Evidence of Renal Dysfunction, and Sclerotic Damage Secondary to Chronic Heart Failure; an Inbred Rat Model of Type 2 Cardiorenal Syndrome

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Abstract

Cardiovascular disease is the leading killer within the western world. Direct pathological interactions have been shown to exist between cardiovascular disease, and the development of renal disease, and *vice versa*. This has become known as cardiorenal syndrome. Type 2 cardiorenal syndrome, a subtype in which chronic heart disease leads to a progressive loss of renal function, and morphological damage, has not been convincingly reproduced in an animal model. The current study addresses this by using a chronic myocardial infarction model in inbred Lewis rats.

Male Lewis rats were subjected to a permanent occlusion of the left anterior descending coronary artery (LAD), or sham surgery (sham), and were maintained for 90 days. This produced infarcts comprising an average of 24.2% of the wet weight of the entire left ventricle at 90 days, and corresponded to increased plasma cardiac troponin I concentrations, measured 4 hours after the ligation procedure. There was no weight difference between sham, and LAD, animals at 90 days. Ventricular function was measured by Langendorff perfusion. LAD occluded animals had significantly decreased left ventricular developed pressure, unpaced *ex vivo* heart rate, and systolic, and diastolic rate pressure derivatives. These findings were indicative of diastolic heart failure. This corresponded to an increase in pro-inflammatory interleukin-1 beta, but no other residual evidence of cytokine activation in myocardial tissue was found at this timepoint. Ventricular hypertrophy, wall thinning, and dilation, was seen through histological staining with Picosirius Red and Fast Green, and supported by physical measurements of ventricular mass. There was no perturbation to haematological parameters in these animals.
Analysis of renal function revealed decreased true creatinine clearance, and increased fractional excretion of sodium, and increased urinary protein. This corresponded to an increase in tissue growth factor beta, and increased renal caspase 3/7 activity. The presence of renal damage was confirmed through histological evidence of glomerulosclerosis, and tubulointerstitial fibrosis in the cortex, and medulla. This evidence shows renal disease formation in rats secondary to heart failure.

This study strongly suggests that this model is able to reliably produce disease closely resembling the clinical manifestation of type 2 cardiorenal syndrome. This provides an experimental framework to study pathophysiological mechanisms, and therapeutic interventions, of type 2 cardiorenal syndrome.
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Nomenclature

\[ \Delta P \cdot \Delta t_{\text{max}} \] Rate of contraction

\[ \Delta P \cdot \Delta t_{\text{min}} \] Rate of relaxation

\[ \Delta \] Change in

Adr Adrenaline

AEC Animal Ethics Code

AKI Acute kidney injury

AT_1R Type 1 angiotensin receptor

BWf Fasted body weight

CF Coronary flow

CHF Chronic heart failure

CKD Chronic kidney disease

COHb Carboxyhaemoglobin

CON Control
CrCl  Creatinine clearance

CRS  Cardiorenal Syndrome

cTnI  Cardiac troponin I

ECM  extracellular matrix

eGFR  Estimated glomerular filtration rate

ET-1  Endothelin-1

ET\textsubscript{A}R  Type A endothelin receptor

ET\textsubscript{B}R  Type B endothelin receptor

EtOH  Ethanol

FE\textsubscript{Na}  Fractional excretion of sodium

GFR  Glomerular filtration rate

HazR  Hazard ratio

Hb  Haemoglobin

Hct  haematocrit

HF  Heart failure

HIF-1  Type 1 Hypoxia-inducible factor

HR  Unpaced \textit{ex vivo} heart rate

IL  Interleukin
IVC  Inferior Vena Cava
IV   Intravenous
KHB  Krebs-Henseleit-Ringer Buffer
LADCA Left anterior coronary artery
LAD  Left anterior descending coronary artery occlusion group
LN   Liquid nitrogen
LVDP Left ventricular developed pressure
LV   Left ventricle
methHb Methaemoglobin
MI   Myocardial infarction
N2O  Nitrous oxide
NAdr Noradrenaline
NO   Nitric oxide
O2   Oxygen
OR   Odds ratio
PBS  Phosphate Buffered Saline
RAS  Renin angiotensin system
RCS  Renocardiac syndrome
rpm  Revolutions per minute
SCL  Southern Community Laboratories
SCr  Serum creatinine
SC   Subcutaneous
sham Sham surgery animal group
SNS  Sympathetic nervous system
TGF-β Tissue growth factor beta
TMS  Tris-Mannitol-Sucrose Buffer
TNF-α Tissue necrosis factor alpha
UnX  Unilateral nephrectomy
Chapter 1

Introduction

1.1 The emergence of cardiorenal syndrome.

Within the field of medicine, and human society as a whole, the quest for improving quality of life is of utmost importance. Cardiovascular disease is responsible for more deaths than any other organic, or exogenous cause, in the developed world, with risk increasing with age [1]. In New Zealand, chronic heart disease, including ischaemic disease, is responsible for 22% of all mortality annually [2]. This puts chronic heart disease second only to all cancers combined (30% of annual mortality). The Māori population has almost twice the risk of chronic heart disease causing mortality, compared to the European population, with 201 per 100,000 Vs. 104 per 100,000 for men, and 114 per 100,000 Vs. 67 per 100,000 for women [2]. Cardiovascular disease is of great concern as there is an increasing proportion of elderly within the population, and thus it is a problem set to dramatically increase in the coming decades [3]. However,
recent research has uncovered a very deleterious interaction between the
heart and the kidneys, where the function of one is highly dependent on
the other, and disease of either can result in *de novo* disease of the other
[4, 5, 6, 7].

This interaction between the heart and the kidney has been termed card-
diorenal syndrome (CRS) [5]. It is a broad grouping of pathologies which
all involve progressive, intrinsically generated, dysfunction of the card-
diorenal axis. Owing to the varied pathological states which exist within
the heart, and kidney, CRS has been divided into multiple subtypes.
Pathologies originating from primary renal damage are occasionally re-
ferred to as renocardiac syndrome (RCS) [5]. It is also known, in an
expanded context, as cardiorenal-anaemia syndrome [6, 8]. This is an
extension of the disease state in which anaemia is present, in addition
to CRS, and is able to worsen both cardiovascular, and renal function.
In the context of CRS, anaemia is of greatest importance when it is the
primary disease state, or is secondary to chronic renal failure, especially
once the disease progresses to the point where dialysis is required as part
of life preserving therapies, due to impairment of normal erythropoiesis,
and is highly correlated to the degree of renal injury [6, 7, 8]. Anaemia
may also occur as a potential side effect of pharmacological intervention,
and as such should regularly be tested for, in conjunction with other func-
tional assessment. It is important to be aware of, but is not the primary
focus of this body of work, and thus will not be dealt with in depth.
1.1.1 Implications of CRS

With the suggestion of deleterious interactions within the cardiorenal axis after dysfunction of any component, it is important to consider both organs together, investigating both correlative, and causative, interactions between the myocardium, and kidneys. This can be further investigated to uncover possible interactions, attributing causation to the dysfunction of either organ, and the secondary dysfunction of the other. It is possible that this interaction is unidirectional, or bidirectional. Unidirectionality would imply that one of the organs is able to cause dysfunction in the other, but without the reverse being true. A truly bidirectional interaction would result in a functional deficit in either organ, leading to declining function in the other, and, in turn, further dysfunction of the primary organ. This would result in a positive feedback loop which would result in an accelerated functional decline in patients. It is of great importance to elucidate these interactions in order to best provide treatment strategies geared towards patient survival, and quality of life, and for this, an animal model is needed to investigate possible mechanisms of propagation of damage from a damaged myocardium to the kidneys.

1.2 Classification, and epidemiology, of clinical cardiorenal syndrome

Recent discussion, and research, surrounding the topic of damage propagation within the cardiorenal axis, has lead to the creation of a classifica-
tion system where CRS, and RCS can be differentiated by various pathophysiological criteria [4, 5]. This classification, groups CRS, and RCS, under a single nomenclature, CRS. This gives clinicians a discernable disease framework, aiding in the selection of specialized interventions, with specific pathophysiological targets in mind. Within this framework, it is possible to dissect all aspects of these disease states, so as to shed light on the most significant aspects of the pathology presented within any individual patient with CRS. This classification also assist in determining whether there are fundamental differences between the pathophysiology of each of the syndrome grouping, giving insight into either a common process in all, or divergent processes with convergent symptomology. Accordingly there are five distinct presentations of CRS which have been put forward by Ronco et al. [4]. Type 1 CRS is a scenario where acute cardiac injury leads to acute renal injury. Type 2 CRS describes chronic cardiac dysfunction (such as chronic heart failure (CHF) leading to progressive chronic kidney disease (CKD). Type 3 CRS (previously termed RCS) is where acute kidney injury (AKI) leads to acute cardiac dysfunction. Type 4 CRS is classified as CKD leading to progressive cardiac dysfunction. Type 5 CRS is where there exists co-committant acute, or chronic, renal and cardiac damage generated as result of another disease state, or unrelated disease state, such as type 2 diabetes mellitus, or sepsis.
1.2.1 Type 1 cardiorenal syndrome

Rapidly declining cardiac function, such as acute heart failure, and acute cardiac syndrome, has been associated with the clinical appearance of AKI. Acute heart failure can be further divided into subtypes such as hypertensive pulmonary edema with preserved left ventricular (LV) systolic function, acutely decompensated heart failure, cardiogenic shock, and predominant right ventricular failure. Pre-morbid renal dysfunction, or failure, is common and presents an elevated risk of developing AKI.

Due to the very recent identification of the CRS phenomena, there is a great lack of clinical trials that have looked at this pathological interaction directly, with many CHF trials excluding renally compromised patients, as it was previously believed to only represent a confounding variable [9]. Thus the vast majority of epidemiological findings are post hoc analysis of data from trials not designed to measure this interaction, leaving any epidemiological findings somewhat variable, and likely to be changed as clinical insight evolves.

A recent meta analysis by Bagshaw et al. [9] has shown a consensus that there is a strong association between acute myocardial injury, and incidence of AKI. Serum creatinine (SCr) elevations were used to estimate that AKI was present in 20-24% (six studies using SCr >44.2 μmol.L⁻¹ as the inclusion criteria), 28 and 34% (two studies using SCr >26.5 μmol.L⁻¹ as the inclusion criteria), and 75% (one study using >8.8 μmol.L⁻¹ as
the inclusion criteria), of patients, presenting with acutely decompensated heart failure. In patients presenting with acute cardiac syndrome, SCr elevations were associated with 9.6% and 9.7% (two studies using >44.2 μmol.L⁻¹ as the inclusion criteria), and 12% (one study using >26.5 μmol.L⁻¹ as the inclusion criteria), incidence of AKI. Two further studies showing that 43.2%, and 19.4% of patients developed AKI when change in (Δ) SCr was reported between 0.1 μmol.L and 3.0 μmol.L, and between 0.3 μmol.L and ≥1.0 μmol.L, respectively. The need for uniformity in methodology of measuring potential CRS interactions becomes obvious rather quickly.

There is a diverse group of processes that may lead to, or exacerbate, renal dysfunction. Decreased cardiac output can lead to decreased renal perfusion, and can also lead to venous congestion which further impairs renal function [10, 11, 12]. Increased cytokine release triggered by vascular damage may lead to immunological damage in the kidneys, caspase activation, fibrosis, and apoptosis [13, 14, 15, 16]. Exogenous factors, such as pharmacotherapy, or the use of radiographic contrast dyes, can also worsen renal function, through renal vasoconstriction [17, 18, 19, 20]. These cascades can lead to increased sympathetic activation, and increased renin angiotensin system (RAS) signalling, leading to Na⁺ and water retention, and vasoconstriction [21, 22, 23, 24, 25]. This vicious neuroendocrine signalling can increase demand on the already strained heart, leading to declining function, and subsequently, renal dysfunction [26, 27, 28].
1.2.2 Type 2 cardiorenal syndrome

Chronic cardiac dysfunction, such as with CHF, is associated with increased risk of kidney dysfunction [29, 30]. This worsening renal function then causes accelerated myocardial dysfunction, when compared to patients with no renal dysfunction [31]. This interaction has serious prognostic implications [31]. In a study conducted by Pons et al. [32], it was found that of 960 heart failure (HF) patients enrolled in the study (median follow up duration of 36 months), 351 died (36.6 %), with cause of death being attributable to cardiovascular events in 230 patients (65.5 % of total deaths). Of these deaths, it was found that chronic kidney failure was present in 68.3 %, and anaemia present in 40.4 %, of patients who died of cardiovascular events. Through multivariate analysis, it was shown that creatinine clearance (CrCl) was a significant predictive factor for mortality. In an earlier meta analysis by Smith et al. [33], it was shown that there may be a dose dependent increase in mortality risk in HF patients with renal impairment. This was found to be a 33 % increased risk of mortality for every 1 mg·dL\(^{-1}\) creatinine in a subset of the studied analysed, a 7 % increase in mortality risk for every 10 mL·minute\(^{-1}\) estimated glomerular filtration rate (eGFR), and in one study, a threshold effect where increased risk was only seen at < 50 mL·minute\(^{-1}\) eGFR. This represented a unanimous agreement, between analysed studies, that impaired renal function negatively affects cardiovascular function, and patient survival. In a further meta-analysis undertaken by Damman et al. [34], it was found that 25 % (4734 of 18,634 patients) developed some
level of AKI. AKI (termed worsening renal function in the study) was defined as any renal decline $> 0.2 \text{mg} \cdot \text{dL}^{-1}$, or $> 5 \text{mL} \cdot \text{dL} \cdot 1.73 \text{m}^2$. AKI was associated with an increased odds ratio (OR) of mortality which increased with severity of AKI. This increased risk of death, at 6 months post study, within the AKI group was independent of outpatient, or hospitalized, status during the study (OR = 1.69 Vs. OR = 1.61, respectively). In one of the studies analysed, it was found that improved renal function lead to a decreased risk of mortality[35]. In this single centre, observational, prospective 6 month study, conducted in 1,216 patients, it was found that not only does the severity of AKI affect the prognosis of HF, but that improving renal function increased favorable prognosis in heart failure.

The previously mentioned meta analysis by Bagshaw et al.[9] also suggests the conclusion that CKD is increased in patients presenting with CHF. Studies using eGFR as a marker of renal dysfunction found that there was an incidence of renal dysfunction where 27.4% and 41% (two studies), and 45-63.6% (three studies), of patients had an eGFR of $60-89 \text{mL.minute}^{-1} \cdot (1.73 \text{m}^2)^{-1}$, and $<60 \text{mL.minute}^{-1} \cdot (1.73 \text{m}^2)^{-1}$, respectively. One single study reported that 9% of included patients presenting with adult congenital heart defects had an eGFR of $< 60 \text{mL.minute}^{-1} \cdot (1.73 \text{m}^2)^{-1}$. In another study of CHF, it was reported that 34% of patients had an eGFR which decreased by at least $15 \text{mL.minute}^{-1} \cdot (1.73 \text{m}^2)^{-1}$, with final eGFR dropping below $60 \text{mL.minute}^{-1} \cdot (1.73 \text{m}^2)^{-1}$. It should be noted that these values will
be likely modified by the inadvertant treatment of CRS, as a result of standard CHF treatments, and thus represent the incidence despite therapy. This further demonstrates the importance of establishing a working, reliable, animal model of type 2 CRS to allow the proper elucidation of effective interventions.

Pathophysiological processes likely to be involved in the generation of this type of secondary renal injury are likely to include haemodynamic parameters, such as venous congestion, neurohormonal abnormalities, such as excessive sympathetic stimulation, and impairments of endogenous vasodilatory mechanisms [36, 37, 12, 28, 38, 39]. Imbalances in erythropoietin, separate to anaemic elements, have also been implicated in the genesis of CKD secondary to CHF [40]. It is also a distinct possibility that there may be a role to be played by CHF therapies, such as angiotensin converting enzyme inhibitors, which may disturb normal erythropoiesis [41, 42]. Type 2 CRS, and potential mechanisms of pathogenesis will be explored in depth in the body of this thesis.

1.2.3 Type 3 cardiorenal syndrome

In this classification of CRS, the kidney is the primarily damaged organ. Abrupt worsening of renal function, such as suffered with glomerulonephritis, AKI, or ischaemia, can lead to acute cardiac dysfunction, such as HF, arrhythmia, and ischaemia [43]. This may be caused by various pathophysiological factors such as increased cardiac preload, decreased renal perfusion, Na\(^+\) and H\(_2\)O retention, and accompanying volume ex-
pansion, hypertension, idiopathic renal injury, sympathetic nerve activation, RAS activation, electrolyte imbalance, acid-base imbalance, and cytokine secretion due to endothelial activation [4].

1.2.3.1 Type 4 cardiorenal syndrome

Type 4 CRS is the classification used when the kidney is chronically injured, such as with chronic glomerular disease, and contributes to chronic worsening of cardiac function, cardiac hypertrophy, or other cardiovascular events [5]. This is a serious issue as the rates of CKD are increasing, with an estimated prevalence in the United States of America of 11-12%, and increasing incidence of end stage renal disease [44]. This is further compounded by the greatly increased risk of cardiovascular events, and death, in CKD patients and has been reported as accounting for upwards of 50% of all mortality in renal failure [45, 46, 47, 48]. This is made worse by the propensity to withhold potentially life extending therapies because there may be increased therapeutic risk owing to the patient's impaired renal function, leading to <50% receiving the same care that would be given to non-CKD patients [4]. This is somewhat of a misquided practice as appropriately titrated, and monitored, cardiovascular therapies can be safely given to patients with CKD, bestowing benefits similar to non-CKD patients. A major problem lies within clinical trials. In many of the trials which profile cardiovascular protection from therapies actively exclude CKD patients from the studies, as it is a confounding variable [9]. This leaves clinicians with insufficient evidence on which to base a therapeutic strategy. This needs to be corrected, and guidelines
penned for the treatment of this syndrome variant. Meta-analysis by Bagshaw et al. [9] revealed that the rates, or risk, of cardiac events, cardiac related hospitalization, or death, were highly dependent on not only the presence of CKD, but also the severity of the CKD. Due to the divergent methods of data presentation within the studies, it will be summarized as a generalized rates, and risk/odds ratios. Incidence of cardiac events, or hospitalization for cardiac pathology, was between 3.65% and 59% of study participants (four studies), or as presented by some studies, a hazard ratio (HazR)/OR of between 1.1 and 3.4 (five studies). The relative rate of cardiovascular deaths was between 7.5% and 83% of participants (three studies), depending on the severity of initial disease, rate of worsening of renal function, or length of follow up. The risk ratios of cardiovascular death in 4 other studies were between 1.11 and 1.86, again dependent of the forementioned variables at initial presentation. These Figures represent a blindingly obvious scenario where renal disease precedes cardiovascular dysfunction, and likely plays a causal role in this pathogenesis.

1.2.4 Type 5 cardiorenal syndrome

Type 5 CRS is a scenario where both cardiac, and renal dysfunction exist secondary to a primary systemic disorder, such as sepsis, or an unknown cause [5]. The very nature of this sub type makes it inherently difficult to collect systematically studied data, as there are so many confounding variables in patients with systemic disorders. Once the damage has been
caused, the cycle may continue in the same way as the other types of CRS. This is especially true if the original cause has been resolved, but cardiac, and renal damage remains. Disease states contributing to type 5 CRS include, but are not limited to, diabetes, sepsis, amyloidosis, systemic lupus erythematosus, and sarcoidosis. It is currently unknown whether elimination of the primary disease/stimulus will halt the renal, and cardiac functional decline. It is likely that the cardiorenal interactions of type 5 CRS will be primarily dependent on the level of dysfunction in either organ, with this dysfunction leading to declining function in the other organ, modified by the type of injury endured as result of the initial disease/stimulus. In the clinical setting, type 5 CRS should be addressed by first identifying any damage to the kidney, or heart, and then treating according to generalized therapeutic strategies devised for types 1-4, depending on which common factors are shared with the specific incidence of type 5 within the patient. There needs to also be an effort focused on identifying in which conditions this may present a secondary concern, the manner in which the initial insult occurs, and how dysfunction is propagated between the heart and kidney in disease states that lead to type 5 CRS. This could be accomplished by the examination of cardiac, and renal, data in studies of contributing disease states, with specific aims of the study aimed at interrupting this deleterious cycle. Intervention needs to focus primarily on stopping further companion organ induced dysfunction, and then attempting to promote recovery of healthy function.
1.3 Primary pathophysiology, in the heart, leading to type 2 CRS

It is one thing to find a clinical correlation between co-morbidities, but it is another to imply causality. To imply causality it is required to replicate the effect in a controlled environment, where possible co-founders can be minimised. It is also crucial to be aware of underlying mechanisms that may impact on this cross pathology. These mechanisms may be directly causative of damage to the companion organ, or they may cause damage passively. Passivity would imply that companion organ damage is secondary to a change in a physiological parameter, as result of dysfunction of the damaged organ, such as circulating volume, or perfusion pressure, without influence from circulating mediators, such as angiotensin II (AngII). In order to investigate the origin of cardiorenal interactions, using an animal model, we must identify the pathological processes occurring within the heart after myocardial injury, acute myocardial infarction (MI) in the present study, and then assess the resultant changes occurring within the kidney, identifying potential sources of causation.

1.3.1 Infarction; the initial insult

MI occurs when there is an occlusion of a coronary artery, interrupting oxygen delivery to a region of the myocardium. The extent of the resulting damage is dependent on the area of myocardium that is not being adequately perfused, and the length of oxygen deprivation [49, 50]. The
most effective strategy in the prevention of damage is the reperfusion of oxygen starved regions of the myocardium [50]. Infarct resolution may include thrombolysis, and/or percutaneous coronary intervention, to reinstate flow by. However, this is not always possible, with an optimum intervention window of only 60-90 minutes after onset for thrombolysis, with an exponential loss of benefit for every hour of delay[50]. There is some suggestion that there may also be benefit from thrombolysis at 6-12 hours after onset, but no significant benefit at any timepoint after 12 hours [50]. Although the window of intervention seems large, the longer the delay, the more irreversible damage occurs, and the chance of ischaemia reperfusion injury increases. The infarct severity will be dependent on the initial location of occlusion, the amount of collateral blood supply, the time to reperfusion (if at all), and early therapeutic intervention [50]. All interventions aim to salvage as much at risk myocardium as possible. There is some suggestion that even late reinstatement of flow may have beneficial effects, but is due to effects peripheral to salvage of myocardial tissue [51].

1.3.2 Early Remodelling

In the first few hours following ischaemic insult, necrosis, edema, and inflammatory processes, will have begun within the infarct [49, 52]. Early phase remodelling begins immediately after an ischaemic insult, and lasts for around 72 hours, with any remodelling occurring beyond 72 hours considered late stage remodelling [53]. This time window is the stage
where thrombolytic and/or percutaneous coronary intervention are most effective, with microvascular damage being a very strong predictor of left ventricular remodelling[54, 50, 55]. This involves the thinning, and expansion of the infarct, and compensatory dilation, and hypertrophy in the ventricle [49]. This process is the leading cause of coronary artery disease evolving into CHF[56]. There are severe morphological changes that occur in these hearts, which become enlarged, and adopt a more spherical shape (rather than elliptical) [57, 56]. It has been demonstrated that sphericity is observable immediately after infarct, and is powerful predictive marker of unfavorable remodelling at three months, one year, during the progression of CHF [58, 57, 59, 56].

The early remodelling process is one in which normal cardiovascular homeostatic mechanisms become overwhelmed, and become detrimental to the patient [53]. The main purpose of this mechanism is to preserve stroke volume. The degree to which this mechanism becomes detrimental depends of the location, severity, reperfusion, and myocardial wall stresses [54, 60, 61, 49, 62]. In the case of a minor scar with no transmurality, this system may effectively compensate for tissue loss, and provide stable function. Compensation may not be possible in cases of severe infarcts covering a large area, greater than ≈20% of the ventricular circumference, especially when involving the apical surface of the LV. The importance of the apex is in part due to the increased curvature of this region which increases ventricular wall stresses, compared to other myocardial regions [60]. Pronounced scarring may start a biochemical
cascade, such as the RAS, in an attempt to compensate for the loss of myocardial function, leading towards greater dysfunction, and potentially CHF [60, 49, 53].

1.3.2.1 Compensatory mechanisms

The primary function of the heart is to perfuse the body with oxygen rich blood. A functionally compromised heart will be increasingly stimulated to increase workload, in an attempt to maintain stroke volume [63]. The infarcted area, starved of oxygen, will begin to become dyskinetic, unable to perform at the same level as the uninfarcted myocardium, and may eventually become akinetic [64, 60, 53]. Akinesis is less detrimental than dyskinesias, as it is less likely to retard myocardial haemodynamics [60, 53]. Dyskinesias, or akineties, will augment decreased ejection fraction, triggering myocardial mechanoreceptor dependent compensatory mechanisms, such as increased catecholamine release [64, 60, 63, 53]. The release of noradrenaline (NAdr), from sympathetic nerve terminals, stimulates the release of NAdr, and adrenaline (Adr) from the adrenal gland, in addition to direct release of NAdr on to the myocardium by the sympathetic nervous system (SNS), directly stimulating increased chronotrophic, and inotropic responses within the myocardium, which can contribute to further scar expansion, and ventricular thinning [65, 66, 67, 68, 69]. The circulating Adr acts to regulate vascular patency, modulating cardiac return, as well as working in conjunction with NAdr within the myocardium [70, 71].
This SNS activity will also trigger the RAS through renin release from the juxtaglomerular apparatus, which will result in raised plasma concentrations of Ang II, leading to increased H$_2$O and electrolyte retention, as well as increased vasoconstriction [72, 39]. This adrenergic stimulation also causes the release of natriuretic peptides, such as atrial, and brain natriuretic peptides, which act to decrease intravascular volumes, and cause systemic increases in vascular resistance [53]. Ventricular wall stress increases due to the reduced pump efficiency, compounded by compensatory mechanisms increasing the volume to be pumped, while decreasing the volume to be pumped through, in an attempt to compensate, and further compounded by the ongoing changes in myocardial mechanics [60, 53]. This increased wall stress causes slippage between myocytes to occur as the damaged tissue is stretched, which effectively decreases the myocyte density in the infarcted region, without an increase in sarcomere length [60, 49, 53]. In extreme cases, this infarct may stretch to the extent that it is unable to maintain patency, and the ventricular wall ruptures, leading to death [60, 53].

Acting alongside the forementioned mechanically triggered events, local inflammation will arise due to the necrosis of myocardial cells affected by the ischaemic insult [73, 52, 53]. This promotes infiltration by immune cells, such as neutrophils, which release matrix metalloproteinases [73, 60, 52, 53]. Collagenous struts between myocytes are also being broken down by serine proteases at this point [73, 60, 52, 53]. This is an important part of the healing process, as it allows for the debridement of non viable,
necrotic, myocytes [53]. Scar expansion causes deformation of the peri-infarct region, which alters the Frank/Starling mechanism, a mechanism where increased stretch results in increased contractile force, increases shortening, further driving up intraventricular pressure [60, 53].

Increased ventricular wall stress is a powerful trigger of mechanoreceptor mediated hypertrophy [60, 53]. This involves the non infarcted myocardium, remote to the infarct [74, 75, 76]. Local mechanoreceptor activation is translated to intracellular signal in part by local AngII release [39]. This stimulates an increased synthesis of contractile assembly units within myocytes, augmenting hypertrophy [39]. This is all in an attempt to compensate for the sudden loss of performance, albeit in a transient fashion.

1.3.3 Late stage remodelling

Remodelling that occurs after the first 72 hours is considered late remodelling [53]. This is the phase in which the healing process is continued via ventricular architectural changes to redistribute variances in wall stresses [60]. This occurs when the extracellular matrix lays down a collagenous scar which stabilizes the infarcted area, becoming a rigid scar [64, 60, 74, 53]. This will minimise further deformation of the infarcted area [60, 53]. Similar processes continue to occur as seen in early remodelling, such as wall thinning, and hypertrophy, but with a decreasing importance of mediators such as inflammation due to necrosis [75, 64, 77, 53].
1.3.4 Scar formation

Tissue growth factor beta (TGF-β) is released by necrotic myocytes, and macrophages in the infarcted region [78]. This stimulates macrophage, and fibroblast chemotaxis, and also fibroblast proliferation [79]. Increasing γ-interferon stimulates macrophages to release nitric oxide (NO) which increases vascular permeability, and also contributes to limiting the cellular inflammation in the infarcted region [80]. Activated macrophages also release Ang II, which functions independently to plasma Ang II, locally, functioning in a paracrine fashion [81]. This release of TGF-β also stimulates the differentiation of fibroblasts into myofibroblasts, which express increased numbers of type 1 angiotensin (AT₁R), TGF-β, and endothelin 1, receptors [80, 53].

Scar formation is initiated by the formation of a fibrin-fibronectin matrix, to which myofibroblasts attach [80]. Myofibroblasts produce aldosterone, which shares a powerful co-stimulatory action with atrial naturetic peptides, endothelin, and TGF-β1 [82, 78]. Local aldosterone acts to stimulate the transcription of type I and type III collagen mRNA [83]. The RAS plays a powerful role in amplifying proliferative, and fibrogenic responses in the production of type I and type III collagen [84, 81]. Under normal circumstances, this fibrosis is confined to the infarct region, but in cases of extensive damage, can spread to viable tissue [81]. As myofibroblasts attach to the matrix, and produce collagen, the scar becomes stronger, and more rigid [64, 60, 53]. This not only represents the debridement of necrotic cells, but also an equilibration of wall forces [60].
The associated changes in collagen metabolic markers are highly correlated with the level of myocardial remodelling at six months [85]. Once the scar has matured, most myofibroblasts begin apoptosis [78]. However, hypertrophy can continue to occur past this scar formation in severely damaged hearts [75, 49].

1.3.5 Chronic hypertrophy

In severe cases of infarction, where the innate compensatory, and preparative mechanisms, fail to reinstate, or compensate for the loss of functional myocardium, the myocardial mechanoreceptors will still register a subperformance of the heart, and further stimulate compensatory mechanisms [60, 75, 28]. In this instance, it is the heart's inability to convert preload to cardiac output that triggers a downward spiral leading to CHF [86]. The mechanical stretch endured by a compromised myocardium will further enhance sympathetic signaling, causing hypertrophic responses in myocytes via $\alpha_1$ adrenergic receptor stimulation. NA$\alpha_r$ will also stimulate the $\beta_1$ adrenergic receptor in the juxtaglomerular apparatus to enhance renin release [87]. This leads to increased fluid and salt retention, as well as increased circulation of AngII [39]. AngII is also released locally by cytoplasmic granules in the myocardium in response to mechanical stretch [53]. This dual source of AngII activates AT$_1$R, which promotes further hypertrophy by encouraging proliferation of contractile proteins [88].
1.3.6 Systemic renin-angiotensin system signalling

The systemic RAS is the predominant mechanism by which the body maintains fluid, and salt, homeostasis [21]. This first stage in this hormonal cascade, the release of renin from the juxtaglomerular apparatus, is modulated in four ways: renal arteriole baroreceptor activation in response to decrease renal perfusion pressure; perturbations in NaCl in the distal tubules of the macula densa; sympathetic stimulation of the cells within the kidney; negative feedback by AngII stimulation of the juxtaglomerular cells [21, 87]. Renin is also produced in other cell lines, but these sites serve as sources of renin for local paracrine RAS, rather than systemic RAS [21]. Renin is an enzyme responsible for the cleavage of angiotensinogen, which is produced in the liver, or locally in tissue, to angiotensinI (AngI), which is in turn cleaved to the most potent form of this hormone, AngII, by angiotensin converting enzyme [21].

Following MI, myocardial haemodynamics are compromised, resulting in reduced cardiac output, and thus perfusion pressure of the arterial system [89]. This results in the activation of the RAS via the forementioned pathways. This is likely the first instance of the pathological activation of RAS, as there is no true perturbation in circulating volume, the regular trigger of this response, but rather a perturbation in the function of the pump, the heart, responsible for generation of perfusion pressure [86]. As previously discussed, this leads to volume overload, and increased stress on the already struggling myocardium [86]. As an increase in circulating volume will further retard cardiovascular function, RAS will continuously
be stimulated [86]. AngII also has a stimulatory role at the carotid body, which has the effect of increasing SNS signalling, and thus further promotes increased renin release, and other actions mediated by the SNS, and subsequent elevations in plasma AngII concentration[63]. It is of no surprise that therapeutic inhibition of the RAS has beneficial effects during CHF, with increased plasma concentrations of each component having adverse effects on function, and recovery [23, 90, 91, 92, 89].

1.3.7 Inflammation, and the immune response to infarction.

Inflammation plays a role in the healing, and compensatory, mechanisms of the myocardium following infarction [73]. It is however more important when considering reperfused myocardium [52]. In non reperfused myocardium, the process will depend on collateral circulation for the process of rapid migration of inflammatory cell lines into the infarcted regions of the myocardium [74]. In regions of infarct without any remaining vascular perfusion, the process will depend on the slow diffusion through tissue [74]. This will put more emphasis on the debridement of cellular debris as the infarct heals, rather than immediate inflammatory responses which may lead to further cell death and infarct expansion [74]. In this context, inflammatory actions in the myocardium will be discussed as an ideally reperfused tissue, leaving the reader to use discretion when considering how important these processes will be, depending on the level of reperfusion that can be achieved, in their specific cases.
After a prolonged ischaemic insult, myocytes within the ischaemic zone begin to become necrotic, and release complement factors (C1-C5a) [52]. This complement promotes neutrophil, and monocyte, recruitment to the site on injury [52]. Recruitment of immune cell lines is greatly augmented with the reinstatement of flow via thrombolytic resolution, or percutaneous coronary intervention [93]. Neutrophils are a source of damaging reactive oxygen species, and proteases that break down extracellular matrix (ECM) components, and clear out dead cells [80, 94]. Neutrophils can however cause further damage by becoming lodged in the microvasculature, impairing reperfusion of oxygen starved tissue, or in the case of non reperfused tissue, limiting the effectiveness of collateral vasculature in compensation of lost perfusion [95]. Immune cells, such as activated monocytes, are a source of inflammatory cytokines such as tissue necrosis factor alpha (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, and IL-8 [52]. These cytokines will further upregulate neutrophil recruitment, and thus, any damage by the infiltrating neutrophils will be perpetuated [52]. However, this is a part of the normal healing process of damaged tissue, with devastating results occurring when immune components are inhibited [80]. The goal of any therapeutic approach which targets these pathways is to instill the correct balance of response, and counter response, which quells the pro-inflammatory immune response, while maintaining the positive aspects of remodelling.
1.4 Currently available models

In order to study a disease in depth, a reliable animal model must be established. This allows researchers to probe the pathophysiology in ways that are not possible with humans, and to remove as many confounding variables as possible. In the realm of CRS, there exist many models to examine type 3 and type 4. There are no working models that have been able to show de novo renal dysfunction and morphological changes consistent with damage, following heart failure.

Renal dysfunction has been, in part, observed in animal models of CHF for some time. Bauersachs et al. [96], demonstrated decreased renal function secondary to MI. This was in the context of examining the effect of endothelin A receptor antagonist LU 135252 on renal function during CHF. Adult male Wistar rats with CHF, induced by coronary artery ligation, were maintained for 12 weeks following MI, with LU 135252 administered for the last 11 weeks of the survival period. They observed a decrease in renal function, indicated by decreases in creatinine clearance, but no increase in FE_{Na}, or proteinuria, was observed. Staining with haematoxylin/eosin, periodic acid Schiff, and Masson’s trichrome, no morphological changes were observed. The treatment with LU 135252 was able to ameliorate the renal impairment, but was most likely due to drug induced vascular changes, rather than true protection.

In a later study by van Dokkum et al. [97] researchers were able to enhance the progression of renal damage through MI, but were only able
to show modest de novo changes in renal morphology secondary to MI. In this study, animals were subjected to laparotomy to remove the right kidney (UnX; no removal in renal sham control), and then left sided thoractomy to induce MI via coronary artery ligation (no ligation in cardiac sham controls; CON for double shams). UnX animals all exhibited renal dysfunction, and physical changes, such as increased kidney weight. This increase in renal weight was not observed in MI animals. Increased proteinuria was only observed in UnX + MI animals, and GFR was decreased in all groups except in the CON group. Histological examination revealed a modest increase in focal glomerulosclerosis, and α-smooth muscle actin. This suggests that there is a cardiorenal interaction, but only modestly demonstrates de novo renal damage.

1.5 Aims & hypothesis

It is hypothesised that the induction of progressive cardiac dysfunction in inbred Lewis rats, with our methods, will lead to de novo renal dysfunction and histological damage, in excess to anything previously published. This will be done by inducing infarcts, greater than 20% of the total weight of the left ventricle, including the septum, in animals through permanent occlusion of the left anterior descending coronary artery, and allowing the disease state to develop for 90 days before organ harvest for analysis. It is believed that 90 days post infarction will be enough time for this renal damage to develop, and be histologically evident. This thesis aims to address the lack of a reliable animal model of type 2 CRS
by demonstrating *de novo* renal dysfunction and morphological changes in renal structures consistent with cellular damage, and a progressive pathology through the induction of CHF via coronary artery ligation. The successful execution of this study will allow for future studies of therapeutic interventions against type 2 CRS.
Chapter 2

Materials and Methods

2.1 Materials

All organic, and inorganic, reagents used were purchased from Sigma-Aldrich (Auckland, New Zealand), or BDH laboratory supplies (Palmerston North, New Zealand) unless otherwise stated. All prescription animal remedies were dispensed from the Animal Welfare Office (University of Otago, Dunedin, New Zealand). These included heparin (Multiparin®, 500 USP units·kg$^{-1}$), halothane, bupivacaine, and mineral oil. All medical grade gasses were obtained from BOC (Dunedin, New Zealand).

2.2 Animals, animal care, and ethics

Male Lewis rats (250 - 270 g) were sourced locally from the University of Otago Hercus-Taeri Animal Unit in Dunedin, New Zealand. All procedures were carried out in accordance with University of Otago “Code
of Ethical Conduct for the Manipulation of Animals in Experimental Research”, with ethical approval (AEC 86/08001). Rats were housed individually, with a 12/12 light/dark cycle, both pre-, and post-, operatively. Water, and food were provided ad libitum, except where experimental protocol otherwise specifies. An acclimatisation period of at least one week, depending on rate of growth, was given between animal arrival, and first experimental manipulation.

Animals were randomly divided between full surgical (LAD), and sham surgical (sham) groups prior to surgery. Final inclusion into the LAD group was dependent on adequate disease state development, determined via troponin I based prediction (2.5.6), and/or direct measurement (2.8.3), of myocardial infarct scar size three months following infarction. Both methods were not available in all animals due to experimental protocol. Animals were monitored for four days after surgical manipulation, using standard University of Otago score sheets. During the 90 day ambulatory survival period, rats were visually monitored daily, and weighed weekly (monitoring data not reported). Visual signs of distress, or illness, were followed up with intensive monitoring for two days, followed by euthanization if no improvement, or further worsening of condition was observed. Saline was given to animals if animals showed signs of dehydration.
2.3 Brief experimental outline

A model of CHF secondary to AMI, well established within the literature [98], and our own lab group, was modified, and assessed for its ability to create a pathophysiological state in which the failing myocardium triggers a decline in renal function, resulting in renal impairment, and potentially RF. In order for renal impairment to develop, it was hypothesised that the myocardial damage would need to be severe. To achieve this, a non reperfusion model of heart failure was employed, and adequate time given for the post infarction renal damage to manifest. Animals with inadequate disease progression, or non renal co-morbidities, were also excluded (2.14). Inbred male Lewis rats were used to minimise genetic, and estral, variation.

HF was surgically induced (2.5.4), with effects on myocardial (2.8.2), and renal, function (2.9.1) assessed 90 days later. Functional, morphological, and biochemical markers were used to confirm, and measure progression of the disease state. Troponin I was used as a predictive biomarker of HF. Metabolic cages were employed at day 88 / 89 to measure urine output, electrolyte balance, and excretion or markers of renal function. On day 90, animals were sacrificed, and organs removed. The heart was functionally assessed in the Langendorff mode, and then processed for biochemical, or histological analysis [99]. The kidneys were removed, with one kidney perfused fixed in situ for histology, and the other prepared for biochemical analysis. Animals were randomly divided between
LAD and sham surgery groups. Venous blood was also collected, and centrifuged for plasma extraction. The brains were also taken from these animals, but are not the subject of this thesis, being part of a wider study (principal investigator Dr. Ivan Sammut, University of Otago, Dunedin, New Zealand).

2.4 General sample acquisition and preparatory techniques

2.4.1 Plasma preparation

All whole blood samples were collected either in heparinized tubes (2.5.3, 2.5.6), or from heparinized animals (2.7). Whole blood was centrifuged at 2,200 g, for 5 minutes, at 4°C. Supernatant (plasma) was recovered from the pellet (erythrocytes), aliquoted to assay specific volumes, and stored at -80°C for further biochemical analysis.

2.4.2 Blood gas analysis

Blood gas analysis was performed on a Radiometer ABL800 Flex blood gas analyser. This was done using heparinized capillary tubes (95 µL) for arterial samples (2.5.3), and heparinized blood in a syringe (≈300 µL) for venous samples (2.7). Analysis was performed in the clinical chemistry unit of Southern Community Laboratories (SCL) within the Dunedin hospital.
2.5 Surgical induction of myocardial infarction

2.5.1 Preparation of surgical equipment and tools

Aseptic technique was observed at all times during surgical protocols. All equipment, and surgical areas, were sterilized with 97% ethanol (EtOH; Department of Chemistry, University of Otago, Dunedin, New Zealand). Surgical instruments were all submerged in 97% EtOH for ≥15 minutes prior to surgery. On animals, all incision sites were shaved clean of fur, and sterilized with 97% EtOH. A surgical drape was used to isolate the surgical fields.

2.5.2 Anesthesia

Animals were fasted overnight before anaesthesia, and surgical manipulation. Anaesthesia was induced in Male Lewis rats (280-300 g) via a bell jar containing halothane (15 mL, evaporated), and then rapidly intubated (14 GA x 2” polyurethane catheter; Braun Safety IV Catheter), and maintained with a small animal ventilator (SAR-830 series, Stoelting Co., Chicago, United States of America) with a mix of 70% oxygen (), 30% nitrous oxide (N₂O), 1-4% halothane (reduced after induction until minimal concentration to maintain full ablation of pain reflex). Flow parameters were as follows: 2.3 mL tidal volume; 50 breaths.min⁻¹ respiratory rate; 230 mL.min⁻¹ inspiratory flow; 0.60 second inspiratory time). Toe pinch limb withdrawal reflex was used to assess depth of anaesthesia, and ablation of pain reflex pathways. Anesthesia was adjusted to
maintain the minimal depth required for full loss of pain reflex response. Mineral oil was applied to the corneas to prevent drying, and animals were maintained at 36°C ± 1°C via heating pad with rectal temperature feedback (Homethermic Blanket Control Unit, Harvard Apparatus). Gas ratios were adjusted as per protocol, as stated in specific methodologies (2.5.4, 2.5.5).

2.5.3 Arterial blood gas analysis; ligation of the femoral artery

This surgery was performed immediately prior to LAD ligation surgery (2.5.4), without the animal being removed from anaesthesia. In order to obtain a sample for blood gas analysis, the femoral artery was ligated. This arterial sample was used to monitor oxygen saturation, and make baseline measurements of various parameters (see 2.4.2).

A 3 cm incision was made along the distal border of the abdominal muscle of the left side of the abdomen, at the level of the thigh. Tissue was separated in layers, via blunt dissection, until the triad of the femoral artery, femoral vein and femoral nerve could be visualized. The femoral artery was isolated, via blunt dissection, and ligature (4-0 braided silk, Ethicon, New Jersey, United States of America) passed around it, proximally, and distally. The artery was tied distal to the intended incision point. Femoral artery blood flow was suspended via temporary occlusion proximal to the cannulation site, using the second ligature, by applying an upward force. A small incision was made in the artery, and heparinized cannula inserted. The vessel was then clamped around the cannula, and
tension on the proximal tie released to reinstate blood flow. Blood gas samples were collected in heparinized capillary tubes (95 μL), and taken for blood gas analysis (2.4.2). The proximal suture was tied, and cannula removed. There was no reinstatement of blood flow to the limb except that afforded by collateral blood flow. The risk of bleeding secondary to this procedure was deemed too high to attempt this procedure and later reinstate blood flow through this artery. This permanent ablation of flow was consistent within all experimental animals, with no signs of impairment of limb function following this procedure. The abdominal incision was then closed in layers with 6-0 prolene sutures (Ethicon, New Jersey, United States of America). Bupivacaine (0.1 mL) was injected evenly around the wound site by subcutaneous (SC) injection. The depth of anaesthesia was tested again via toe pinch, and the LAD surgery commenced.

2.5.4 Induction of heart failure; ligation of the left anterior descending coronary artery

The midpoint between the xiphoid process, and the manubrium was located, and a 3 cm transverse incision made towards the rat’s left flank. A 1 cm radius of epidermis was then separated from the underlying musculature surrounding the incision. The pectoralis profundus was then separated from the xiphimumeralis, and underlying musculature, via blunt dissection, until the anterior aspect of scalenus was visualized, and then retracted rostrally. The anterior aspect of the scalenus was then sepa-
rated from the surrounding musculature, and retracted caudally. This exposed the 5th to 6th intercostal space. The intercostal tissue was separated, and retracted (to absolutely no more than ≈1 cm wide). The pericardial sac was cut, exposing the heart. A 7-0 prolene suture (Ethicon, New Jersey, United States of America) was superficially (1.0 - 1.5 mm depth, 5 mm width) passed through the muscle of the LV between the margin of the atria, and the origin of the pulmonary artery. The ligature was either tied tight (LAD), or removed (Sham). Occlusion of the left anterior descending coronary artery (LADCA) was confirmed via visualization of LV blanching. If this was not seen to be sufficient, a second ligature was applied. In cases of severe infarction, pronounced bradycardia could be observed, with periods of fibrillation in the most severe cases. Cardiac massage was used in an attempt to stabilize rhythm, or restart hearts that had ceased to beat. At this point, N\textsubscript{2}O was withdrawn, and compensated for by an equipercen tile increase in O\textsubscript{2} delivery. This decrease in overall anaesthesia, and increase in O\textsubscript{2} was employed to increase survival rates, ensuring maximal O\textsubscript{2} delivery immediately post insult, as well as reducing the anaesthetic load. This modification did not affect the ablation of then limb withdrawal reflex within the remaining 5 to 7 minutes that it took to complete the abdominal wound closure and apply local anaesthesia. Complete withdrawal of N\textsubscript{2} should be reconsidered if surgical manipulations cannot be completed within the short period before the return of the limb withdrawal reflex, and should be monitored accordingly. This grace period does not last long, with halothane alone unable to maintain ablation of the limb withdrawal reflex for extended periods,
without an increase in the concentration of halothane administered. The chest cavity was irrigated with a small amount (2-3 drops) of phosphate buffered saline (PBS) to prevent drying, and minimise adhesions. With the aid of a 17 GA cannula placed within the ribspace, the intercostals space closed with a 5-0 prolene suture (Ethicon, New Jersey, United States of America), and the lungs were reinflated using the ventilators manual inspiration mode. Bupivacaine (0.1 mL) was distributed evenly between the intercostal muscles of the intercostal regions immediately rostral, and caudal, to the opening. The remaining wound was closed in layers, approximating any separated musculature, or tissue, with 5-0 prolene sutures. Halothane was withdrawn immediately before the final epidermal suture line (5-0 prolene sutures) was completed. This minimised time spent under anaesthesia, while proving adequate residual anaesthesia to complete the procedure. This also ensured faster post-operative recovery, and turn around. Bupivacaine (0.1 mL) was injected around the suture line for post operative local anaesthesia.

2.5.5 Peri, and post, surgical care

Animals were kept on 100 % O₂ until spontaneous breathing, and righting reflex, were regained. 5 mL warmed PBS was injected SC into the nape of the rat, with the rat being returned to individual housing, containing only shredded paper as bedding, once ambulatory. This 5 mL of warmed saline rehydrated the animals, and aided in maintenance of core body temperature while recovering from anaesthesia. Quantitative analysis of
predicted infarct size was done using a cardiac troponin I assay (cTnI; (2.5.6)). Post-surgical monitoring took place 4 hours post surgery, and again between 5 p.m. and 7 p.m. Animals were monitored once daily for a further 3 days post surgically. Moribund animals were euthanized. Animals were maintained for 90 days post surgically, allowing for disease development.

### 2.5.6 Cardiac Troponin I

cTnI is a specific marker of myocardial damage which is released from the myocardium after insult [100]. Plasma concentrations peak at 4 hour after an acute insult in the rat [100]. 4 hours after the ligation, or sham ligation, of the LADCA, a tail vein (IV) blood sample was taken from conscious animals. This was preferred to re-anesthetizing the animals due to the major surgery, and potentially weakened cardiovascular parameters, recently undergone. A conical polythene restraint bag was used to restrain the animals during sampling. 0.5 - 1.0 mL of blood was collected in a heparinized syringe. For samples exceeding 0.5 mL, equal volume of PBS was given, IV, to compensate for loss in circulatory volume. Plasma was obtained by the method outlined in 2.4.1.

cTnI concentration was measured using a commercially available High Sensitivity ELISA for Determination of Cardiac Troponin-I in Rat Plasma assay kit (Life diagnostics, Pennsylvania, United States of America). This assay works on the principle of recognition of specific epitopes on the cTnI isoform. Two different affinity purified antibodies are used in this assay.
The assay instructions were followed. Wash solution (provided within the kit) was prepared by diluting the 20x stock (50 mL) with 950 mL 18.3 MΩ H₂O. Standards were prepared by reconstituting lyophilised cTnI stock with 400 µL of 18.3 MΩ H₂O. The stock was then serially diluted, with standard diluent, to give a range of standards between 0.078, and 5 ng.mL⁻¹. A blank (0 ng.mL⁻¹) was also prepared. 100 µL of each plasma sample was diluted with 300 µL of plasma diluent. 100 µL of horse radish peroxidase conjugate was added to every well, followed by 100 µL of standards/samples. All standards/samples were plated in duplicate. Wells were mixed at 150 rpm for 60 minutes at room temperature. The incubation mixture was removed and then washed six times with wash solution (400 µL per well). 100 µL of tetramethylbenzidine reagent was added to each well, and incubated at room temperature for 20 minutes at 150 rpm. The reaction was stopped via the addition of 100 µL of stop solution to each well. The wells were gently mixed and absorbance read at λ=450 nm using a Bio-Rad Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Inc. California, United States of America).

2.6 Urine collection and analysis

2.6.1 Collection

In order to collect urine for electrolyte, protein, and creatinine analysis, rats were kept in metabolic cages for 24 hours immediately prior to endpoint organ retrieval. This employed a clear plastic housing with
Figure 2.1: Metabolic cage used for urine collection. (A) Profile view shows the general construction with food hopper, water bottle, animal housing area, concentrating funnel, filter bulb, as well as the collecting container. The diameter of the collecting container is less than the diameter of the filter bulb, and thus prevents solid matter contaminating the collected urine. (B) This Figure shows an angled top down point of view. This highlights the wire mesh flooring of the animal housing area, the food hopper, and spout of the water bottle. Different grades of mesh were employed to minimise food pellet contamination.
a wire mesh floor (Figure 2.1). A funnel/filter system was utilised to minimise contamination with food, or fecal, matter. This worked by using a dome system that collected fluid, but deflected solid material. Rats were weighed prior to this collecting period. Water intake was also monitored. Urine volume was calculated by weight, as a more accurate, and consistent, method. Urine samples were centrifuged at 2,200 g, at 4 °C, for 10 minutes (Eppendorf centrifuge 5810R; Global Science, New Zealand), to remove any solid impurities. A 50 μL aliquot was diluted (1:200) in 18.3 mΩ H₂O containing 7.5 mM Li₂CO₃ (Li⁺ concentration of 15 mEq.L⁻¹) as an internal standard for electrolyte analysis. Aliquots (2 x 1 mL) were also kept for protein, and creatinine biochemistry. Urine samples were stored at -80 °C until analysis.

2.6.2 Electrolyte analysis

Urinary Na⁺ ion excretion was measured using flame photometry (FP20 SEAC, Italy). Diluted urine samples 2.6.1 were prepared, and measured in duplicate. A 7.5 mM Li₂CO₃ solution was used as a blank, and standardized against a synthetic urine standard provided (SEAC Na⁺ 100 mEq.L⁻¹). Samples were vortexed, and then read via flame photometry as mEq.L⁻¹ for Na⁺, a concentration equivalent to mM. This concentration was then multiplied by the total urine excretion in 24 hours to give the total amount of Na⁺ excreted as μmol.24 hours⁻¹.
2.7 Organ harvest

All animals were fasted overnight before scheduled organ retrieval. This provided a more stable metabolic baseline for comparison between animals. Animals were weighed, and then induced via a bell jar containing halothane (15 mL evaporated) in a fume hood, and maintained with 60% O₂, 40% N₂O, and 3% halothane, via nose cone. A toe pinch reflex test was used to determine when pain reflexes had been lost. The abdominal cavity was opened via midline incision, and the bifurcation of the inferior vena cava (IVC) located. 0.1 mL heparin was injected into the IVC, and allowed to circulate. 4 mL of whole blood was drawn from the IVC for further analysis.

The heart was rapidly excised via thoractomy, and transferred to ice cold Krebs-Henseleit-Ringer buffer (KHB; (A.1)). The heart was then immediately mounted in the Langendorff mode for functional analysis 2.8.1. The IVC was cut below the liver, to facilitate drainage, and the thoracic aorta cannulated (16 GA cannula). The inferior organs were immediately in situ perfused with 60 mL of 4°C Tris-Mannitol-Sucrose buffer (TMS; (A.2)). Once perfused, the left kidney was excised, weighed, and held in ice cold TMS buffer for further processing. The left kidney was then cut down the sagittal plane, and the cortex and medulla separated. Separated regions were then snap frozen in liquid nitrogen (LN). The snap frozen regions were finely crushed using a cold, flat, mortar and pestle, then stored at -80°C for future biochemical analysis. The right kidney
was concurrently in situ perfuse-fixed with 4% paraformaldehyde (4°C; A.4), dissected out, weighed, and divided along the sagittal plane. The sections were post-fixed in 4% paraformaldehyde overnight, before being transferred to 70% EtOH, and stored at 4°C for later histological examination.

2.8 Myocardial function

2.8.1 Langendorff

Hearts were immediately mounted on a Langendorff perfusion rig upon excision from the animal [99]. Aortas were cannulated, and perfused in the Langendorff mode with oxygenated (95% O₂, 5% CO₂), 37°C KHB at 100 mm H₂O. The pulmonary cone was cut to reduce pulmonary back pressure. Excess tissue was removed (such as lungs, thymus, fat, etc), and an intraventricular balloon was inserted via a small incision in the left atria, and threaded into the left ventricle via the mitral orifice. Pressure on the left ventricular balloon was translated by a physiological pressure transducer (Memscap SP 844), passed through a bridge amp (ADInstruments), PowerLab/4sp (ADInstruments), and measured using Chart 5.4.2 for Windows (ADInstruments). Traces were calibrated using 2 points (30 mmHg, and 160 mmHg, via sphygmomanometer). Hearts were equilibrated for a period of 20 minutes before testing. The intraventricular balloon was filled to baseline. This was achieved by filling the balloon with 10 µL increments of fluid, until the slack in the balloon was
accounted for. This presents as a stable diastolic pressure, with a systolic pressure that increases with increasing balloon volume.

2.8.2 Myocardial functional measurement

Intraventricular balloon volume was increased in 50 µL increments until diastolic pressure increased to 10 mmHg above baseline. Volume increases were only made when the diastolic pressure had stabilized. This point, a 10 mmHg increase in diastolic pressure, was used for all functional measurements. Derived values included left ventricular developed pressure (LVDP), rate of contraction \( \Delta P \cdot \Delta t_{max}^{-1} \), rate of relaxation \( \Delta P \cdot \Delta t_{min}^{-1} \), un-paced \textit{ex vivo} heart rate (HR). Physical measurements included coronary flow (CF). LVDP was derived as the mean difference between systolic pressure, and diastolic pressure. \( \Delta P \cdot \Delta t_{max}^{-1} \) is representative of the inclining pressure slope as the LV contracts, with \( \Delta P \cdot \Delta t_{min}^{-1} \) being representative of the declining pressure slope as the LV relaxes. HR was calculated as the amount of ventricular contractions per minute. CF represents the volume of pulmonary effluent collected in 60 seconds.

2.8.3 Post Langendorff tissue preparation

After final measurements had been taken from the \textit{ex vivo} heart, the heart dissected for biochemical analysis. The atria, and major vessels emanating from the superior aspect of the heart, were removed, and discarded, the remaining ventricles were weighed (ventricular weight). The RV free wall was removed, weighed, and discarded. The septum was
separated from the LV free wall, weighed, and snap frozen in L. The scar (if present) was dissected from the remaining free wall, with a 2 mm border of peri-infarct tissue. The remaining free wall was snap frozen in L. In sham animals, the scar was approximated (for biochemical analysis, not presented in this thesis) by using healthy ventricle, all other steps were identical. The peri-infarct tissue was dissected from the scar, and snap frozen in L. The scar was weighed, and then snap frozen in L (biochemical analysis not presented in this thesis). All samples were then stored at -80 °C until further biochemical analysis was performed. The weights obtained were used to calculate various parameters such as combined ventricular weight to fasted body weight (BWf; obtained in section 2.7) ratios, scar size (presented as % wet weight of left ventricle, including the septum), and relative mass of each ventricular region (RV, LV, scar, and septum, expressed as a percentage of entire ventricular mass).

2.9 Renal function

2.9.1 Glomerular filtration rate

Glomerular filtration rate (GFR) was estimated (eGFR) using CrCl. CrCl is a clinical measure of renal function, based on the limited tubular secretion that endogenous creatinine undergoes. For this purpose, both plasma (150 μL), and urine (1 mL), from the same 24 hour period, were collected, and analysed for creatinine concentration. These analysis were
performed by SCL, located within the Dunedin Hospital, on a Synchron Cx7 Beckman Analyser, using a modified colourimetric Jaffe method. From these concentrations, CrCl was calculated using the following formula:

\[
\text{CrCl} = \frac{[\text{Cr}]_{\text{urinary}} \cdot \text{UV}_{24 \text{hour}}}{[\text{Cr}]_{\text{plasma}} \cdot 1440 \text{ minute}}
\]

This value is then expressed as mL.min\(^{-1}\).100 g BW\(^{-1}\).

2.9.2 Fractional excretion of sodium

The Fractional excretion of sodium (\(\text{FE}_{\text{Na}}\)) is a representative measure of the amount of the total sodium filtered by the kidney that is excreted in the urine. It is used clinically as a representative measure which can indicate renal tubular impairment, with >1% used as a marker of renal failure, and >2% potentially indicating acute tubular necrosis, or other acute renal damage. It is important to note that this data was obtained from conscious animals, rather than anesthetised animals, as anaesthesia is known to increase \(\text{FE}_{\text{Na}}\) values in rats. It is calculated from data collected in 2.9.1 using the following formula:

\[
\text{FE}_{\text{Na}} = 100 \cdot \frac{[\text{Na}]_{\text{urinary}} \cdot [\text{Cr}]_{\text{plasma}}}{[\text{Na}]_{\text{plasma}} \cdot [\text{Cr}]_{\text{urinary}}}
\]

\(\text{FE}_{\text{Na}}\) is expressed as a %. 
2.10 Biochemical assays

2.10.1 Tissue homogenisation

Tissue samples, where appropriate (2.10.2, 2.10.3, 2.10.4), were removed from -80 °C storage, and transferred to lN. Samples were freeze crushed using a flat, ice cold, mortar and pestle, three times. The resultant powder was then transferred to a glass-Teflon homogeniser (Potter - Elvejhem; clearance 50 µm), and homogenised manually with assay specific buffer (300 µL) on ice. The homogenisation procedure consisted of three slow strokes, the first at 700 rpm, and the second two at 1,500 rpm. The raw homogenates were then centrifuged (Beckman J2-MC, CA, United States of America) at 4,680 g for 5 minutes, at 4 °C. The pellet, consisting primarily of heavier cell debris (collagen, membranes and non-fractured cell tissue) was kept, and stored at -80 °C. The supernatant was removed, and filtered using 2.0 mL Spin-X tubes (0.22 µm cellulose acetate filters; Bonnet Equipment, Auckland, New Zealand), and centrifuged (Beckman J2-MC, California, United States of America) again at 13,000 g for 1 hour, at 4 °C, filtering out cell organelles and debris from the sample filtrate. The sample filtrate was then stored at -80 °C until biochemical analysis. Protein concentrations were standardized using the method described in section 2.10.2.
2.10.2 Protein determination

Determination of protein concentration was achieved using a BioRad DC Protein Assay kit (Bio-Rad Laboratories Inc., New Zealand), a modified spectrophotometric Lowry assay. A standard curve was constructed using bovine serum albumin (2 mg·mL\(^{-1}\)) diluted with the same buffer that samples were prepared in. See specific assays for buffer used. The standard curve consisted of protein concentrations of 0.2, 0.4, 0.6, 1.2, and 1.8 mg·mL\(^{-1}\). Samples (5-10 μL) were diluted 1 in 10 initially, and then 1 in 50, or 1 in 100, if they remained above the detectable limits of the assay. Urine samples were diluted to 1 in 5. The use of Lowry based protein determination for urine protein has been previously established [101]. The same buffer as samples were prepared in was used for these dilution steps in all cases.

Standard assay protocol was followed. Briefly, samples, and standards (5 μL for both) were plated into 96 well microtitre plates (Becton Dickinson, United States of America), in triplicate. Samples/standards were plated, followed by alkaline copper tartrate solution (25 μL), and then dilute Folin reagent (200 μL). The plates were mixed, and incubated at room temperature for 15 minute. Maximum absorbance was measured at \(\lambda=750\) nm using a Bio-Rad Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Inc. California, United States of America). The protein concentration of samples was calculated using the standard curve prepared on each plate. This was repeated until all samples had been measured within the measureable range, diluting samples as re-
quired.

2.10.3 Renal Caspase-3/7 Activity

Caspase-3/7 activity was assessed, in both renal cortex, and renal medulla, homogenates using a commercially available caspase assay kit (Anaspec EnsoLyte™ Homogeneous AFC caspase-3/7 assay kit; San Jose, California, United States of America). This assay works by measuring the AFC fluorophore (blue fluorescence) generated when the assay substrate (peptide sequence: Z-Asp-Glu-Val-Asp-AFC) is cleaved by caspase-3.

Samples were homogenised by the aforementioned method (2.10.1), using TMS buffer. The assay reagents were prepared as per the included instructions, and instructions followed for assay procedure. The enzyme reaction mixture was made up by combining 42.5 μL of caspase substrate, 340 μL of dithiothreitol (1 M), and made up to 8.5 mL with EnsoLyte™ assay buffer. Black walled, clear bottomed 96 well plates were used for this assay. 100 μL of each standard/sample (1mg.mL\(^{-1}\)protein) was pipetted in duplicate, followed by 50 μL of enzyme reaction mixture. The plate was incubated for 14 hours at room temperature (21 °C) on a plate shaker at 200 rpm. The plate was then read on a Gemini-EM fluorometric plate reader (Molecular Devices Corporation, California, United States of America) at excitation/emission of \(\lambda=380\) nm / 500 nm, with results expressed as relative fluorescent units per mg of protein (RFU.mg\(^{-1}\)protein).
2.10.4 Cytokine assays

To assess cytokine concentrations within myocardial, and renal, tissue, commercially available Milliplex\textsuperscript{TM} MAP Rat Cytokine Assay kits (Millipore\textsuperscript{TM}, Massachusetts, United States of America) were utilised. In myocardial peri-infarct tissue IL-1\textbeta, IL-4, IL-6, IL-10, and TNF-\textalpha were measured. In renal tissue, TGF-(b), and TNF-\textalpha were measured. The kits comprised of reagents allowing the measurement of numerous cytokines, within a single sample, in a capture sandwich immunoassay format. The kits work on the principle of fluorescent colour-coded beads, each conjugated to a different antibody specific to the biomarker of interest. These beads are reacted with the sample, and after conjugation to the target molecule, incubated with biotinylated antibody specific to the molecule of interest. Streptavidin-phycocerythrin is then coupled to the biotinylated antibodies, allowing detection. The Luminex 100\textsuperscript{TM} analyser (Luminex Corp, Austin, Texas, United States of America) employs a dual laser, and associated optics, setup which allows for the differentiation between colour-codes. The fluorescence output is managed by a high-speed digital signal processor. These combined technologies allow the quantification of cytokines in the samples. These assays are normally used for cell culture, or plasma, analysis, but have been adapted in the literature, as well as within our own lab group, to cater work with homogenised tissue samples [102].
2.10.4.1 Assay of IL-1β, IL-4, IL-6, IL-10, and TNF-α

Homogenised, filtered, samples (2.10.1) prepared in TMS buffer were used for this assay. Samples consisted of cardiac peri-infarct tissue. Kit instructions were followed. Briefly, all reagents were reconstituted, as specified in manufacturer’s instructions, with mΩ H₂O, and thoroughly vortexed. Vials containing antibody-immobilized beads were sonicated for 30 seconds to disperse beads, and then vortexed. Bead aliquots were taken (60 μL) from each of the cytokines been assessed, combined, diluted to 3.0 mL with Bead Diluent (provided with the kit), and vortexed. Provided cytokine standards were diluted serially with the proprietary Assay Buffer included in the kit. This produced a range of concentrations between 4.88, and 5000 pg.mL⁻¹. Two sets of internal quality controls were also included.

A 96 well filter plate was used for this assay. The plate was pre-wetted with 200 μL Assay Buffer, shaken at 250 rpm for 10 minutes at room temperature, and then removed via vacuum filtration. Standards, controls, and neat filtered homogenate were plated (25 μL for all), and followed by 25 μL of Assay Buffer, and then the 25 μL of the previously mixed beads. The plate was sealed, and agitated on a plate shaker, at 250 rpm for 16 hours in the dark at 4°C.

Fluid contents of the wells were then vacuum filtered. Each well was washed with Wash Buffer (200 μL) and vacuum filtered, twice. Detection Antibody (25 μL) was then added to each well, the plate sealed,
and agitated at 250 rpm for 1 hour at room temperature. The wells were then vacuum filtered, and washed twice with 200 µL of Assay buffer, as previously described. Streptavidin-Phycoerythrin (25 µL) was added to wells containing detection antibody, and incubated on a plat shaker (250 rpm) for 30 minutes. The wells were then vacuum filtered, and washed twice with 200 µL of Assay buffer, as previously described. Sheath Fluid (150 µL) was added to each well, and the plate agitated for 5 minutes at 250 rpm. Fluorescence was then measured by a Luminex 100™ analyser (Luminex Corp., Texas, United States of America). The standard curve was internally derived using Bio-Plex Manager™ software, and used to convert each measurement to pg/mL⁻¹. These concentrations were then standardized to against protein concentrations calculated by the previously outlined method (2.10.2).

2.10.4.2 Assay of TGF-β isoforms.

Homogenised, filtered, samples (2.10.1) prepared in TMS buffer were used for this assay. Samples consisted of renal medulla, as well as three spiked samples consisting of 25 µL of a sham sample, and 25 µL of TGF-β standard solution, resulting in a sample consisting of 78 pg·mL⁻¹ of each isoform. Kit instructions were followed. Briefly, all reagents were reconstituted, as specified in manufacturer’s instructions, with mΩ H₂O, and thoroughly vortexed. Vials containing antibody-immobilized beads were sonicated for 30 seconds to disperse beads, and then vortexed for 1 minute. Provided standards were diluted serially with the proprietary Assay Buffer included in the kit. This produced a range of concentrations
between 9.8, and 10,000 pg.mL$^{-1}$. Two sets of internal quality controls were also included. These were reconstituted with 250 µ of Assay Buffer, inverted several times, and mixed by vortexing for 1 minute. Wash Buffer was prepared by diluting the 10X stock (30 mL) with mΩ H$_2$O (270 mL).

A 96 well filter plate was used for this assay. The plate was pre-wetted with 200 µL Assay Buffer, shaken at 250 rpm for 10 minutes at room temperature, and then removed via vacuum filtration. Standards, controls, and neat filtered homogenate were plated (25 µL for all), and followed by 25 µL of Assay Buffer, and then the 25 µL of the previously mixed beads. The plate was sealed, and agitated on a plate shaker, at 250 rpm for 18 hours, 12 minutes, in the dark at 4°C.

Fluid contents of the wells were then vacuum filtered. Each well was washed with Wash Buffer (200 µL) and vacuum filtered, twice. Detection Antibody (25 µL) was then added to each well, the plate sealed, and agitated at 250 rpm for 1 hour at room temperature. Streptavidin-Phycoerythrin (25 µL) was added to wells containing detection antibody, and incubated on a plat shaker (250 rpm) for 30 minutes. The wells were then vacuum filtered, and washed twice with 200 µL of Assay buffer, as previously described. Sheath Fluid (150 µL) was added to each well, and the plate agitated for 5 minutes at 250 rpm. Fluorescence was then measured by a Luminex 100™ analyser (Luminex Corp., Texas, United States of America). The standard curve was internally derived using Bio-Plex
Manager™ software, and used to convert each measurement to pg.mL$^{-1}$. These concentrations were then standardized against protein concentrations calculated by the previously outlined method (2.10.2).

2.11 Cardiac histology

2.11.1 Tissue fixation and slide preparation

Hearts were arrested in diastole using 20 mM KCl KHB (A.1), and ex vivo perfused with 60 mL neutral buffered formalin at a pressure of 1 m of H$_2$O. Atrial appendages, and major vessels were removed, and the remaining ventricles were stored in 10% neutral buffered formalin overnight, and then transferred into 70% EtOH until histological staining. The ventricles were sliced coronally into four blocks, at 1.5 mm intervals. The apex of the heart was labelled as level one, with the base of the heart being level four. This resulted in the apical region (level one) corresponding to 0-1.5 mm, level two corresponding to 1.5-3mm, and so forth. Blocks were permeated in an xylene bath, and paraffin wax embedded. Using a microtome (Lecia Jung Autocut, Germany), 10 µm sections were cut, and mounted on slides for staining. This procedure has been completed for three hearts to date, and is not yet complete.

2.11.1.1 Picrosirius Red with Fast Green contrast stain

Sections were stained with Picrosirius Red, and Fast Green contrast stain, to produce a strong distinction between viable myocardium (blue green),
collagenous scar tissue (red). Sections, were deparaffinised, and fixed in pre-warmed Bouins' solution (55°C) for 1 hour, and then washed in running tap water until the yellow disappeared. The slides were then stained with 0.1% Fast green for 10 minutes, and then soaked in 1% acetic acid for 2 minutes. The slides were rinsed in tap water, and the stained with 0.1% Picro-Sirius red for 30 minutes. Slides were dehydrated with EtOH, cleared with xylene, and mounted using Entellan medium.

This resulted in collagen staining red, with viable myocardium staining a blue green. Images were captured using a slide scanner.

2.12 Renal histology

2.12.1 Slide preparation

Following *in situ* perfuse fixation with 4% paraformaldehyde, and storage in 70% EtOH (2.7), kidneys were dehydrated using a series of increasing EtOH concentrations, permeated in an xylene bath, and then paraffin wax embedded. Using a microtome, 3 μm sections were cut, and mounted on slides for staining.

2.12.2 Histological staining

2.12.2.1 Masson’s trichrome

Sections were stained using a Masson’s trichrome stain [103]. Sections, were deparaffinised, stained in celestine blue for 5 minutes, rinsed in dis-
tilled water, and then stained with Gill’s No. 2 Haematoxylin for 5 minutes. Slides were then washed in Blue in Scott’s tap water, stained in 0.5% acidified Acid Fuchsir solution (B.1.1) for three minutes, and then rinsed in distilled water. Sections were placed in 1% phosphomolybdic acid solution (B.1.2) for three to five minutes, stained in 2% acidified Methyl Blue solution (B.1.3), and rinsed again in distilled water and 1% acetic acid (B.1.4) for two minutes. Slices were dehydrated with EtOH, cleared with xylene, and mounted using Entallan medium.

This process resulted in nuclei being stained blue black, and collagen blue. Cytoplasm, muscle, and erythrocytes, all stained red [103].

2.12.2.2 Martius Scarlet Blue

Sections were stained using a Martius Scarlet Blue stain [103]. Sections, were deparaffinised, stained in celestine blue for 5 minutes, rinsed in distilled water, and then stained with Gill’s No. 2 Haematoxylin for 5 minutes. Slides were then washed in Blue in Scott’s tap water, stained in Martius yellow solution (B.2.1) solution for 2 minutes, and then rinsed in distilled water. Brilliant crystal scarlet solution (B.2.2) was then added for 10 minutes, rinsed with distilled water, and treated with phosphotungstic acid solution (B.1.2) until no red remained in the collagen, usually for around 4 minutes. The slides were then rinsed in distilled water, stained in methyl blue solution (B.2.4) for 1 minute, rinsed in 1% acetic acid (B.2.5), rapidly dehydrated, cleared, and mounted.
This process resulted in nuclei being stained blue, erythrocytes yellow, muscle red, and collagen blue. Fibrin stained red, with early fibrin staining yellow, and old fibrin staining blue.

2.12.3 Microscopy

Sections were visualized using a Zeiss AxioPlan 2 microscope, and images captured using Axiovision 4.6 software. Each section was visualized under direct light, examining them to identify morphological features in both the medulla, and cortex, and photographed for visual reference. The observer was blinded to the animal groupings. No quantification has been able to be carried out at this point, but qualitative analysis of the histological morphology of collagen deposition was performed in five separate fields per section.

2.13 Statistics

Analysis between two groups (single variable) was performed using one-tailed unpaired T-tests with Welch’s correction, which does not assume both populations have equal variance. Significance was considered if $P \leq 0.05$. Analysis was displayed as mean $\pm$ SEM. GraphPad Prism® 5.03 for Windows was used for statistical analysis. Each group consisted of at least $n = 5$, with some groups containing more replicates. This discrepancy was due to either exclusion criteria ((2.14)), due to samples being retained for future analysis, or different analysis requiring the same aliquot, thus precluding all analysis from being performed on all tissues.
2.14 Exclusions

Rats with an infarct <20% weight of the left ventricle, including the septum, were excluded from any analysis that was done between sham and LAD. 20% was chosen as a cut off as it represented an injury severity that corresponded with adequate impairment of normal haemodynamic function as assessed by Langendorff perfusion. This was derived from within the total group of rats that underwent the aforementioned surgical manipulations to be able to assign rats as showing evidence of cardiac dysfunction consistent with heart failure. Rats that showed morbidity separate from the intended disease state were also included. As specific examples, one sham animal that was observed to have a distended bladder, and found to be containing seven large renal caliculi, with another single sham animal which was severely underweight, due to a failure to put on weight, rather than losing weight, despite all efforts of augmented feeding. Animals whose hearts failed to show an increase in diastolic pressure above baseline, despite large volumes being added to the ventricular balloons, were also excluded from cardiac functional analysis. Some urine samples were excluded on the basis of contamination with food, or fecal matter. Other samples were not included in analysis due to difficulties, or malfunctions, encountered while collecting, or processing them.
2.15 Validation of experimental aims

In order to validate this model, as a working example of type 2 CRS, there are several criteria that must be met. It must first be established that the surgical techniques used are able to adequately induce left ventricular dysfunction, and tissue remodelling, consistent with left ventricular failure. This will include the presence of markers of myocardial ischaemic injury, such as elevated cTnI, an established, specific, marker of infarction in humans, and more importantly, the rat.

Once cardiac dysfunction has been confirmed, and profiled, renal function can be investigated. In order to demonstrate type 2 cardio renal interaction, there must be significant renal impairment, compared to in naïve controls. This needs to be confirmed by demonstrating decreases in established functional markers of renal dysfunction, such as GFR, and be further supported by morphological changes meeting the criteria for renal damage, such as interstitial fibrosis.

Blood gas measurement at the time of sacrifice will be used to provide additional data able to highlight haematological changes, such as decreased erythrocyte concentration. Decreased erythrocyte concentration is not required for confirmation of type 2 cardiorenal interactions, but is a significant aspect of end stage renal disease, and also presents a contributory factor to the decline in function of the primary organs of interest within this model.
Chapter 3

Cardiac, Haematological, and General Results

3.1 Mortality reports

During the development of this model, mortality rates varied. This was due to a combination of factors, including training, refinement of techniques, and calibration of techniques to acquire desired levels of injury. This presented initially with a mortality rate <15 %, but with the consequence of a high rate of sub threshold injuries, consisting of left ventricular scars that were below 20 % wet weight of the left ventricle including the septum, and required modification of technique. Ligation of the left anterior descending coronary artery closer to its origin resulted in much larger scars, but also resulted in >50 % of animals dying immediately after ligation. With further adjustment of ligature placement a high rate of myocardial scaring >20% wet weight, was achieved with an accompany-
ing mortality rate of 28%. It should also be noted that these figures are based around the entire cohort of animals used in the presented study, and other therapeutic studies not presented in this thesis, as this thesis comprised a subset of data from a wider, yet to be completed, study. It was not possible to measure, or predict, the infarct severity of animals that died during surgery.

3.2 Prediction, and measurement of infarction, and other physical parameters

Cardiac troponin I was used as a biomarker predictive of myocardial damage. cTnI was significantly elevated \((P = 0.0006)\), at 4 hours post ligation, in LAD animals, compared to sham animals \((4.456 \pm 0.4129 \text{ ng}\cdot\text{mL}^{-1} (n = 5) \text{ Vs.} 1.346 \pm 0.5322 \text{ ng}\cdot\text{mL}^{-1} (n = 13)\), respectively) (Figure 3.1).

Gross myocardial scaring was visually apparent upon organ harvest. This can be seen in Figure 3.2, which shows a healthy sham heart, and a severely infarcted LAD heart. Scaring was prominent, spanning from the myocardial apex to the atrioventricular junction, and indicative of successful LADCAO during surgical induction of HF. The overall shape of the myocardium was changed in severely infarcted hearts, becoming more apparent as scar size increased. In the infarcted hearts, sphericity could be seen, and is prominent in the LAD example shown. This was also accompanied by impaired contraction, and the scar tissue was visually non-contractile. The impaired left ventricular haemodynamics is
Figure 3.1: Plasma concentration of cardiac troponin I (cTnI). Data presented as individual data points, with plotted mean ± SEM. n = 5, and n = 13, for Sham, and LAD, respectively. *** P = 0.0006 Vs. Sham.
Figure 3.2: Representative images of a sham, and a severely infarcted heart. (A) Sham operated heart showing the typical appearance of a healthy myocardium mounted in the Langendorff mode. (B) LAD heart. Myocardial sphericity can be observed, along with a very prominent scar, which was transmural. This view is side on with the left ventricle on the right, and the right ventricle on the left of the images. In this instance, almost the entire free wall of the ventricle was infarcted, and comprised of collagenous scar.
quantified in section 3.3.

There was no difference between sham and LAD groups in terms of whole animal BWf at time of sacrifice (423.9 ± 9.7 g Vs. 417.3 ± 16.4 g, respectively). There was, however, a significant (P = 0.0027) increase in total ventricular mass per 100 g of BWf when sham and LAD were compared (0.34 ± 0.02 g,100 g$^{-1}$BWf ($n=5$) Vs. 0.50 ± 0.04 g,100 g$^{-1}$BWf ($n=6$), respectively; Figure 3.3). Average scar size in the LAD group was 24.2 ±1.5% wet weight of the LV mass, including the septum, of hearts not processed for histology.

When individual regions of the myocardium were examined in a subset ($n=4$) of rats (Table (3.1)), it was found that LV free wall mass (inclusive of scar, if appropriate) was significantly (P = 0.0103) increased between groups. This was also true for the right ventricular free wall, but not the septum. This represents a scenario where the proportion of total ventricular mass is comprised of approximately 37.9% LV, 32.1% RV, and 30% septum, in sham animals, and 53.4% LV, 30% RV, and 16.6% septum, for LAD animals. In this subset, LAD animals had an average scar of approximately 23.7% of the LV and septum. This regional breakdown was only available in $n=4$ for each group, as the left ventricle, and septum, were not weighed separately in the early stages of the study.

Examination of the apex also reveals obvious thinning of the myocardium, with the heart wall becoming translucent within the scarred region. Slices of infarcted myocardium were stained with Picrosirius-Red, with a Fast
Figure 3.3: Ventricular mass adjusted for bodyweight. Measured as heart weight per 100 g of fasted bodyweight. Data presented as individual data points, with plotted mean ± SEM. \( n = 5 \), and \( n = 6 \), for Sham, and LAD, respectively. BWf = fasted heart weight. ** \( P = 0.0027 \) Vs. Sham.
Table 3.1: Regional breakdown of myocardial regional mass in a subset of animals. This complete regional breakdown was only available in four of the analysed animals, in each group, as the left ventricle, and septum, were not weighed separately in the early stages of the study.

<table>
<thead>
<tr>
<th>Regional breakdown</th>
<th>Sham</th>
<th>LAD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ventricular free wall mass (including scar) (g)</td>
<td>0.53 ± 0.10</td>
<td>1.03 ± 0.11</td>
<td>0.0103</td>
</tr>
<tr>
<td>Scar mass (g)</td>
<td>0 g</td>
<td>0.32 ± 0.02</td>
<td>N/A</td>
</tr>
<tr>
<td>Septum mass (g)</td>
<td>0.42 ± 0.11</td>
<td>0.32 ± 0.05</td>
<td>0.2366</td>
</tr>
<tr>
<td>Right ventricular free wall mass (g)</td>
<td>0.45 ± 0.04</td>
<td>0.58 ± 0.04</td>
<td>0.0381</td>
</tr>
</tbody>
</table>
Figure 3.4: Representative Picrosirius Red stained myocardial sections with a Fast Green contrast stain taken at 90 days post occlusion, from sham, and LAD, ligated animals, respectively. Viable myocardium stains blue/green, and Collagen is stained red (A-D) Sections taken at 1.5 mm intervals in a healthy sham heart showing left and right ventricles. (A) Level 1; apex. At this level, the apex of a healthy heart can be seen, comprising of healthy myocardial wall. (B & C) Level 2, and 3. Healthy ventricular tissue can be seen showing normal morphology. The sections showed no evidence of extra collagen deposition. (D) Level 4; base of the myocardium. At the level of this final slice, there is still no sign of injury, and appearance is healthy. (E-H) Sections taken at 1.5 mm intervals of an LAD heart estimated to have an infarct of 29% volume of the entire left ventricle. In other hearts (not shown) there was also evidence of collagen deposition within the septum. (E) Level 1; apex. At this level, substantial thinning of the LV free wall, and collagen deposition in the LV free wall can be observed. (F) Level 2. The collagen deposition, and ventricular thinning observed in the apical slice can be seen to be extensive, persisting in the LV free wall. The resultant scar was found to be transmural, across the free wall of the LV. (G) Level 3. As seen with level 3, the transmural, collagenous scar, of the LV free wall, persists even further towards the base of the myocardium. (H) Level 4; base of the myocardium. At the level of this final slice, the scar can be seen to be less dramatic. This was close to the origin of the infarction.
Green counter stain, to show the cellular changes in tissue composition between the infarcted regions, and the healthy myocardium (Figure 3.4). This revealed a substantial ventricular enlargement, seen as an increased intraventricular volume, when viewed alongside a sham heart, with substantial wall thinning, and a very prominent red stained collagenous scar.

3.3 Myocardial left ventricular haemodynamic function

LAD surgery was shown to significantly (P = 0.0013) decrease LVDP from $119.3 \pm 5.173 \text{ mmHg}$ to $85.51 \pm 7.681 \text{ mmHg}$ (Figure 3.5). The rate of contraction and relaxation of the LV myocardium was also affected by LAD surgery. There was a significant (P = 0.0141) decrease in the rate of myocardial relaxation, from $-1389 \pm 106.9 \text{ mmHg.s}^{-1}$ to $-1076 \pm 73.70 \text{ mmHg.s}^{-1}$ (Figure 3.7). There was however only a trend towards significance (P = 0.0547) when rate of contraction was measured in the LV, dropping from $2265 \pm 240.3 \text{ mmHg.s}^{-1}$ to $1742 \pm 195.0 \text{ mmHg.s}^{-1}$.

There was also a significant (P = 0.231) decrease in spontaneous HR, dropping from $162.6 \pm 8.5 \text{ bpm}$ to $136.6 \pm 8.6 \text{ bpm}$ (Figure 3.8). There was no change in CF between Sham ($8.343 \pm 1.087 \text{ mL.minute}^{-1}$) and LAD animals ($7.800 \pm 0.9522 \text{ mL.minute}^{-1}$).
Figure 3.5: Left ventricular developed pressure (LVDP) of sham operated, and LAD operated animals. Data represented as individual data points with mean ± SEM. $n = 11$, and $n = 9$, for Sham, and LAD, respectively. ** $P = 0.0013$ Vs. Sham.
Figure 3.6: $\Delta P \cdot \Delta t_{\text{max}}^{-1}$ representing the rate of left ventricular contraction. Data is represents individual data points with mean ± SEM. $n = 11$, and $n = 9$, for Sham, and LAD, respectively. # P = 0.055 Vs. Sham.
Figure 3.7: $\Delta P \cdot \Delta t^{-1}_{\text{min}}$ representing the rate of left ventricular relaxation. Data is represents individual data points with mean ± SEM. $n = 11$, and $n = 9$, for Sham, and LAD, respectively. * $P = 0.014 \text{ Vs. Sham.}$
Figure 3.8: Heart rate of denervated *ex vivo* myocardium mounted in the Langendorff mode. Data represented as individual data points with mean ± SEM. $n = 11$, and $n = 9$, for Sham, and LAD, respectively. **$P = 0.023$ Vs. Sham.**
3.4 Myocardial cytokine profiles

In the myocardium, IL-1β was significantly (P = 0.0082) increased in LAD animals, compared to sham animals, with values of 35.74 ± 6.877 and 15.40 ± 2.106, respectively (Figure 3.9). IL-4 was not significantly affected by LAD surgery (13.62 ± 2.664, and 12.09 ± 2.721, for sham and LAD, respectively) in the myocardium. IL-6 was not significantly affected, in the myocardium, either (95.82 ± 33.62, and 95.76 ± 34.79, for Sham and LAD, respectively). This was also the case for myocardial IL-10 (145.2 ± 81.22, and 118.3 ± 39.31, for Sham, and LAD, respectively). Concentrations of IFN-γ, and TNF-α, in the myocardium, were below the detectable limits of the multiplex assay.

3.5 Blood gas analysis

Arterial blood gas samples were taken at the time of LAD surgery from the femoral artery (section 2.5.3), and venous blood gas samples were taken at the time of organ harvest (section 2.7), and analysed using SCL equipment (section 2.4.2). There was no evidence of anaemia in any of the animals, with no significant difference between haemoglobin (Hb) concentration, or haematocrit (Hct), between sham, and LAD animals, at time of surgery, or organ harvest (Table 3.2). There was also no disturbance in blood pH, or circulating electrolyte balance, as denoted by the lack of significant change seen in pH, Ca^{2+}, Na^+, or K^+, measurements between groups, at either time point (Table 3.2). Oxygen carrying
Figure 3.9: IL-1β concentration in the myocardium. Data is representative of individual data points with mean ± SEM. n = 9, and n = 11, for Sham, and LAD, respectively. ** P = 0.0082 Vs. Sham.
Table 3.2: Haematological parameters measured by blood gas analysis. Arterial samples were obtained at the time of LADCAO, and venous samples were obtained, 90 days later, at the time of sacrifice. All values represent mean ± SEM, with no significant results obtained between sham, and LAD, animals at either timepoint, for any measurements.

<table>
<thead>
<tr>
<th>Arterial blood gas</th>
<th>Sham</th>
<th>LAD</th>
<th>P</th>
<th>Venous blood gas</th>
<th>Sham</th>
<th>LAD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g·L⁻¹)</td>
<td>143.00 ± 4.95</td>
<td>151.60 ± 3.99</td>
<td>0.1073</td>
<td>Hb (g·L⁻¹)</td>
<td>132.60 ± 8.03</td>
<td>133.90 ± 3.13</td>
<td>0.4448</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>43.92 ± 1.48</td>
<td>46.46 ± 1.17</td>
<td>0.1083</td>
<td>Hct (%)</td>
<td>40.72 ± 2.43</td>
<td>41.14 ± 0.97</td>
<td>0.439</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 ± 0.03</td>
<td>7.34 ± 0.02</td>
<td>0.4197</td>
<td>pH</td>
<td>7.30 ± 0.01</td>
<td>7.27 ± 0.02</td>
<td>0.2039</td>
</tr>
<tr>
<td>Ca²⁺ (mmol·L⁻¹)</td>
<td>0.68 ± 0.12</td>
<td>0.74 ± 0.16</td>
<td>0.3847</td>
<td>Ca²⁺ (mmol·L⁻¹)</td>
<td>1.22 ± 0.07</td>
<td>1.08 ± 0.09</td>
<td>0.1251</td>
</tr>
<tr>
<td>Na⁺ (mmol·L⁻¹)</td>
<td>139.00 ± 3.08</td>
<td>140.30 ± 0.97</td>
<td>0.3555</td>
<td>Na⁺ (mmol·L⁻¹)</td>
<td>134.20 ± 1.24</td>
<td>134.30 ± 2.18</td>
<td>0.4867</td>
</tr>
<tr>
<td>K⁺ (mmol·L⁻¹)</td>
<td>4.960 ± 0.47</td>
<td>5.143 ± 0.45</td>
<td>0.3930</td>
<td>K⁺ (mmol·L⁻¹)</td>
<td>3.94 ± 0.28</td>
<td>4.29 ± 0.10</td>
<td>0.1504</td>
</tr>
<tr>
<td>COHb (%)</td>
<td>-0.44 ± 0.13</td>
<td>-0.11 ± 0.42</td>
<td>0.241</td>
<td>COHb (%)</td>
<td>-0.40 ± 0.38</td>
<td>-0.16 ± 0.39</td>
<td>0.3313</td>
</tr>
<tr>
<td>metHb (%)</td>
<td>-0.60 ± 0.25</td>
<td>-0.39 ± 0.32</td>
<td>0.3053</td>
<td>metHb (%)</td>
<td>0.28 ± 0.23</td>
<td>0.449 ± 0.24</td>
<td>0.3183</td>
</tr>
</tbody>
</table>
potential of blood samples was not hindered either, with no significant changes in carboxyhaemoglobin (COHb), or methaemoglobin (metHb) concentration, between groups, at either time point (Table 3.2).
Chapter 4

Renal Results

4.1 Renal function

Renal function was calculated using components from the ambulatory disease progression phase of the study, and the peri-mortem stage of the study. This gave measures of renal performance.

4.1.1 Creatinine clearance

Creatinine clearance, used as a measure of GFR, was shown to be significantly ($P < 0.0001$) lower in LAD animals (0.6480 ± 0.04748 mL minute$^{-1}$100g$^{-1}$ $BW_f$) compared to sham animals (1.425 ± 0.06664 mL minute$^{-1}$100g$^{-1}$ $BW_f$; Figure 4.1). This was calculated as actual CrCl, as opposed to estimated CrCl.
Figure 4.1: True creatinine clearance. Derived from urinary, and plasma creatinine measurements. Data represented as individual data points with mean ± SEM. $n = 8$, and $n = 8$, for Sham, and LAD, respectively. *** $P > 0.0001$ Vs. Sham.
Figure 4.2: Fractional excretion of sodium ($\text{FENa}$). Data is represents individual data points with mean ± SEM. $n = 6$, and $n = 7$, for Sham, and LAD, respectively. ** $P = 0.001$ Vs. Sham.
4.1.2 Fractional excretion of sodium

\( \text{FE}_{\text{Na}} \) was significantly (\( P = 0.0011 \)) increased in LAD animals (\( 0.1264 \pm 0.01498 \% \)) compared to sham animals \( 0.05067 \pm 0.005920 \% \); Figure 4.2). This value was however still below 1\%, the cut off used to indicate potential renal tubular impairment which may indicate a progression towards of renal failure.

4.1.3 Urine protein

There was significantly (\( P < 0.0001 \)) elevated urine protein excretion over 24 hours in conscious animals within the LAD group, compared to the sham group, with \( 101.00 \pm 3.73 \text{ mg.24hours}^{-1} (n = 9) \) Vs. \( 47.85 \pm 5.15 \text{ mg.24hours}^{-1} (n = 10) \), respectively (Figure 4.3).

4.2 Renal markers of fibrosis

There was a significant increase in TGF-\( \beta_2 \) in the renal medulla, with values of \( 0.7887 \pm 0.5378 \text{ pg.mg}^{-1}_{\text{protein}}, N=4 \), and \( 12.27 \pm 4.578 \text{ pg.mg}^{-1}_{\text{protein}}, N=5 \), for Sham, and LAD respectively (Figure 4.4). TGF-\( \beta_1 \), and TGF-\( \beta_3 \), isoforms were below the detectable limits of the assay. Recovery of TGF-\( \beta \), via spiked samples (\( n=3 \)) proved variable, with 0\%, 25\%, and 59\%, with TGF-\( \beta_1 \), 68\%, 56\%, and 46\% recovery of TGF-\( \beta_2 \), and all three below detectable limits with TGF-\( \beta_3 \). The standard curves were within acceptable bounds (\( R^2 \geq 0.9 \)).
Figure 4.3: Urine protein excreted in 24 hours. Data is represents individual data points with mean ± SEM. \( n = 10 \), and \( n = 9 \), for Sham, and LAD, respectively. *** \( P < 0.0001 \) Vs. Sham.
Figure 4.4: TGF-β2 concentration in the renal medulla. Data is representative of individual data points with mean ± SEM. $n = 4$, and $n = 5$, for Sham, and LAD, respectively. * $P = 0.0337$ Vs. Sham.
4.3 Renal histology

As to date, no quantitative measurements of collagen deposition have been made concerning the renal histology. Qualitatively, it can be seen that there is a substantial increase in collagen staining (Blue) in LAD animals, compared to Sham animals (Figure 4.5, and 4.6). The staining shows that the collagen deposition is mainly concentrated in the tubulointerstitium, and in, and around the glomeruli. Glomerulosclerosis is seen as collagen deposition around the glomerular blood vessels, and around the basement membrane. There is also evidence of interstitial fibrosis around proximal, distal, and convoluted tubule elements of the renal nephron. This is true for both Masson’s trichrome, and Martius Scarlet Blue, stains.

4.4 Renal caspase 3/7 activity

Caspase 3/7 activity was significantly \((P = 0.0006, \text{ and } P = 0.007)\) increased in both the medulla, and cortex, respectively. This was reflected as a cortical increase in caspase activity in LAD animals, with values of \(4353 \pm 526.0 \text{ RFU}\) for shams, and \(6877 \pm 723.3 \text{ RFU}\) for LAD animals (Figure 4.7). The medullary increase in caspase activity was of a much larger magnitude than seen in the cortex, with values of \(5959 \pm 608.4 \text{ RFU}\) for sham, and \(15642 \pm 1762 \text{ RFU}\) for LAD animals (Figure 4.8).
Figure 4.5: Masson’s trichrome stained renal cortex, and medulla, sections. These slides are representative of what was seen across all animals. Nuclei are stained blue/black, cytoplasm, and muscle are stained red, and collagen is stained blue. 
(A) Renal cortex of a sham operated animal, showing normal morphology.  
(B) Renal cortex of a LAD operated animal, showing increased collagen deposition around glomeruli, and in the tubulointerstitium.  
(C) Renal medulla of a sham operated animal, showing normal morphology.  
(D) Renal medulla of a LAD operated animal, showing increased tubulointerstitial collagen deposition.
Figure 4.6: Martius Scarlet Blue stained renal cortex, and medulla, sections. These slides are representative of what was seen across all animals. Nuclei are stained blue, muscle is stained red, and Collagen is stained blue. Fibrin stained red, with early fibrin staining yellow, and old fibrin staining blue. (A) Renal cortex of a sham operated animal, showing normal morphology. (B) Renal cortex of a LAD operated animal, showing increased collagen deposition around glomeruli, and in the tubulointerstitium. (C) Renal medulla of a sham operated animal, showing normal morphology. (D) Renal medulla of a LAD operated animal, showing increased tubulointerstitial collagen deposition.
Figure 4.7: Caspase-3/7 activity in the renal cortex. Data is represents individual data points with mean ± SEM. $n = 9$, and $n = 8$, for Sham, and LAD, respectively. 

** $P = 0.0072$ Vs. Sham.
Figure 4.8: Caspase-3/7 activity in the renal medulla. Data is represents individual data points with mean ± SEM. n = 9, and n = 7, for Sham, and LAD, respectively. *** P = 0.0006 Vs. Sham.
Chapter 5

Discussion

5.1 Summary of results

In the course of this study it has been shown that a non-reperfused occlusion of the left anterior descending coronary artery can create a substantial scar of > 20% wet weight of the entire left ventricle at 90 days, as well as an increase in ventricular mass. There is also a corresponding ventricular dysfunction manifesting as decreased left ventricular developed pressure (LVDP), decreased rate of ventricular contraction ($\Delta P \cdot \Delta t_{max}^{-1}$), and relaxation ($\Delta P \cdot \Delta t_{min}^{-1}$), and decreased intrinsic heart rate (ex vivo, denervated, and unpaced). This was achieved with a final mortality rate of 28%. The ventricular haemodynamic changes seen within this study are described as diastolic heart failure [104].

This cardiac dysfunction was accompanied by the association of elevated cardiac troponin I (4 hours post ligation) with the presence of myocardial
scarring (90 days post infarction). Cardiac troponin I represents a specific biomarker of myocardial injury. This may be useful as a predictor of infarct severity, measurable early after infarction, and act as a further predictor of renal dysfunction and damage. There were also gross myocardial anatomical changes, such as increased ventricular volume and mass, and scar thinning, with histological evidence of the collagenous nature of the scar resulting from this surgical induction of heart failure. This LAD occlusion procedure did not result in any significant perturbations in haematological parameters, such as electrolyte balance, pH, or oxygen carrying potential, but did result in substantial renal impairment, and morphological changes consistent with damage.

At 90 days post infarction, there was significant impairment of renal function in the myocardial infarction group. This was demonstrated by the decrease in true glomerular filtration rate (GFR), and increase in $F_{E_{Na}}$. Proteinuria, another indicator of renal damage, was also observed in the LAD group. This impaired function was further supplemented with increased caspase 3/7 activity, a marker of apoptosis, in both the cortex, and medulla, of the kidney. This was most substantial in the medulla. Histological evidence of renal sclerosis was consistent with functional, and biochemical data. Renal sections from LAD occluded animals showed an increased level of tubulointerstitial, peri glomerular, and intra glomerular, collagen deposition, demonstrated using both Masson’s trichrome, and Martius scarlet blue, stains.
5.2 Myocardial damage, and function

5.2.1 Cardiac Troponin I

Cardiac troponin I plasma concentration was increased in the LAD group. This plasma sample was obtained 4 hours after the induction of infarction, and represents a specific marker of myocardial damage. This is important as a biomarker which can be measured clinically, indicating the severity of cardiac damage likely to develop, within hours of onset/insult. Troponin I served to confirm the success of the myocardial infarction procedure, and further studies will correlate the severity of scarring at 90 days with cTnI levels measured in the plasma 4 hours after LADCA occlusion [105, 100].

5.2.2 Physical differences between sham and LAD animals

The post infarct mortality rate was higher than that reported in other studies, such as Ye et al. [98], but was comparable to previous studies using similar procedures within this lab group. This is likely due to the more aggressive approach taken in this study, ligating closer to the origin of the left anterior descending coronary artery. For a surgeon learning these procedures, 20-30% mortality is an acceptable, and expected, Figure. This procedure also demonstrated increased ventricular mass, unlike the study of Ye et al. [98], which is a further indicator that the mortality rate is closely coupled to infarct severity produced by this surgical procedure. This was observed within the present study, as a high rate of insufficient scar formation (< 20% left ventricular wet weight), associated
with a low (<15%) mortality rate at the early stages of this study.

At the conclusion of the in vivo studies, 90 days post infarction, there was no change in animal weight, which concurred with previous work by Ye et al. [98], who reported no significant changes in weight 16 weeks after ligation surgery. The significant change in ventricular mass, was not found in the study by Ye et al. [98], but they did report that they observed some increase in mass in some animals, although this did not reach significance when group data was combined. This may have been due to an effect of infarct size, which may have resulted in this trend being insignificant. This may also indicate that the methodology in the current study was able to create infarcts of greater severity, leading to remodelling, and ventricular hypertrophy. The regional breakdown further supports the notion of this increase in ventricular mass being due to enlargement of the free walls, in particular, the left ventricular free wall. The observations of a morphological change towards sphericity in these infarcted hearts further bolsters this finding, although not measured quantitatively in the current study. This all combines to show strong evidence for gross morphological changes associated with cardiac dysfunction [106, 107, 108].
5.2.3 Myocardial function derived by Langendorff

The myocardium is a dynamic pump that must maintain proper function in order to provide adequate perfusion of end organs. Within the current study, left ventricular developed pressure (LVDP), systolic rate of contraction \( \Delta P \cdot \Delta t^{-1}_{max} \), diastolic rate of relaxation \( \Delta P \cdot \Delta t^{-1}_{min} \), and unpaced \textit{ex vivo} heart rate (HR), were all compromised. The reduction of LVDP, \( \Delta P \cdot \Delta t^{-1}_{max} \), and \( \Delta P \cdot \Delta t^{-1}_{min} \) have also been previously reported in other similar studies of myocardial infarction, and were of similar magnitude [109]. The trend towards decreased functionality in infarcted hearts is consistent. Reduced left ventricular function is a well established prognostic indicator associated with greater risk of developing congestive heart failure [59].

This represents a scenario where the damage to the myocardium has rendered the myocardium unable to sustain normal pump function. This type of dysfunction is indicative of reduced cardiac output, reduced ejection force, and impaired ventricular filling [110]. The rate of diastolic shortening is compromised, which means that the heart does not fully relax, which prevents complete filling of the ventricle [110]. This is further evidence of functional worsening associated with myocardial stiffening, and hypertrophy, as a scar replaces nonviable myocardium, and the ventricles undergo pathological remodelling in an attempt to maintain function [53]. The reduced heart rate is a likely consequence of damage to the conductive pathways of the ventricle wall responsible for regulation of synchronous cardiac contraction [111, 61]. It was also observed
that severely infarcted hearts were more likely to experience episodes of ventricular fibrillation. The episodes occurred spontaneously, and in response to increased intraventricular balloon pressure loading. This further supports, even if only inadvertently at this juncture, that the ventricular conduction pathways were damaged in these hearts, and represent a vulnerability to similar, possibly life threatening, episodes \textit{in vivo}.

In the live animal, it is unlikely that any amount of myocardial modulation by circulating humoral factors, such as adrenaline, or stimulation by the sympathetic nervous system, acting through noradrenaline release, would be able to compensate for the functional impairment resulting from the gross damage and remodelling of myocardial tissue seen here. The cTnI elevations also reinforce that this dysfunction is due to acute myocardial damage sustained secondary to experimental LADCA occlusion. The remodelling occurring in the following 90 days, is consistent with our understanding of deleterious compensatory mechanisms, such as SNS stimulation, or increased renin release existing in the pathogenesis of ischaemic heart failure [23, 39].

5.2.4 Myocardial inflammatory markers

The only cytokine, of those measured at 90 days post infarction, that was significantly altered was IL-1β. The rest showed no difference between sham and LAD. This may be indicative of the resolution of any inflammatory reaction between the time of infarction, and tissue harvest 90 days post. In the case of IL-1β, it was a 2.4 fold increase compared to sham
operated animals. IL-1β is known to play an role in infarct healing, and is a known modulator to the type one angiotensin receptor, which plays a direct role in fibrosis [112, 113].

5.2.5 Myocardial histology

Myocardial sections stained with picrosirius red and fast green as a control stain revealed profound collagenous scar formation, ventricular enlargement, and wall thinning, in severely infarcted animals. This further supports the ventricular haemodynamic data, which showed dysfunction consistent with ventricular enlargement. The difference between infarcted, and sham hearts is visually profound, but has not been measured to date. This will be the subject of follow up analysis. The observed staining was in line with what would be expected, and quantification of observed characteristics is to be the subject of future studies [114].

5.3 Haematological parameters

5.3.1 Blood gas measurements

The lack of haematological changes in the LAD group compared to the sham group is evidence that the source of damage in either organ is not likely to be of haematological origins. Measured haemotological factors are also unlikely to be driving renal dysfunction secondary to CHF. This does not preclude other factors which have not, to date, been measured. Haemoglobin levels were not perturbed, and thus it is likely
that renal dysfunction is not severe enough to disrupt normal erythropoietin signalling, and red blood cell turnover, and thus no disturbance in haemoglobin was noted. There was also no systemic disturbance of blood electrolytes, indicating that electrolyte imbalance cannot be a causative factor within this model. This is consistent with the observed renal dysfunction being an event secondary to CHF. Coupled with the lack of any changes in metHb, or fCOHb, it is safe to assume that the oxygenation potential of circulating whole blood has not been compromised in any way, apart from the loss of perfusion pressure, and efficiency, due to cardiac dysfunction resulting in reduced cardiac output.

5.3.2 Effect of anaemia on renal and heart function

Anaemia is a physiological factor that is very important prognostic factor in cardiorenal-anaemia syndrome, but, from literature review, and experimental data discussed later, appears to be of most concern when the primary disease state is renal, or anaemic, in origin, or manifest by an unrelated pathological, or iatrogenic means [6, 7, 8]. Anaemia can worsen both HF and RF, and can itself be exacerbated by HF, and RF [6, 7, 8]. When considering anaemia, it is highly indicative of the end stage of RF [115]. This indicates that anaemia can be effectively ignored, unless found to be present, within controlled animal models of CRS or cardiovascular origin.
5.4 Renal Parameters

5.4.1 Evidence of renal dysfunction

With the establishment of myocardial impairment, and progression towards myocardial failure, any renal damage seen in the LAD group can be assumed to be secondary to the myriad of post infarction events. Reduction in CrCl is a known consequence of renal hypoperfusion, and renal damage in more severe circumstances [116]. Decreased CrCl is indicative of impaired renal filtration of plasma creatinine, and is used as a representative measure of GFR [117]. The observed decrease in creatinine clearance, accompanied by an increased FE\textsubscript{Na} is indicative of renal dysfunction, and likely renal damage. The levels seen within this study were decreased further than those seen in a study by Bauersachs et al., [96], previously described in section 1.4, with CrCl of LAD animals in the present study being decreased to 45 % of that measured in sham animals, as compared to a reduction of 60 % in the forementioned study. The magnitude of the decline in CrCl is similar to that seen in renal mass ablation models, where renal mass is removed via surgical resection, or infarction [118]. There was no evidence of renal damage in the study by Bauersachs et al., [96], which is in contrast to the present study. In our Lewis rat animal model, we were able to generate a greater decrease in CrCl, corresponding to morphological, biochemical, and functional, evidence of damage. This will be discussed further in section 5.4.2.
Interpretation of $\text{FE}_{\text{Na}}$ is not so straightforward. $\text{Na}^+$ is freely excreted by the proximal, loop of Henle, distal, and convoluted, tubules of the nephron. $\text{FE}_{\text{Na}}$ is a representative measure of the proportion of $\text{Na}^+$ excreted by the kidney, which is not reabsorbed by the nephron. It can be diagnostic of acute tubular necrosis, damage to the tubules will impair resorption, increasing the amount excreted. The small increase is not discouraging, as it reinforces the decreased CrCl as representative of decreased GFR, to which $\text{FE}_{\text{Na}}$ is dependent [119]. It would only be expected to see a substantial increase in $\text{FE}_{\text{Na}}$ as GFR drops to around 20% of normal GFR, with an exponential rise beginning at a GFR $< 15\%$ of normal GFR [119]. This means that a very modest increase in $\text{FE}_{\text{Na}}$ is exactly what should be expected, when considering CrCl only dropped to 45% of control. It is also the nature of type 2 CRS, being a progressive disease, and thus fits well with established disease parameters, representing a relatively early stage of renal decline [5]. Decreased GFR is also associated with the progression of chronic kidney disease to end stage renal disease, especially when combined with the presence of microalbuminuria [44]). To elucidate the cause of this impairment of renal function, we must look to markers of renal injury using histological, and biochemical means.
5.4.2 Evidence of renal damage

5.4.2.1 Increased protein excretion in urine

There was evidence of increased urine protein, likely indicative of microalbuminuria, in rats with myocardial infarcts. Microalbuminuria is a biomarker associated with cardiovascular disease, and increased risk cardiovascular mortality[120, 121, 44, 122, 123]. Coupled with the decline in GFR (via measurement of CrCl), microalbuminuria is predictive of the progression of chronic renal disease to end stage renal disease, and provides further evidence that this model is able to not only cause renal damage, but progressive renal damage [44, 123]. Renal damage was found in the current study, presenting as increased collagen deposition in the tubular interstitium, and as glomerulosclerosis. This was also confirmed using two different staining techniques, and will be discussed in section 5.4.2.3.

In the study conducted by Bauersachs et al., [96], there was no evidence of microalbuminuria, further supporting the notion that the present study has succeeded where they failed; in the generation of renal injury secondary to CHF. Proteins in the urine have several sources. They may enter the ultrafiltrate via the glomerulus, passing through the glomerular barrier, or tubular secretion from the blood. Tubular cells also have the capability to reabsorb some proteins from the filtrate within the tubules [124]. There also exists the possibility of protein entering the urine from the prostate gland, being a mechanisms restricted to males only [124].
The most well documented cause of protein elevation during renal disease is through modification of the selectivity of the glomerular barrier, or through direct damage to the glomerular barrier [124, 125]. It has been demonstrated that acute renal ischaemia-reperfusion has the effect of changing the permeability of glomerular capillaries, which would contribute to an increase in proteinuria [125]. Podocytes, a component of the glomerular barrier, are responsible for forming a selective barrier between the glomerular space, and the afferent arterioles [126]. If podocytes become damaged, they are unable to regenerate, much like cardiomyocytes. This means that gaps in the layer of podocytes must be filled via hypertrophy of neighbouring podocytes. If damage is severe enough, this selective barrier may become broken, and capillaries may adhere directly to the basement membrane, once occupied by podocytes, or be filled with extracellular matrix [126]. This impairs the function of the nephron, and may lead to tubular atrophy. These breaks in the podocyte wrapper around the efferent capillaries may then lead to contents spilling directly into the glomerulus, leading to increased protein content in the filtrate, and subsequently, the urine, as well as glomerular fibrosis [126].

5.4.2.2 Renal apoptosis

As microalbuminuria is not directly diagnostic of renal injury, other evidence of tissue injury was investigated, to provide more weight to our findings of renal disease. This was conducted by measuring caspase 3/7 activity in homogenised renal cortex, and renal medulla. This revealed a
significant increase in caspase 3/7 activity, which is representative of the activation of the apoptotic cascade. The increase in activity was greatest in the renal medulla. The fact that apoptotic activity was significantly increased at 90 days post infarction is a strong indicator that the observed renal damage is ongoing, and likely to be progressive. However, without localization studies, the source of this apoptosis within the vascular, and nephron elements of the kidney cannot be determined.

Apoptosis is a critical feature of normal cell physiology, as it represents a mechanism of controlled cell death associated with progressive tissue dysfunction. The caspases operate as the intracellular mediators of this programmed death [127]. In models of unilateral ureteral obstruction it has been found that there is increased apoptosis of tubular and interstitial cells, and that this interaction is Ang II modulated [128]. This suggests that within the present study, the apoptosis may be representative of apoptosis of tubular, and interstitial cells. This would account for much of the evidence addressed already. It has been previously reported that stimuli such as hypoxia can lead to both apoptosis, and fibrosis [129, 130, 131].

However, without the use of cell proliferation studies, using histological markers such as BrdU, it cannot be confirmed if the observed apoptosis represented a net loss of cells, or just represented a regenerative process ongoing in the renal tissue. There also remains the likely possibility that any proliferation of cells may actually represent pathological fibrotic
remodelling within the tissue [129, 130]. Without specific analysis, we cannot be certain of the origin of the observed apoptosis, or if there is a net loss of cells within the renal tissue. In order to attempt to further elucidate the likely source of this apoptosis, and the associative processes, such as fibrosis, we can turn to the histological sections stained specifically to show changes in collagen (α smooth muscle actin, collagen I, collagen IV) deposition.

5.4.2.3 Histological analysis of renal sections

Morphological changes consistent with fibrotic remodelling were confirmed through histological analysis of the kidneys. Two different stains were used to confirm histological evidence of damage; Masson’s trichrome, and Martius scarlet blue. Visual interpretation of these histological slides revealed an increased deposition of collagen around the glomerulus, inside the glomerular space, consistent with glomerulosclerosis, and also in the tubulointerstitium. Increased collagen deposition was uniformly found in both the renal cortex, and renal medulla of animals suffering myocardial infarcts > 20% of the entire left ventricle. This is an established pattern of damage seen in animal models of chronic renal failure, with tubular damage being closely associated with renal dysfunction [132, 133, 118]. The apparent glomerulosclerosis likely reflects damage to podocytes which allows protein to leak into the renal filtrate, bypassing the normal filtration mechanisms. The evidence of microalbuminuria supports this assertion. This also represents a mechanism by which plasma contents may leak into the interstitial space, triggering further fibrosis
[126]. There has been no quantification of this data, as it is the focus of future research, and analysis. This was further supported by the evidence of TGF-β2, a promoter of fibrosis, found within the medulla [13]. The negative result for the presence of TGF-β1, and TGF-β3, may represent a problem with those beads, potentially aggregating.

The cumulative evidence presented in this study supports our original aim, and represents direct evidence of renal damage secondary to heart failure, indicative of success in creating a model of type 2 CRS. The following sections puts forward several mechanisms which are likely to contribute to the genesis of the forementioned pathophysiological state.

5.5 Proposed mechanisms of renal dysfunction, and subsequent damage, secondary to heart failure

Hypoxia represents a physiological state in which oxygen delivery is unable to meet demand. Under normal physiological conditions, intrinsic homeostatic mechanisms are able to compensate for transient periods of hypoxia, and if anything, make the tissue more resilient to future episodes of hypoxia, such as those experienced during intense physical burden [134, 135, 136]. However, in states of chronic disease, prior substantial organ damage (i.e. sclerotic lesions), or an acute injury, endogenous homeostatic mechanisms may become overwhelmed, unable to fully compensate for decreased cardiac, or renal, function[137]. In this state mechanisms that were once protective become deleterious, leading
to dysfunction, cellular loss, and fibrotic lesions [138, 139, 140, 141].

5.5.1 Perturbations of renal oxygenation

During CHF, within this model, cardiac performance was compromised within the assessed parameters. This creates a scenario where the kidneys are deprived of the oxygen which they require to function normally, as the kidney is second only to the myocardium in terms of oxygen requirement, accounting for around 20% of cardiac output [142, 143]. Renal hypoperfusion is associated with decreased performance, even after short periods of hypoxia by experimental impairment of renal blood flow [144]. Reduced cardiac output has also been associated, along with venous congestion, with renal decline, and potentially represents a trigger for renal damage resulting in fibrosis, and nephron loss [10, 11]. The reverse is also true, with continuous flow cardiac support, or the use of left ventricular assist devices, able to normalize perfusion to organs [145]. Such procedures may be utilised during the final stages of myocardial failure, improving renal function[145]. Experimental findings have shown that hypoperfusion can indeed cause tubulointerstitial injury, as observed in the present study [11]. This was achieved through the induction of an irreversible glomerulonephritis, impeding blood flow within the kidney. This led to hypoxia, tubulointerstitial fibrosis, and peritubular capillary loss. There is also evidence for decreased oxygen within the kidney after ischaemia-reperfusion, lending weight to the idea that hypoxia, or ischaemia, can create a scenario where hypoxia is further accentuated in
the absence of the initial stimulus, such as, occlusion of a renal pedicle [146]. This leads us to the interesting notion that renal injury can become self perpetuating under the right circumstances, and will only accentuate any other hypoxic mediators further.

In cases where cardiac output has not decreased enough to trigger de novo renal damage on its own, there are other humoral factors which may contribute to the generation of an oxygen poor environment within the kidney. Pathological upregulation of renin release, and subsequently circulating Ang II is a known phenomena in CHF [39]. Ang II has been demonstrated to cause constriction of the pre glomerular afferent, and post glomerular efferent, arterioles of the outer cortex [138, 139]. This is more pronounced in the afferent arterioles, but when the increases in vascular resistance, associated with Ang II administration, are incorporated, the effective reduction in flow is greater in the efferent arterioles [138, 139]. This mechanisms would be beneficial under normal physiological circumstances, where renal perfusion pressure needs to be maintained for adequate filtration to occur, but in the case of CHF, where the RAS is overactive, this becomes a detrimental element to the already struggling kidney, and is modulated through the AT_1R [138, 139]. These vasoconstrictive effects can be further accentuated by the action of endothelial derived endothelins with endothelin-1 (ET-1) being the most potent endogenously derived systemic, and renal, vasoconstrictor yet discovered [147, 148]. ET-1 exerts a vasoconstrictive action through the ET_A R, while also being able to exert a vasodilatory effect through the ET_B R.
It has also been demonstrated that there is a preferential distribution of each subtype within the kidney, where the ET₄R is primarily expressed within the cortex, in the peritubular capillaries, and the ET₅R is primarily expressed within the medulla, mainly in the vasa recta bundles [147]. During CHF, the regulation of these receptors changes, with the vasoconstriction mediating ET₄R upregulated, and the vasodilation mediating ET₅R downregulated, which further accentuates pathological vasoconstriction within the kidney, with ET-1 production increased in CHF [147, 149, 150, 151]. Sympathetic innervation, and subsequent NA release, or alternatively, circulating catecholamines, have also been demonstrated to play a role in the perpetuation of a hypoxic state within the kidney, with sympathetic pressor effects being augmented by AngII [25, 24, 152, 22]. This is further accentuated by pathological AngII stimulation which also results in increased ROS formation, namely ·O₂⁻, and H₂O₂. The formation of ·O₂⁻ can directly react, via redox chemistry, with NO. NO is an important vasodilator which can mediate increased renal blood flow [153, 154]. This reaction forms another radical, ONOO⁻, which does nothing to reduce cellular oxidative stress, but instead depletes cellular NO, reducing vasodilation [153, 154].

These processes all represent therapeutic targets which have all, to some degree, shown success in minimising disease progression when blocked pharmacologically [96, 148, 155, 156, 24, 157, 158]. It becomes obvious that this diverse range of homeostatic mediators needs to be controlled using pharmacological agents if the kidneys stand any chance of main-
taining normal function during heart failure. It is likely that the coinci-
dental side effect of many cardiovascular interventions employed during
CHF may also be simultaneously protecting the kidney from progressive
injury, especially when the severity of the myocardial damage, and sub-
sequent dysfunction are less severe [155]. The generation of this hypoxic
environment is not the end of the story. Hypoxia triggers a cascade of
intrarenal signalling which can lead to an overall change in the renal
landscape.

5.5.2 From hypoxia to dysfunction, cellular loss, and fibrosis

Downstream intrarenal signalling pathways must be examined to explain
how a chronic hypoxic stimulus may be translated into increased apop-
tosis, fibrotic damage, and to provide insight into potential therapeu-
tic targets. During hypoxia, cells are forced to shift away from aerobic
metabolism to anaerobic metabolism [159]. One of the results of this
transition is the increase in ROS production, which will supplement the
oxidative stress induced by pathological AngII stimulation [153]. ROS
can directly damage cellular components, leading to apoptosis, or act as a
mediator in the initiation of other dysregulative cellular cascades. From
this point we can draw many parallels between the cellular pathogenesis
occurring in the myocardial tissue, and the pathogenesis in renal tissue,
the major difference being the initial injury stimulus, with the stimulus
in the heart being acute, and the kidney chronic.
5.5.2.1 Apoptosis

Apoptosis is a known phenomena during periods of ischaemia [160, 161, 162]. The increased level of caspase-3/7 activity in the current study may have been generated during the loss of cells within the kidney, as consequence of the hypoxic state that is associated with the pathological homeostatic signalling seen in CHF [138, 23]. The persistence of caspase activity in LAD animals, even at 90 days after infarction, suggests that the dysfunction observed within this study is progressive, and given time, would progress to renal failure. The increase in apoptosis observed in the renal cortex, and medulla may represent the progressive death of renal cells as they atrophy due to damage progression, and is consistent with the increased glomerular, and tubulointerstitial collagen deposition observed histologically in this study [161, 162].

5.5.2.2 Fibrosis and inflammation

The increase in fibrosis secondary to CHF observed in this study is likely consequent to the myriad of profibrotic pathways at play, as indicated within this study by finding pro-fibrotic TGF-β to be elevated within the renal tissue. During hypoxia in the tubulointerstitium fibroblasts, the predominant ECM promoting cells in that region, show increased propensity towards a fibrogenic phenotype [137]. This involves enhanced myofibroblast differentiation, proliferation, and alterations in ECM metabolism towards ECM accumulation [163]. There is also the possibility that collagen may be altered post transcriptionally, resulting
in a matrix that is resistant to degradation [163]. A further factor which promotes ECM accumulation is the suppression of matrix metalloproteinases expression, and activity, during hypoxic insult [163]. There is also evidence that the oxygen sensing molecule hypoxia-inducible factor 1 (HIF-1), once thought to be beneficial, can actually be profibrotic in pathological states [164, 130, 165, 166, 167]. However, this is controversial, and likely represents the influence of yet to be described variables. There is evidence to suggest that endothelial cells have the capacity to transdifferentiate into fibroblasts [167, 168]. There is also the possibility of contribution by contractile pericytes, which surround endothelial cells, and are also able to transdifferentiate into fibroblasts [169, 79]. This presents an interesting notion, as transdifferentiation would have the combined effect of worsening hypoxia by disrupting peritubular vascularization, as well as increasing the number of ECM producing cells. The forementioned scenario is one where normal homeostatic mechanisms have been shunted towards fibrosis, and further hypoxia promoting pathology. Inflammation is an intrinsic part of fibrosis, and hypoxia acts as an attractive stimulus inflammatory cells [137, 79]. The inflammatory cells accumulate within the damaged tissue, and perpetuate damage in a similar manner as previously discussed in section 1.3.7.
5.6 Experimental strengths, limitations, and methodological commentary

This model provides a robust methodology to produce type-2 CRS in a rat, giving the researchers the ability to dissect, in depth, the transition from CHF, to renal injury. It would be more pertinent to use a model of nephropathy that mimics the damage seen in this model to dissect out the return mechanism, where chronic renal failure leads to cardiovascular injury. This is required as it is likely that similar stimuli play a role in the propagation of injury between organs. It would also be of advantage to examine liver enzyme activities, and hepatic fibrotic pathways. This model has the potential to uncover yet another factor in this complex series of pathologies. Early studies by our group using losartan within this model (data not shown) have provided little evidence of protection. There appears to be a paradox in that drugs such as RAS inhibitors used clinically to attenuate heart, or renal, failure have at best little therapeutic value. A recent review by Ronco et al. [5], of clinical studies, has even gone as far as to state that RAS inhibitors may actually promote, or worsen, the pathology existing in type 2 CRS.

5.6.1 Surgical commentary

5.6.1.1 Mortality

In order to use this model correctly, there will always be some mortality, or some failure to achieve sufficient damage. This is because there is
always inter animal variability, even when inbred rats are used. There is also an unavoidable amount of variance that is resultant in the surgery on any particular day. The nature of ligating an artery, using only landmarks, on a beating myocardium, is one that would produce variation in efficacy, even with perfect technique. The use of a binocular dissecting microscope, or surgical loupes, are of value to surgeons attempting this procedure. A mortality rate, arising from acute heart failure after infarction, of \( \approx 25\% \) should be aimed for, as it will represent a reasonable compromise between conservative, and liberal, approaches to generating myocardial damage. Using tissue blanching as a visual reference of sufficient occlusion of the LADCA is useful, but should not be relied on, as it is easy to make subjective estimates, rather than objective estimates, causing the surgeon to accrue false positives. Visual estimation of occlusion via blanching is a skill that takes time to develop, and this should be noted by surgeons attempting this surgical model. As a rule of thumb, it may be advisable to place a second ligature around the LAD. While this procedure may increase the risk of morbidity, or mortality, in the ligated animal, the value of producing a reproducible, sizable, infarct outweighs the wastage, and exclusion, of animals though insufficiently sized infarcts. The risk of complications is of lesser concern than a false positive, and, in turn, an animal that is unfit for further study.

5.6.1.2 Surgical technique

During this work, several modifications were made in order to optimize the surgeries. These modifications ensured more consistent outcomes,
and minimised post ligation deaths. The primary adaptations were in the context of anaesthesia. These modifications included a protocol of variable gas delivery. This was to ensure that anaesthesia depth was optimal, and post surgical recovery from anaesthesia maximised. This was achieved through practice, effectively shortening the length of the surgeries, and modulation of the gas mixture in response to observed, and imposed cues. This resulted in halothane use being decreased to just maintain loss of pain reflexes, N₂O was withdrawn, and compensated for by an equivolume increase in O₂, upon LADCAO. These modifications allowed for less anesthetic load, and maximized surgical recovery. Cardiac massage was also utilised in cases of arrhythmia, or even severe brady-cardia, immediately after LADCAO, which effectively increased survival in severely infarcted animals.

5.7 Future directions

The immediate future of this model is to continue with biochemical analysis of tissues obtained during this study. This will provide further insight into pathophysiological mechanisms within this model, and give detailed evidence as to what damage has occurred, and how this may be prevented. It will also be pertinent to follow up with therapeutic compounds which may prevent de novo renal damage, or even reverse it. It will also be of great importance to conclude ongoing studies involving the effect of losartan within this model. These results are only partially completed, and not currently ready for presentation. They do however suggest pow-
erful reno-protective properties in this model. There is also the potential to extend this model by including subgroups that have co-committant renal dysfunction, or other compounding variables such as hypertension, and diabetes. Monitoring blood pressure over the course of the study would be of great value, as well as weekly, or at least monthly estimation of renal function by utilizing metabolic cages, and urine analysis. This will provide a more detailed understanding of the progression of renal dysfunction in these animals. It may also be of use to assess timepoints shorter than 90 days, and longer than 90 days to further analyse the pathophysiological timecourse. This could potentially lead to the discovery of windows of opportunity for intervention, or sensitive periods where small changes in function may have long term potential for massive injury.

5.8 Closing remarks

From the issues, and evidence, addressed in this thesis, it is apparent that this model represents a solid foundation from which to build a reliable, and useful, animal model of type 2 CRS. It has been concluded that there is substantial renal dysfunction that is associated with a single cardiovascular insult sufficient to cause myocardial dysfunction, and failure. The data obtained was in agreement with previously published literature that covered various aspects addressed in this work. It was also found that the pathophysiology observed in this study is of a similar nature to that clinically observed in cases of type 2 CRS. This represents a syndrome
with a pathomechanism that is likely convergent with other forms of renal dysfunction, and represent a scenario in which manual control of normally autoregulatory mechanisms must be taken through therapeutic interventions to provide more normal functionality. It is with confidence that this model can be used as a tool in the elucidation of therapeutic strategies to combat the pathophysiology inherent in CRS. It will provide a framework to investigate the base mechanisms that lead to this dysfunction, and develop therapies to eliminate a source of complications for sufferers of HF. This will aid progress towards curative interventions in HF, maximizing time remaining for patients to spend with loved ones.
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Appendix A

Buffers, and general solutions

A.1 Krebs-Henseleit-Ringer buffer

In order to make 1.0 L of Krebs-Henseleit-Ringer Buffer, the following dry reagents were combined with 500 - 700 mL 18.3 mΩ H₂O, in a 1.0 L volumetric flask, and mixed until dissolved.

6.92 g Sodium Chloride (NaCl)

2.1 g Sodium hydrogen carbonate (NaHCO₃)

0.35 g Potassium chloride* (KCl)

0.3 g Magnesium sulphate 7-hydrate (MgSO₄·7H₂O)

0.16 g Potassium dihydrogen orthophosphate (KH₂PO₄)

2.0 g D-glucose (C₆H₁₂O₆)
The precursor buffer was then bubbled with carbogen (95% O\textsubscript{2}, 5% CO\textsubscript{2}) for 15 minutes. Following this,

0.21 g Calcium chloride 2-hydrate (CaCl\textsubscript{2}·2H\textsubscript{2}O)

was then added, and mixed via inversion five times. 18.3 mΩ H\textsubscript{2}O was then added until the total volume was 1.0 L

* 2.98 g of KCl was used instead when 20 mM KCl (cardioplegic) KHB (cKHB), for inducing cardiac arrest in diastole, was required.

### A.2 Tris-mannitol-sucrose buffer

In order to make 1.0 L of Tris-Mannitol-Sucrose buffer, the following reagents were combined with 500 - 700 mL 18.3 mΩ H\textsubscript{2}O, in a 1.0 L volumetric flask, and mixed until dissolved:

41 g D-mannitol (C\textsubscript{6}H\textsubscript{14}O\textsubscript{6})

28.7 g Sucrose (C\textsubscript{12}H\textsubscript{22}O\textsubscript{11})

1.2 g Trizma base (C\textsubscript{4}H\textsubscript{11}NO\textsubscript{3})

175 μL PMSF (400 nM)

This solution was then cooled to 4°C, and pH’d using 4°C 1 M Trizma hydrochloride (C\textsubscript{4}H\textsubscript{11}NO\textsubscript{3}·HCl) to achieve 7.17 ≥ pH ≤ 7.24. 18.3 mΩ H\textsubscript{2}O was then added until the volume was 1.0 L
A.3 Phosphate buffered saline

In order to make 1.0 L of Phosphate buffered saline, the following reagents were combined with 500 - 700 mL 18.3 mΩ H₂O, in a 1.0 L volumetric flask, and mixed until dissolved:

- 8.00 g Sodium chloride (NaCl)
- 0.20 g Potassium chloride (KCl)
- 1.44 g Disodium hydrogen orthophosphate (Na₂HPO₄)
- 0.24 g Phosphoric acid (KH₂PO₄)

This solution was then pH'd using 1 M hydrochloric acid (HCl), or 1 M sodium hydroxide (NaOH), depending on the initial pH, to achieve pH 7.4. 18.3 mΩ H₂O was then added until the volume was 1.0 L.

A.4 4% paraformaldehyde

In order to make 100 mL of 4% paraformaldehyde solution, the following reagents were mixed at a temperature of 52-58°C, in a fume hood:

- 4g Paraformaldehyde powder (OH(CH₂O)ₙH (ₙ = 8 - 100))
- 100 mL Phosphate buffered saline (appendix A.3)

Concentrated NaOH was then carefully dropped into the solution until the solution turned clear. The solution was then cooled, to ambient room temperature, and stored at 4 °C until use, for a maximum of two days.
Appendix B

Histological solutions

B.1 Masson’s trichrome

B.1.1 Acid Fuchsin solution
0.5g Acid fuchsin
0.5mL Glacial acetic acid
100mL Distilled water

B.1.2 Phosphomolybdic acid solution
1g Phosphomolybdic acid
100mL Distilled water

B.1.3 Methyl Blue solution
2g Methyl blue
2.5 mL Glacial acetic acid
100 mL Distilled water

**B.1.4 1% Acetic acid**
2 mL Glacial acetic acid
100 mL distilled water

**B.2 Martius scarlet blue**

**B.2.1 Martius yellow solution**
0.5 g Martius yellow
2 g Phosphotungstic acid
100 mL 95% EtOH

**B.2.2 Brilliant crystal scarlet solution**
1 g Brilliant crystal scarlet
2 mL Glacial acetic acid
100 mL Distilled water

**B.2.3 Phosphotungstic acid solution**
1 g Phosphotungstic acid
100mL  Distilled water

B.2.4 Methyl blue solution

0.5g  Methyl blue

1mL  Glacial acetic acid

100 mL  Distilled water

B.2.5 1% Acetic acid

2mL  Glacial acetic acid

100mL  distilled water