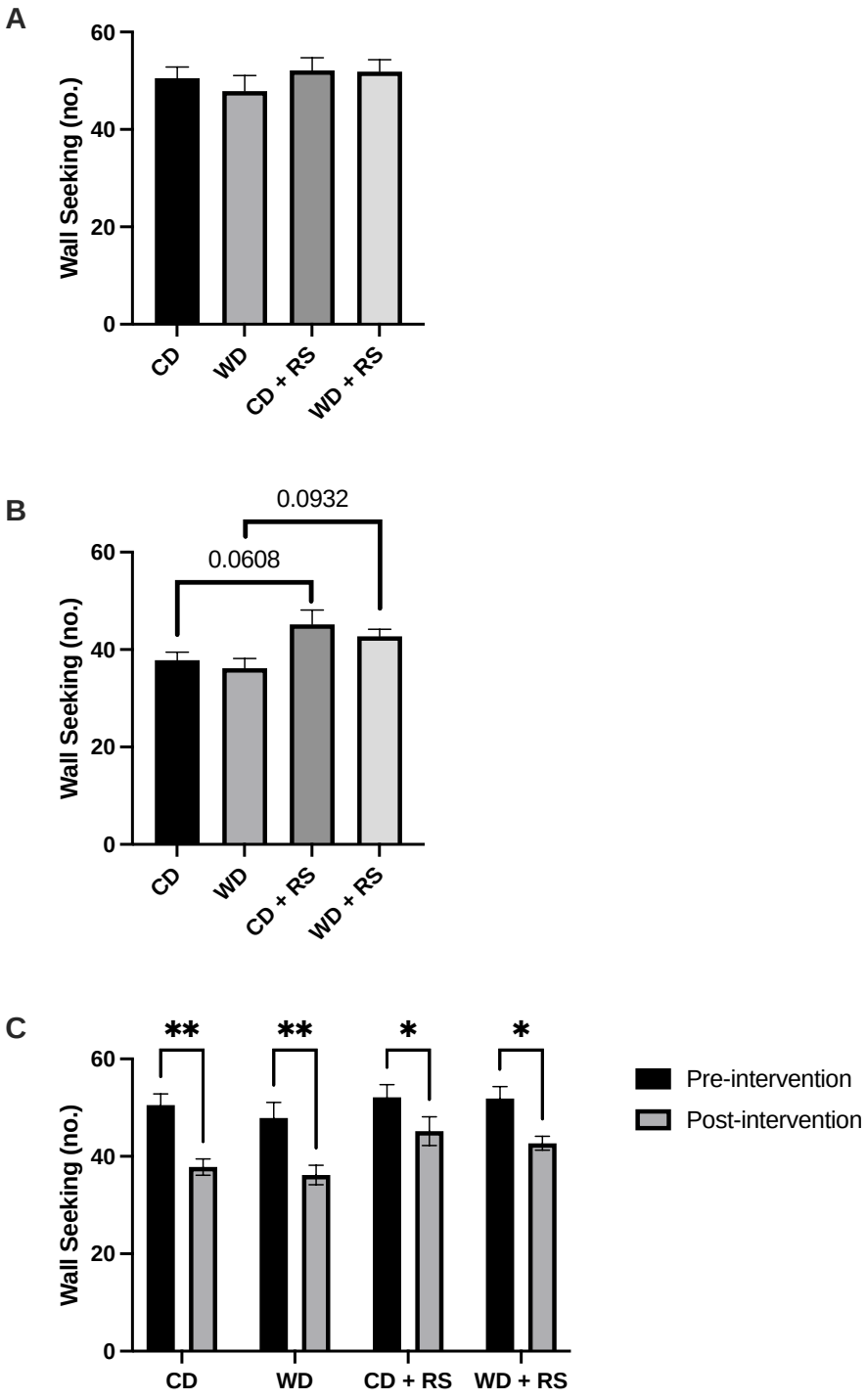

Figure 9: Frequency of Wall-Seeking Behaviour during the Open Field Test. A) Wall seeking behaviour (number) at pre-intervention. **B)** Wall seeking behaviour (number) at post-intervention. **C)** Wall seeking behaviour (number) at pre- vs post-intervention. *p < 0.05, **0.01. Results presented at mean±SEM. n = 16. Pre-intervention week started at week 0. Post-intervention week commenced at week 17 of study.

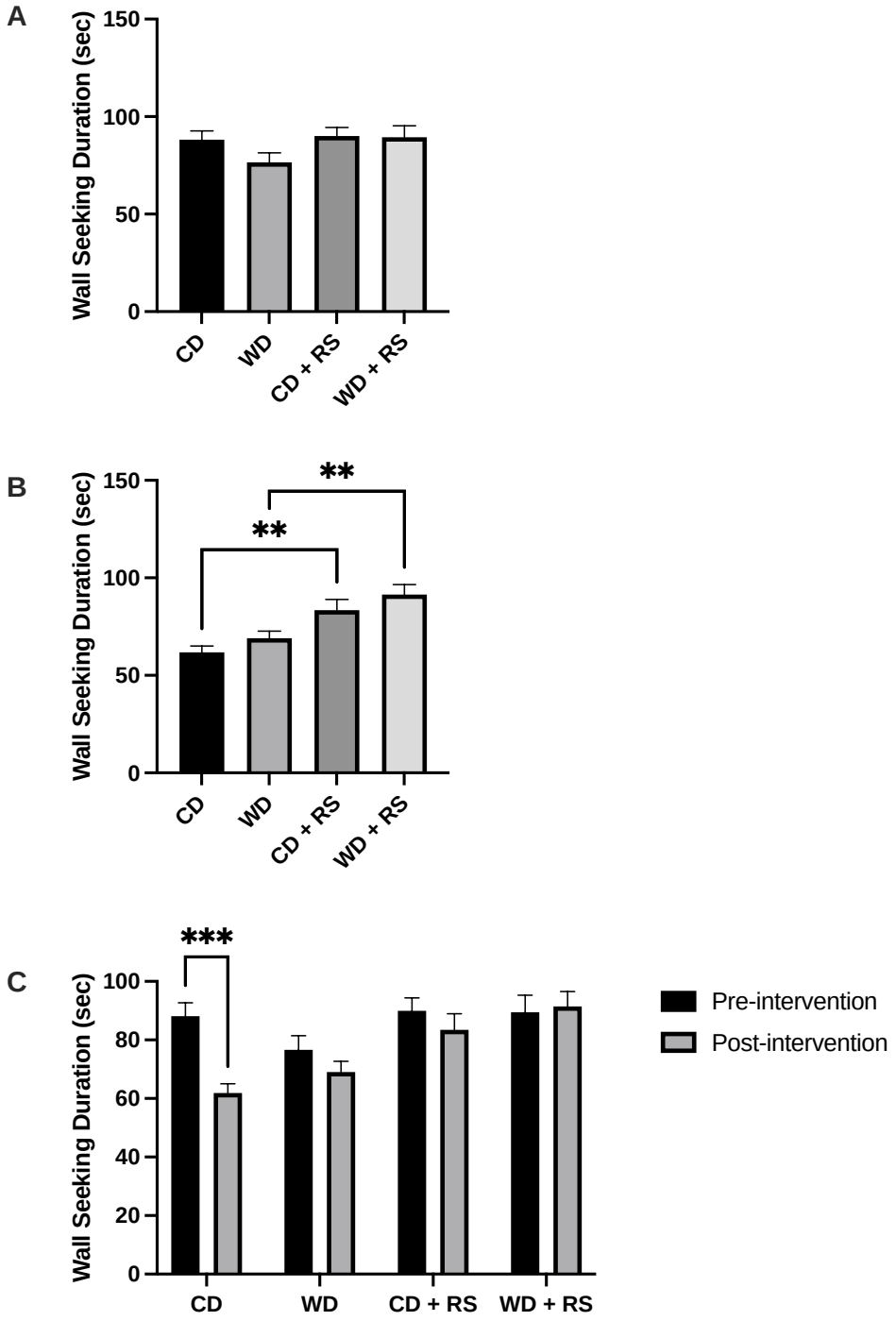

Figure 10: Wall-Seeking Duration during the Open Field Test. A) Wall seeking behaviour (seconds) at pre-intervention. **B)** Wall seeking behaviour (seconds) at post-intervention. **p < 0.01. **C)** Wall seeking behaviour (seconds) at pre- vs post-intervention. ***p < 0.001. Results presented at mean±SEM. n = 16. Pre-intervention week started at week 0. Post-intervention week commenced at week 17 of study.

No significant differences were found between groups at the pre-intervention OFT (figures 8-10). After the interventions (the exposure to the WD or RS, or their combination), the RS

groups travelled significantly more distance ($p < 0.0001$), and displayed increased wall seeking activity ($p < 0.05$) and duration at the walls ($p < 0.01$) compared to CD and WD groups. The CD and WD groups covered significantly less distance ($p < 0.0001$) in the post-intervention OFT than during the pre-intervention OFT. All groups displayed significantly reduced wall-seeking behaviour during the post-intervention OFT compared to the pre-intervention OFT, consistent with an age or habituation effect (evident in both control and intervention groups). In addition, the CD group spent less time at the wall (wall seeking) at the post-intervention OFT ($p<0.001$).

4.3 Blood Metabolic Parameters

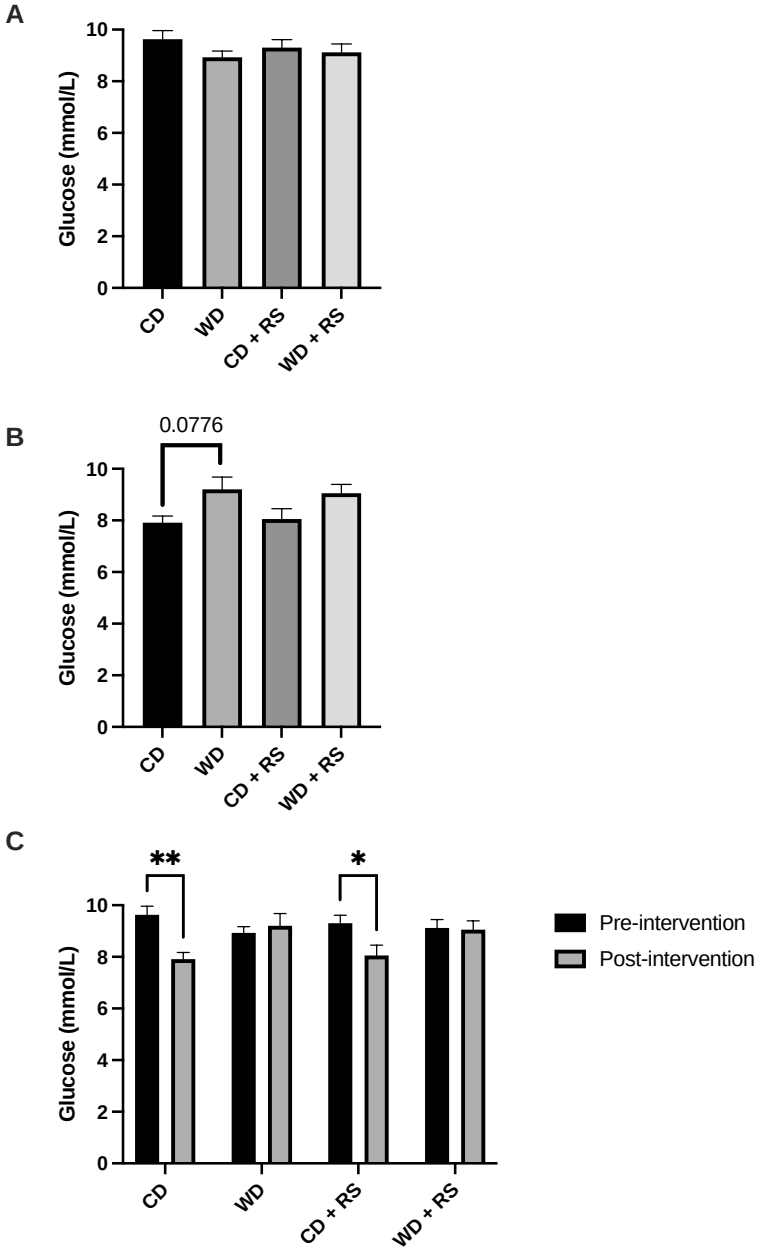

Figure 11: Fasted Blood Glucose. A) Pre-intervention Fasted Blood Glucose. **B)** Post-intervention Fasted Blood Glucose. **C)** Pre-intervention vs Post-intervention Fasted Blood Insulin. * $p < 0.05$, ** $p < 0.01$. Data presented as mean ± SEM. n = 16.

Fasted blood glucose levels are displayed in figure 11. No significant intergroup differences in blood glucose levels were found in the pre- (A) or post-intervention (B) samples. However, a trend for increased blood glucose ($p = 0.0776$) was found between CD and WD groups post-intervention. Pre- vs post-intervention glucose values (C) shows that there were significantly higher fasted glucose levels pre-intervention compared to post-intervention in the CD ($p < 0.01$) and CD + RS ($p < 0.05$) groups.

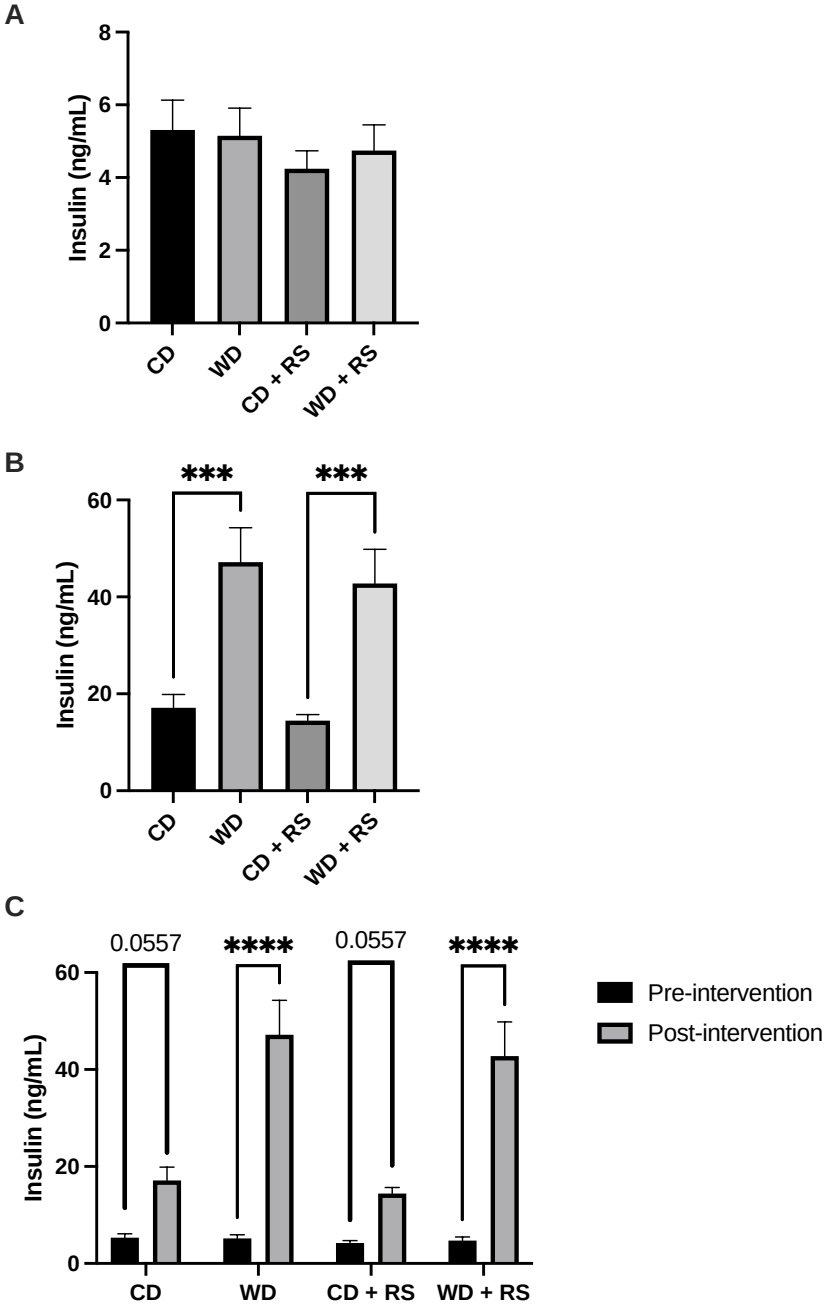

Figure 12: Fasted Blood Insulin. A) Pre-intervention Fasted Blood Insulin. **B)** Post-intervention Fasted Blood Insulin. *$p < 0.05$, ***$p < 0.001$. **C)** Pre-intervention vs Post-intervention Fasted Blood Insulin. ****$p < 0.0001$. Data presented as mean ± SEM. n = 16.

No significant differences in fasted blood insulin levels were found at pre-intervention (figure 12A). However, the WD fed groups had significantly higher post-intervention fasted insulin levels (figure 12B) compared to their control diet counterparts ($p < 0.001$). Figure 12C shows that WD fed groups had significantly higher fasted insulin when comparing post-intervention to pre-intervention values ($p<0.001$). Trends were found in the CD fed groups ($p = 0.0557$) when comparing pre- vs post-intervention levels.

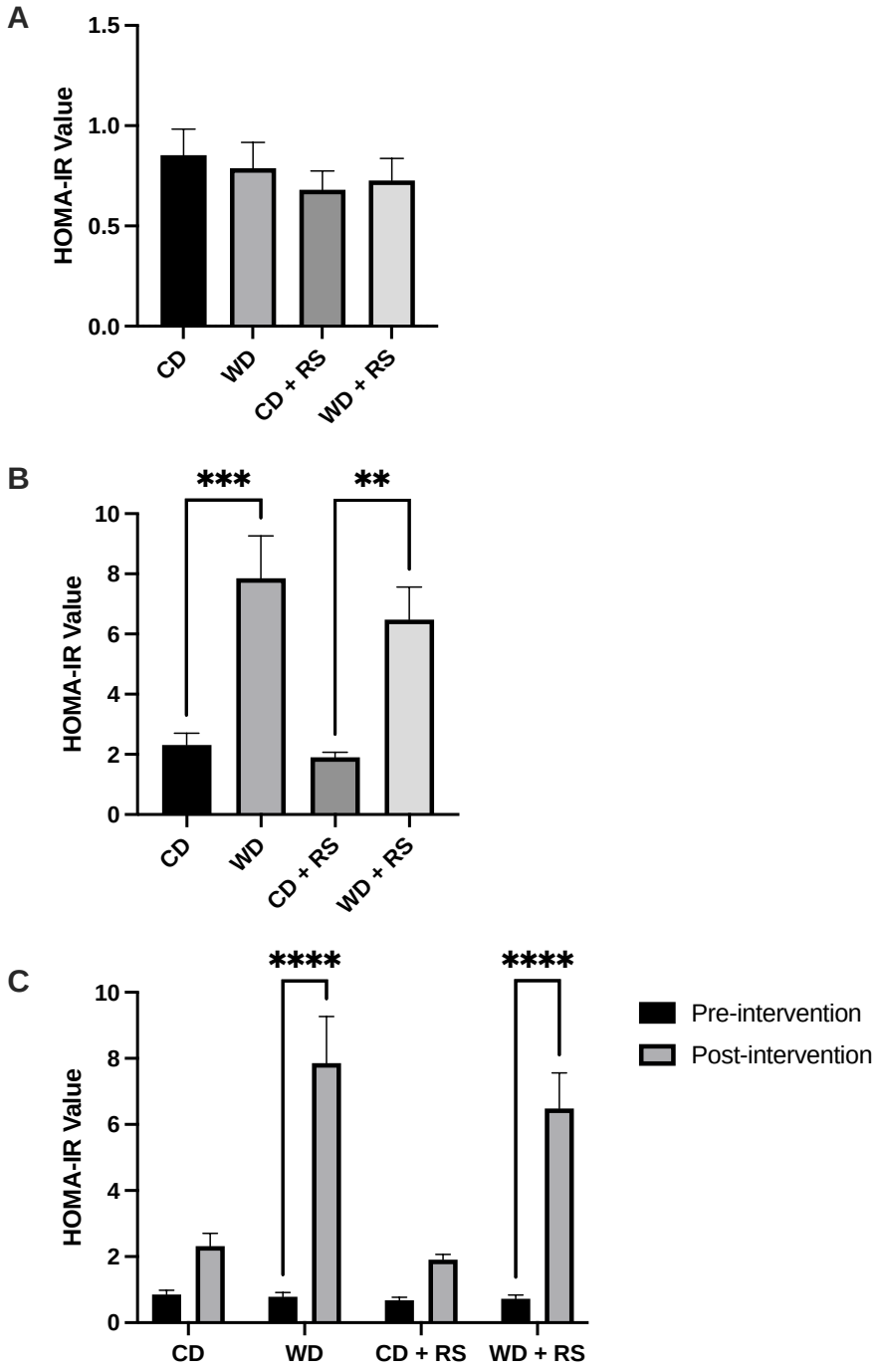

Figure 13: HOMA-IR Values in Fasted Serum. A. Pre-intervention Serum HOMA-IR Values. **B.** Post-intervention Serum HOMA-IR Values. **p < 0.01, ***p < 0.001. **C.** Pre- vs Post-intervention Serum HOMA-IR Values. ****p < 0.0001. Results presented as Mean±SEM. n = 15/16.

No significant differences for HOMA-IR values were found in pre-intervention fasted blood. Significant increases were found in WD ($p < 0.001$) and WD + RS ($p < 0.01$) HOMA-IR values compared to the CD groups (figure 13B) post-intervention. Figure 13C shows significant increases ($p < 0.001$) in HOMA-IR values in the WD and WD + RS groups in post-intervention blood when compared with their pre-intervention values.

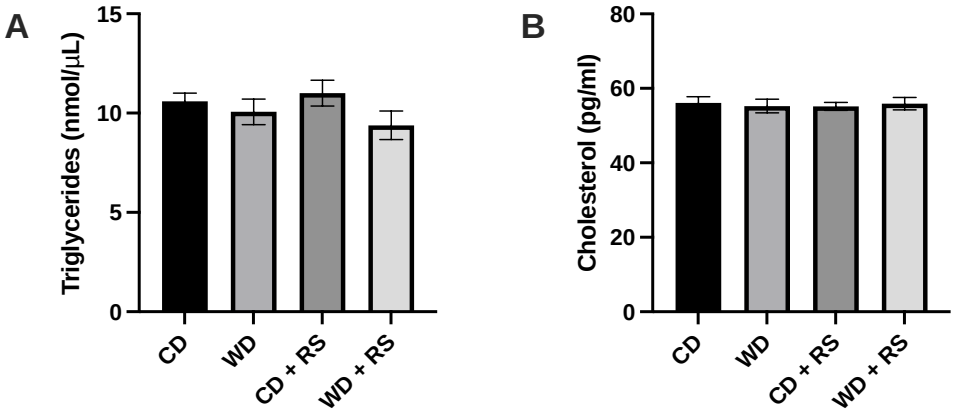

Figure 14: Fasted Blood Lipid Concentration. A. Fasted Blood Trigylceride Concentration. **B.** Fasted Blood Cholesterol Concentration. Results Presented as mean±SEM. n = 15/16.

No significant differences were found in either the fasted triglyceride or cholesterol levels at 17 weeks (post-intervention) (figure 14).

4.4 Circulating Cytokine and Hormone Levels

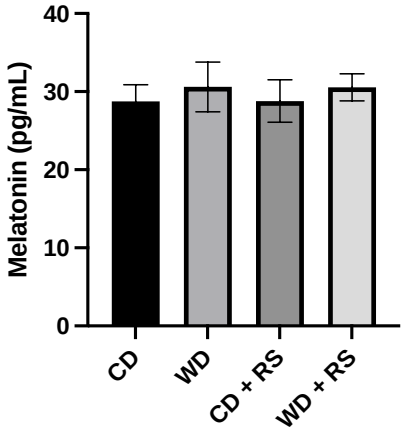

Figure 15: Melatonin in non-fasted blood. Results presented as mean±SEM. n = 8-13.

Melatonin in unfasted serum is presented in figure 15. No significant intergroup differences were found.

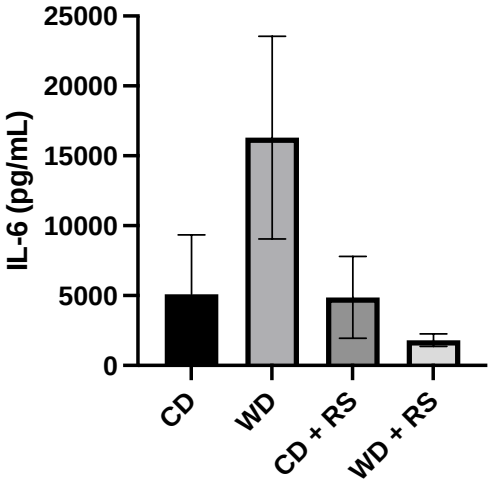

Figure 16: IL-6 in non-fasted blood. Results presented as mean±SEM. n = 3-7.

IL-6 concentration in unfasted serum is shown in figure 16. No significant differences were found between groups.

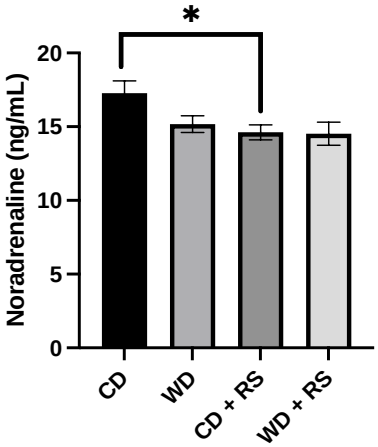

Figure 17: Circulating noradrenaline in non-fasted blood. *$p < 0.05$. Results presented as mean±SEM. n = 12.

The CD + RS group had a significantly lower ($p < 0.05$) concentration of noradrenaline than the CD group.

4.5 Post-ischaemic Cardiac Outcomes

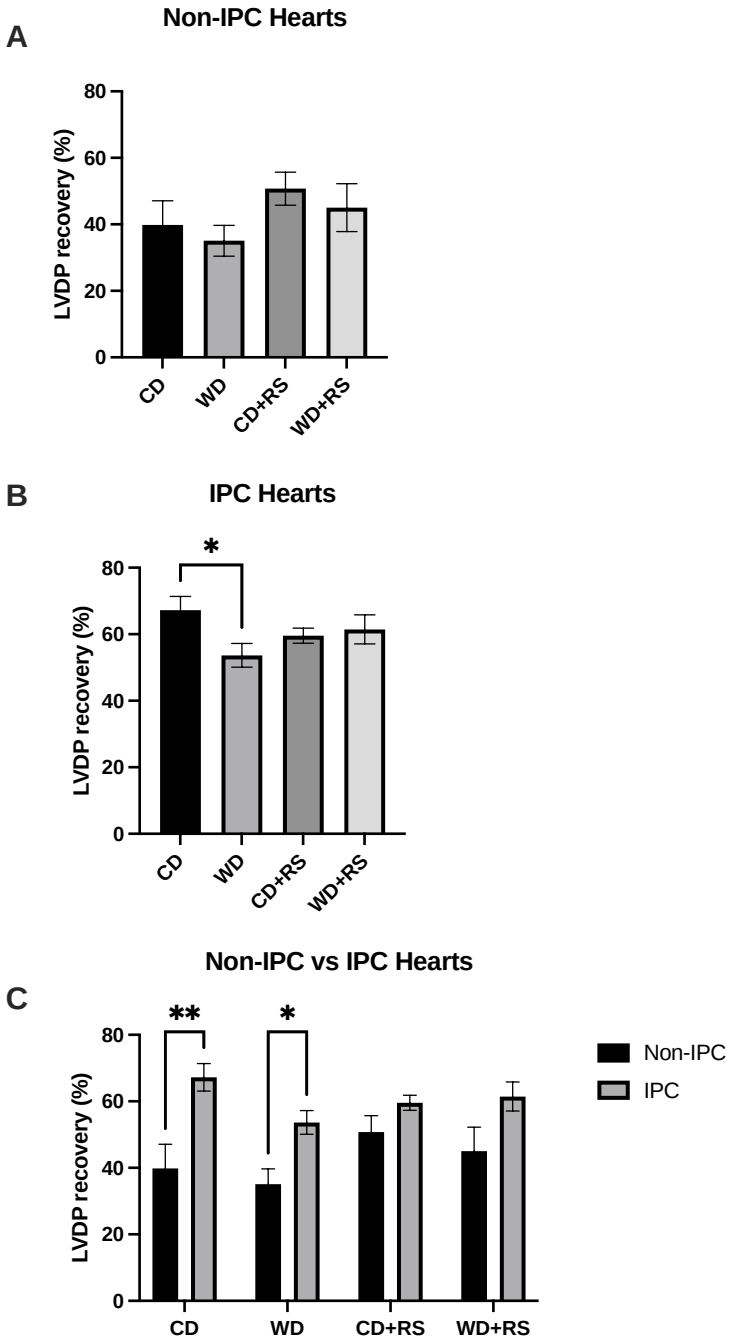

Figure 18: Left Ventricular Developed Pressure Recovery Post-Ischemia. A. Left ventricular developed pressure recovery in non-IPC hearts at 45 minutes reperfusion. **B.** Left ventricular developed pressure recovery in IPC hearts at 45 minutes reperfusion. *$p < 0.05$. **C.** Left ventricular developed pressure recovery in non-IPC vs IPC hearts at 45 minutes reperfusion. *$p < 0.05$, **$p < 0.01$. Results presented as mean±SEM. n = 5-8.

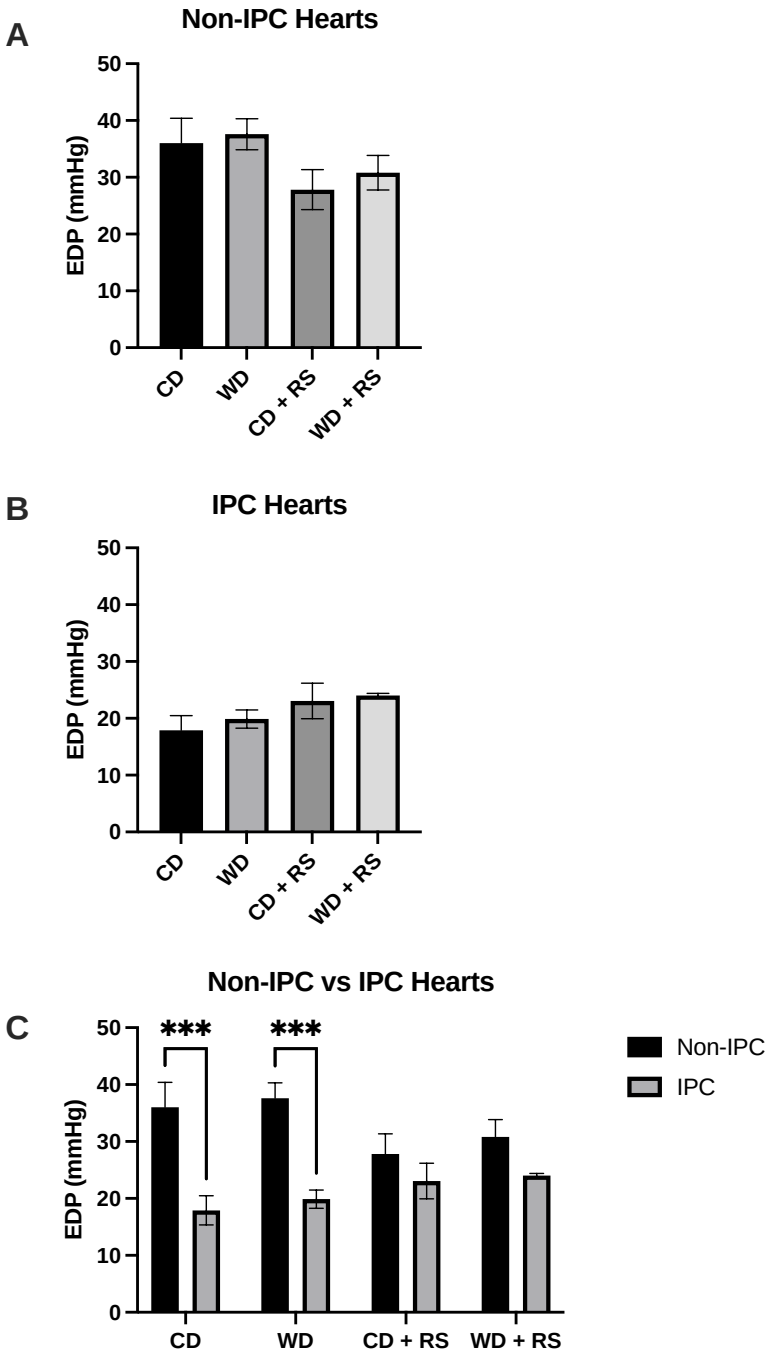

Figure 19: End-Diastolic Pressure in Post-Ischemic Hearts. A. End-diastolic pressure in non-IPC hearts. **B.** End-diastolic pressure in IPC hearts. **C.** End-diastolic pressure in non-IPC vs IPC hearts. ***p < 0.001. Results presented as mean±SEM. n = 5-8.

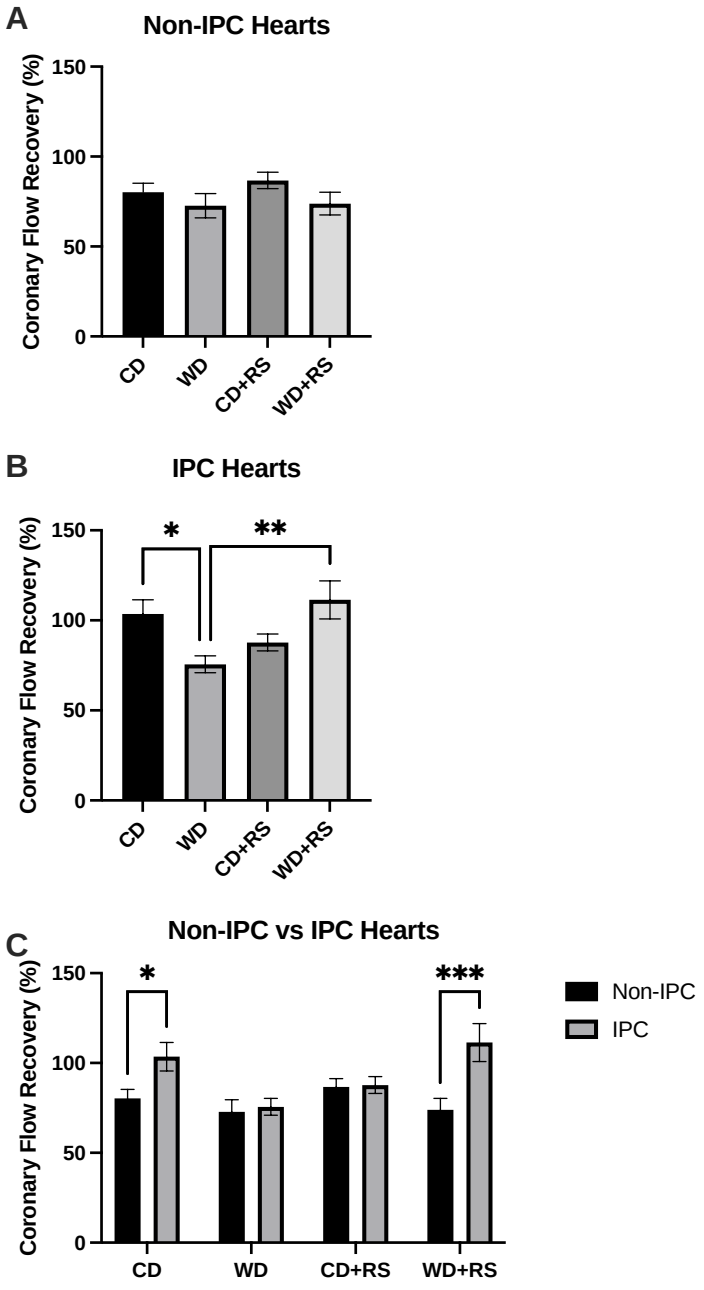

Figure 20: Coronary Flow Recovery in Post-Ischemic Hearts. A. Coronary flow recovery in non-IPC hearts. **B.** Coronary flow recovery in IPC hearts. *p < 0.05, **p < 0.01. **C.** Coronary flow recovery in non-IPC vs IPC hearts. *p < 0.05, ***p < 0.001. Results presented as mean±SEM. n = 5-8.

Langendorff heart perfusion data is shown in figures 18 (left ventricular developed pressure (LVDP) recovery), 19 (end diastolic pressure (EDP)), and 20 (coronary flow recovery). No significant differences were found in non-IPC hearts for LVDP, EDP, and coronary flow. However, when we look at the IPC hearts, the WD IPC group had a significantly lower LVDP

recovery compared to the CD IPC hearts ($p < 0.05$). When comparing LVDP recovery of the non-IPC and IPC hearts, CD ($p < 0.01$) and WD ($p < 0.05$) IPC hearts had a greater recovery compared to their non-IPC counterparts. Supporting this data, CD and WD IPC hearts also had significantly reduced EDP ($p < 0.001$) compared to their non-IPC groups. Thus, ventricular functional protection with IPC was not markedly altered by a WD. In contrast, protective effects of IPC on both LVDP and EDP was largely eliminated by RS (±WD). Coronary flow was significantly increased in CD ($p < 0.05$) and WD + RS (0.001) IPC hearts, which follows LVDP trends.

4.6 Cell Damage Marker Levels

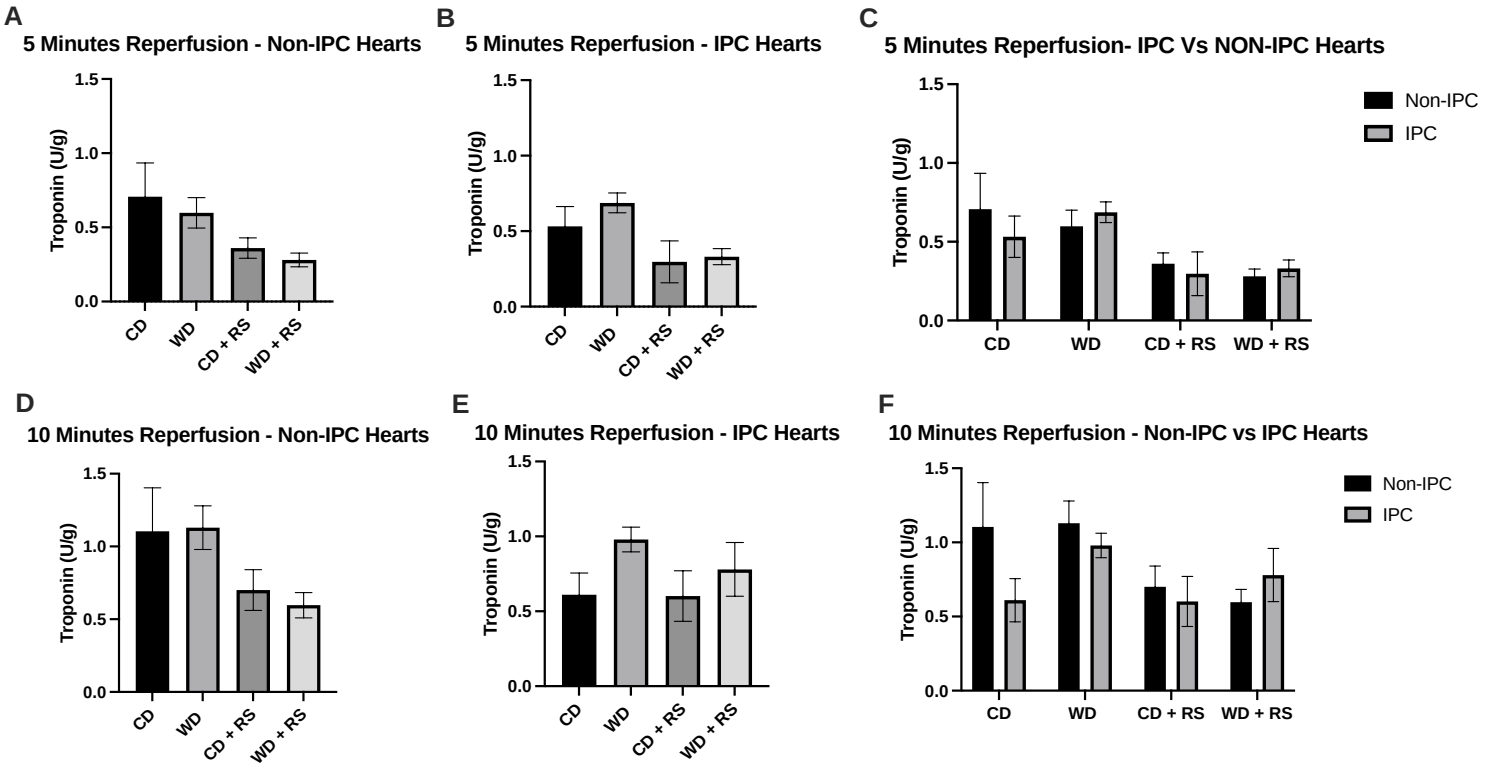

Figure 21: Troponin Release into Coronary Effluent. A. Troponin release into coronary effluent at 5 minutes reperfusion in non-IPC hearts. **B.** Troponin release into coronary effluent at 5 minutes reperfusion in IPC hearts. **C.** Troponin release into coronary effluent at 5 minutes reperfusion in non-IPC vs IPC hearts. **D.** Troponin release into coronary effluent at 10 minutes reperfusion in non-IPC hearts. **E.** Troponin release into coronary effluent at 10 minutes reperfusion in IPC hearts. **F.** Troponin release into coronary effluent at 10 minutes reperfusion in non-IPC vs IPC hearts. Results presented as mean±SEM. n = 5-10.

Troponin I release was measured in coronary effluent from 0-5 minutes and 5-10 minutes reperfusion. Figure 21 shows that there were no significant differences in cardiac troponin release in non-IPC, IPC, or non-IPC vs IPC hearts.

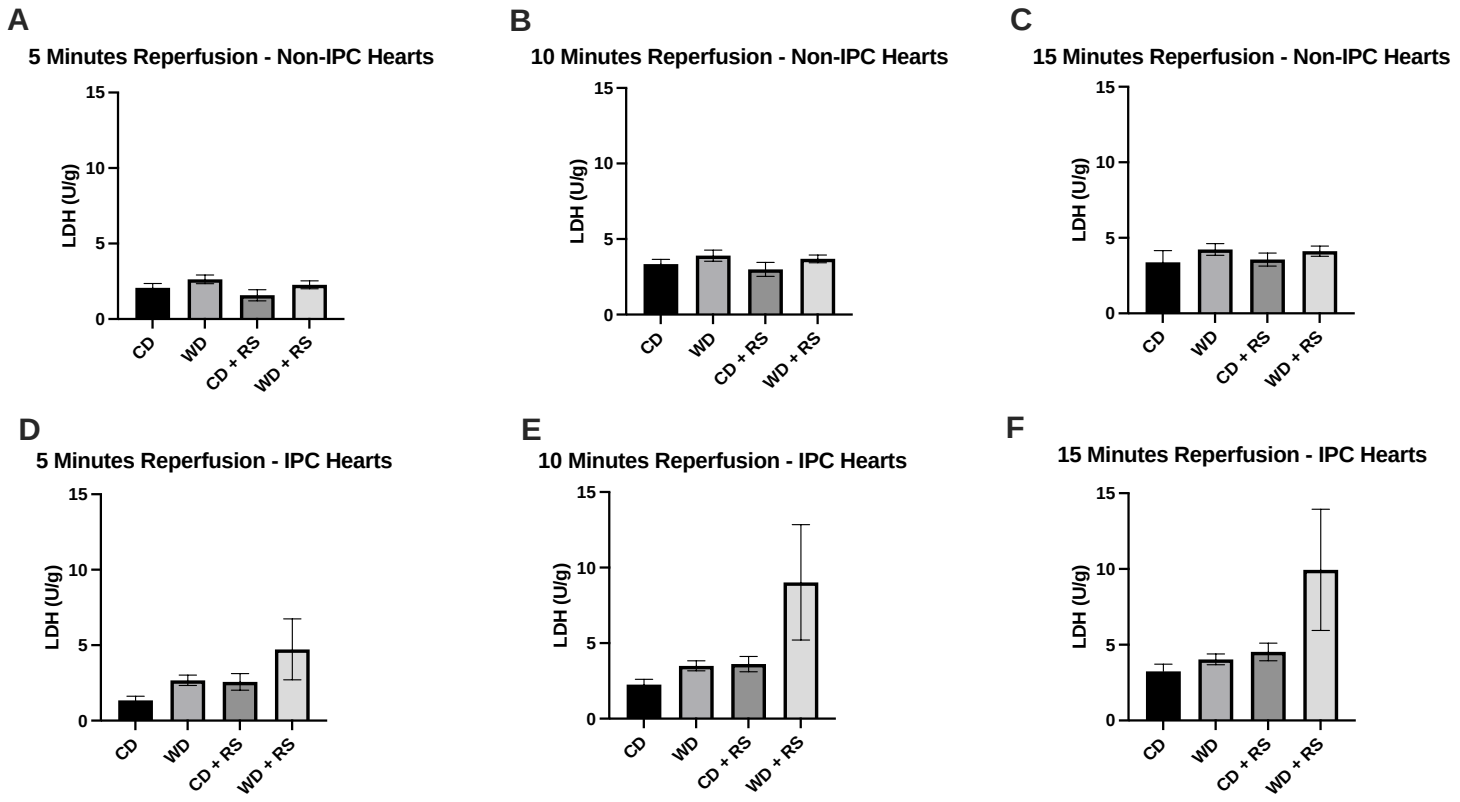

Figure 22: LDH Release into Coronary Effluent. A) LDH release into coronary effluent at 5 minutes reperfusion in non-IPC hearts. **B)** LDH release into coronary effluent at 10 minutes reperfusion in non-IPC hearts. **C)** LDH release into coronary effluent at 15 minutes reperfusion in non-IPC hearts. **D)** LDH release into coronary effluent at 5 minutes reperfusion in IPC hearts. **E)** LDH release into coronary effluent at 10 minutes reperfusion in IPC hearts. **F)** LDH release into coronary effluent at 15 minutes reperfusion in IPC hearts. Presented as mean±SEM. n = 5-10

Lactate dehydrogenase (LDH) release during the first 5 minutes, 5-10 minutes and 10-15 minutes reperfusion is presented in figure 22. No significant differences in LDH release were found in non-IPC hearts or IPC hearts at 5, 10 or 15 minutes reperfusion.

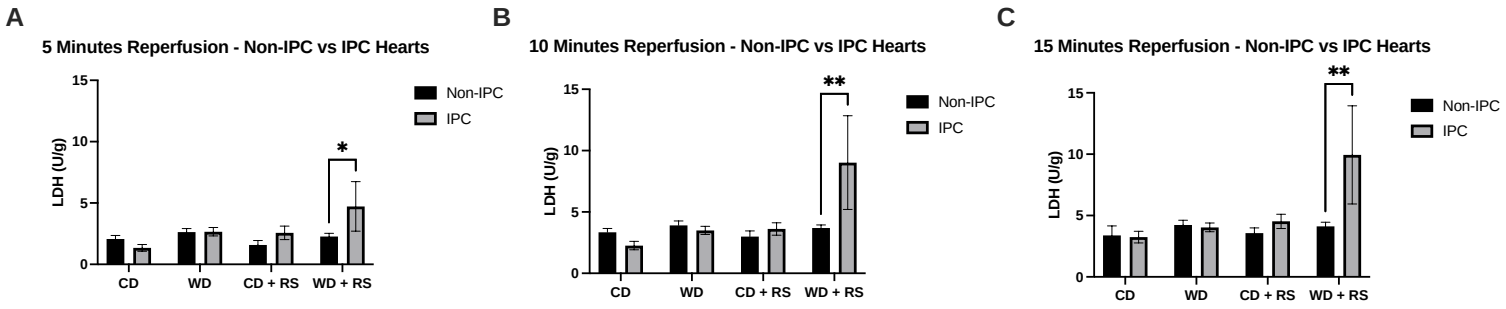

Figure 23: LDH Release into Coronary Effluent Pre- vs. Post-Intervention. A) LDH release into coronary effluent at 5 minutes reperfusion. *p < 0.005. **B)** LDH release into coronary effluent at 10 minutes reperfusion. **p < 0.001. **C)** LDH release into coronary effluent at 15 minutes reperfusion. **p < 0.001. Presented as mean +/- SEM. N = 5-10.

LDH release into coronary effluent in non-IPC vs IPC hearts is presented in figure 23. WD + RS IPC hearts released significantly more LDH at 5 ($p < 0.05$), 10 ($p < 0.01$), and 15 ($p < 0.01$) minutes reperfusion compared to WD + RS non-IPC hearts.

4.7 Myocardial Tissue Cytokine Levels

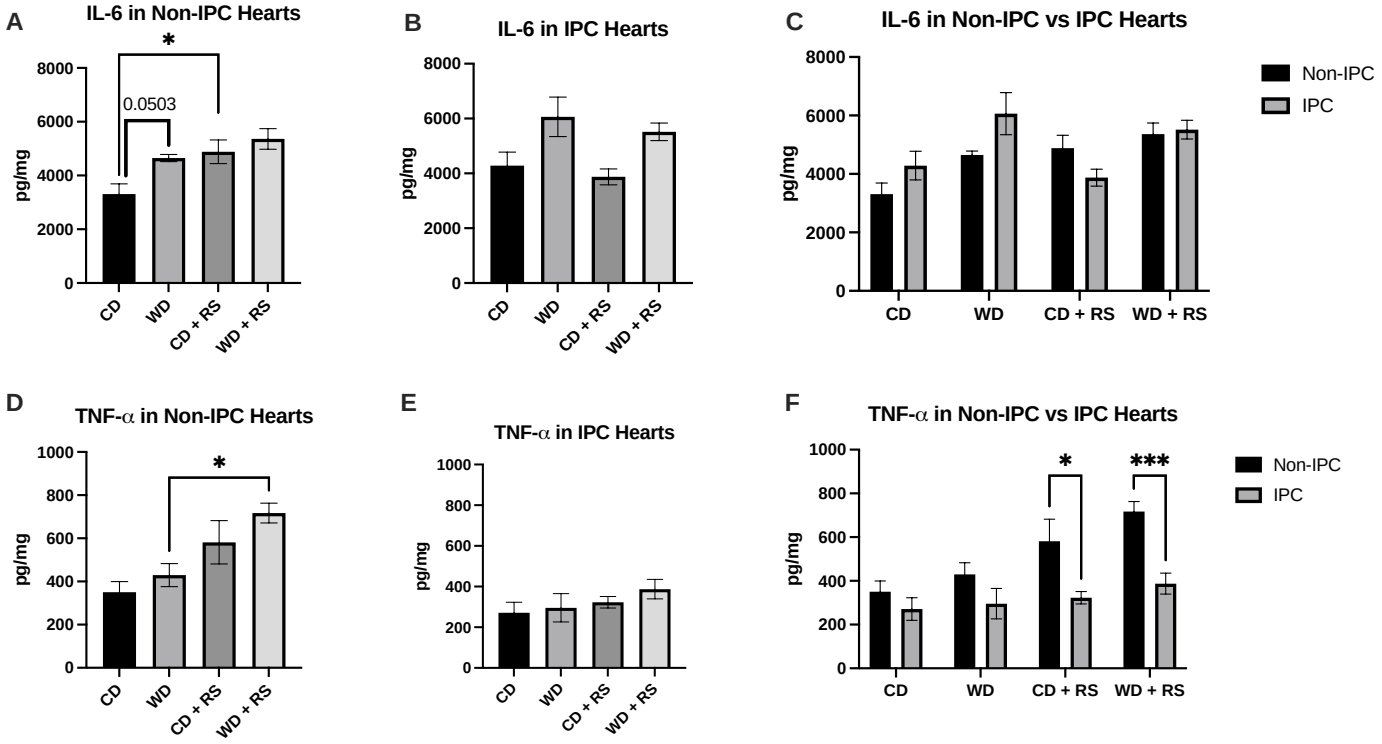

Figure 24: Cytokines in left ventricle post-I/R. A. IL-6 in non-IPC hearts. *$p < 0.05$. **B.** IL-6 in IPC hearts. **C.** IL-6 in non-IPC vs IPC hearts. **D.** TNF-α in non-IPC hearts. *$p < 0.05$. **E.** TNF-α in IPC hearts. **F.** TNF-α in non-IPC vs IPC hearts. *$p < 0.05$, ***$p < 0.001$. Results presented as mean±SEM. n = 7-8.

Cytokine concentration in post-ischemic myocardial tissue is shown in figure 24. CD + RS non-IPC hearts had significantly higher ($p < 0.05$) levels of IL-6 in the left ventricle compared to CD non-IPC hearts. The WD non-IPC group had significantly lower ($p < 0.05$) levels of TNF-α than the WD + RS non-IPC group. When comparing TNF-α levels in non-IPC vs IPC hearts, both non-IPC RS groups had significantly higher ($p < 0.05$, $p < 0.001$) levels of TNF-α than their IPC counterparts. No other significant differences were found. Thus, stress or a combination of WD+RS appears pro-inflammatory, increasing Il-6 and TNF-a in non-IPC

hearts, while IPC itself also appeared anti-inflammatory in the hearts of animals subjected to stress ±WD feeding.

4.8 RISK Pathway Protein Expression as Determined using Western Blots

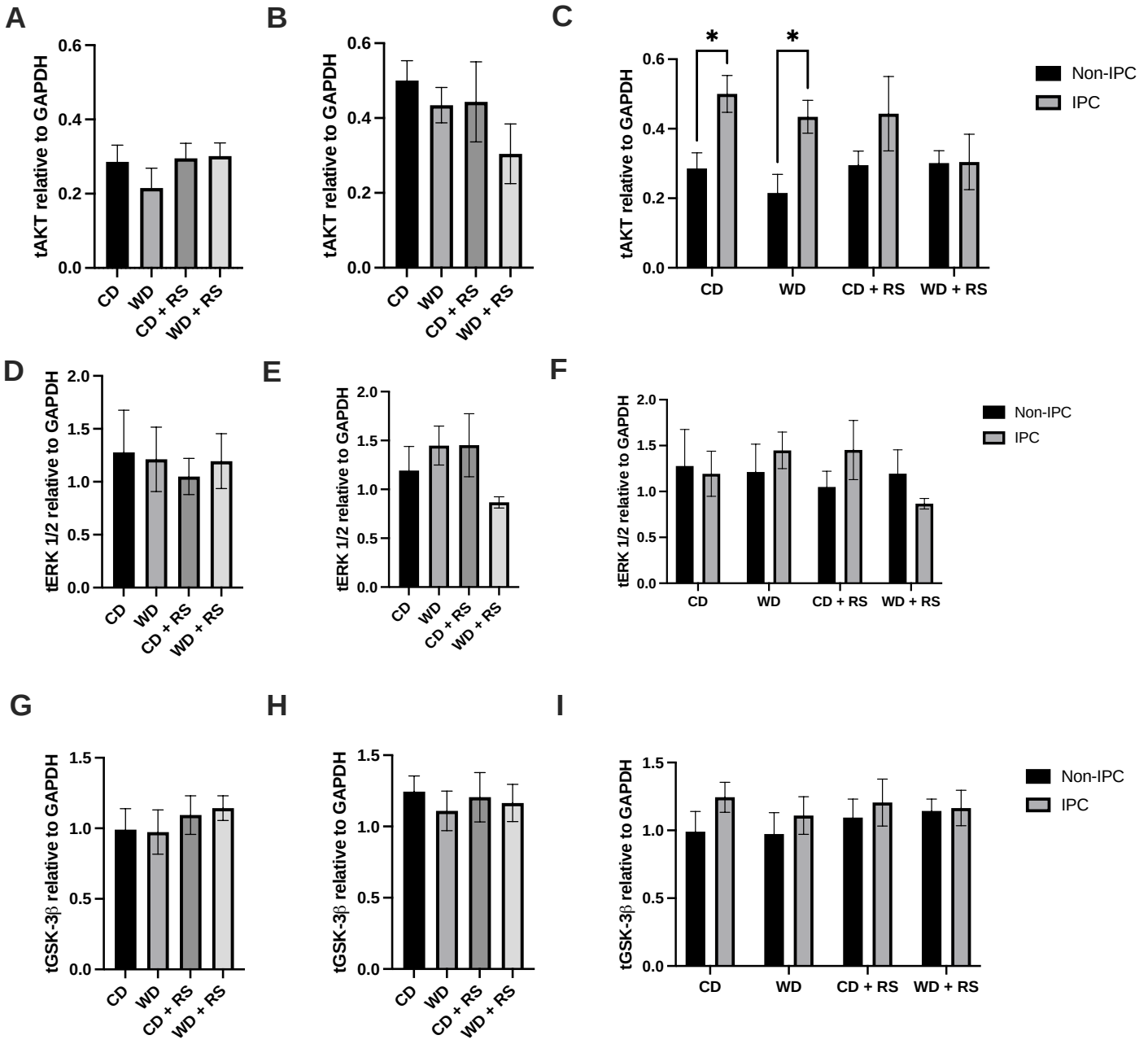

Figure 25: Total levels of RISK pathway proteins in the left ventricle via Western Blot Analysis. A. t-AKT relative to GAPDH in non-IPC hearts. **B.** t-AKT relative to GAPDH in IPC hearts. **C.** t-AKT relative to GAPDH in non-IPC vs IPC hearts. *p < 0.05. **D.** t-ERK 1/2 relative to GAPDH in non-IPC hearts. **E.** t-ERK 1/2 relative to GAPDH in IPC hearts. **F.** t-ERK 1/2 relative to GAPDH in non-IPC vs IPC hearts. **G.** t-GSK-3β relative to GAPDH in non-IPC hearts. **H.** t-GSK-3β relative to GAPDH in IPC hearts. **I.** t-GSK-3β relative to GAPDH in non-IPC vs IPC hearts. Results presented as mean±SEM. n = 6-10

Total levels of AKT, ERK 1/2, and GSK-3β are presented in figure 25. The CD and WD IPC groups had significantly increased ($p < 0.05$) total AKT. Thus, AKT appears up-regulated with IPC, an effect unaltered by a WD but eliminated by RS (±WD), mirroring changes in functional protection. No other significant differences were found.

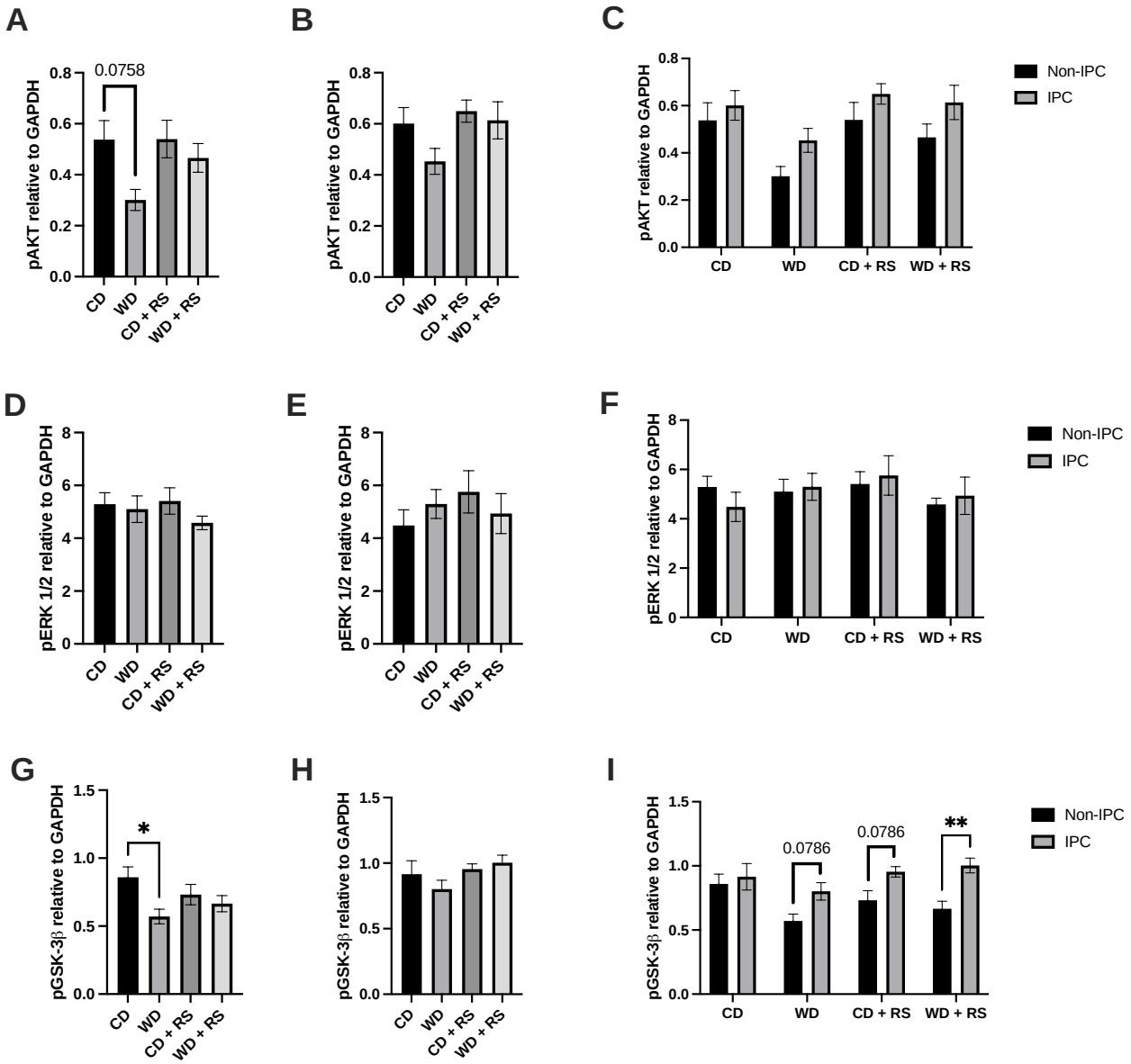

Figure 26: Phoshorylated levels of RISK pathway proteins in the left ventricle via Western Blot Analysis. A. p-AKT relative to GAPDH in non-IPC hearts. **B.** p-AKT relative to GAPDH in IPC hearts. **C.** p-AKT relative to GAPDH in non-IPC vs IPC hearts. **D.** p-ERK 1/2 relative to GAPDH in non-IPC hearts. **E.** p-ERK 1/2 relative to GAPDH in IPC hearts. **F.** p-ERK 1/2 relative to GAPDH in non-IPC vs IPC hearts. **G.** p-GSK-3β relative to GAPDH in non-IPC hearts. **H.** p-GSK-3β relative to GAPDH in IPC hearts. **I.** p-GSK-3β relative to GAPDH in non-IPC vs IPC hearts. **$p < 0.01$. Results presented as mean±SEM. n = 6-10

Phosphorylated levels of AKT, ERK 1/2, and GSK-3β relative to GAPDH are shown in Figure 26. Interestingly, the WD reduced cardiac pAKT and pGSK3β levels in non-IPC hearts, whereas RS had no effect. The WD + RS IPC group had significantly higher ($p < 0.01$) pGSK-3β at post-ischemia than the WD + RS non-IPC group. There were notable trends ($p = 0.0786$) to increased GSK-3β phosphorylation in the WD and CD + RS IPC groups (vs respective non-IPC groups).

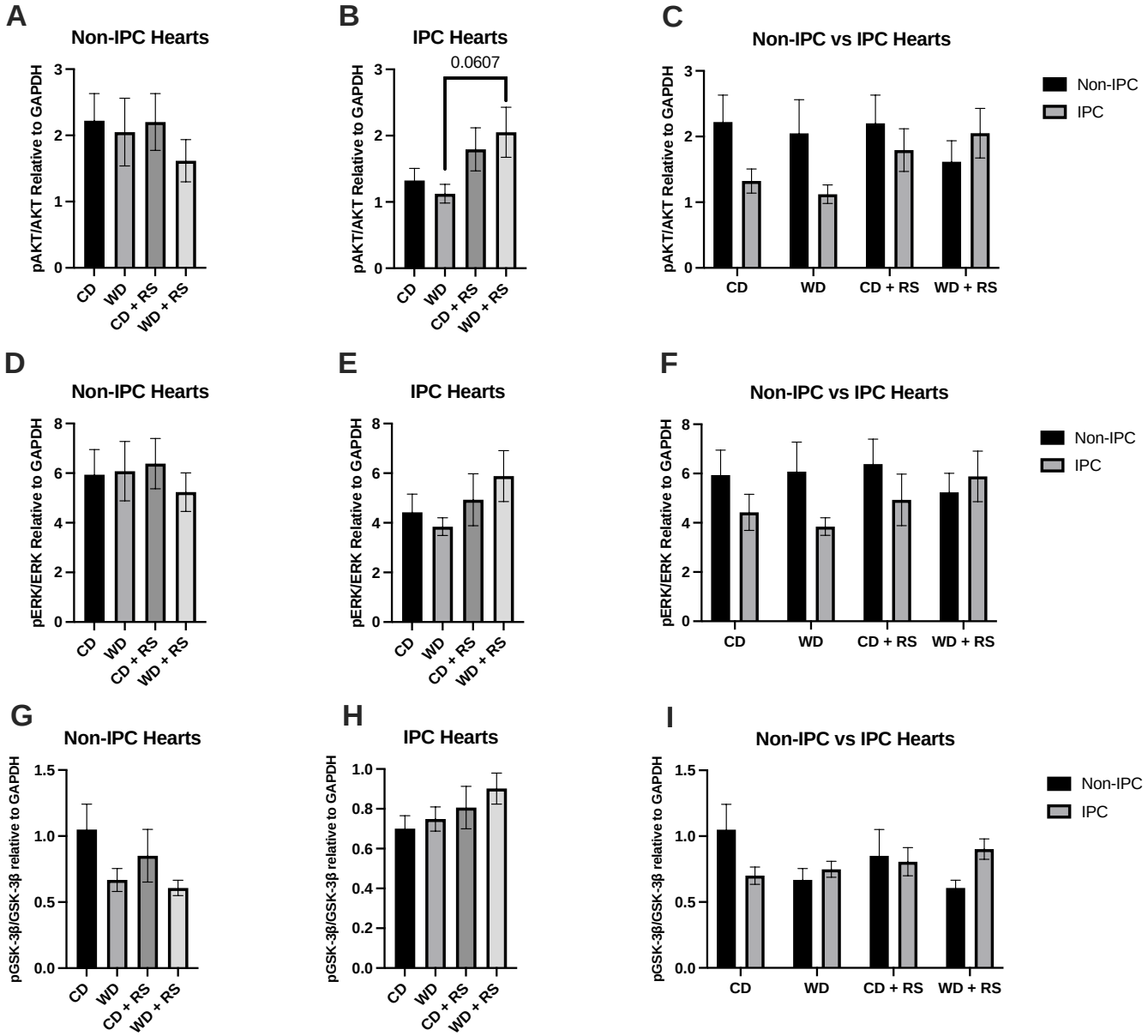

Figure 27: Phoshorylated to total protein ratio of RISK pathway proteins in the left ventricle via Western Blot Analysis.. A. p-AKT/t-AKT ratio relative to GAPDH in non-IPC hearts. **B.** p-AKT/t-AKT ratio relative to GAPDH in IPC hearts. **C.** p-AKT/t-AKT ratio relative to GAPDH in non-IPC vs IPC hearts. **D.** p-ERK 1/2/t-ERK 1/2 ratio relative to GAPDH in non-IPC hearts. **E.** p-ERK 1/2/t-ERK 1/2 ratio relative to GAPDH in IPC hearts. **F.** p-ERK 1/2/t-ERK 1/2 ratio relative to GAPDH in non-IPC vs IPC hearts. **G.** p-GSK-3β/t-GSK-3β ratio relative to GAPDH in non-IPC hearts. **H.** p-GSK-3β/t-GSK-3β ratio relative to GAPDH in IPC hearts. **I.** p-GSK-3β/t-GSK-3β ratio relative to GAPDH in non-IPC vs IPC hearts. Results presented as mean±SEM. n = 6-10

Figure 27 shows the results for Western blot analysis of ERK, AKT, and GSK-3β in post-ischemic heart tissue. No significant differences were found between non-IPC hearts, IPC hearts, or non-IPC vs IPC hearts for any of the three RISK pathway proteins investigated. However, statistical analysis of pAKT/tAKT ratio in IPC hearts revealed a trend ($p = 0.0607$) when comparing WD to WD + RS hearts (Figure 23B).

5.0 Discussion

In this study we investigated the effects of a WD or low-level CS and their combined effect on myocardial tolerance to I/R injury and myocardial responsiveness to a cardioprotective pre-conditioning stimulus. It was predicted that both the WD and the low-level CS would cause mild metabolic dysregulation, and that the WD and CS would act synergistically to cause greater impairment of myocardial infarct tolerance and responsiveness to IPC. The WD was effective in inducing obesity and causing insulin resistance in the WD fed groups. Triglyceride and cholesterol levels were however unchanged by the diet, CS exposure, or the combination of both. Behaviour assessments using the OFT indicated that the two hours of daily RS caused mild mood disturbance in these mice. The WD or CS intervention, or the combined effect of these interventions did not have an adverse effect on intrinsic ischemic tolerance (in non-preconditioned hearts). Hearts from the CD and WD fed animals could be pre-conditioned, while cardioprotective effects of IPC were negated by stress in both dietary groups. This effect was linked to a reduction in IPC dependent up-regulation of AKT. CS was also shown to be pro-inflammatory in the heart, increasing Il-6 and TNF-α levels in non-IPC hearts. However, although IPC reduced post-ischaemic myocardial inflammation, this was not accompanied by improved post-ischaemic functional outcomes.

5.1 Effect of the WD and RS on Metabolic Profile

After 12 weeks of feeding, the WD significantly increased body weight in WD fed mice, when compared to the CD mice. This indicates that the WD was successful in promoting an overweight/obese phenotype in these rodents, which is consistent with current findings in the literature[192,193]. At week 16, a significant decrease in body weight was seen following the introduction of the two hours daily RS protocol at week 15. Body weight did not recover over the next two weeks of chronic RS. This decrease in body weight is likely due to the stress induced reduction in food and therefore kilojoule (kJ) consumption in the RS groups in week

16. Average daily energy intake increased during week 17 for the RS groups, which is likely the reason that the body weight for the RS groups began to stabilise in week 17. Previously, RS has been found to cause weight loss and impaired body weight gain, with the probable driver of this weight loss suggested to be decreased kJ intake (as a result of increased corticotropin releasing hormone in response to stress)[194,195].

The C57Bl/6J mouse is often used in dietary studies since they are prone to obesity and metabolic dysregulation when fed HF or Western diets[196,197]. Significant decreases in fasted glucose in our CD mice, along with trends for a post-intervention rise in insulin levels and higher HOMA-IR values indicate that there may be some evidence of an age-related increase in insulin resistance in these mice. Age related impairments in glucose tolerance and insulin resistance have been reported previously, but often these studies are comparing age related changes between 6-month-old (similar to the age our mice were at the conclusion of this study) and 12–24-month-old mice[198,199]. These studies support our findings, with our data suggesting that these age-related changes may start occurring earlier in life. We recorded elevated fasting insulin levels post-intervention in the WD fed mice. The HOMA-IR values were higher in both WD fed groups at the end of the study (post-intervention) when compared to the CD. These results indicate that the WD promoted a greater degree of insulin resistance in these mice over the 17-week period. No significant changes in triglyceride or cholesterol concentrations were found in any of the groups. Because of this, the metabolic dysregulation caused by the WD is mild. This diet has been successful in causing mild metabolic disturbance in prior studies[12]. Previously, an insulin resistant phenotype was achieved using this diet, however no significant differences in body weight, glucose, or insulin levels were observed. This study also did not find changes in triglyceride or cholesterol concentration, supporting our serum lipid findings. HOMA-IR values confirmed insulin resistance in the WD mice, and the WD and CRS had a synergistic effect to further exacerbate insulin resistance[12]. This additive effect of the diet and

RS in promoting insulin resistance was not evident in our study. These difference in outcomes may relate to subtle differences in the chronic stress model used for these two studies. The restraint stress protocol in this previous study was only 30 minutes for 21 days, while we used 2 hours RS for 14 days in the current study. Because of this, the CS protocol in the previous study did not illicit the same severe body weight loss we saw in the current study. In the literature it has been established that Western or HF diets induce metabolic disturbances, including obesity, hyperglycaemia, insulin insensitivity/T2DM, and/or dyslipidaemia[99,172,200,201]. Our current research supports many of these findings. Marwitz, et al. [192] found insignificant weight increases in the WD fed mice at 9 weeks of feeding, along with hyperinsulinemia and insulin resistance 10 weeks into the diet. These findings support ours where mice did not display significant weight changes until week 12. Some studies report more pronounced metabolic dysregulation than what has been documented in this study[201]. This is often because these diets are given for longer periods of time, with the goal of these researchers to achieve marked metabolic disturbance.

In this study, RS alone did not appear to impact metabolic homeostasis, despite previous reports often linking chronic stress to insulin resistance[202]. While hyperglycaemia, insulin resistance, and increased circulating lipids have been reported in chronically stressed rodents, research outcomes are conflicting with hypoglycaemia and reduced cholesterol also reported post-chronic RS exposure[203,204]. Our study further adds to these conflicting findings with our stress protocol found to have no impact on these metabolic parameters. Both RS protocols in these studies went for 30 days, with one study inducing stress for one hour daily, while the other was for two hours daily. The difference in metabolic response to stress may be because the daily duration of these stress protocols varied. Differences in findings between these studies and our own may be attributed to the number of days that the RS protocol was implemented, with Choudhary, et al. [203] and Nagaraja and Jeganathan [204] exposing the mice to

RS for 30 days, which is double the period of time mice in our study were exposed to stress. These contrasting findings may also be attributed to the different rodents used in these studies, and the differing innate stress thresholds these rodents have regarding how stress impacts their metabolic homeostasis (e.g. gluconeogenesis and insulin production are promoted during the stress response), or other subtle differences in these rodents' metabolism[13]. Supporting our findings, Rostamkhani, et al. [205] reported that while chronic psychological stress initially promoted higher glucose levels, it did not result in insulin resistance when compared to the control group, with no significant changes in lipids observed.

5.2 Impacts of a WD, RS or their Combination on Systemic Inflammation

Chronic systemic inflammation is associated with obesity and MetS, further promoting the disease state and progression. During obesity, M1 macrophages in adipocytes release pro-inflammatory TNF-α and IL-6 and decrease their production of anti-inflammatory adiponectin. IL-6 promotes CRP production from the liver (in addition to fibrinogen), which is a biomarker of systemic inflammation and promotes CVD risk. Inflammation via raised IL-6 levels promotes atherosclerosis, insulin resistance/T2DM, heart failure, CAD, and MetS[206-208]. Like metabolic disorders, mood disorders such as MDD and CS are also strongly linked to increased IL-6. These high levels of circulating IL-6 promote HPA axis overactivation and further drive inflammation and disease state[13,116,209]. In the current study, circulating IL-6 was unaltered by the RS or a WD, however there was a trend of increased circulating IL-6 in the WD mice. There were also no trends towards increased IL-6 in the RS mice, regardless of diet. These observations do not corroborate previous studies looking at chronically stressed phenotypes[210]. Voorhees, et al. [211] showed that during a 6-hour RS protocol, IL-6 levels significantly increased. It is possible that our WD mice were not obese enough to evoke pronounced systemic inflammation, and that our RS protocol was not stressful enough to cause an increase

in IL-6. In addition, our stress protocol also concluded four days before animal sacrifice and blood sample collection. This delay in sample collection may have allowed any significant stress induced increases in circulating IL-6 to disappear in the RS mice. This theory is supported by Voorhees, et al. [211] who saw IL-6 levels recover to normal levels quickly after their stress protocol was ended. Technical factors may include the possibility that there was not enough power in our statistical analysis due to low n values, which may have resulted in no statistical differences between groups. Our data did however show trends that are supported by previous data that have found an increase in IL-6 in WD fed/obese mice and humans[208,212,213].

5.3 Diet, Stress and Behavioural Phenotype

Mood disorders are one of the leading causes of burden of disease worldwide, with anxiety and depressive disorders at the forefront of this mental health epidemic. In contemporary society, the presence of underlying chronic stress in everyday life has become more common, with this type of stress linked to increased risk of deleterious cardiovascular outcomes[104,214-216]. Moreover, links between chronic stress promoting obesity and vice versa have been found[116]. The SPT is used to investigate the presence of depressive behaviour (anhedonia) in mice[189]. In our study we saw reduced sucrose consumption in mice fed the WD and the WD + RS animals post-intervention. While reduced sucrose consumption is indicative of anhedonia and a hallmark of depressive behaviour, it is possible that the WD had an impact on sucrose consumption in these mice due to the additional sugar content of the diet. As the WD fed to these mice had a high sugar concentration, the combination of the diet with the sucrose water may have been too sweet for their palette, resulting in these mice having a preference for normal, unsweetened water. Alternatively, the WD may have contained enough sugar in the diet, which negated the mice's need/desire to have sugar from the water. Therefore, the SPT

may not be an accurate behavioural assessment in this type of study where sugar content of the food is elevated above normal values.

The OFT measures anxiety-like behaviours, locomotor activity, and exploratory behaviour in mice. CRS protocols have been widely deployed to induce an anxious or depressed phenotype in laboratory animals[217-220]. In the current study, increased distance travelled and wall seeking behaviour (both regarded as locomotor measures in the OFT) were observed in our mice, along with significant weight loss (due to decreased kJ intake) during the stress protocol. Our findings suggest that 2-hour daily RS was effective in causing mild mood disturbance in our mice, independent of diet. Previously, CS protocols have been found to not only induce anxiety, but also nonspecific behavioural alterations such as hyper- or hypo-locomotion, which can interfere with the interpretation of the behaviour and assessment of anxiousness in the OFT. It has been suggested that hyperlocomotion is triggered in mice exposed to CS under brightly or moderately lit test conditions. The moderate to bright light is considered an acute stressor and can interfere with the evaluation of anxiety-like behaviour in the OFT[221]. This behaviour was found to be abolished with anti-anxiety medication. Ito, et al. [222] investigated the impacts of CRS in C57BL/6J mice. This group found that increased locomotion was a stress response associated with the CRS phenotype. It was suggested that this was due to synaptic plasticity in the anterior cingulate cortex, which is involved in mood disturbances, due to CS reducing presynaptic GABAergic interneuron activity (reducing inhibition). Other studies investigating different types of stressors have also found CS linked to hyperactivity in mice[223]. Ieraci, et al. [224] investigated the effects of social isolation on mice and suggested that hyperlocomotive behaviour could be due to the social impacts of the stressor. These findings from previous studies support the idea that increased locomotion is potentially a stress response resulting from CS exposure in rodents. This data, along with the reduced caloric intake and subsequent weight drop at the induction of the RS protocol, allow

the conclusion that our RS protocol effectively resulted in mild mood disturbance in these mice. These mood disturbances may be due to impairments in the anterior cingulate cortex caused by CS, which should be investigated in future studies.

Previously, a study investigating the effects of low-level restraint stress and/or a WD found that 30 minutes of restraint stress for 21 days induced anxiety, however when this stress protocol was combined with a WD, it induced depression[12]. It is likely these results differ due to the decreased daily RS time and the prolonged duration of the protocol (21 days as opposed to 14). Additionally, the number of days a stress protocol is applied is believed to play a greater role in determining the extent of mood disturbance that manifests (i.e., depression, anxiety) rather than the period of time per day the rodents are exposed to the stress[225]. It has been suggested that inconsistencies in locomotor activity findings in the literature (i.e., some chronically stressed mice presenting with hyperactivity in the OFT, while others are hypolocomotive) may be attributed to CS exposure decreasing depressive and anxious behaviours in mice due to adaptations of the HPA axis to consistent prolonged stress (like the stress protocol undertaken in this current study). However, this adaption has not been found when using unpredictable stress protocols[187]. Furthermore, the aim in this study was not to create an overstated depressed phenotype, but rather expose the mice to subclinical stress to cause mild mood disturbance. Therefore, an unpredictable stress protocol was not suitable for this study due to the increased likelihood of it inducing overt depression.

We found that circulating noradrenaline was reduced by RS. Elevated catecholamines have been well documented to be increased under CS conditions[226]. We suggest that perhaps under predictable low-level chronic stress conditions basal noradrenaline decreases due to sympathetic-adrenal-medullary axis adaption to the long-term stress.

It was also observed that distance travelled decreased in the CD and WD groups when comparing pre- vs. post-intervention results. This is likely an age-related change with Shoji, et

al. [227] finding that age influenced distance travelled in the OFT, with decreases in hyperactivity found in the first 5 minutes of the test as the mice increasingly aged. These findings are supported by several other findings in the literature[228,229].

5.4 Effect of a WD and/or CS on Circulating Melatonin

Melatonin secreted by the pineal gland regulates the circadian rhythm, playing an important role in sleep-wake cycles[230]. Melatonin is a marker of mood disorders, with altered melatonin levels and secretion seen in depression and anxiety[231,232]. Circulating melatonin was unaltered by RS or a WD. Our mouse model did not display behavioural signs of depression or anxiety but did display behavioural changes that are indicative of mood disturbances. The absence of overt changes in melatonin levels are in line with the behavioural changes we documented in our mice. Our stress protocol was likely insufficiently severe to induce changes in melatonin or overt anxiety/depression. Another explanation for the absence of difference in circulating melatonin levels across groups could relate to C57Bl/6J mice being reported to be largely melatonin deficient due to impaired synthesis of the hormone[233,234]. CS has previously been reported to cause melatonin abnormalities, resulting in an increase in circulating melatonin levels[235]. However, this study measured the concentration of melatonin at various times during the day, with only one time point showing a significant difference between control and stressed mice[235]. Cano, et al. [236] investigated the effects of overt obesity on melatonin levels over a 24-hour period, with a significant decrease in melatonin in obese rats found at one timepoint. In addition to our study taking serum for melatonin analysis at different times throughout the day, we did not sample melatonin at several specific timepoints, nor during the dark cycle. This may be a reason we did not see any significant changes in melatonin levels, as both studies only observed a significant difference at one timepoint, which was during the dark phase.

5.5 Impacts of a WD and/or Stress on Myocardial Function, Ischaemic Tolerance, and Response to IPC

5.5.1. Effects of WD and CS on Myocardial Ischemic Tolerance

Reperfusion function was not changed by the WD or RS in this study. There were no significant differences in LDH (cell death marker) or troponin I (myocyte death marker) efflux from the hearts of the four different groups, suggesting there were no differences in cardiac injury between groups. Despite no robust changes in post-ischaemic cardiac outcomes, hearts from the RS (±WD) mice had a trend for improved LVDP recovery, reduced EDP, and lower troponin I release. These trends for improved reperfusion recovery may be due to the weight loss induced by the stress protocol in the final 14 days of the study. RS (±WD) did not promote changes in innate RISK pathway signalling. CS has previously been found to not result in an increase in AKT phosphorylation post I/R in normotensive rodents[237]. This supports our current findings. Despite these trends in the RS (±WD) hearts, the WD group had a trend for decreased myocardial pAKT and significantly decreased pGSK-3β expression. It is expected that these changes in protein expression levels would reduce the ability of pGSK-3β to prevent mPTP opening at reperfusion and hence reduce ischemic tolerance. However, this was not the case with no improvements found in reperfusion function in the WD group. These findings are supported by Wensley, et al. [97] who also found reduced phosphorylation of cytosolic AKT and GSK-3β in obese, insulin insensitive rodents. Oi, et al. [98] found that obesity and insulin resistance caused by a 16-week high fat diet in rats worsened myocardial tolerance to ischemia, with these animals presenting with larger infarct sizes post-ischemia. While both this study and our study had a dietary intervention of approximately the same period of time, it is possible the small differences in the dietary composition in the Oi, et al. [98] study resulted in a more

substantial metabolic dysregulation, resulting in worsened infarction. Conversely, hearts from WD fed mice have also been described to have improved post-I/R function[99]. It is likely that we found no differences in ischaemic tolerance between groups because we did not elicit robust metabolic changes in our mice due to our shorter dietary intervention. Du Toit, et al. [12] investigated the cardiovascular impacts of low-level stress and a WD. This group found a synergistic interaction between the WD and low-level stress, which caused worsened myocardial post-I/R outcomes and higher oncotic death than what either of the interventions induced individually. In the current study, no synergistic effect of a WD and RS was seen. Low-level stress alone wasn't reported to have detrimental impacts on myocardial recovery in Du Toit, et al. [12], although RS mice in our study exhibited myocardial inflammation (discussed later). This data supports what was found in our non-IPC RS hearts. Myocardial intolerance to ischemia has been established to occur in chronically stressed phenotypes, so it is likely that the RS protocol was too mild to induce a detrimental effect, regardless of diet[103]. Rats exposed to chronic unpredictable stress were found to have worsened infarct tolerance when compared with control mice[103,113]. It is possible that our findings differed due to variance in the stress protocols, as the protocols in these two studies were unpredictable in nature. However, studies utilising predictable RS for 11-12 days have also found worsened myocardial tolerance to ischemia, with these hearts presenting with larger infarct sizes[113]. This group may have observed worsened post-ischemic outcomes because they subjected the hearts to an ischaemic event the day after ceasing the stress protocol. In our study the RS protocol was stopped four days prior to exposure to I/R which may have allowed the stress response to wane before the hearts were subjected to the ischaemic insult.

5.5.2. Effects of WD and CS on Myocardial Response to IPC

IPC and myocardial reperfusion function: IPC is an experimental, therapeutic technique aimed to reduce the infarct size and improve myocardial tolerance to prolonged I/R.

IPC protocols utilise several brief cycles of ischemia followed by reperfusion to stimulate endogenous cardioprotective kinases before the major, lethal ischemic event. While experimental IPC is one of the most effective interventions to reduce myocardial damage and death post I/R, these findings have not translated well into clinical practice. IPC has been found to be impaired when used in clinical practice, potentially due to underlining health conditions and comorbidities in these patients[238-241]. In the current study, the hearts from animals fed the WD were not responsive to IPC when compared to the CD group, presenting with reduced LVDP recoveries and coronary flows. There were however no differences in coronary effluent cell death markers (LDH, troponin), myocardial inflammatory markers (IL-6 and TNF-α) or the expression of RISK pathway proteins between IPC groups. The WD hearts had a trend towards a reduced AKT phosphorylation, which may suggest that RISK pathway downregulation or dysfunction made the hearts of these animals unresponsive to IPC. The detrimental impact insulin resistance has on myocardial tolerance to I/R and responsiveness to IPC has previously been documented, with it being suggested to be the primary cause of impaired ischemic tolerance in obesity[97-99,185,200]. The overt insulin resistance in the WD group may have resulted in this insensitivity to IPC and the impairment of reperfusion functional recovery, with RS playing a protective role to prevent worsened functional outcomes in the WD + RS IPC hearts. Obese, insulin insensitive rats have previously been reported to abolish the cardioprotective effects of pre-conditioning, resulting in larger infarct sizes in these hearts compared to metabolically healthy mice[99,185]. These findings support our results.

The impact of diet on differences in myocardial reperfusion function in non-IPC vs. IPC hearts: The CD and WD hearts subjected to IPC had improved LVDP recovery and reduced EDP during reperfusion, compared to their respective non-IPC groups. The CD IPC group also displayed increased reperfusion coronary flow. These hearts from the CD and WD

fed animals were responsive to classical IPC stimuli and had improved reperfusion function. Despite these improvements in function, no significant differences were seen in troponin or LDH release into coronary effluent, indicating no differences in myocardial injury between the groups. There was however a trend for reduced troponin-I release (indicating less cardiac injury) in the CD IPC group compared to the non-IPC CD group, which supports the improved functional recoveries seen in this group. Collectively, this data indicates that the CD and WD hearts were more responsive to myocardial ischemic pre-conditioning stimuli, and therefore suffered less injury post-ischemia. Our findings will be discussed further in relation to other studies below.

IPC, Diet, and the RISK pathway: IPC resulted in an increase in total AKT levels in CD and WD hears - an effect eliminated by RS. However, IPC failed to increase post-ischaemic AKT phosphorylation in these hearts, resulting only in trends for reduced pAKT/tAKT ratios. It is well documented that IPC increases AKT phosphorylation and thereby induces cardioprotection through RISK pathway activation[242,243]. Donner, et al. [99] reported that pre-conditioning was abolished by obese, insulin resistant rats. They however described no differences in cytosolic pAKT or pGSK-3β levels when comparing WD non-conditioned with WD pre-conditioned hearts. Nonetheless, conditioning increased pERK levels in WD rats (compared to non-conditioned WD)[99]. We found a trend in increased pGSK-3β in the WD IPC hearts, despite no changes in the phosphorylated to total ratio. Contrary to Donner, et al. [99], our WD IPC hearts had improved recovery (compared to non-IPC WD hearts), and we did not find any differences in ERK 1/2 phosphorylation in the WD IPC group. The rats in that paper were fed an obesogenic diet for 32 weeks, whereas our mice were exposed to the WD for half that period of time. It is therefore possible that our mice were less insulin resistant, with the degree of insulin insensitivity an important determinant in how well the heart responds to IPC to

increase ischemic tolerance. This relatively modest insulin resistance may have resulted in the hearts being responsive to IPC when compared to the non-IPC WD group, however it not being effective in increasing ischemic tolerance to the same extent as the healthy CD IPC hearts. LDH release into coronary effluent was reduced in CD hearts conditioned via δ-opioid receptor agonist, however the δ-opioid receptor agonist had no effect on LDH efflux in obese, insulin insensitive rats[99]. In the current study, the WD alone did not have differences in LDH, however it was elevated in the WD + RS hearts, indicating that IPC was ineffective in preventing oncosis.

The impact of CS on myocardial reperfusion function and RISK pathway expression in IPC hearts, and their comparison to non-IPC CS hearts: IPC did not improve contractile function or reduce cell death in the RS groups, indicating that RS made the hearts refractory to IPC, without altering baseline I/R tolerance. The WD + RS hearts subjected to IPC also had higher levels of LDH release compared to the non-IPC WD + RS hearts, indicating that IPC may worsen tolerance to I/R in conditions where a WD and stress are comorbidities. However, IPC did reduce myocardial inflammation (TNF-α) post-I/R in these groups, which follows trends of increased LVDP recovery and reduction of EDP in the RS groups, along with improved coronary flow in the WD + RS IPC group. Additionally, the WD + RS hearts exposed to IPC had increased phospho-inhibition of GSK-3β (despite showing no significant differences in the pGSK-3β to tGSK-3β ratio). The WD and RS did not have an additive effect on cardiac functional recovery in the IPC hearts. These findings suggest that, regardless of diet, chronic subclinical stress may impair myocardial contractile function after exposure to I/R (as suggested by the impaired reperfusion recovery and increased oncosis seen in these hearts). However, not all cardioprotective effects of IPC are abolished (reduced inflammation, greater phosphorylation of GSK-3β). These functional impairments are likely due to intracellular

calcium accumulation in cardiomyocytes. Unresponsiveness to IPC in RS hearts could also reflect reduced myocardial AKT upregulation. To elaborate, RS IPC hearts had no difference in total AKT expression levels when comparing IPC vs. non-IPC hearts, unlike the non-stressed hearts (CD and WD). While the pAKT/tAKT ratio is unchanged in the non-stressed IPC groups, these hearts had significantly increased total AKT. Meaning, although there were no significant differences in phosphorylated to total AKT ratios in any intervention group, there was a greater amount of total AKT in the non-stressed IPC groups that could then be phosphorylated during RISK pathway signalling. Hence, a smaller AKT pool with no changes in phosphorylation may have contributed to impaired responsiveness to IPC in the RS hearts, and therefore resulted in impaired functional recovery and increased cell death. The efficacy of IPC has not been well defined in models of CS, with no known studies directly investigating its relationship. However, one article has investigated the cardiovascular impacts of ischemic post-conditioning in a CMS-induced depression model[175]. This study found that non-stressed mice exposed to IPC had higher levels of pAKT than the control and stressed (depressed) mice, and that IPC was ineffective in improving functional recovery and reducing troponin release from hearts of depressed animals. The absence of increased phosphorylation of AKT in our RS mice and the diminished protective effect of the conditioning response are supported by this data. Our data indicate that these changes may occur during low-level stress, and a full depressive phenotype is not necessary to induce these pathological changes.

IPC and myocardial inflammation: An inflammatory response is triggered after a myocardial infarction, with increases in many pro-inflammatory cytokines including TNF-α and IL-6. When there is chronic systemic inflammation (i.e., due to stress, metabolic syndrome, infection, or other disease), this myocardial inflammatory response is often prolonged and exaggerated, resulting in pathological remodelling due to cytokine promotion of impaired

contractility (through impairing calcium release/sensitivity) and myocyte apoptosis[244,245]. Myocardial TNF-α and IL-6 levels are increased in models exposed to CS, as seen in this study[105]. IPC has previously been found to be effective in reducing post-ischaemic myocardial TNF-α levels[246]. In this study, we found that IPC reduced post-ischemic inflammation as reflected by myocardial cytokine levels in the RS hearts. Although the LVDP and EDP data for the RS hearts showed no significant changes, the RS (±WD) hearts exposed to IPC followed a similar pattern of improved reperfusion recovery, with trends matching the improved post-ischemic TNF-α data. Inflammation is a key mechanism implicated in stress related mood disorder development and progression[247]. However, the pro-inflammatory effect of CS does not appear to be overly relevant in the heart, with IPC able to reduce myocardial inflammation in RS mice. In addition, there was a marginal reduction in TNF-α levels in CD and WD IPC mice, compared to non-IPC, which may have contributed to improved functional recovery in these IPC hearts. Previously, myocardial TNF-α levels have been reported to be unchanged post-I/R in WD, stressed, and the combination of WD and stressed mice[12]. These results are unlike the ones found in the study and may differ because the restraint stress protocol in those mice was shorter per day and longer in length than this protocol.

5.6 Concluding Remarks

The present study indicates that a WD was effective in promoting the development of obesity and metabolic dysregulation, while also inducing mild systemic inflammation. These changes were confirmed by the body weight, insulin, glucose, and HOMA-IR data, with subtle shifts in circulating cytokines (IL-6). Exposure to chronic low-level stress caused mild mood disturbance in our mice, which was exhibited by significant weight loss and hyperlocomotion. This stress protocol was however likely too mild to elicit pronounced MDD or anxiety. Intrinsic tolerance to myocardial I/R was essentially unaltered by these interventions, although trends

suggest that RS may have improved myocardial functional recovery, despite evidence for increased post-ischaemic myocardial inflammation. While consumption of a WD impaired IPC responses compared to the CD group, the WD hearts remained responsive to pre-conditioning, with improved functional outcomes found in the WD IPC group vs. the WD non-IPC group. In contrast, RS negated the protective effects of IPC on cardiac function, despite reducing myocardial inflammation and increasing levels of pGSK-3β (albeit no changes in phosphorylated to total GSK-3β ratio). This impairment of IPC responses with chronic stress was associated with reduced AKT expression, although relative kinase phosphorylation was not improved. Nonetheless, a smaller AKT pool (with similar phosphorylation status) will limit protective signalling. If the impact of stress on IPC is similar in humans, it could assist in explaining why clinical cardioprotection remains a challenge in patients with heart diseases that are often linked to chronic stress and MDD (among other co-morbidities).

Limitations for this study include the exclusive use of male mice, given evidence of sexual dimorphisms in cardiovascular and metabolic systems and disease development, along with differences in responses to stress[248-252]. Future work would be directed at examining potentially distinct response in males and females. A second limitation relates to some groups having small n values due to the loss of hearts during Langendorff perfusion, which may have impacted statistical power. Further time and financial support would permit more extensive analysis of this nature (which is both time and funding intensive). In addition, there was a four day gap between the last exposure to RS and the day of sacrifice, potentially resulting in some of the effects of RS waning before the hearts underwent Langendorff perfusions. However, this gap was unavoidable due to necessary behavioural testing and blood collection prior to animal sacrifice, which could not be impacted by running the stress protocol at the same time.

6.0 Reference List

1. Khan, M.A. et al. Global Epidemiology of Ischemic Heart Disease: Results from the Global Burden of Disease Study. *Cureus* **12**, e9349 (2020).
2. World Health Statistics 2021: Monitoring Health for the SDGs. (World Health Organisation, Geneva, 2021).
3. Saklayen, M.G. The Global Epidemic of the Metabolic Syndrome. *Current hypertension reports* **20**, 1-8 (2018).
4. Slade, T., Johnston, A., Oakley Browne, M.A., Andrews, G. & Whiteford, H. 2007 National Survey of Mental Health and Wellbeing: methods and key findings. *Aust N Z J Psychiatry* **43**, 594-605 (2009).
5. Kessler, R.C. et al. Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry* **62**, 593-602 (2005).
6. Nemati, M., Zardooz, H., Rostamkhani, F., Abadi, A. & Foroughi, F. High-fat diet effects on metabolic responses to chronic stress. *Archives of Physiology and Biochemistry* **123**, 182-191 (2017).
7. Patist, C.M., Stapelberg, N.J.C., Du Toit, E.F. & Headrick, J.P. The brain-adipocyte-gut network: Linking obesity and depression subtypes. *Cognitive, Affective, & Behavioral Neuroscience* **18**, 1121-1144 (2018).
8. Nicholson, K. et al. Multimorbidity and comorbidity revisited: refining the concepts for international health research. *Journal of clinical epidemiology* **105**, 142-146 (2019).
9. Navickas, R., Petric, V.-K., Feigl, A.B. & Seychell, M. Multimorbidity: What Do We Know? What Should We Do? *Journal of Comorbidity* **6**, 4-11 (2016).
10. Blakely, T., Kvizhinadze, G., Atkinson, J., Dieleman, J. & Clarke, P. Health system costs for individual and comorbid noncommunicable diseases: An analysis of publicly funded health events from New Zealand. *PLOS Medicine* **16**, e1002716 (2019).
11. Hall, M. et al. Multimorbidity and survival for patients with acute myocardial infarction in England and Wales: Latent class analysis of a nationwide population-based cohort. *PLoS Medicine* **15**, e1002501 (2018).
12. Du Toit, E.F. et al. Synergistic effects of low-level stress and a Western diet on metabolic homeostasis, mood, and myocardial ischemic tolerance. *Am J Physiol Regul Integr Comp Physiol* **319**, R347-r357 (2020).
13. Headrick, J.P. et al. The heartbreak of depression: 'Psycho-cardiac' coupling in myocardial infarction. *Journal of Molecular and Cellular Cardiology* **106**, 14-28 (2017).
14. Tune, J.D., Goodwill, A.G., Sassoon, D.J. & Mather, K.J. Cardiovascular consequences of metabolic syndrome. *Translational research : the journal of laboratory and clinical medicine* **183**, 57-70 (2017).
15. Haby, M.M., Markwick, A., Peeters, A., Shaw, J. & Vos, T. Future predictions of body mass index and overweight prevalence in Australia, 2005-2025. *HEALTH PROMOTION INTERNATIONAL* **27**, 250-260 (2012).
16. Ranasinghe, P., Mathangasinghe, Y., Jayawardena, R., Hills, A.P. & Misra, A. Prevalence and trends of metabolic syndrome among adults in the asia-pacific region: a systematic review. *BMC Public Health* **17**, 101 (2017).
17. Noubiap, J.J. et al. Global, regional, and country estimates of metabolic syndrome burden in children and adolescents in 2020: a systematic review and modelling analysis. *The Lancet Child & Adolescent Health* (2022).

18. Lee, J. et al. Should central obesity be an optional or essential component of the metabolic syndrome? Ischemic heart disease risk in the Singapore cardiovascular cohort study. *Diabetes Care* **30**, 343-347 (2007).
19. Swarup, S. & Zeltser, R. *Metabolic Syndrome*, (StatPearls Publishing, Treasure Island (FL), 2019).
20. Wagh, A. & Stone, N.J. Treatment of metabolic syndrome. *Expert Review of Cardiovascular Therapy* **2**, 213-228 (2004).
21. Eckel, R.H., Grundy, S.M. & Zimmet, P.Z. The metabolic syndrome. *The Lancet (British edition)* **365**, 1415-1428 (2005).
22. Huang, P.L. A comprehensive definition for metabolic syndrome. *Disease models & mechanisms* **2**, 231-237 (2009).
23. Redon, J. et al. Mechanisms of hypertension in the cardiometabolic syndrome. *Journal of hypertension* **27**, 441-451 (2009).
24. Hansen, L. et al. Metabolically Healthy Obesity and Ischemic Heart Disease: A 10-Year Follow-Up of the Inter99 Study. *The Journal of Clinical Endocrinology & Metabolism* **102**, 1934-1942 (2017).
25. Lassale, C. et al. Separate and combined associations of obesity and metabolic health with coronary heart disease : A pan-European case-cohort analysis. *European Heart Journal* **39**, 397-406 (2018).
26. Thomsen, M. & Nordestgaard, B.G. Myocardial Infarction and Ischemic Heart Disease in Overweight and Obesity With and Without Metabolic Syndrome. *JAMA Internal Medicine* **174**, 15-22 (2014).
27. Ninomiya, J.K. et al. Association of the Metabolic Syndrome With History of Myocardial Infarction and Stroke in the Third National Health and Nutrition Examination Survey. *Circulation* **109**, 42-46 (2004).
28. Van Gaal, L.F., Mertens, I.L. & De Block, C.E. Mechanisms linking obesity with cardiovascular disease. *Nature* **444**, 875-880 (2006).
29. Arita, Y. et al. Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. *Biochemical and biophysical research communications* **257**, 79-83 (1999).
30. Achari, A.E., Jain, S.K., Sushil, J. & Arunkumar, A. Adiponectin, a therapeutic target for obesity, diabetes, and endothelial dysfunction. *International journal of molecular sciences* **18**, 1321 (2017).
31. Martín-Timón, I., Sevillano-Collantes, C., Segura-Galindo, A. & Del Cañizo-Gómez, F.J. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World journal of diabetes* **5**, 444-470 (2014).
32. Zanni, M.V., Stanley, T.L., Makimura, H., Chen, C.Y. & Grinspoon, S.K. Effects of TNF-alpha antagonism on E-selectin in obese subjects with metabolic dysregulation. *Clinical endocrinology (Oxford)* **73**, 48-54 (2010).
33. Wyne, K.L. Free fatty acids and type 2 diabetes mellitus. *The American Journal of Medicine* **115**, 29-36 (2003).
34. Lupi, R. et al. Prolonged Exposure to Free Fatty Acids Has Cytostatic and Pro-Apoptotic Effects on Human Pancreatic Islets. *Diabetes* **51**, 1437 (2002).
35. Cnop, M., Hannaert, J.C., Hoorens, A., Eizirik, D.L. & Pipeleers, D.G. Inverse Relationship Between Cytotoxicity of Free Fatty Acids in Pancreatic Islet Cells and Cellular Triglyceride Accumulation. *Diabetes* **50**, 1771 (2001).
36. Bollheimer, L.C., Skelly, R.H., Chester, M.W., McGarry, J.D. & Rhodes, C.J. Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *The Journal of clinical investigation* **101**, 1094-1101 (1998).

37. Kwiterovich, P.O., Jr. Clinical relevance of the biochemical, metabolic, and genetic factors that influence low-density lipoprotein heterogeneity. *Am J Cardiol* **90**, 30i-47i (2002).
38. Kunjathoor, V.V. et al. Scavenger Receptors Class A-I/II and CD36 Are the Principal Receptors Responsible for the Uptake of Modified Low Density Lipoprotein Leading to Lipid Loading in Macrophages*. *Journal of Biological Chemistry* **277**, 49982-49988 (2002).
39. Libby, P., Ridker, P.M. & Maseri, A. Inflammation and atherosclerosis. *Circulation (New York, N.Y.)* **105**, 1135-1143 (2002).
40. Berg, A.H. & Scherer, P.E. Adipose Tissue, Inflammation, and Cardiovascular Disease. *Circulation research* **96**, 939-949 (2005).
41. Sattar, N. et al. Elevated Alanine Aminotransferase Predicts New-Onset Type 2 Diabetes Independently of Classical Risk Factors, Metabolic Syndrome, and C-Reactive Protein in the West of Scotland Coronary Prevention Study. *Diabetes* **53**, 2855 (2004).
42. David, C.W.L., Dhillon, B., Yan, H., Szmitko, P.E. & Verma, S. Adipokines: molecular links between obesity and atheroslcerosis. *American Journal of Physiology - Heart and Circulatory Physiology* **288**, 2031-2041 (2005).
43. Matsuzawa, Y. The metabolic syndrome and adipocytokines. *FEBS letters* **580**, 2917-2921 (2006).
44. Barazzoni, R. et al. Relationships between Desacylated and Acylated Ghrelin and Insulin Sensitivity in the Metabolic Syndrome. *The Journal of Clinical Endocrinology & Metabolism* **92**, 3935-3940 (2007).
45. Tschöp, M. et al. Circulating Ghrelin Levels Are Decreased in Human Obesity. *Diabetes* **50**, 707 (2001).
46. Olavi, U. Ghrelin and Metabolic Disorders. *Current Protein & Peptide Science* **10**, 2-7 (2009).
47. Qader, S.S., Lundquist, I., Ekelund, M., Håkanson, R. & Salehi, A. Ghrelin activates neuronal constitutive nitric oxide synthase in pancreatic islet cells while inhibiting insulin release and stimulating glucagon release. *Regulatory peptides* **128**, 51-56 (2005).
48. Asakawa, A. et al. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* **120**, 337-345 (2001).
49. Chang, L. et al. Protective Effects of Ghrelin on Ischemia/Reperfusion Injury in the Isolated Rat Heart. *Journal of Cardiovascular Pharmacology* **43**(2004).
50. Gnanapavan, S. et al. The Tissue Distribution of the mRNA of Ghrelin and Subtypes of Its Receptor, GHS-R, in Humans. *The Journal of Clinical Endocrinology & Metabolism* **87**, 2988-2991 (2002).
51. Frascarelli, S., Ghelardoni, S., Ronca-Testoni, S. & Zucchi, R. Effect of ghrelin and syntheticgrowth hormone secretagogues in normal and ischemic ratheart. *Basic Research in Cardiology* **98**, 401-405 (2003).
52. Pulkkinen, L., Ukkola, O., Kolehmainen, M. & Uusitupa, M. Ghrelin in Diabetes and Metabolic Syndrome. *International Journal of Peptides* **2010**, 248948 (2010).
53. Tesauro, M. et al. Ghrelin Improves Endothelial Function in Patients With Metabolic Syndrome. *Circulation* **112**, 2986-2992 (2005).
54. Li, W.G. et al. Ghrelin Inhibits Proinflammatory Responses and Nuclear Factor-κB Activation in Human Endothelial Cells. *Circulation* **109**, 2221-2226 (2004).
55. Considine, R.V. et al. Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans. *New England Journal of Medicine* **334**, 292-295 (1996).

56. Li, W.-C. et al. Serum leptin is associated with cardiometabolic risk and predicts metabolic syndrome in Taiwanese adults. *Cardiovascular Diabetology* **10**, 36 (2011).
57. Valle, M. et al. Relationship between high plasma leptin concentrations and metabolic syndrome in obese pre-pubertal children. *International Journal of Obesity* **27**, 13-18 (2003).
58. Tsai, J.P. et al. Hyperleptinaemia positively correlated with metabolic syndrome in hemodialysis patients. *Eur J Intern Med* **22**, e105-9 (2011).
59. Barateiro, A., Mahú, I. & Domingos, A.I. Leptin Resistance and the Neuro-Adipose Connection. *Frontiers in Endocrinology* **8**(2017).
60. Mariella, T. et al. Leptin and Vascular Smooth Muscle Cells. *Current Pharmaceutical Design* **20**, 625-634 (2014).
61. Konstantinides, S., Schäfer, K., Koschnick, S. & Loskutoff, D.J. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. *The Journal of clinical investigation* **108**, 1533-1540 (2001).
62. Parhami, F., Tintut, Y., Ballard, A., Fogelman, A.M. & Demer, L.L. Leptin Enhances the Calcification of Vascular Cells: Artery Wall as a Target of Leptin. *Circulation research* **88**, 954-960 (2001).
63. Oda, A., Taniguchi, T. & Yokoyama, M. Leptin stimulates rat aortic smooth muscle cell proliferation and migration. *Kobe journal of the medical sciences* **47**, 141-150 (2001).
64. Izquierdo, A.G., Crujeiras, A.B., Casanueva, F.F. & Carreira, M.C. Leptin, Obesity, and Leptin Resistance: Where Are We 25 Years Later? *Nutrients* **11**, 2704 (2019).
65. Münzberg, H., Flier, J.S. & Bjørbæk, C. Region-Specific Leptin Resistance within the Hypothalamus of Diet-Induced Obese Mice. *Endocrinology* **145**, 4880-4889 (2004).
66. Schillaci, G. et al. Prognostic value of the metabolic syndrome in essential hypertension. *J Am Coll Cardiol* **43**, 1817-22 (2004).
67. Mattsson, C., Reynolds, R.M., Simonyte, K., Olsson, T. & Walker, B.R. Combined receptor antagonist stimulation of the hypothalamic-pituitary-adrenal axis test identifies impaired negative feedback sensitivity to cortisol in obese men. *The Journal of Clinical Endocrinology & Metabolism* **94**, 1347-1352 (2009).
68. Reynolds, R.M. et al. Altered control of cortisol secretion in adult men with low birth weight and cardiovascular risk factors. *The Journal of Clinical Endocrinology & Metabolism* **86**, 245-250 (2001).
69. Stolk, R., Lamberts, S., De Jong, F., Pols, H. & Grobbee, D. Gender differences in the associations between cortisol and insulin in healthy subjects. *Journal of endocrinology* **149**, 313-318 (1996).
70. Solano, M., Kumar, M., Fernandez, B., Jones, L. & Goldberg, R. The pituitary response to ovine corticotropin-releasing hormone is enhanced in obese men and correlates with insulin resistance. *Hormone and Metabolic Research* **33**, 39-43 (2001).
71. Lee, J. et al. Interaction of IL-6 and TNF-α contributes to endothelial dysfunction in type 2 diabetic mouse hearts. *PLOS ONE* **12**, e0187189 (2017).
72. Eikelis, N., Schlaich, M., Aggarwal, A., Kaye, D. & Esler, M. Interactions between leptin and the human sympathetic nervous system. *Hypertension* **41**, 1072-9 (2003).
73. Cavalera, M., Wang, J. & Frangogiannis, N.G. Obesity, metabolic dysfunction, and cardiac fibrosis: pathophysiological pathways, molecular mechanisms, and therapeutic opportunities. *Translational research : the journal of laboratory and clinical medicine* **164**, 323-335 (2014).

74. Ferron, A.J.T. et al. Association between Cardiac Remodeling and Metabolic Alteration in an Experimental Model of Obesity Induced by Western Diet. *Nutrients* **10**, 1675 (2018).
75. Stahrenberg, R. et al. Association of glucose metabolism with diastolic function along the diabetic continuum (vol 53, pg 1331, 2010). *DIABETOLOGIA* **54**, 990-990 (2011).
76. Cook, S.A. et al. Abnormal myocardial insulin signalling in type 2 diabetes and left-ventricular dysfunction. *European heart journal* **31**, 100-111 (2010).
77. Battiprolu, P.K. et al. Metabolic stress-induced activation of FoxO1 triggers diabetic cardiomyopathy in mice. *J Clin Invest* **122**, 1109-18 (2012).
78. Lee, J. et al. Multiple abnormalities of myocardial insulin signaling in a porcine model of diet-induced obesity. *Am J Physiol Heart Circ Physiol* **298**, H310-9 (2010).
79. Abel, E.D., O'Shea, K.M. & Ramasamy, R. Insulin Resistance: Metabolic Mechanisms and Consequences in the Heart. *Arteriosclerosis, Thrombosis, and Vascular Biology* **32**, 2068-2076 (2012).
80. Sena, S. et al. Impaired insulin signaling accelerates cardiac mitochondrial dysfunction after myocardial infarction. *J Mol Cell Cardiol* **46**, 910-8 (2009).
81. Boudina, S. et al. Contribution of Impaired Myocardial Insulin Signaling to Mitochondrial Dysfunction and Oxidative Stress in the Heart. *Circulation* **119**, 1272-1283 (2009).
82. Hu, P. et al. Minimally invasive aortic banding in mice: effects of altered cardiomyocyte insulin signaling during pressure overload. *Am J Physiol Heart Circ Physiol* **285**, H1261-9 (2003).
83. McQueen, A.P. et al. Contractile dysfunction in hypertrophied hearts with deficient insulin receptor signaling: possible role of reduced capillary density. *J Mol Cell Cardiol* **39**, 882-92 (2005).
84. Gray, S. & Kim, J.K. New insights into insulin resistance in the diabetic heart. *Trends Endocrinol Metab* **22**, 394-403 (2011).
85. Carley, A.N. & Severson, D.L. Fatty acid metabolism is enhanced in type 2 diabetic hearts. *Biochim Biophys Acta* **1734**, 112-26 (2005).
86. Dirkx, E., Schwenk, R.W., Glatz, J.F., Luiken, J.J. & van Eys, G.J. High fat diet induced diabetic cardiomyopathy. *Prostaglandins Leukot Essent Fatty Acids* **85**, 219-25 (2011).
87. Li, Z.-L. et al. Transition From Obesity to Metabolic Syndrome Is Associated With Altered Myocardial Autophagy and Apoptosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **32**, 1132-1141 (2012).
88. Fontes-Carvalho, R., Ladeiras-Lopes, R., Bettencourt, P., Leite-Moreira, A. & Azevedo, A. Diastolic dysfunction in the diabetic continuum: Association with insulin resistance, metabolic syndrome and type 2 diabetes. *Cardiovascular Diabetology* **14**, 4 (2015).
89. Ferrara, L.A., Cardoni, O., Mancini, M., Zanchetti, A. & on behalf of the Gubbio Study Research, G. Metabolic syndrome and left ventricular hypertrophy in a general population. Results from the Gubbio Study. *Journal of Human Hypertension* **21**, 795-801 (2007).
90. Guerra, F. et al. The association of left ventricular hypertrophy with metabolic syndrome is dependent on body mass index in hypertensive overweight or obese patients. *PloS one* **6**, e16630-e16630 (2011).
91. Ahn, M.-S. et al. Cardiovascular parameters correlated with metabolic syndrome in a rural community cohort of Korea: the ARIRANG study. *Journal of Korean medical science* **25**, 1045-1052 (2010).

92. van de Weijer, T., Schrauwen-Hinderling, V.B. & Schrauwen, P. Lipotoxicity in type 2 diabetic cardiomyopathy. *Cardiovascular Research* **92**, 10-18 (2011).
93. Magnani, J.W. et al. P Wave Indices, Obesity, and the Metabolic Syndrome: The Atherosclerosis Risk in Communities Study. *Obesity* **20**, 666-672 (2012).
94. Abed, H.S. et al. Obesity results in progressive atrial structural and electrical remodeling: Implications for atrial fibrillation. *Heart Rhythm* **10**, 90-100 (2013).
95. Pathak, R.K., Mahajan, R., Lau, D.H. & Sanders, P. The Implications of Obesity for Cardiac Arrhythmia Mechanisms and Management. *Canadian Journal of Cardiology* **31**, 203-210 (2015).
96. Gonçalves, N. et al. Early cardiac changes induced by a hypercaloric Western-type diet in "subclinical" obesity. *American Journal of Physiology - Heart and Circulatory Physiology* **310**, H655-H666 (2016).
97. Wensley, I., Salaveria, K., Bulmer, A.C., Donner, D.G. & du Toit, E.F. Myocardial structure, function and ischaemic tolerance in a rodent model of obesity with insulin resistance. *Experimental Physiology* **98**, 1552-1564 (2013).
98. Oi, M. et al. Pravastatin improves risk factors but not ischaemic tolerance in obese rats. *European Journal of Pharmacology* **826**, 148-157 (2018).
99. Donner, D., Headrick, J.P., Peart, J.N. & Du Toit, E.F. Obesity improves myocardial ischaemic tolerance and RISK signalling in insulin-insensitive rats. *DMM Disease Models and Mechanisms* **6**, 457-466 (2013).
100. Charlson, F.J. et al. The contribution of major depression to the global burden of ischemic heart disease: A comparative risk assessment. *BMC Medicine* **11**, 250-250 (2013).
101. Wu, Q. & Kling, J.M. Depression and the Risk of Myocardial Infarction and Coronary Death A Meta-Analysis of Prospective Cohort Studies. *MEDICINE* **95**, 1-9 (2016).
102. Yusuf, S. et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *The Lancet* **364**, 937-952 (2004).
103. Mercanoglu, G., Safran, N., Uzun, H. & Eroglu, L. Chronic emotional stress exposure increases infarct size in rats: The role of oxidative and nitrosative damage in response to sympathetic hyperactivity. *Methods and Findings in Experimental and Clinical Pharmacology* **30**, 745-752 (2008).
104. Moran, K.E., Ommerborn, M.J., Blackshear, C.T., Sims, M. & Clark, C.R. Financial Stress and Risk of Coronary Heart Disease in the Jackson Heart Study. *American Journal of Preventive Medicine* **56**, 224-231 (2019).
105. Wang, R.P. et al. Toll-like receptor 4/nuclear factor-kappa B pathway is involved in myocardial injury in a rat chronic stress model. *Stress* **14**, 567-75 (2011).
106. Xinxing, W. et al. A neuroendocrine mechanism of co-morbidity of depression-like behavior and myocardial injury in rats. *PLoS ONE* **9**, e88427 (2014).
107. Turdi, S., Yuan, M., Leedy, G.M., Wu, Z. & Ren, J. Chronic social stress induces cardiomyocyte contractile dysfunction and intracellular Ca 2+ derangement in rats. *Physiology and Behavior* **105**, 498-509 (2012).
108. Wang, Y., Zhang, H., Chai, F., Liu, X. & Berk, M. The effects of escitalopram on myocardial apoptosis and the expression of Bax and Bcl-2 during myocardial ischemia/reperfusion in a model of rats with depression. *BMC Psychiatry* **14**, 349 (2014).
109. Wang, Y., Xiao, Z., Liu, X. & Berk, M. Venlafaxine modulates depression-induced behaviour and the expression of Bax mRNA and Bcl-xl mRNA in both hippocampus

and myocardium. *Human Psychopharmacology: Clinical and Experimental* **26**, 95-101 (2011).
110. Ravingerová, T. et al. Impaired cardiac ischemic tolerance in spontaneously hypertensive rats is attenuated by adaptation to chronic and acute stress. *Experimental and Clinical Cardiology* **16**, e23-e29 (2011).
111. Rorabaugh, B.R. et al. Sex-dependent effects of chronic psychosocial stress on myocardial sensitivity to ischemic injury. *Stress* **18**, 645-53 (2015).
112. Eisenmann, E.D., Rorabaugh, B.R. & Zoladz, P.R. Acute Stress Decreases but Chronic Stress Increases Myocardial Sensitivity to Ischemic Injury in Rodents. *Frontiers in psychiatry* **7**, 71-71 (2016).
113. Scheuer, D.A. & Mifflin, S.W. Repeated intermittent stress exacerbates myocardial ischemia-reperfusion injury. *Am J Physiol* **274**, R470-5 (1998).
114. Sheng, X. et al. Cardioprotective effects of low-level carotid baroreceptor stimulation against myocardial ischemia-reperfusion injury in canine model. Vol. 45 131-140 (Springer US, New York, 2016).
115. Sher, L.D. et al. Chronic stress and endothelial dysfunction: mechanisms, experimental challenges, and the way ahead. *American Journal of Physiology-Heart and Circulatory Physiology* **319**, H488-H506 (2020).
116. Tomiyama, A.J. Stress and Obesity. *Annual Review of Psychology* **70**, 703-718 (2019).
117. Strekalova, T., Spanagel, R., Bartsch, D., Henn, F.A. & Gass, P. Stress-Induced Anhedonia in Mice is Associated with Deficits in Forced Swimming and Exploration. *Neuropsychopharmacology* **29**, 2007-2017 (2004).
118. Carney, R.M. et al. Association of depression witk reduced heart rate variability in coronary artery disease. *The American Journal of Cardiology* **76**, 562-564 (1995).
119. Grippo, A.J., Moffitt, J.A. & Johnson, A.K. Cardiovascular alterations and autonomic imbalance in an experimental model of depression. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **282**, 1333-1341 (2002).
120. Tachibana, H., Naga Prasad, S.V., Lefkowitz, R.J., Koch, W.J. & Rockman, H.A. Level of β-adrenergic receptor kinase 1 inhibition determines degree of cardiac dysfunction after chronic pressure overload-induced heart failure. Vol. 111 591-597 (LIPPINCOTT WILLIAMS & WILKINS, PHILADELPHIA, 2005).
121. Matsuura, N. et al. Restraint stress exacerbates cardiac and adipose tissue pathology via β-adrenergic signaling in rats with metabolic syndrome. *American Journal of Physiology - Heart and Circulatory Physiology* **308**, H1275-H1286 (2015).
122. Wang, Y. et al. β1-adrenoceptor stimulation promotes LPS-induced cardiomyocyte apoptosis through activating PKA and enhancing CaMKII and IκBα phosphorylation. Vol. 19 76 (BioMed Central, England, 2015).
123. Hu, A. et al. Chronic β-adrenergic receptor stimulation induces cardiac apoptosis and aggravates myocardial ischemia/reperfusion injury by provoking inducible nitric-oxide synthase-mediated nitrative stress. Vol. 318 469-475 (AMER SOC PHARMACOLOGY EXPERIMENTAL THERAPEUTICS, BETHESDA, 2006).
124. Colucci, W.S., Sawyer, D.B., Singh, K. & Communal, C. Adrenergic overload and apoptosis in heart failure: Implications for therapy. Vol. 6 1-7 (W B SAUNDERS CO, PHILADELPHIA, 2000).
125. Remondino, A. et al. β-Adrenergic Receptor–Stimulated Apoptosis in Cardiac Myocytes Is Mediated by Reactive Oxygen Species/c-Jun NH2-Terminal Kinase–Dependent Activation of the Mitochondrial Pathway. Vol. 92 136-138 (American Heart Association, Inc, PHILADELPHIA, 2003).

126. Anisman, H. & Merali, Z. Cytokines, stress and depressive illness: brain-immune interactions. *Ann Med* **35**, 2-11 (2003).
127. Johnson, R.W., Kelley, K.W., O'Connor, J.C., Dantzer, R. & Freund, G.G. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature Reviews Neuroscience* **9**, 46-56 (2008).
128. Leff, P. et al. The Interacting Neuroendocrine Network in Stress-Inducing Mood Disorders. *The Open Neuroendocrinology Journal* **3**(2010).
129. Dantzer, R.D.V.M.P. Cytokine, Sickness Behavior, and Depression. *Immunology and Allergy Clinics of North America* **29**, 247-264 (2009).
130. Shao, M. et al. Depression and cardiovascular disease: Shared molecular mechanisms and clinical implications. *Psychiatry Research* **285**, 112802 (2020).
131. Roth, L. et al. Chronic intermittent mental stress promotes atherosclerotic plaque vulnerability, myocardial infarction and sudden death in mice. *Atherosclerosis* **242**, 288-294 (2015).
132. Jackson, J.S., Knight, K.M. & Rafferty, J.A. Race and unhealthy behaviors: Chronic stress, the HPA Axis, and physical and mental health disparities over the life course. *American Journal of Public Health* **100**, 933-939 (2010).
133. Schneiderman, N., Ironson, G. & Siegel, S.D. Stress and health: Psychological, behavioral, and biological determinants. *Annual Review of Clinical Psychology* **1**, 607-628 (2005).
134. Pariante, C.M. & Lightman, S.L. The HPA axis in major depression: classical theories and new developments. *Trends in Neurosciences* **31**, 464-468 (2008).
135. Oken, B.S., Chamine, I. & Wakeland, W. A systems approach to stress, stressors and resilience in humans. *Behavioural brain research* **282**, 144-154 (2015).
136. Lazarus, R.S. *Psychological stress and the coping process*, (McGraw-Hill, New York, NY, US, 1966).
137. Sambhi, M.P., Weil, M.H. & Udhoji, V.N. Acute Pharmacodynamic Effects of Glucocorticoids; Cardiac Output and Related Hemodynamic changes in Normal Subjects and Patients in Shock *Circulation* **31**, 523-530 (1965).
138. Spranger, J. et al. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* **52**, 812-7 (2003).
139. Willerson, J.T. & Ridker, P.M. Inflammation as a Cardiovascular Risk Factor. *Circulation* **109**, II-2-II-10 (2004).
140. Sapolsky, R.M., Romero, L.M. & Munck, A.U. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *ENDOCRINE REVIEWS* **21**, 55-89 (2000).
141. Marcondes, A.K. et al. Dyslipidemia induced by stress. (2012).
142. Asmat, U., Abad, K. & Ismail, K. Diabetes mellitus and oxidative stress-A concise review. *Saudi Pharm J* **24**, 547-553 (2016).
143. Raghav, A., Ahmad, J. & Naseem, I. Chronic unpredictable environmental stress impair biochemical and physiological homeostasis: Role in diabetes mellitus. *Diabetes Metab Syndr* **13**, 1021-1030 (2019).
144. Burguera, B., Muruais, C., Peñalva, A., Dieguez, C. & Casanueva, F.F. Dual and Selective Actions of Glucocorticoids upon Basal and Stimulated Growth Hormone Release in Man. *Neuroendocrinology* **51**, 51-58 (1990).
145. Colao, A. The GH–IGF-I axis and the cardiovascular system: clinical implications. *Clinical Endocrinology* **69**, 347-358 (2008).
146. Stapelberg, N.J.C., Neumann, D.L., Shum, D. & Headrick, J.P. Health, pre-disease and critical transition to disease in the psycho-immune-neuroendocrine network: Are

there distinct states in the progression from health to major depressive disorder? *Physiology & Behavior* **198**, 108-119 (2019).
147. Verduijn, J. et al. Pathophysiology of major depressive disorder: mechanisms involved in etiology are not associated with clinical progression. *Translational Psychiatry* **5**, e649-e649 (2015).
148. Stapelberg, N.J.C. et al. From feedback loop transitions to biomarkers in the psycho-immune-neuroendocrine network: Detecting the critical transition from health to major depression. *Neuroscience and Biobehavioral Reviews* **90**, 1-15 (2018).
149. Aschbacher, K. et al. Chronic stress increases vulnerability to diet-related abdominal fat, oxidative stress, and metabolic risk. *Psychoneuroendocrinology* **46**, 14-22 (2014).
150. Finger, B.C., Dinan, T.G. & Cryan, J.F. The temporal impact of chronic intermittent psychosocial stress on high-fat diet-induced alterations in body weight. *Psychoneuroendocrinology* **37**, 729-741 (2011).
151. Kai, K. et al. Environmental stress modifies glycemic control and diabetes onset in type 2 diabetes prone Otsuka Long Evans Tokushima Fatty (OLETF) rats. *Physiology & Behavior* **68**, 445-452 (2000).
152. Fu, J.H. et al. The combination of a high-fat diet and chronic stress aggravates insulin resistance in Wistar male rats. *Exp Clin Endocrinol Diabetes* **117**, 354-60 (2009).
153. Agrimi, J. et al. Obese mice exposed to psychosocial stress display cardiac and hippocampal dysfunction associated with local brain-derived neurotrophic factor depletion. *EBioMedicine* **47**, 384-401 (2019).
154. Finger, B.C., Dinan, T.G. & Cryan, J.F. High-fat diet selectively protects against the effects of chronic social stress in the mouse. *Neuroscience* **192**, 351-360 (2011).
155. da Costa Estrela, D. et al. Predictive behaviors for anxiety and depression in female Wistar rats subjected to cafeteria diet and stress. *Physiol Behav* **151**, 252-63 (2015).
156. Welfare, A.I.o.H.a. National Hospital Morbidity Database (NHMD). (2020).
157. Ferdinandy, P., Schulz, R. & Baxter, G.F. Interaction of Cardiovascular Risk Factors with Myocardial Ischemia/Reperfusion Injury, Preconditioning, and Postconditioning. *Pharmacological Reviews* **59**, 418 (2007).
158. Crompton, M. The mitochondrial permeability transition pore and its role in cell death. *Biochemical Journal* **341**, 233-249 (1999).
159. Halestrap, A.P., Clarke, S.J. & Javadov, S.A. Mitochondrial permeability transition pore opening during myocardial reperfusion—a target for cardioprotection. *Cardiovascular research* **61**, 372-385 (2004).
160. Pacher, P., Csordás, G. & Hajnóczky, G. Mitochondrial Ca2+ signaling and cardiac apoptosis. *Neurosignals* **10**, 200-223 (2001).
161. Di Lisa, F., Menabò, R., Canton, M., Barile, M. & Bernardi, P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *Journal of Biological Chemistry* **276**, 2571-2575 (2001).
162. Schott, R.J., Rohmann, S., Braun, E.R. & Schaper, W. Ischemic Preconditioning Reduces Infarct Size in Swine Myocardium. *Circulation research* **66**, 1133-1142 (1990).
163. Li, Y., Whittaker, P. & Kloner, R.A. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *American Heart Journal* **123**, 346-353 (1992).
164. Dong, J.H., Liu, Y.X., Ji, E.S. & He, R.R. [Limb ischemic preconditioning reduces infarct size following myocardial ischemia-reperfusion in rats]. *Sheng Li Xue Bao* **56**, 41-6 (2004).

165. Peart, J.N. & Headrick, J.P. Clinical cardioprotection and the value of conditioning responses. *American Journal of Physiology - Heart and Circulatory Physiology* **296**, 1705-1720 (2009).
166. Heusch, G. Molecular Basis of Cardioprotection: Signal Transduction in Ischemic Pre-, Post-, and Remote Conditioning. *Circulation Research* **116**, 674-699 (2015).
167. Alkhulaifi, A.M., Pugsley, W.B. & Yellon, D.M. The influence of the time period between preconditioning ischemia and prolonged ischemia on myocardial protection. *Cardioscience* **4**, 163-9 (1993).
168. Matsubara, T. et al. Three minute, but not one minute, ischemia and nicorandil have a preconditioning effect in patients with coronary artery disease. *J Am Coll Cardiol* **35**, 345-51 (2000).
169. Sandhu, R., Diaz, R.J., Mao, G.D. & Wilson, G.J. Ischemic preconditioning: differences in protection and susceptibility to blockade with single-cycle versus multicycle transient ischemia. *Circulation* **96**, 984-95 (1997).
170. Schulz, R., Post, H., Vahlhaus, C. & Heusch, G. Ischemic Preconditioning in Pigs: A Graded Phenomenon. *Circulation* **98**, 1022-1029 (1998).
171. Heusch, G. & Rassaf, T. Time to Give Up on Cardioprotection?: A Critical Appraisal of Clinical Studies on Ischemic Pre-, Post-, and Remote Conditioning. *Circulation Research* **119**, 676-695 (2016).
172. Russell, J.S. et al. Chronic type 2 but not type 1 diabetes impairs myocardial ischaemic tolerance and preconditioning in C57Bl/6 mice. *Experimental Physiology* **104**, 1868-1880 (2019).
173. Zhuo, C. et al. Cardioprotection by ischemic postconditioning is abolished in depressed rats: role of Akt and signal transducer and activator of transcription-3. *Molecular and Cellular Biochemistry* **346**, 39-47 (2011).
174. Wagner, C., Kloeting, I., Strasser, R.H. & Weinbrenner, C. Cardioprotection by Postconditioning Is Lost in WOKW Rats With Metabolic Syndrome: Role of Glycogen Synthase Kinase 3β. *Journal of Cardiovascular Pharmacology* **52**, 430-437 (2008).
175. Bouhidel, O. et al. Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice. *American Journal of Physiology - Heart and Circulatory Physiology* **295**, 1580-1586 (2008).
176. Salie, R., Huisamen, B. & Lochner, A. High carbohydrate and high fat diets protect the heart against ischaemia/reperfusion injury. *Cardiovascular Diabetology* **13**, 109 (2014).
177. Ueda, Y. et al. Pravastatin restored the infarct size-limiting effect of ischemic preconditioning blunted by hypercholesterolemia in the rabbit model of myocardial infarction. *Journal of the American College of Cardiology* **34**, 2120-2125 (1999).
178. Ferdinandy, P. et al. Loss of Pacing-induced Preconditioning in Rat Hearts: Role of Nitric Oxide and Cholesterol-enriched Diet. *Journal of Molecular and Cellular Cardiology* **29**, 3321-3333 (1997).
179. Giricz, Z. et al. Hyperlipidemia Attenuates the Infarct Size-Limiting Effect of Ischemic Preconditioning: Role of Matrix Metalloproteinase-2 Inhibition. *Journal of Pharmacology and Experimental Therapeutics* **316**, 154 (2006).
180. Tosaki, A., Engelman, D.T., Engelman, R.M. & Das, D.K. The evolution of diabetic response to ischemia/reperfusion and preconditioning in isolated working rat hearts. *Cardiovascular Research* **31**, 526-536 (1996).
181. Kristiansen, S.B. et al. Ischaemic preconditioning does not protect the heart in obese and lean animal models of Type 2 diabetes. *Diabetologia* **47**, 1716-1721 (2004).

182. del Valle, H.F., Lascano, E.C., Negroni, J.A. & Crottogini, A.J. Absence of ischemic preconditioning protection in diabetic sheep hearts: Role of sarcolemmal KATP channel dysfunction. *Molecular and Cellular Biochemistry* **249**, 21-30 (2003).
183. Tsang, A., Hausenloy, D.J., Mocanu, M.M., Carr, R.D. & Yellon, D.M. Preconditioning the Diabetic Heart. *Diabetes* **54**, 2360 (2005).
184. Wider, J. et al. Remote ischemic preconditioning fails to reduce infarct size in the Zucker fatty rat model of type-2 diabetes: role of defective humoral communication. *Basic Research in Cardiology* **113**, 16 (2018).
185. Katakam, P.V.G. et al. Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **292**, R920-R926 (2007).
186. Hurst, J.L. & West, R.S. Taming anxiety in laboratory mice. *Nat Methods* **7**, 825-6 (2010).
187. Zhu, S.S., Shi, R., Wang, J., Wang, J.-F. & Li, X.-M. Unpredictable chronic mild stress not chronic restraint stress induces depressive behaviours in mice. *Neuroreport* **25**(2014).
188. Seibenhener, M.L. & Wooten, M.C. Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. *J Vis Exp*, e52434 (2015).
189. Liu, M.-Y. et al. Sucrose preference test for measurement of stress-induced anhedonia in mice. *Nature Protocols* **13**, 1686-1698 (2018).
190. Reichelt, M.E., Willems, L., Hack, B.A., Peart, J.N. & Headrick, J.P. Cardiac and coronary function in the Langendorff-perfused mouse heart model. *Experimental Physiology* **94**, 54-70 (2009).
191. Hoe, L.E.S. et al. Sarcolemmal cholesterol and caveolin-3 dependence of cardiac function, ischemic tolerance, and opioidergic cardioprotection. *American Journal of Physiology-Heart and Circulatory Physiology* **307**, H895-H903 (2014).
192. Marwitz, S., Woodie, L. & Blythe, S. Western-style diet induces insulin insensitivity and hyperactivity in adolescent male rats. *Physiology & behavior* **151**(2015).
193. Bortolin, R.C. et al. A new animal diet based on human Western diet is a robust diet-induced obesity model: comparison to high-fat and cafeteria diets in term of metabolic and gut microbiota disruption. *International Journal of Obesity* **42**, 525-534 (2018).
194. Zardooz, H., Zahedi Asl, S., Gharib Naseri, M.K. & Hedayati, M. Effect of chronic restraint stress on carbohydrate metabolism in rat. *Physiology & Behavior* **89**, 373-378 (2006).
195. Makino, S., Asaba, K., Nishiyama, M. & Hashimoto, K. Decreased type 2 corticotropin-releasing hormone receptor mRNA expression in the ventromedial hypothalamus during repeated immobilization stress. *Neuroendocrinology* **70**, 160-7 (1999).
196. Wang, C.-Y. & Liao, J.K. A mouse model of diet-induced obesity and insulin resistance. *Methods in molecular biology (Clifton, N.J.)* **821**, 421-433 (2012).
197. Fisher-Wellman, K.H. et al. A Direct Comparison of Metabolic Responses to High-Fat Diet in C57BL/6J and C57BL/6NJ Mice. *Diabetes* **65**, 3249-3261 (2016).
198. Houtkooper, R.H. et al. The metabolic footprint of aging in mice. *Scientific reports* **1**, 134-134 (2011).
199. Reynolds, T.H. et al. The impact of age and sex on body composition and glucose sensitivity in C57BL/6J mice. *Physiological reports* **7**, e13995-e13995 (2019).

200. Toit, E.F.d. et al. Myocardial susceptibility to ischemic-reperfusion injury in a prediabetic model of dietary-induced obesity. *American Journal of Physiology-Heart and Circulatory Physiology* **294**, H2336-H2343 (2008).
201. Donner, D.G., Elliott, G.E., Beck, B.R., Bulmer, A.C. & Du Toit, E.F. Impact of Diet-Induced Obesity and Testosterone Deficiency on the Cardiovascular System: A Novel Rodent Model Representative of Males with Testosterone-Deficient Metabolic Syndrome (TDMetS). *PLOS ONE* **10**, e0138019 (2015).
202. Yan, Y.-X. et al. Investigation of the Relationship Between Chronic Stress and Insulin Resistance in a Chinese Population. *Journal of epidemiology* **26**, 355-360 (2016).
203. Choudhary, S., Mourya, A., Ahuja, S., Sah, S.P. & Kumar, A. Plausible anti-inflammatory mechanism of resveratrol and caffeic acid against chronic stress-induced insulin resistance in mice. *Inflammopharmacology* **24**, 347-361 (2016).
204. Nagaraja, H.S. & Jeganathan, P.S. Effect of short & long term restraint stress on some physiological & biochemical parameters in rats. *Indian Journal of Medical Research* **109**, 76 (1999).
205. Rostamkhani, F., Zardooz, H., Zahediasl, S. & Farrokhi, B. Comparison of the effects of acute and chronic psychological stress on metabolic features in rats. *Journal of Zhejiang University. B. Science* **13**, 904-912 (2012).
206. Ellulu, M.S., Patimah, I., Khaza'ai, H., Rahmat, A. & Abed, Y. Obesity and inflammation: the linking mechanism and the complications. *Archives of medical science : AMS* **13**, 851-863 (2017).
207. Stępień, M. et al. Obesity indices and inflammatory markers in obese non-diabetic normo- and hypertensive patients: a comparative pilot study. *Lipids in health and disease* **13**, 29-29 (2014).
208. Nettleton, J.A. et al. Dietary patterns are associated with biochemical markers of inflammation and endothelial activation in the Multi-Ethnic Study of Atherosclerosis (MESA). *Am J Clin Nutr* **83**, 1369-79 (2006).
209. Alesci, S. et al. Major depression is associated with significant diurnal elevations in plasma interleukin-6 levels, a shift of its circadian rhythm, and loss of physiological complexity in its secretion: clinical implications. *J Clin Endocrinol Metab* **90**, 2522-30 (2005).
210. Tsigos, C. & Chrousos, G.P. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research* **53**, 865-871 (2002).
211. Voorhees, J.L. et al. Prolonged restraint stress increases IL-6, reduces IL-10, and causes persistent depressive-like behavior that is reversed by recombinant IL-10. *PloS one* **8**, e58488-e58488 (2013).
212. Napier, B.A. et al. Western diet regulates immune status and the response to LPS-driven sepsis independent of diet-associated microbiome. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 3688-3694 (2019).
213. Bastard, J.P. et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* **85**, 3338-42 (2000).
214. Rosengren, A. et al. Association of psychosocial risk factors with risk of acute myocardial infarction in 11119 cases and 13648 controls from 52 countries (the INTERHEART study): case-control study. *Lancet* **364**, 953-62 (2004).
215. Bosma, H., Peter, R., Siegrist, J. & Marmot, M. Two alternative job stress models and the risk of coronary heart disease. *Am J Public Health* **88**, 68-74 (1998).
216. Matthews, K.A. & Gump, B.B. Chronic Work Stress and Marital Dissolution Increase Risk of Posttrial Mortality in Men From the Multiple Risk Factor Intervention Trial. *Archives of Internal Medicine* **162**, 309-315 (2002).

217. Solberg, L.C., Horton, T.H. & Turek, F.W. Circadian rhythms and depression: effects of exercise in an animal model. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **276**, R152-R161 (1999).
218. Liu, W.-Z. et al. Identification of a prefrontal cortex-to-amygdala pathway for chronic stress-induced anxiety. *Nature Communications* **11**, 2221 (2020).
219. Willner, P., Towell, A., Sampson, D., Sophokleous, S. & Muscat, R. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology (Berl)* **93**, 358-64 (1987).
220. Rosenkranz, J.A., Venheim, E.R. & Padival, M. Chronic Stress Causes Amygdala Hyperexcitability in Rodents. *Biological Psychiatry* **67**, 1128-1136 (2010).
221. Strekalova, T., Spanagel, R., Dolgov, O. & Bartsch, D. Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. *Behavioural Pharmacology* **16**(2005).
222. Ito, H., Nagano, M., Suzuki, H. & Murakoshi, T. Chronic stress enhances synaptic plasticity due to disinhibition in the anterior cingulate cortex and induces hyper-locomotion in mice. *Neuropharmacology* **58**, 746-57 (2010).
223. Levine, J.B. et al. Isolation rearing and hyperlocomotion are associated with reduced immediate early gene expression levels in the medial prefrontal cortex. *Neuroscience* **145**, 42-55 (2007).
224. Ieraci, A., Mallei, A. & Popoli, M. Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. *Neural plasticity* **2016**, 6212983-6212983 (2016).
225. Kim, K.-S. & Han, P.-L. Optimization of chronic stress paradigms using anxiety- and depression-like behavioral parameters. *Journal of Neuroscience Research* **83**, 497-507 (2006).
226. McCarty, R., Horwatt, K. & Konarska, M. Chronic stress and sympathetic-adrenal medullary responsiveness. *Soc Sci Med* **26**, 333-41 (1988).
227. Shoji, H., Takao, K., Hattori, S. & Miyakawa, T. Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. *Molecular Brain* **9**, 11 (2016).
228. Lalonde, R. & Strazielle, C. Exploratory activity and motor coordination in old versus middle-aged C57BL/6J mice. *Archives of Gerontology and Geriatrics* **49**, 39-42 (2009).
229. Shoji, H. & Miyakawa, T. Age-related behavioral changes from young to old age in male mice of a C57BL/6J strain maintained under a genetic stability program. *Neuropsychopharmacology reports* **39**, 100-118 (2019).
230. Tordjman, S. et al. Melatonin: Pharmacology, Functions and Therapeutic Benefits. *Current neuropharmacology* **15**, 434-443 (2017).
231. Srinivasan, V. et al. Melatonin in mood disorders. *The World Journal of Biological Psychiatry* **7**, 138-151 (2006).
232. Sundberg, I. et al. Daytime melatonin levels in saliva are associated with inflammatory markers and anxiety disorders. *Psychoneuroendocrinology* **112**, 104514 (2020).
233. Zhang, Z., Silveyra, E., Jin, N. & Ribelayga, C.P. A congenic line of the C57BL/6J mouse strain that is proficient in melatonin synthesis. *Journal of pineal research* **65**, e12509-e12509 (2018).
234. Vivien-Roels, B. et al. Daily Variations in Pineal Melatonin Concentrations in Inbred and Outbred Mice. *Journal of Biological Rhythms* **13**, 403-409 (1998).
235. Dagnino-Subiabre, A. et al. Chronic stress decreases the expression of sympathetic markers in the pineal gland and increases plasma melatonin concentration in rats. *Journal of Neurochemistry* **97**, 1279-1287 (2006).

236. Cano, P. et al. Effect of a high-fat diet on 24-h pattern of circulating levels of prolactin, luteinizing hormone, testosterone, corticosterone, thyroid-stimulating hormone and glucose, and pineal melatonin content, in rats. *Endocrine* **33**, 118-125 (2008).
237. Ledvényiová-Farkašová, V. et al. Effect of crowding stress on tolerance to ischemia–reperfusion injury in young male and female hypertensive rats: molecular mechanisms. *Canadian Journal of Physiology and Pharmacology* **93**, 793-802 (2015).
238. Yellon, D.M., Baxter, G.F., Garcia-Dorado, D., Heusch, G. & Sumeray, M.S. Ischaemic preconditioning: present position and future directions1. *Cardiovascular Research* **37**, 21-33 (1998).
239. Iliodromitis, E.K., Lazou, A. & Kremastinos, D.T. Ischemic preconditioning: protection against myocardial necrosis and apoptosis. *Vascular health and risk management* **3**, 629-637 (2007).
240. Ghosh, S., Standen, N.B. & Galiñianes, M. Failure to precondition pathological human myocardium. *J Am Coll Cardiol* **37**, 711-8 (2001).
241. Goyal, A. & Agrawal, N. Ischemic preconditioning: Interruption of various disorders. *Journal of the Saudi Heart Association* **29**, 116-127 (2017).
242. Rossello, X., Riquelme, J.A., Davidson, S.M. & Yellon, D.M. Role of PI3K in myocardial ischaemic preconditioning: mapping pro-survival cascades at the trigger phase and at reperfusion. *Journal of cellular and molecular medicine* **22**, 926-935 (2018).
243. Yang, C., Talukder, M.A.H., Varadharaj, S., Velayutham, M. & Zweier, J.L. Early ischaemic preconditioning requires Akt- and PKA-mediated activation of eNOS via serine1176 phosphorylation. *Cardiovascular Research* **97**, 33-43 (2012).
244. Nian, M., Lee, P., Khaper, N. & Liu, P. Inflammatory Cytokines and Postmyocardial Infarction Remodeling. *Circulation Research* **94**, 1543-1553 (2004).
245. Saini, H.K. et al. Role of tumour necrosis factor-alpha and other cytokines in ischemia-reperfusion-induced injury in the heart. *Experimental and clinical cardiology* **10**, 213-222 (2005).
246. Ma, J., Qiao, Z. & Xu, B. Effects of ischemic preconditioning on myocardium Caspase-3, SOCS-1, SOCS-3, TNF-α and IL-6 mRNA expression levels in myocardium IR rats. *Mol Biol Rep* **40**, 5741-8 (2013).
247. Lee, C.-H. & Giuliani, F. The Role of Inflammation in Depression and Fatigue. *Frontiers in immunology* **10**, 1696-1696 (2019).
248. Mauvais-Jarvis, F. et al. Sex and gender: modifiers of health, disease, and medicine. *The Lancet* **396**, 565-582 (2020).
249. Gao, A. et al. Sexual dimorphism in glucose metabolism is shaped by androgen-driven gut microbiome. *Nature Communications* **12**, 7080 (2021).
250. Velasco, M. et al. Sexual dimorphism in insulin resistance in a metabolic syndrome rat model. *Endocrine connections* **9**, 890-902 (2020).
251. Verma, R., Balhara, Y.P.S. & Gupta, C.S. Gender differences in stress response: Role of developmental and biological determinants. *Industrial psychiatry journal* **20**, 4-10 (2011).
252. Moisan, M.P. Sexual Dimorphism in Glucocorticoid Stress Response. *Int J Mol Sci* **22**(2021).