Improving cardioprotection during cardiac bypass surgery

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DECLARATION

I, Edney Boston-Griffiths, confirm that the work presented in this thesis is my own. Information that has been derived from other sources has been clearly indicated and referenced within my thesis. The thesis presented is the one on which I expect to be examined.

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ABSTRACT

Ischaemic heart disease (IHD) is the leading cause of death worldwide and according to the World Health Organisation the number of patients with IHD will reach 19 million by 2020 if current trends continue. While coronary artery bypass graft (CABG) surgery remains the treatment of choice for the severest form of the disease, the detrimental effects of peri-operative myocardial injury particularly in the form of myocardial ischaemia-reperfusion injury (IRI) accounts for significant levels of morbidity and mortality particularly in high-risk patients.

The past four decades have seen advances in cardioprotective strategies especially within the disciplines of cardioplegia and anaesthesia. Despite this, improvements in patient survival have been limited. Researchers and clinicians alike have called for novel ways of protecting the heart, directing their attention to cellular and mitochondrial pathways which may hold the key to improving survival.

This thesis covers a fascinating exploration into the cardioprotective effects brought about by the inhibition of the mitochondrial permeability transition pore (mPTP) using cyclosporin A (CsA), as well as the role of remote ischaemic preconditioning (RIPC) in limiting the extent of myocardial injury in the setting of complex cardiac bypass surgery.

In summary, this thesis examines both pharmacological and non-pharmacological strategies for protecting the heart in the setting of cardiac surgery. Despite decades of advancement in research within this field, the consequences of ischaemia-reperfusion injury remain ever-present. As a result, it is hoped that the research in this thesis will make a positive contribution to the body of evidence currently available for the benefit of patients with IHD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AAA- abdominal aortic aneurysm

ACS- acute coronary syndrome

ADP- adenosine diphosphate

AF- atrial fibrillation

AIF- apoptosis inducing factor

AKI- acute kidney injury

AMP- adenosine monophosphate

ANT- adenine nucleotide translocase

ASD- atrial septal defect

ATP- adenosine triphosphate

AUC- area under the curve

Ca^{2+}- calcium ions

CABG- coronary artery bypass graft

CAD- coronary artery disease

cGMP- cyclic guanosine monophosphate

CK-MB- creatine kinase MB (isoenzyme)

COREC- Central Office for Research Ethics Committees

CPB- cardio-pulmonary bypass

CPBT- cardiopulmonary bypass time

CPK- creatine phosphokinase

CsA- cyclosporin A

CyP- cyclophilins

dATP- deoxyadenosine triphosphate

DNP- 2,4-dinitrophenol

EDP- end-diastolic pressure.

EDTA- ethylenediaminetetraacetic acid

eGFR- estimated glomerular filtration rate

EGTA- ethylene glycol tetra-acetic acid

eNOS- endothelial nitric oxide synthase

Epo- erythropoietin

ERK- extra-cellular receptor kinase

EuroSCORE- European system for cardiac operating risk evaluation

FAPB- fatty acid binding protein

FK506- tarcrolimus

GM-CSF- granulocyte-macrophage colony-stimulating factor

GPCRs- G-protein-coupled receptors

HSP-70- heat shock protein 70

IABP- intra-aortic balloon pump

ICCF- intermittent cross-clamp fibrillation

ICH-GCP- International Conference on Harmonisation- Good Clinical Practice

ICU- intensive care unit

IF-γ – interferon gamma

IHD- ischaemic heart disease

IL- interleukin

IMM- inner mitochondrial membrane

iNOS- inducible nitric oxide synthase

IPC- ischaemic preconditioning

IPostC- ischaemic post-conditioning

IRI- ischaemia-reperfusion injury

IVC- inferior vena cava

JNK- c-jun kinase

K_{ATP}- Potassium ATP channel

LAD- left anterior descending artery

LBBB- left bundle branch block

LDH- lactate dehydrogenase

LV- left ventricle

LVDP- left ventricular diastolic pressure

LVEF- left ventricular ejection fraction

MAPK- mitogen-activated protein kinase

MI- myocardial infarction

mitoK_{ATP}- mitochondrial-potassium ATP channel

MMC- mitochondrial mega-channel

MPO- myeloperoxidase

mPTP- mitochondrial permeability transition pore

MRI- magnetic resonance imaging

NADH- nucleotide adenine dehydrogenase

NO- nitrous oxide

NRES- National Research Ethics Service

NSTEMI- non-ST-elevation myocardial infarction

OPCAB- off-pump coronary artery bypass

PI3K- phosphatidylinositol 3-kinase

PKC- protein kinase C

PKG- protein kinase G

PTCA- percutaneous trans-luminal coronary angioplasty

RACK- receptor for activated kinase C

RIPC- remote ischaemic preconditioning

RISK- reperfusion injury salvage kinase

ROS- reactive oxygen species

SfA- sanglifehrin A

STEMI- ST-elevation myocardial infarction

SWOP- second window of protection

TNF-α - tumour necrosis factor alpha

TnI- Troponin I

TnT- Troponin T

TOE- trans-oesophageal echocardiography

TPP+- triphenylphosphonium

VDAC- voltage-dependent anion channel

VSD- ventricular septal defect

WHO- World Health Organisation

XCT- aortic cross-clamp time

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CHAPTER 1

1. The Evolution of Cardiac Surgery in the Management of Coronary and Valvular Heart Disease

1.1. Introduction

1.1.1. A global perspective of ischaemic and valvular heart disease

Ischaemic heart disease (IHD) is the leading cause of death in most industrialised countries. In the UK, it alone accounts for 22% of all deaths in men and 17% of all deaths in women[1]. Although much of the Western World has seen a decrease in IHD rates over the last three decades- largely due to the modification of secondary preventative measures, the World Health Organisation (WHO) has predicted an increase in the number of sufferers from 9 million to 19 million by 2020[2;3]. Coronary artery bypass graft (CABG) surgery has remained the treatment of choice particularly for the severest form of the disease, with more than 30,000 patients operated upon across thirty-eight units within the UK each year[4].

In addition to this, the EuroHeart survey suggests that valvular heart disease represents a substantial burden to the public health of nations within Europe[5]. The impact of this burden is linked with the increase in prevalence of the degenerative form of the disease, an elderly population and an increase in life expectancy[6].

Due to the demographic changes in life expectancy and the benefits of performing more than one procedure at the same sitting, the number of coronary artery bypass grafts with concomitant valve operations is set to rise.

With this in mind, protecting the heart during cardiac bypass surgery is likely to have an even bigger impact on both the short term and long term survival for prospective patients.

1.1.2. A historical perspective of cardiac surgery and the drive for cardioprotection

Since John Gibbon paved the way for cardiac surgery in 1953 after performing the first atrial septal defect (ASD) closure, the specialty has seen great advances in the surgical technique and myocardial preservation[7]. Cardiac surgeons in that era were faced with a number of obstacles that impeded operative success. They soon established that the main challenges were the occurrence of air embolisation compounded with having to perform complex operations in a blood-filled operative field. It was thought that these obstacles would be best addressed by arresting the heart and stopping the circulation to the myocardium. Clamping the aorta during intra-cardiac repair proved a significant step, but as patient mortality continued to rise, focus was directed towards the need for myocardial protection(reviewed by Cordell[8]). The introduction of 'practical heat exchangers' allowed for the rapid induction of hypothermia in an effort to protect the myocardium[9;10]. Melrose and co-workers realized the potential of potassium-containing solutions in arresting and restarting the heart[11]. At that time however, the occurrence of myocardial necrosis limited the popularity of this method. By the 1970s intermittent cross-clamping fibrillation (ICCF) was established as a safer alternative for cardioprotection in the US, while the use of potassium-containing cardioplegic formulations grew in prominence in Europe[12].

The 1980s witnessed a surge in the use of various forms of cardioplegia ranging from: hypothermic, normothermic, crystalloid, substrate-enhanced and blood; along with variations

in its delivery with the use of the anterograde approach, retrograde approach or a combination of both[13]. This surge was driven mainly by the increase in morbidity seen particularly in high risk CABG patients as a result of a condition referred to as 'low output syndrome'. Post-operative 'low cardiac output syndrome', now defined as the need for postoperative intra-aortic balloon pump (IABP) or inotropic support for over 30 minutes in the intensive care unit[14], was thought to occur as a result of inadequate myocardial protection. It directed the attention of researchers into reassessing pre-existing methods of protection while looking for the optimal cardioplegic temperature, composition and method of delivery.

Earlier techniques using cold crystalloid solutions allowed good visualisation of the operating field but adversely caused the inhibition of enzymatic activity resulting in the delay in cardiac recovery[15]. This was followed by the use of blood as a cardioplegic agent as it had the advantage of being an oxygen carrier[16]. It was soon shown to be superior to crystalloid due to its ability to facilitate aerobic myocardial metabolism thereby reducing lactate production[17;18]. By the early 1990s, optimal myocardial protection was shown to be achieved with intermittent cold- blood cardioplegia as this enabled adequate visualisation of operative field as well as allowing early resumption of temperature-dependent mitochondrial enzymatic function with a speedy return of aerobic metabolism and ATP production[19].

1.2. Current strategies for cardioprotection during cardiac surgery

1.2.1. Cardioplegia

i) Cold crystalloid cardioplegia

Cold crystalloid cardioplegia was introduced into clinical practice in the mid 1960s[20;21] and has since existed in two forms:

The intracellular form consists of a low sodium and calcium content (Bretschneider's solution); while the extracellular form consists of high magnesium, sodium and calcium content (St Thomas' Hospital No.2). Both forms contain about 10-20 mmols of potassium and can be manufactured to have increased osmotic activities through the addition of substances such as mannitol, lidocaine, procaine as well as buffers such as amino acids and bicarbonate[22].

Cardioprotection is brought about through the induction of hypothermia and the maintenance of electrochemical arrest. In operations where a prolonged aortic cross-clamp time (XCT) is anticipated, additional doses of cardioplegic solution may be administered at set intervals. This can however inadvertently contribute substantially to haemodilution during cardiopulmonary bypass[22].

ii) Blood cardioplegia

The mid 1990s saw a surge in the use of blood as the preferred cardioplegic agent replacing crystalloid in most parts of the western world.

A recent survey conducted in the UK and Ireland in 2004 showed that 84.3% of surgeons used cardioplegia and 15.7% used intermittent cross-clamp fibrillation (ICCF) techniques for on-pump CABG. Of those who opted for cardioplegia, 83.5% used blood and 16.5% used crystalloid[23].

After numerous experimental studies and the retrospective analysis of clinical data, the application of this form of cardioplegia has evolved, and can now be divided into: multi-dose cold blood cardioplegia, warm blood cardioplegic reperfusion, warm induction, antegrade and retrograde delivery, continuous cold blood perfusion and intermittent warm blood cardioplegia[24].

The advantages of blood over crystalloid cardioplegia include: its versatility in being able to maintain oncotic balance; its buffering properties; its anti-oxidant benefits; its ability to augment O_2 delivery, and its role in preventing ischaemic injury thereby limiting reperfusion damage[24].

The constituents of blood cardioplegia

Blood cardioplegia constitutes the following properties:

- Hyperkalaemia- allows the induction and maintenance of cardioplegic arrest.
- Hypocalcaemia- limits mitochondrial calcium overload preventing apoptosis and necrosis of cardiomyocytes.

- Tris buffer- prevents tissue acidosis.
- Hyperosmolarity and hyperglycaemia- prevents myocardial oedema.
- Glutamate and aspartate- replenishes deleted amino acids that are essential for Krebs's cycle. This enhances aerobic respiration particularly during ischaemia[24].

Blood cardioplegia consists of native blood and a commercially available crystalloid solution in a ratio of 4:1. It is delivered via a double-headed roller pump and guided through a special heat exchanger before it enters the patient's heart. The perfusionist ensures that the delivery system and the aortic root remains air-free by a careful de-airing technique, essential to avoid coronary artery air embolism[24].

Cardiopulmonary bypass (CPB) for routine cardiac surgery is performed with a linear flow at 2.6 l/min per m^2 while maintaining a perfusion pressure of 60-80mmHg and a systemic blood temperature of 35 ^{o}C[24].

The delivery of blood cardioplegia

A number of advance strategies exist for the delivery of blood cardioplegia:

Warm cardioplegic induction- this aims to actively resuscitate the heart which is by this time not only ischaemically-damaged, but also both energy and substrate-depleted. It maximises the kinetics of repair and minimises oxygen demand[25]. It contains the Krebs's cycle intermediates glutamate and aspartate, both of which are depleted in compromised hearts. This method of delivery tends to be used in patients with acute myocardial infarction, cardiogenic shock and a poor ejection fraction[24].

Controlled reperfusion- is a strategy aimed at reducing reperfusion injury in patients undergoing urgent CABG for acute coronary occlusion[24]. A study comparing the use of this method during CABG with percutaneous trans-luminal coronary angioplasty (PTCA) found that overall mortality was reduced from 8.7% to 3.9%[26].

Blood cardioplegic leukocyte filtration- we know that myocardial ischaemia and reperfusion cause the activation of neutrophils and the expression of adhesion molecules on the myocardial endothelial surface. In complex cardiac surgery which invariably involves longer cross-clamp times, activated leukocytes in the blood cardioplegia can result in severe myocardial damage. Clinical studies support the benefit of leukocyte depletion particularly in high risk patients. It has been shown that at least 90% of leukocytes must be removed to attenuate reperfusion injury enough to gain clinical benefit[27-29].

Standard techniques of blood cardioplegia

Standard techniques of blood cardioplegia have been modified to address different clinical situations:

- Continuous warm blood cardioplegia- aims to limit injury through continuous delivery of warm cardioplegic blood. Although a common mode of delivery, there is a potential for cardioplegic overdose with this method[30].

- Intermittent antegrade warm blood cardioplegia- first published by Calafiore in 1995[31], this technique aims to improve visualisation of the operating field. Delivery is limited in cases of critical coronary stenosis where cardioplegia is prevented from reaching ischaemic regions of the heart, resulting in the induction of warm ischaemic injury[31].

- Tepid blood cardioplegia- this technique combines the advantages of warm and cold cardioplegia. The temperature of the heart is reduced from 37^{o}C to 29^{o}C which helps in the reduction of myocardial lactate release[32].

iii) Anaesthesia

Since the conception of cardiac surgery, anaesthetic drugs have played a vital role in reducing morbidity and promoting survival by not only maintaining haemodynamic stability but also in providing pain relief and anaesthesia. In conjunction with this, good experimental evidence now supports a cardioprotective role of some anaesthetic agents and as such, a number of possible mechanisms have been proposed. Some authors have alluded to a preconditioning-like effect; others have speculated on a role in blocking calcium overload in combination with an anti-oxidant effect. The most promising however, is that of a neutrophils/platelet-endothelium interaction. Incidentally, it is the multiplicity of mechanisms proposed that has hampered the adaptation of anaesthesia as cardioprotective agents in their own right.

Non-volatile anaesthesia

Fentanyl

Fentanyl is an opioid that has been linked with an anti-inflammatory action in its role in cardioprotection. It reduces the inflammatory response that occurs as a result of the bypass circuit as well as that imposed by ischaemia-reperfusion injury (IRI) during cardiac surgery[33]. It has been shown to oppose the negative inotropic effects of inflammatory mediators in the rat ventricular myocytes[34] and its effect can lead to improved intracellular calcium mobilisation[35].

Propofol

Propofol is commonly used for anaesthetic induction as well as maintaining anaesthesia peri- and post-operatively (reviewed Bryson 1995[36]). Propofol has been shown using experimental models to protect the heart through its action as a free-radical scavenger[37]; enhancing tissue antioxidant capacity[38;39]; and inhibiting plasma membrane Ca^{2+} channels[40].

Some evidence have suggested a role in the inhibition of the mitochondrial permeability transition pore (mPTP) [discussed later] in Langendoff perfused rat heart[41] and in the activation of prosurvival kinases[42].

Etomidate (carboxylated imidazole)

Etomidate is used intravenously to induce anaesthesia particularly in patients with impaired left ventricular (LV) function as it does not alter haemodynamic parameters. Unlike the agents previously mentioned, it has not been shown to be effective in cardioprotection.

Volatile anaesthesia

Isoflurane

Isoflurane is arguably the most extensively studied volatile agent in cardioprotection today and is thought to act via a number of different mechanisms. It blocks L-type calcium channels[43]; preserves energy-rich phosphates[44]; causes the vasodilatation of coronary vessels[45]; and reduces the expression of adhesion molecules[46].

The role of isoflurane as a possible preconditioning mimetic was suggested by Kersten and colleagues[47] after demonstrating that it activates ATP-dependent K+ channels, incurring

protection via the mitochondrial ATP-dependent potassium (mitoK $_{ATP}$)channel opening [discussed later] along with reactive oxygen species (ROS) generation and subsequent protein kinase C (PKC) activation[48-50]. Despite all the promise however, Preckel and colleagues[51] failed to show protection against IRI using isoflurane.

Sevoflurane

Sevoflurane is reported to induce a preconditioning-like effect via mitoK $_{ATP}$ channel opening[52;53]. There is also some evidence of an anti-inflammatory effect, suppressing production of IL6 and IL8, inhibiting neutrophil activity[54], and modulating pro- and anti-inflammatory cytokines[55].

In recent years, randomised clinical trials conducted in patients undergoing cardiac bypass surgery have demonstrated cardioprotection mostly through the attenuation of cardiac troponin release and the improvement of post-operative cardiac function. However, they have lacked the statistical power to demonstrate an advantage in terms of morbidity or mortality. A recent meta-analysis (reviewed by Landoni[56]), conducted after pooling data on the use of Desflurane and Sevoflurane, found significant reductions of in-hospital mortality, myocardial infarction rates, intensive care unit and hospital stay, time on mechanical ventilation and incidence of long term cardiac events. Most recently, a systematic review by Yu and colleagues included 32 randomised controlled trials encompassing 2,841 patients[57]. After examining the composite outcome of death or acute MI, volatile anaesthetics did not appear to be associated with a reduced frequency of events. The studies included in this meta-analysis were indeed small in size, conducted in low risk population groups and so lacked sufficient power to

demonstrate effects on mortality. A study by Garcia and colleagues included in the meta-analysis looking at 72 patients undergoing elective CABG was the only study to demonstrate a reduction in the incidence of late cardiac events[58]. It is clear that a large multi-centred clinical study is required to determine whether volatile anaesthetics impact on long-term clinical outcomes post-cardiac surgery.

iv) Off-pump cardiac surgery

Off-pump coronary artery bypass (OPCAB) was first performed in the late 1960s[59] but soon went out of favour as the use of CPB and cardioplegic arrest became routine[60]. Its re-emergence as a safer form of surgical revascularisation is thought to have been precipitated by the need to avoid the unwanted complications of CPB particularly in the increasingly complex elderly patients referred for operation. Haemodynamic instability had posed an obstacle in OPCAB, preventing grafting of the posterior wall, thus compromising the basic principle of complete revascularisation. However the introduction of the Octopus, a cardiac stabilisation device, has allowed surgeons to access all sides of the beating heart allowing OPCAB to be performed in patients with triple vessel disease (Reviewed by Hijazi[61]).

The implementation of CPB, before and after cardioplegia, exposes the heart and other major organs to micro-emboli, protease and chemical cytotoxins as well as regional hypoperfusion[62]. Furthermore, myocardial oedema and the distension of the cardioplegic heart results in the reduction of myocardial contractility[63]; raised ventricular end-diastolic volumes, an increase in myocardial wall stress and oxygen consumption[62]. Researchers have shown that by allowing continuous perfusion of the beating heart, OPCAB should reduce the occurrence of myocardial

injury. Angelini and colleagues demonstrated that the frequency of myocardial infarction was reduced by 2% in OPCAB at 2 years of follow-up[64]; while more recently, Keenan and colleagues provided further evidence of this reduction in myocardial injury using cardiac MRI[65].

The aetiology for acute kidney injury post-operatively is multi-factorial particularly in patients with co-morbid conditions such as diabetes and pre-existing renal impairment[66]. The use of CPB results in renal hypoperfusion and direct inflammatory damage and is now widely regarded as the most important cause of acute renal failure in this setting[67]. Beating Heart versus Cardioplegic Arrest Studies (BHACAS-1 study) demonstrated a significant reduction in both glomerular and tubular function in on-pump compared to off-pump surgery[68]. In a retrospective study by Magee and colleagues, they were able to show that the reduction in the frequency of renal failure in patients undergoing OPCAB was present even in those with pre-existing renal impairment[69].

Impaired pulmonary function is seen up to 4 months after cardiac surgery. However, when compared with on-pump cardiac surgery, OPCAB showed a reduction in ventilation duration allowing for early extubation with the benefits more pronounced in patients with pre-existing lung disease[70].

Permanent neurological dysfunction after conventional CABG can result in significant disability, carrying a stroke risk of 3% and up to 60% of patients showing varying degrees and durations of cognitive decline[71]. The pathogenesis of cerebral injury and dysfunction is multifactorial, with an increasing body of evidence pointing towards the role of micro-embolisation from the ascending aorta, cardiac chambers and the bypass circuit[72]. Despite a marked reduction in

cerebral embolisation when CPB is avoided, this does not seem to translate to a reduction in incidence of stroke and post-operative neurological dysfunction[70]. This could be explained by the fact that aortic manipulation still occurs in OPCAB particularly during construction of the proximal anastomosis[73]. The adoption of the aortic 'no touch' technique, which aims to avoid intraoperative atheromatous embolisation from the diseased aorta, may represent a significant step in improving neurological outcomes after OPCAB[74].

The advantages of OPCAB have been shown to extend beyond major organ protection. The benefits of avoiding CPB with the subsequent systemic inflammatory response has been shown to reduce post-operative gastrointestinal complications[75]; reduce the incidence of AF[70]; reduce post-operative bleeding and the need for blood transfusion[64].

Finally, studies have shown comparable mortality rates after OPCAB with that of patients who undergo on-pump bypass surgery[76]. However, the reduction in morbidity rates with OPCAB and a decrease in the incidence of complications are associated with decreased length of ICU and hospital stay with favourable economic outcomes[77]; prompting researchers to contemplate the adoption of beating-heart CABG surgery for all surgical revascularisations.

1.3. The unresolved complication of cardiac bypass surgery

Researchers and clinicians alike have spent the last four decades trying to understand the complexities of myocardial injury in this setting and have made considerable in-roads in tackling what is clearly a significant problem. Despite relatively low 30-day mortality rates following cardiac surgery in general, the morbidity and mortality rates remain unacceptably high in the

subset of patients undergoing complex surgery (redo operations and CABG+ valves) and those with severe aortic stenosis and left ventricular failure[78].

Along with the other mechanisms of myocardial injury [discussed later] these patients are particularly susceptible to the lethal effects of myocardial IRI through the prolongation of aortic-cross-clamp times commonly seen in complex cardiac surgery. Ischaemia-reperfusion injury is a major cause of myocardial injury during cardiac surgery, and studies have demonstrated its correlation with longer hospital stays and worsening long term outcomes [79].

1.4. Sources of peri-operative myocardial injury

The mechanism of myocardial injury in the setting of cardiac surgery is multi-factorial with variable detrimental effects:

1.4.1. Technical inadequacy

Technical inadequacy is an important source of myocardial injury and its impact on patient outcomes is often underestimated. In the setting of cardiac surgery, it usually results in the failure of aorto-coronary bypass grafts due to poorly constructed distal anastomosis and prosthetic valve regurgitation due to inadequate placement of aortic valve prosthesis[80].

Errors in intra-operative judgment including decisions on whether or not to revascularise an occluded coronary artery or deciding to repair a regurgitant mitral valve rather than replace it will have a significant bearing on peri-operative and long-term outcomes[81].

1.4.2. Ischaemic injury

Aortic cross-clamping or coronary artery/graft occlusion leads to progressive pathological ischaemic changes that start within minutes, resulting in a time-dependent hazard for myocardial injury. The current cardioprotective strategies [previously discussed] aim to reduce this mode of injury. It is now recognized that these techniques are limited in their ability to offer complete protection particularly in complex operations requiring prolonged cross-clamp times[82].

Despite the improvement of cardioplegic techniques and the use of newer perfusion systems, the issue of optimising cardioprotection remains topical. Prolonged cross-clamp times result in an increase in myocardial injury, low output syndrome and the need for inotropic support. Studies have gone further and identified ischaemic injury as an independent predictor of immediate and long-term morbidity and mortality. Nissinen and colleagues found that the type of cardiac surgery performed did not significantly impact on 30-day mortality[83]. However a strong association was seen between the number of procedures performed in one sitting and subsequent 30-day mortality. While one procedure carried two percent 30-day mortality, this increased to as high as 25% after three to four procedures. It so follows that it is in this group of patients who undergo prolonged cross-clamping that would benefit most from cardioprotective interventions. They noted that durations of less than 150 minutes and 240 minutes for aortic cross-clamp time (XCT) and cardiopulmonary bypass-times (CPBT) respectively were associated with a lower risk of morbidity and mortality independent of the operative risk to the patient and the complexity of the surgery[84].

While it is clear that patients with impaired ventricular function are more susceptible to myocardial ischaemia, the overall impact of prolonged cross-clamp times is more complex compared to patients with normal left ventricular function. While clearly demonstrating prolonged cross-clamp time as an independent predictor of mortality in patients with a LVEF > 40%, Doenst et al were not able to show similar trends in patients with LVEF < 40%[85]. Patients with poorer ejection fractions had higher mortality overall and at both ends of the spectrum. These patients were more likely to be affected by the effects of preconditioning, as they had suffered from previous myocardial infarction, triple vessel disease and unstable angina[86]. The increase in mortality seen as a result of short cross-clamp times were thought to be due to incomplete revascularization, another independent predictor of mortality[87].

1.4.3. Ischaemia-reperfusion injury

Clamping the aorta during cardiac surgery renders the myocardium ischaemic by compromising its blood supply, subsequently resulting in a reduction of oxygen and nutrients to the heart. This leads to a reduction in energy production by the mitochondria which in turn results in an abnormal accumulation of lactate, sodium and calcium ions (reviewed by Suleiman[88]). The eventual metabolic imbalance causes a disruption to the ionic pumps and channels resulting in the depolarisation of cellular membranes and loss of excitability. A rapid restoration of blood flow can correct ionic haemostasis with the preservation of cellular structure and function. However, in the event of prolonged ischaemia, reperfusion tends to have detrimental effects on the heart. Numerous studies have confirmed that reperfusion triggers a further increase in cytosolic calcium and the generation of reactive oxygen species (ROS)[89;90]. This in turn triggers a conformational change within the inner mitochondrial membrane causing the opening of the

mitochondrial permeability transition pore [discussed later]. Mitochondrial swelling subsequently ensues with eventual cell death through necrosis or apoptotic pathways[91].

The destruction of myocardial cells lead to the activation and accumulation of neutrophils at the sites of ischaemia or myocardial damage[92]. The simultaneous release of inflammatory mediators and cytokines, in particular interleukin-6 (IL6) play a significant role in the amplification of this devastating cascade of events perpetuating further cell damage[93]. Interleukin-8 (IL8) stimulates the upregulation of adhesion molecules which in turn encourage the adhesion of neutrophils to myocytes were their release of proteolytic enzymes can be most damaging[94]. The release of IL6 has been shown to be most evident in the coronary beds of CABG patients[95] and in experimental models using cold crystalloid cardioplegia[96]. Direct myocardial damage has also be shown to occur with IL6 and IL8; the former causing negative inotropic effects as a result of increase nitric oxide and activation of cGMP leading to the inhibition of L-type calcium channels[97].

1.4.4. Myocardial stunning

Myocardial stunning is defined as reversible contractile dysfunction with (near) normal blood flow to the stunned region. In the cardiac surgical setting, it occurs as a result of reperfusion following global ischaemia after cross-clamping of the aorta. Previous studies described significant but temporary declines in left and right ventricular ejection fractions after CABG despite 'uncomplicated' procedures[98]. Impaired contractility was notable approximately 4 hours postoperatively, affecting both systolic and diastolic functions, with a gradual recovery

occurring over the subsequent 24 hours[99]. After the exclusion of other causes, it is likely that a temporary period of myocardial stunning was responsible for impaired function.

1.4.5. Inadequate revascularisation and aorto-coronary bypass conduit failure

Failure to achieve complete revascularisation is a recognisable cause of myocardial infarction and death post-operatively. This usually occurs due to failure to recognise the presence of significant disease and also in advanced diseases that do not lend themselves to revascularisation[100].

Current vein harvesting techniques have been shown to be injurious to the endothelium and are thought to play a significant role in aorto-coronary bypass conduit failure[101]. Currently the point of focus of much research is in improving graft harvest techniques. Until improvements materialise however, one must appreciate this as an important mode of injury.

1.4.6. Systemic inflammatory response

The implementation of cardiopulmonary bypass (CPB) triggers a systemic inflammatory response as blood comes into contact with foreign surfaces leading to the activation of complement pathways[102]. The response is then propagated by the release of inflammatory mediators leading to the activation of a massive defence reaction. This includes the release of: hormones, cytokines, chemokines, vasoactive substances, cytotoxins, reactive oxygen species and proteases. The detrimental effects of consumptive coagulopathy, interstitial fluid shifts and micro-emboli eventually lead to multi-organ dysfunction (reviewed by Raja et al[103]).

A similar inflammatory response is triggered peri-operatively by direct surgical trauma, blood loss and hypothermia. At instances where splanchnic hypo-perfusion occurs, subsequent

mucosal damage leads to the release of endotoxins from the gut resulting in further organ damage[104].

1.4.7. Genetic predisposition

It is estimated that 10% of patients who undergo cardiac surgery suffer peri-operative myocardial injury[105]. The relationship between myocardial infarction in the non-surgical population and genetic variants on chromosome 9p21 has been well documented[106]. It was recently shown however that a similar relationship exists between genetic variants in 9p21 and peri-operative myocardial injury after CABG[107]. These genetic variants are adjacent to genes for cyclic-dependent kinases CDKN2A/B[108] which regulate cell aging, cell proliferation and apoptosis as well as ANRIL[109], a large anti-sense non-coding RNA gene expressed in cell types involved in atherosclerosis. This relationship was shown to be independent from type 2 diabetes and the severity of coronary artery disease[110]. Variants in 9p21 have also been shown to be associated with carotid atherosclerosis, progression of atherosclerosis[111], abdominal aortic aneurysm[112] and intracranial aneurysm[113].

1.4.8. Identifying key contributors to myocardial injury

Identifying the factors that contribute most to myocardial injury in cardiac surgery is complex task. Clearly, with the multitude of factors previously discussed, it would be difficult to measure the contribution of each one individually. However inferences could be made as to which would be the major contributor and this would largely depend on the type of cardiac surgery being carried out.

In the setting of on-pump cardiac surgery, ischaemia-reperfusion injury as a result of aortic cross-clamping gives rise to a global myocardial ischaemia. With the addition of the systemic inflammatory response of CPB, these factors would be the larger contributors to myocardial injury. However in the setting of off-pump surgery, local myocardial ischaemia-reperfusion injury occurs after graft anastomosis has taken place contributing mostly to myocardial injury.

When CABG surgery is carried out concomitantly with mitral valve replacement, a comparatively higher release of cardiac enzymes is often seen, reflecting an additional increase in myocardial injury. This is because the replacement of the mitral valve involves more direct trauma to the myocardium (through the incision of the left atrium- please refer to chapter 3) unlike other forms of cardiac surgery.

The impact of ischaemia-reperfusion injury and its contribution to myocardial injury is evident particularly when the key factors are explored. In addition, the type of cardiac surgery performed has a significant bearing on the mode of cardioprotection that becomes most relevant. A third variable that would be discussed in the next section is the susceptibility of the hypertrophied heart to reperfusion injury and its response to cardioprotection.

1.5. Cardiac hypertrophy and its susceptibility to reperfusion injury

Cardiac hypertrophy is an adaptive response to pressure overload commonly seen clinically in systemic hypertension and aortic stenosis[114]. The left ventricular chamber stiffness and hypertrophy that occurs as a result of pressure overload correlates well with accumulated collagen content and has been identified as an independent risk factor for sudden cardiac death, myocardial infarction and congestive cardiac failure[115]. The hypertrophied heart acts as

an arrhythmogenic substrate[116], in addition, there is the growing evidence supporting its predisposition to IRI due to its reduced capillary density[117].

1.5.1 Cell signalling in cardiac hypertrophy

The triggers for pathological cellular hypertrophy can be divided broadly into neurohumoral signalling and stress signalling. Neurohumoral signalling involves the activation of G-protein-coupled receptors by hormones (angiotensin II, endothelin I, catecholamines, insulin-like growth factor-1) with the activation of Protein kinase C further downstream. Downstream triggers result in the release of Ca^{2+} from the sarcoplasmic reticulum which in turn activates calcineurin. Cacineurin dephosphorylates nuclear factor of activated T cell (NFAT) transcription factors permitting the translocation of NFAT to the nucleus where they participate the hypertrophic gene expression (reviewed by Suleiman et al[118]).

The stimulation of myocardial stress receptors within the myocardium can also induce pathological hypertrophy. Two important proteins that act as stretch sensors in cardiomyocytes are melusin and 'muscle LIM protein'. They both act via focal adhesion kinase (FAK) and the calcineurin-NFAT signalling pathways respectively, with the end-effect being the induction of hypertrophy (reviewed by Suleiman et al[118]).

Under the conditions of severe cardiac hypertrophy, the energy production within cardiomyocytes is impaired, resulting in the worsening of cardiac contractility. Important metabolic alterations have also been described, including the use of carbohydrate metabolism in preference to fatty acids for energy production. It is likely that cardiomyocytes adopt this

mechanism purely for survival and this modulation of myocardial metabolism can certainly act as a key target for therapeutic intervention[119].

The hypertrophied heart is more susceptible to the effects of IRI due to its reduced capillary density which hinders the diffusion of nutrients and oxygen to energy production sites[120]. The activation of the renin-angiotensin-aldosterone pathway is thought to further perpetuate myocardial injury as the occurrence of systemic vasoconstriction results in an increase in afterload with oxygen demand far outstripping supply[121]. Some reports have held an opposing view, suggesting that the hypertrophied heart is more resistant to ischaemia- this could be related to the extent of the hypertrophied heart, as severely hypertrophied hearts were thought to be more susceptible to reperfusion injury than moderately hypertrophied hearts[122].

1.5.2 Cardioprotection of the hypertrophied heart

Several signalling pathways have been identified in playing a role in protecting the hypertrophied heart. The stimulation of TNF receptor type 2 results in the production of a lower TNF-α concentration which improves the recovery of the hypertrophied heart[123]. Inhibition of Na/H+ exchanger in hypertrophied rat myocardium has also been shown to confer significant protection[124]. Increased availability of cellular substrates including aspartate and glutamate have also been shown to be cardioprotective[125]. Interventions during cardioplegic arrest are less protective in the hypertrophied heart compared with the normal heart with slower recovery rates and more unstable haemodynamic parameters seen compared with normal hearts[126].

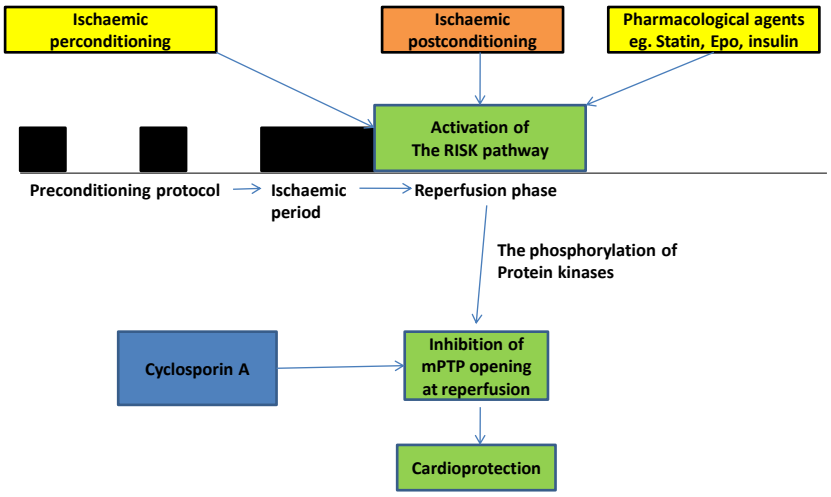
Ischaemic post-conditioning has been shown to attenuate IRI in isolated hypertrophied rat hearts with its mode of action partly mediated through the PI3K/Akt/GSK-3b signalling pathway[127]. (See below for more on 'conditioning' and the RISK pathway).

1.6. Novel cardioprotective strategies

1.6.1. 'Conditioning' the heart for protection

The concept of 'conditioning' the human heart in order to endogenously protect it from the detrimental effects of lethal ischaemia-reperfusion injury has been known to researchers for almost three decades. This rather novel endogenous cardioprotective phenomenon is brought about by applying brief non-lethal episodes of ischaemia and reperfusion either directly to the heart (by clamping the aorta) or to an organ or tissue remote to it, RIPC (remote ischaemic pre-conditioning)[128]. This strategy can either be applied prior to the index ischaemic insult (ischaemic preconditioning); after ischaemia (ischaemic perconditioning) or at the onset of reperfusion (ischaemic postconditioning).

Figure 1.01: *Variations in the timing of 'conditioning' all of which ultimately bring about cardioprotection. The preconditioning protocol consists of short episodes of ischaemia and reperfusion that is instituted prior to the index ischaemic period. Ischaemic perconditioning commences after the onset of myocardial ischaemia but prior to myocardial reperfusion, while ischaemic post-conditioning commences at the start of the reperfusion phase. Pharmacological agents including statins, erythropoietin and insulin have been shown to activate the RISK pathway (discussed further later), while inhibiting the opening of the mitochondrial permeability transition pore either directly using CsA or through the phosphorylation of prosurvival kinases represents the final stage of cardioprotection.*



Key: Epo- erythropoietin, RISK- reperfusion injury salvage kinase, mPTP- mitochondrial permeability transition pore.

i) Background of IPC

Establishing the vital role of reperfusion in the treatment of myocardial ischaemia remains one of the most significant discoveries in cardiovascular medicine[129]. The introduction of clot-dissolving drugs, followed by the use of percutaneous coronary angioplasty and stenting, have

revolutionised the management of acute coronary syndromes (ACS). Despite this, clinicians were still left bemused as the rates of heart failure and death from ischaemic heart disease continued to rise.

Up until the mid 1980s, it had remained unclear whether or not it would be possible to limit infarct size therapeutically. However, in 1986 Murry and colleagues made the crucial discovery of an intrinsic mechanism of profound endogenous protection which they named ischaemic preconditioning (IPC)[130]. Using a canine experimental model, they showed that exposure of the circumflex coronary artery territory to brief periods of ischaemia (4 cycles of 5 minute ischaemia followed by reperfusion) before 40 min of complete ischaemia substantially reduced the size of the resultant infarct after restoration of blood flow[130]. There was no differences in coronary collateral blood flow between the groups, suggesting that the mechanism of preconditioning was independent of collateral recruitment [130]. It turns out that for each sub-lethal episode of ischaemia and reperfusion prior to the index lethal ischaemic event, a minimum period of 30 seconds to 1 minute of reperfusion was required to see protection[131].

Whether this phenomenon follows an all-or-nothing response or a graded response is still unclear. Although evidence exists for and against either outcome, it is likely that IPC is graded as this would comply with nature (reviewed by Yellon and Downey)[132].

Other organs have been shown to be amenable to protection including the kidneys, liver, brain and intestine. The clinical application of IPC in the setting of cardioprotection will be reviewed later in this introduction.

ii) A brief overview of the mechanism of IPC

In the ground-breaking publication by Murry and colleagues, a 75% reduction in infarct size was demonstrated[130]. This strategy has been subsequently reproduced by numerous researchers using various animal models all of which have shown similar anti-infarct effects[133]. It is now regarded as the strongest form of protection against myocardial infarction after reperfusion itself[134].

Ischaemic preconditioning changes the physiology of the heart rendering it resistant to infarction. The mechanism of preconditioning exists in two phases. The first phase, also known as "early" or "classical"[130] preconditioning is the more potent of the two phases, commencing immediately after the IPC stimulus and lasting for 1-2 hours[135]. The second phase referred to as the second window of protection (SWOP) commences after 12-24 hours following the IPC stimulus and lasts up to 3-4 days[136].

Signal transduction pathways which underlie preconditioning can be divided into: triggers, mediators, memory and end-effectors. The pathways are activated by triggers known as autacoids which consist of catecholamines, opioids, adenosine and bradykinin[132]. The memory element keeps the heart in a preconditioned state but how the heart remembers that it has been preconditioned is still a mystery. However, because IPC starts within 10-15 minutes, it is likely that the memory effect occurs as a result of reversible post-translational modification of pre-existing proteins rather than from gene expression[132]. Steps distal to the memory step are now referred to as mediators and steps proximal to the memory step are termed triggers[132].

The second phase of ischaemic preconditioning is thought to share similar trigger substances to 'classical' preconditioning and its underlying mechanism is likely to be related to protein synthesis, post-translational protein modification and a change in the compartmentalisation of existing proteins[132].

The signal transduction pathways

The mechanism underlying IPC is receptor-mediated and was first demonstrated by Downey and colleagues who implicated the role of the adenosine A1 receptor[137]. They were able to show that A1 receptor blockers inhibit protection and agonists conferred the protection.

We now know that any G- protein coupled receptor (GPCRs) can trigger preconditioning; in fact multiple receptors work in parallel to stimulate the prosurvival pathway[132].

After the release of autocoids during the brief ischaemic period, their respective receptors trigger IPC via the activation of GPCRs. Blockage of a single receptor acts to increase the ischaemic threshold rather than completely blocking it[132].

There are a number of other potential triggers of IPC that have been identified. Elevated calcium levels are thought to bring about protection via a protein kinase C dependent pathway and verapamil has been shown to blocks both Ca- induced preconditioning and ischaemic preconditioning[138;139]. Free radicals have been shown to act directly to trigger IPC via their action on protective kinases[140]. Other triggers include hyperthermia[141], hypoxia, ethanol[142] and pacing[143] but their mechanism of action remains unclear. Nitric oxide has also been shown to have a role in IPC particularly in the SWOP phase [for detailed review refer to Yellon and Downey][132] .

iii) Some important kinases

The signal transduction pathways consists of highly important kinases that act as mediators such that when activated, interact with effectors to bring about protection.

Protein kinase C

Protein kinase C (PKC) is a serine threonine kinase whose role in IPC was co-discovered by Mitchell and Ytrehus in 1994[144;145]. It is activated by lipid cofactors derived from the breakdown of membrane lipids by phospholipase C[132]. It exists as multiple isoforms that can be classified as: classical, novel and atypical[132]. These isoforms bind to RACK (receptor for activated C kinase) -which are strategically located near specific organelles within the cell. The activation of PKC then leads to the phosphorylation of specific protein substrates that lie within the cell[132].

Tyrosine kinase and the mitogen-activated protein kinases

Maulik identified the presence of tyrosine kinase using genistein, a broad-spectrum tyrosine kinase inhibitor and concluded that at least one tyrosine kinase was present in the overall pathway[146]. Others have suggested that a tyrosine kinase was likely to be downstream[147] or at least in parallel to PKC[148]. It was Maulik who confirmed the identification of this tyrosine kinase as p38 MAPK.

MAPKs are activated by dual phosphorylation of a serine and a threonine. The MAPK is a tyrosine kinase and at least the ones targeting p38 MAPK can be blocked by genistein[149].

Sub-families of MAPK have also been suggested to play a role in IPC. They include:

- ERK (extracellular receptor kinase)- ERK1/2: ERK 1 activity is said to increase in the ischaemically preconditioned myocardium[150].

- JNK (*c-jun* kinase)- JNK 46 and JNK 54 are present in the heart and are strongly activated during reperfusion after ischaemia[151].

- P38 MAPK: exists in at least five different isoforms all of which mediate different biological functions[152]. It has two amino acids which must be phosphorylated for activation[132]. The importance of p38 activation for cardioprotection remains unclear and this is mainly due to the variety of isoforms in different species and the selectivity of the different inhibitors that have been used.

Phosphatidylinositol 3-kinase (PI3K) has been shown to have a definitive role within the pathway. Using a PI3K inhibitor, Tong and colleagues demonstrated the blockade of protection using myocardial contractile dysfunction as the end point[153]. Mocanu went on to confirm this result using an infarct size model[154].

K_{ATP} channels

K_{ATP} channels have been shown to be important mediators of cardioprotection in a variety of different models. They were first described by Noma using cardiac ventricular myocytes and the name is derived from the fact that they can be inhibited by physiological levels of ATP[155].

K_{ATP} channels are modulated by pH, fatty acids, NO, SH-redox state, various nucleotides, G proteins and various ligands[156]. It is the opening of the channels that bring about protection in IPC, a phenomenon first proposed by Gross and colleagues[157] and further substantiated through subsequent studies[158;159].

Two types of K_{ATP} channels have been identified; the sarcolemmal (surface K_{ATP}) and mitochondrial (mitoK_{ATP})[160]. Garlid and Liu confirmed that it was mitoK_{ATP} channel that was responsible for protection[161;162]. The exact composition of the channel is unknown.

Terzic and colleagues found that opening the mitoK_{ATP} channels made isolated mitochondria more resistant to Ca^{2+} entry therefore promoting cell survival[163]. Data exists suggesting that transient opening of mitoK_{ATP} channels put the heart into a preconditioned state that continues long after the channel is closed.

Experiments by Pain had suggested that the role of the mitoK_{ATP} channels is as a trigger[164]. However, Wang disputed this, suggesting that the channels played a dual role both as a trigger and a mediator[165]. Current evidence supports Wang's proposal suggesting that mitoK_{ATP} channel opening triggers a kinase cascade that feeds back in a positive manner to keep the channel open during the index ischaemia.

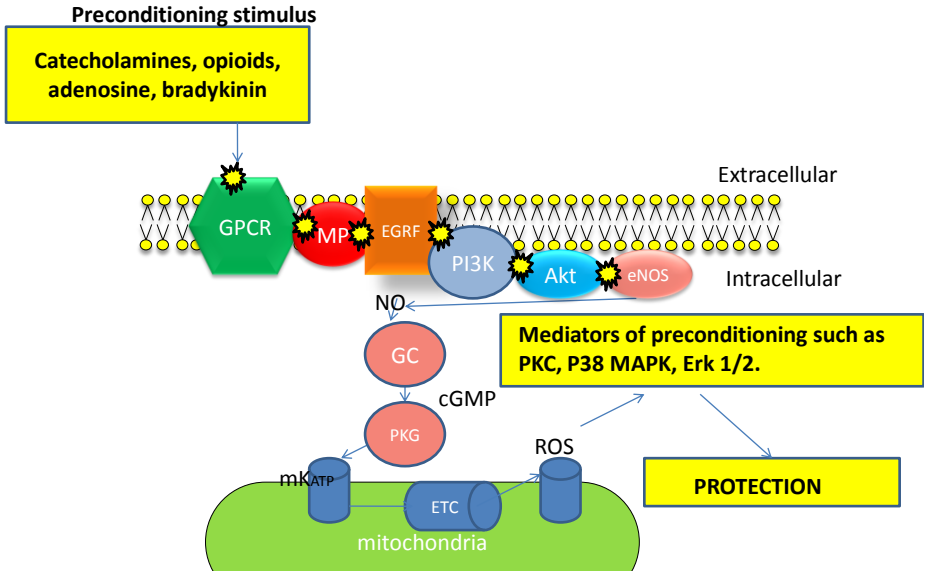
Pain and colleagues proposed a new view of the pathway suggesting a role for free radicals and mitoK_{ATP} channels. They suggested that receptor binding led to mitoK_{ATP} channel opening that resulted in the release of reactive oxygen species (ROS). The free radicals would then activate kinases downstream that would then modulate the end-effectors. The blockage of protection by free radical scavengers supported this theory[166].

The protection triggered by adenosine however, seems to be elusive to K_{ATP} blockage or free radical scavengers. This led Downey and colleagues to propose that the adenosine pathway must be parallel to the RISK pathway[167].

Ischaemia allows autacoids to populate GPCRs, which subsequently trigger the opening of mitoK_{ATP} channels. At this stage, because oxygen is lacking, the signal would eventually die. However, reperfusion allows for a burst of ROS which ultimately results in signal transduction pathway activation bringing about protection[132].

The final step in the preconditioning pathway has eluded researchers for decades and still remains an enigma. Many theories have been considered, the oldest being that preconditioning improves the energy balance in the cell by inhibiting mitochondrial ATPase activity[168]. However this theory has since been disproven and more viable candidates for end-effectors including the mitoK_{ATP} channels and the mitochondrial permeability transition pore (mPTP) have received much attention. In fact, Hausenloy et al[169] described a vision whereby the opening of the mitoK_{ATP} channel acted to inhibit the mPTP.

Figure 1.02: *The RISK pathway- activation of the G-coupled protein receptor by an IPC stimulus leads to the trans-activation of the epidermal growth factor receptor via the matrix metalloproteinase. This then leads to the activation of PI3K-Akt and ErK 1/ 2 both of which are thought to run in parallel. After the subsequent activation of eNOS, guanylate cyclase and protein kinase G are activated via nitric oxide and cGMP respectively. Protein kinase G then stimulates the opening of the mitoK$_{ATP}$ channel and this leads to the generation of ROS via the electron transfer chain.*



Key: GPCR- G-coupled protein receptor, MP- metalloproteinase, EGFR- epidermal growth factor receptor , (PI3K)-Akt-phosphatidylinositol-3 kinase- OH, eNOS- extra-cellular nitric oxide synthase, NO- nitric oxide, GC- guanylate cyclase, PKG- protein kinase G, cGMP- cyclic guanine-5-monophosphate.

1.6.2. Inhibiting mitochondrial permeability transition pore (mPTP) for protection

In recent years the mPTP has presented itself as a major potential pharmacological target for cardioprotection as researchers have become more aware of the role the mitochondria plays in cellular injury. Pharmacological agents like cyclosporin A that already play a significant role in transplant medicine have been shown to prevent cellular injury by inhibiting the formation of

this pore through the inhibition of cyclophilin D, one of its major components. In order to appreciate the mitochondria's involvement in cellular injury, we must first understand the nature of calcium transport within the cell.

i) Calcium transport

The physiology of calcium regulation has been well known for over 20 years. The three main carriers involved in calcium transport are: the Ca^{2+} uniporter, Na^{+}/Ca^{2+} carrier and the Na^{+}/H^{+} antiporter[170]. Calcium enters the mitochondria electrophoretically via the Ca^{2+} uniporter and exits via the Na^{+}/ Ca^{2+} carrier in exchange for Na^{+}[170]. This transport cycle allows the changes in cytosolic Ca^{2+} to be relayed to the mitochondrial matrix, establishing a Ca^{2+} concentration $[Ca^{2+}]$ ranging between 0.2-10µM. It is at this range that calcium-sensitive enzymes especially those involved in oxidative metabolism (pyruvate dehydrogenase, oxoglutarate dehydrogenase and isocitrate dehydrogenase) are most effective[171].

When the myocyte contracts, there is an increase in cytosolic $[Ca^{2+}]$. This leads to an increase in mitochondrial $[Ca^{2+}]$ which in turn activates the tricarboxylic acid cycle. This is followed by an increase in oxidative phosphorylation and ATP production, allowing the ATP/ADP ratio to remain unchanged[172].

It was initially thought that certain types of cellular injury occurred as a result of significant energy utilization brought about by rapid mitochondrial calcium transport. However, the rate of Ca^{2+} cycling cannot exceed that of the Ca^{2+} uniporter so much so that an exposure of say a 10-fold increase in cytosolic $[Ca^{2+}]$ would only cause a 2% increase in respiration[173]. In other words, pathological insults that result in large mitochondrial $[Ca^{2+}]$ do not result in significant

energy dissipation from the cell to affect its viability. Another pathway must therefore exist to account for the accumulation of Ca^{2+} within the mitochondria resulting in injury.

During ischaemia, cytosolic calcium slowly and progressively accumulates, but it alone is not sufficient to cause cellular injury. The absence of adenine nucleotides is also required for cellular injury to occur. If ATP were present in abundance during cellular injury, the ATP/ADP ratio would be maintained and cells would remain viable. We now know that the presence of Ca^{2+} overload, high phosphate or oxidative stress coupled with an absence of adenine nucleotides lead to pore formation within the inner mitochondrial membrane(IMM) ultimately leading to cell death[174].

ii) The mitochondrial permeability transition pore

Alterations in the integrity of the mitochondria following incubation in isotonic solution containing phosphate and calcium have been observed since the 1950s[175]. The Ca^{2+} -dependent pore known to be integral to this change, results in an increase in permeability of the IMM with eventual swelling and disruption of mitochondrial function.

This unique property of the IMM was confirmed in 1979 and coined the "Ca^{2+} -induced transition"[176]. Three years prior to this study, while looking at the mechanism and function of calcium uptake, Hunter et al noted that in the presence of as little as 10nmol/mg of calcium, the IMM underwent a configurational transition followed by an increase in permeability to solutes. This effect which occurred in an all-or-nothing manner, was largely nonspecific (both neutral and charged molecules permeate) and was linked to a reversible disruption in respiration[177].

Hunter and colleagues looked specifically at how this transition could be controlled. They discovered that the increase in permeability of the IMM and subsequent swelling of the mitochondria could be inhibited by ADP, NADH and Mg^{2+} [178],as well as cations including H^{+} (low pH), strontium (Sr^{2+}),manganese (Mn^{2+}), ethylenediaminetetraacetic acid (EDTA) and lanthanum(La^{3+})[179;180]. The authors' own suggested mechanism for this phenomenon involved the binding of Ca^{2+} to units within the IMM in the absence of any endogenous inhibitory agent which then led to the opening of a transmembrane hydrophilic channel[181]. They deduced that the size of solutes permeable through this membrane would have a molecular weight no more than 1000Da[182].

Further confirmation of the reversibility of Ca^{2+} induced permeability came in 1986. Al Nasser and Crompton showed that after permeability had been induced by Ca^{2+} and phosphate ions in liver mitochondria, resealing could be brought about by ethylene glycol tetra-acetic acid (EGTA) in a biphasic fashion, with a rapid initial phase followed by a slow second phase[183]. Using the heart mitochondria, Crompton showed that the requirements for permeability included calcium and phosphate ions or calcium and hydroperoxide ions and estimated a pore diameter of about 2.2nm[184].

In summary, calcium regulation is clearly essential in cardiomyocyte contraction and has a vital role in cellular injury. Moreover, calcium accumulation is imperative for the formation of the mPTP particularly in the setting of ischaemia. However, it was the timely discovery of cyclosporin A and its possible role in cardioprotection that really catapulted our understanding of the components of the mPTP.

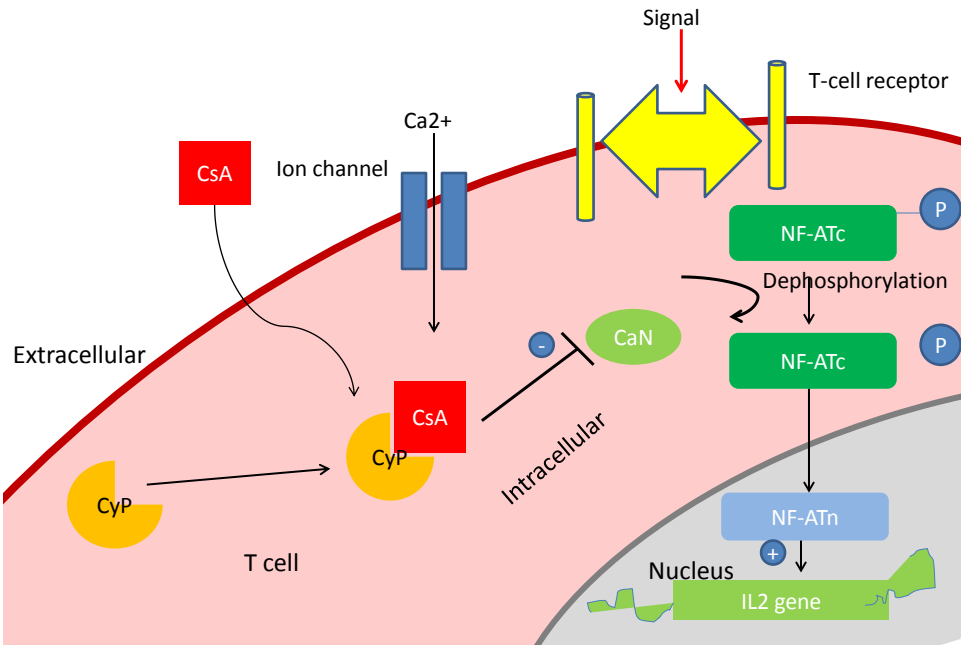
iii) A historical perspective of cyclosporin

First isolated from a Norwegian soil plant in the late 1960s[185], cyclosporin A is now widely known for both its immunosuppressive properties and as an anti-rejection agent[186]. The immunosuppressive effect of cyclosporin A was first discovered in 1972 by the pharmaceutical company Sandoz (now Novartis) and was approved for clinical use in 1983[185].

Cyclosporin A is a lipophilic cyclic peptide of 11 amino acids. It has a high affinity to a family of cytoplasmic proteins known as cyclophilins (CyPs) which are found in most cells. The cyclophilins share similar binding residues but play different roles in cell metabolism. CyP-A is located within the cytosol and participates in the translocation of apoptosis-inducing factor (AIF) in the nucleus and in protecting against O_2 stress. CyP-B resides within the endoplasmic reticulum and has a role in suppressing apoptosis associated with O_2 stress and altered Ca^{2+} metabolism.

When CsA binds to CyP-A, it forms a drug-protein complex which binds to and inhibits calcineurin (CaN), a calcium and calmodulin dependent phophatase[187-189]. This in turn inhibits the translocation of a family of transcription factors, NF-AT, leading to reduced transcriptional activation of early cytokine genes for IL2, tumour necrosis factor alpha (TNFα), IL3, IL4, CD40L, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma (IF-γ)[188-190]. In addition to the calcineurin/NF-AT pathway, cyclosporin A inhibits the activation of T-cell transcription factors AP-1 and NF-k B through blocking of JNK and p38 signalling pathways[191]. CyP-D resides within the mitochondrial matrix and has recently been confirmed as having a key role in ischaemia-reperfusion injury.

Figure 1.03: *The mechanism through which CsA enters the T cell- The lipophilic CsA molecule enters the cell by passive diffusion. It then forms a complex with the immunophilin, cyclophilin (CyP). The CsA-CyP complex binds to and inhibits the enzyme calcineurin (CaN), which has a serine/ threonine phosphatase activity. As a result, CaN fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc). The transport of NF-ATc into the nucleus and its subsequent binding to the nuclear component of the nuclear factor of activated T cells does not occur. As a result, T cells do not produce IL-2, which is necessary for full T cell activation. ref*



Key: CsA- cyclosporin A, CyP- cyclophilin, CaN- calcineurin, NF-ATc- nuclear factor of activated T cells (cycloplasmic component), NF-ATn- nuclear factor of activated T cells (nuclear component).

iv) Confirming the properties of the pore using CsA

The effect of cyclosporin A (CsA) on the mitochondria was first considered by Fournier in 1987. He showed that the fluxes of calcium were inhibited by CsA, promoting retention of accumulated calcium in the isolated mitochondria[192]. Crompton went on to investigate the effect of CsA on the Ca^{2+}- induced pore. He was able to demonstrate using isolated mitochondria from the female Sprague-Dawley rat heart, that CsA suppressed the instability of

the mitochondria in the presence of calcium and phosphate loading[193] and that this was due to its action on the pore.

The turn of the decade sparked a race for the determination of the components of the Ca^{2+}-induced pore. So far it had been understood that Ca^{2+} influx had a detrimental effect on the mitochondria and ultimately the cell.[194;195] The Ca^{2+}- induced pore opening was triggered when calcium levels reached a certain threshold[183];[196]and resulted in the non-specific permeability of the IMM. It was also clear from previous studies that injuries that involved calcium release were particularly troublesome due to their detrimental effects on mitochondrial energy transduction which tended to contribute to the progression of the injury[197-200]. It became apparent that by determining the components of the pore, one might be on the first steps to finding pharmacological targets and as a result prevent the occurrence of reperfusion injury.

Leading the way among others was Crompton, who in 1990 sought to understand the relationship between the Ca^{2+}-induced pore and reperfusion-induced injury. He showed that EGTA-induced resealing of the pore was stimulated by ADP. He reconfirmed the size of the pore (2.3nm) and determined that the pore was proteinaceous and not phospholipid based[201] as it was then thought. He concluded by hypothesising that the action of CsA must involve either directly binding to the pore or binding to a protein involved in pore opening[202].

The use of electrophysiological patch clamp experiments on the 'contact points' of the mitochondrial membrane led to the identification of channels in the IMM[203]. The channels were said to be of two types- low conductance and high conductance. Later, the high conductance channels, referred to as the mitochondrial mega-channel (MMC), were shown to:

- become activated by calcium ions;
- become inhibited by divalent cations;
- demonstrate competitive action between calcium and cyclosporin A;
- be modulated by pH levels;
- have similar kinetic behaviour as the permeability pore[204].

It was concluded that the structure considered as being the Ca^{2+}- induced pore was in fact the mitochondrial mega-channel which was later referred to as the *mitochondrial permeability transition pore (mPTP)*.

v) Determining the constituents of the pore using CsA

The exact structural components of the pore still remain an enigma. The voltage-dependent anion channel (VDAC), located within the outer mitochondrial membrane[205], was previously thought of as a key component of the mPTP as it seemed to exhibit similar electrophysiological properties. Crompton demonstrated in 1998 that CyP-D-glutathione S-transferase would bind to both VDAC and adenine nucleotide translocase (ANT) in solubilised heart mitochondria and that the VDAC-ANT-CyP-D complex acted as a calcium-dependent pore which was sensitive to CsA[193]. However, recent genetic manipulations have disputed this model. Baines et al was able to show that mitochondria lacking all the isoforms of VDAC showed similar pore opening capabilities as normal mitochondria. He showed that fibroblasts lacking all isoforms of VDAC were more susceptible to cell death induced by oxidative stress. It seemed therefore that the role of VDAC was more to promote survival rather than as a structural component of the mPTP[206].

Studies have shown that when the nucleotides ADP, ATP and dADP bind to ANT located in the inner membrane of the mitochondria, the pore opens[207]. ANT tends to act as a gated pore, alternating between two conformational states when substrates bind to the ADP/ATP binding sites. ANT can either present its binding site on the matrix side of the inner membrane (m-state) or on the cytoplasmic side (c-state). Ligands that bind while in the m-state inhibit the pore (bongkrekic acid) by decreasing its sensitivity to [Ca^{2+}] and those that bind while in the c-state activate it (atractylate) by sensitising the pore to [Ca^{2+}][208].

Recent knockout studies however, have disputed the role of ANT as being essential for pore opening. Rather, a regulatory role has been attributed to it, leaving the role of a *phosphate carrier* as the most definitive component to date[209;210](see below).

As with ANT, the possibility of the other component of the pore was considered after the effect of particular ligands. The involvement of cyclophilin D(CyP-D) was suggested after it was noted that the concentration of CsA needed to inhibit the pore was the same as that needed to inhibit the enzymatic activity of mitochondrial cyclophilin D[193;211;212]. CyP-D is a nuclear encoded mitochondrial isoform of the cyclophilins with a molecular mass of 18kDa[213]. The effect of CsA is mediated through the inhibition of the peptidyl-propyl cis-trans-isomerase (PPIase) activity of CyP-D[193;214;215]. Similar sensitivities of the pore and CyP-D have been shown with CsA analogues[216-218]. Sanglifehrin A, a non-immunosuppressive analogue of CsA has been shown to inhibit the pore without interacting with calcineurin[219]. Further evidence confirming the role of CyP-D as a component of the pore has been shown with the use of CyP-D knockout mice. Mitochondria isolated from the knock-out group demonstrated a low sensitivity to calcium

resulting in delayed mPTP opening which was subsequently insensitive to CsA[220-223]. CyP-D is thought to cause pore opening by facilitating the Ca^{2+}-induced conformational change[224].

It has been long recognised that pore opening is activated in the presence of high phosphate ions. However it was only recently that Leung and colleagues showed that CyP-D had an affinity for a phosphate carrier and that binding occurred in a CsA sensitive manner[225]. This interaction tended to increase under conditions of oxidative stress, which sensitised pore opening to Ca^{2+}. Further studies have presented the model of a phosphate carrier as a component of the pore by showing that pore inhibition through the blocking of CyP-D can only occur in the presence of phosphate ions[226].

vi) The relationship between mPTP opening and ischaemia-reperfusion injury

So far we can appreciate that mPTP opening, triggered by conditions of high Ca^{2+} ions, phosphate ions and oxidative stress in the absence of adenine nucleotides is detrimental to the cell[192]. We also know that after reperfusing an organ that had been exposed to a period of prolonged ischaemia, the presence of calcium, phosphate and oxidative stress play a major role in inducing subsequent injury. Poole-Wilson demonstrated that the influx of calcium occurred late during ischemia followed by a more rapid influx early in the reperfusion phase and that this was related to cell necrosis [227]. This rapid influx was triggered by the depletion of two-thirds of the cell's ATP reflecting failure of the calcium pumps both on the plasma membrane and on the sarcoplasmic reticulum. Intracellular acidification from increased lactate production led to further pump failure by increasing intracellular Na^+ (impaired Na^+/H^+ antiporter) and this in turn led to the impairment of the Na^+/Ca^{2+} carrier[228]. Cobbold et al noted that when the cytosolic

[Ca^{2+}] was below 1-2µM, cell viability was restored during reperfusion. However if this critical limit was exceeded during ischaemia, reperfusion would not restore calcium homeostasis and the cell would die. This finding suggested that Ca^{2+} overload and pore opening may be a precondition for this form of cell death[229].

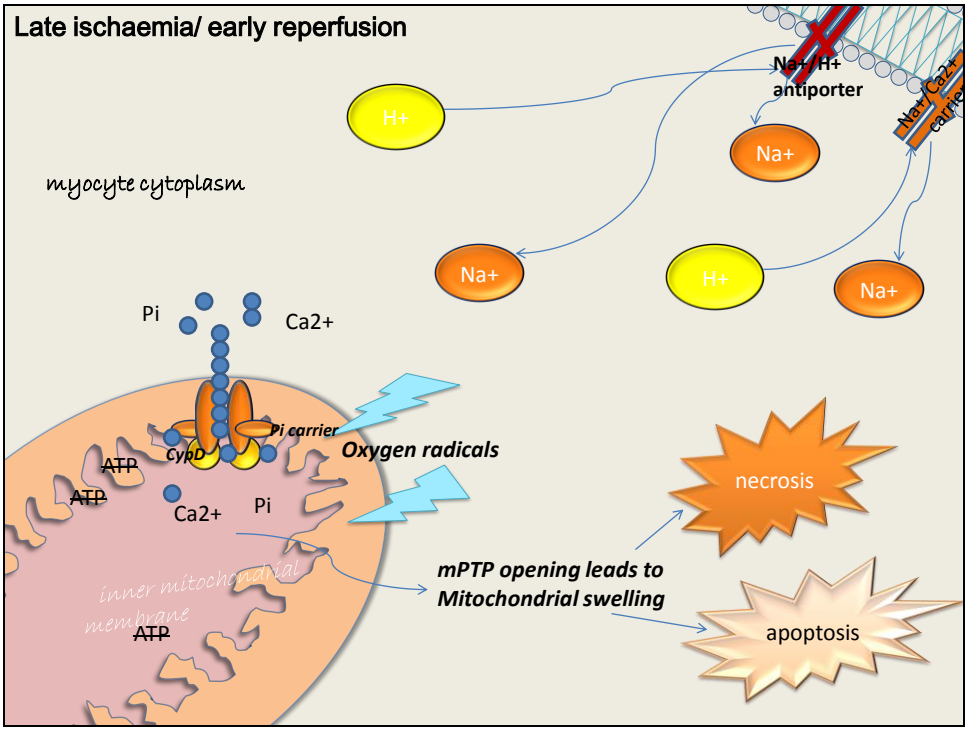
Reperfusion after a period of prolonged ischaemia gives rise to the production of oxygen species through a number of mechanisms. One such mechanism involves the enzyme xanthine dehydrogenase which is found in endothelial cells. This enzyme is converted to the oxidase form by a calcium-dependent protease[230]. During reperfusion, xanthine oxidase accumulates and reacts with hypoxanthine, a by-product of adenine nucleotide degradation, leading to the production of hydrogen peroxide[231]. Other sources of oxygen species include neutrophils, which are recruited to sites of ischaemia by the endothelial cell expression of surface adhesion molecules[232]. Platelets and mitochondria also produce superoxides during reperfusion which are converted to hydrogen peroxide by superoxide dismutase located in the intracellular and extracellular compartments[233;234].

While necrosis occurs after substantial ATP depletion, apoptosis is thought to be an energy-dependent process[235]. If a cell was to experience a partial ischaemic insult, some of the mPTPs may be open while others may remain closed with continued ATP production. Cell death is then directed down an apoptotic pathway[236]. As molecules of a low molecular weight enter the matrix, equilibration occurs with the solutes outside the IMM. Larger molecules remain within the matrix generating an osmotic gradient with subsequent mitochondrial swelling. The IMM has a larger surface area than the outer membrane so the outer membrane tends to rupture

leaving the IMM intact. Rupture of the outer membrane leads to the release of proapoptotic proteins into the cytoplasm, inducing apoptosis[237].

Cellular breakdown during apoptosis is governed by a complex array of proteins known as caspases. They are expressed as inactive proenzymes and become activated after proteolytic cleavage[238]. Liu et al was the first to discover that caspase activation required dATP and cytochrome c[239]. Cytochrome c binds to apoptotic-inducing factor forming a complex which in turn leads to the recruitment of further procaspases that present in their active form[240]. The role of the mitochondria in apoptosis was established after the confirmation that cytochrome c was part of the pathway. Further work has shown that cytochrome c translates into the cytosol from the intermembrane space a few hours into the apoptotic process[241].

***Figure 1.04**: The intra-cellular and mitochondrial changes that occur during late ischaemia/ early reperfusion. Impaired cell membrane receptors due to low pH result in influx of calcium and phosphate ions. The accumulation of ROS and the absence of ATP lead to the formation of the mPTP. Subsequent mitochondrial swelling leads to eventual cell death via necrosis and apoptosis.*



Key: H+- hydrogen ions, Na+- sodium ions, Pi- phosphate ions, ATP- adenosine triphosphate]

1.6. Novel cardioprotective strategies

1.6.1. Cyclosporin A as a cardioprotective agent

The discovery by Crompton and Broekemeier in the late 1980s of the capacity of CsA to inhibit the opening of the mPTP[184;242], led to a surge in interest that continued through the turn of the century. Halestrap and Davidson showed that the swelling of the heart mitochondria after exposure to Ca^{2+} was inhibited by both bongkrekic acid and CsA through their interaction with cyclophilin[243]. It then followed that analogues of CsA could also protect against IRI via a similar

mechanism although the effect was less pronounced[244]. Nazareth et al were able to show that by preserving the ATP/ADP ratio, CsA was able to reduce necrosis in ventricular myocytes, preserving cell viability[245]. Some years later Borutaite et al showed that by preventing mPTP opening during reperfusion, CsA prevented cytochrome c release from the mitochondria therefore preventing cell death via the apoptotic pathway[246].

In our laboratory, we confirmed that only by intervening in the first fifteen minutes of reperfusion was it possible to inhibit pore formation. Using isolated male Sprague-Dawley rat hearts, we showed that by giving sanglifehrin A (an agent pharmacologically similar to CsA that does not inhibit calcineurin) within the first fifteen minutes of reperfusion, it was possible to reduce the infarct risk ratio by as much as twenty percent[247]. The administration of SfA after fifteen minutes of reperfusion had no significant effect on infarct size[248].

As noted previously, the action of CsA has an immunosuppressive component so it is possible that its action could occur via calcineurin inhibition. Since FK506 is also a calcineurin inhibitor, studies were done looking at the effects of this agent against IRI. FK506 was found to be at best only marginally protective in this setting reconfirming that protection occurred via mPTP inhibition[249;250]. The use of NIM811, a non-immunosuppressive and more specific derivative of CsA further reaffirmed the cardioprotective properties as it was demonstrated to be even more effective than CsA [251].

Over the years other mechanisms for cardioprotection were postulated. Using H9c2 embryonic rat heart-derived cell lines, Huei-Wen et al examined the mechanism through which CsA regulated the cardiomyocytes against stress-induced apoptosis[252]. They first demonstrated that

cells exposed to hydrogen peroxide exhibited morphological signs of apoptosis (cell shrinkage, apoptotic body formation and DNA fragmentation) similar to those seen after exposure to hypoxia/ re-oxygenation injury[253]. They were able to show that pre-treatment using CsA at a concentration of 0.1μM provided protection against the effects of peroxide-induced apoptosis. They went on to postulate that the 'delayed protection' that is usually observed in preconditioning may be due to the expression of cardio-protective proteins namely, *heat-shock protein 70* (HSP-70) and *induced-nitric oxide synthase* (iNOS) which were observed in cells exposed to CsA[254].

The effect of reactive oxygen species in the context of CsA was regarded as a double-edged sword. At small doses, CsA produces a small amount of ROS which was cardio-protective; however at larger doses or in the presence of hydrogen peroxide, large amounts of ROS were generated promoting cell death[255]. Huei-Wen and colleagues were the first team to look at the cardio-protective effects of CsA in the context of its relationship with ROS as well as the first to consider the up-regulation of cardio-protective proteins and their role in delayed protection.

Other possible mechanisms shown to be responsible for cardioprotection included immune-modulation[256]; modulation of cardiac metabolism[257]; interaction with the mitoK_{ATP} channel[258] and direct, pleiotropic effect against metabolic, structural and stress signalling changes which resulted in cardioprotection[259].

It was not until 2004 that the cardio-protective effects of CsA were first demonstrated in the human cardiac muscle. Shanmuganathan et al looked at human atrial appendages taken from patient undergoing coronary artery bypass surgery[260]. Experiments were also carried out on

atrial myocytes isolated from the atrial appendages and subjected to IRI. Their aim was to see whether CsA and sanglifehrin, another known inhibitor of the mPTP, offered any cardio-protection and the mechanism by which protection occurred[261].

They were able to show significant improvement in the force of contraction of human appendages treated with both CsA and SfA compared with control. The treated group showed a higher percentage of cell survival along with a significant reduction in the percentage of necrotic cells. They also demonstrated that after laser-induced oxidative stress, the time taken for mPTP opening was significantly prolonged in cells exposed to CsA or SfA [262].

These findings were very significant at the time, as they further reaffirmed the mPTP as a viable target for cardio-protection. The study also went on to demonstrate not only that CsA was cardio-protective, but by using Sanglifehrin-A they showed that the benefits were not due to immuno-suppression but almost certainly due to inhibition of mPTP opening as SfA is a more specific inhibitor of mPTP and has no effect on calcineurin[263].

Over recent years, numerous experimental data have been published which have demonstrated as high as forty-percent reduction of infarct size through pharmacological manipulation of the mPTP by maintaining its closure during reperfusion. This has been supported by a landmark proof-of-concept clinical study conducted by Ovize et al. They looked at 58 patients from multiple centres who had all presented within 12 hours of an ST-elevation myocardial infarction[264]. Ten minutes before direct stenting, patients in the treatment group received 2.5mg/kg of Cyclosporin A intravenously while the control group received normal saline. They were able to demonstrate approximately a 40% reduction in infarct size in the CsA group,

shown through a reduction in the 'area under the curve' (AUC) for serum creatinine kinase[265]. A subgroup of 27 patients underwent cardiac MRI five days after their initial presentation. In the cyclosporin group, the absolute mass of the area of hyper-enhancement was significantly reduced- a reduction of 20%. This study was the first of its kind to suggest the occurrence of IRI in humans and has once again shown that cyclosporin A can offer protection against this phenomenon likely through its action on the mPTP[266].

Table 1.01*: A summary of studies investigating the cardioprotective effects of CsA.*

Source	Aim /intervention	Mechanism	Outcome
Crompton et al 1988[193]	Heart mitochondria; action of CsA on Ca^{2+} dependent pore.	Inhibition of Ca^{2+} dependent pore.	CsA potent inhibitor of pore opening.
Halestrap and Davidson 1990[267]	Heart and liver mitochondria; inc dose of CsA.	Preventing Ca^{2+}-induced pore formation.	CsA inhibited mitochondrial swelling.
Griffiths and Halestrap 1991[268]	IRI on isolated rat hearts; 0.2micro M CsA; G and H less effective	Prevention of interaction of ANT and matrix PPIase	CsA showed cardioprotection through improved LV developed pressure.
Nazareth et al 1991[269]	Ventricular cardiomyocytes from Sprague-Dawley rats. CsA 200nM	Limitation of decrease of ATP/ADP concentrations	Substantial reduction in necrosis.
Massoudy et al 1997[270]	IRI on isolated guinea pig hearts; 0.8micro M CsA	CsA elevates the level of nitric oxide thereby reducing O_2 stress.	CsA showed cardioprotection.
Halestrap et al 1997[271]	Isolated Langendoff hearts; CsA 0.2µM	mPTP inhibition	Greater recovery of LVDP and ATP/ADP ratios. Lower AMP levels and EDP. Analogues of CsA were also used and they too showed protection.

Weinbrenner et al 1998[272]	Isolated rabbit hearts; CsA and FK506	Inhibition of phosphatases and prolongation of phosphorylation state in ischaemic cells.	Reduction of infarct size particularly after administration prior to or 10min after ischaemic insult.
Squadrito et al 1999[273]	Rat hearts CsA 1mg/kg	Anti-inflammatory	Reduced myocardial necrosis, MPO and CPK activity, inc myocardial contractility.
Minners et al 2000[274]	Langendorff- perfused isolated rat hearts; CsA	Modulation of mitochondrial homeostasis.	DNP and CsA were protective. Trimetazidine attenuated preconditioning.
Griffiths et al 2000[275]	Isolated adult rat ventricular myocytes; 0.2 and 1microM CsA	Suggested mechanisms: low dose CsA via mPTP inhibition; high dose CsA via calcineurin inhibition.	Cell recovery and protection seen in CsA treated group.
Nieman et al 2002[276]	Male Sprague-Dawley rats; CsA 0-25mg/kg	Modulation of cardiac metabolism	CsA showed cardioprotection
Huei-Wen et al 2002[277]	H9c2 embryonic rat heart-derived cell lines; CsA 0.1μM	1. Reversal of collapse of mitochondrial membrane potential. 2. up-regulation of HSP-70 and iNOS.	CsA showed cardioprotection.
Hausenloy et al 2002[278]	Isolated rat hearts; IPC, 0.2 μmol/l CsA, FK506, atractyloside, diazoxide, atractyloside, CCPA	mPTP inhibition via activation of mitochondrial K(ATP) channel.	Reduction of infarct size seen in: CsA, IPC, diazoxide.
Hausenloy et al 2003[279]	Isolated perfused rat hearts; 1 μM SfA.	Inhibition of mPTP opening in the first 15min of reperfusion.	Limitation of infarct size and protection against O2 stress.
Borutaite et al 2003[280]	Langendorff-perfused rat hearts; CsA and FK506	Inhibition of mPTP prevents cytochrome c release.	CsA prevented ischaemic changes and thus apoptosis. FK506 not protective.
Schneider et al 2003[281]	Isolated atrial appendage; CsA 0.2microM/L and insulin 5mU/mL	CsA causes slight decrease in ATP production which inhibits mitoK_{ATP} (as mitoK_{ATP} is inhibited by ATP). Opening of mitoK_{ATP} is protective.	Both agents protective, insulin more so than CsA.
Ganote et al 2003[282]	CCCP ischaemia on Isolated rabbit	Reduction of mitochondrial swelling by	Cardioprotection- additive with both; swelling

	cardiomyocyte; CsA and IPC, NIM 811	pore inhibition.	reduced by CsA; NIM 811 also protective.
Hausenloy et al 2004[283]	Adult rat myocytes; 0.2µM CsA and 0.4 µM N-methyl-4-valine-CsA	Suppression of mPTP in the presence of O2 stress.	Promotes cell survival.
Bes et al 2005[284]	Rat cardiomyocytes; CsA (100 and 1000 ng/ml)	Direct, pleiotropic protection against metabolic, structural and stress signalling changes.	Preservation of mitochondrial function and cell viability.
Shanmuganathan et al 2005[285]	IRI on human atrial tissue; CsA 0.2micromol/l and SfA 1micromol/l	Inhibition of mPTP during reperfusion	Cardioprotective via improved survival.
M. Nathan et al 2005[286]	FK506 (0.2µM/l) vs CsA(0.2µM/l)- isolated perfused rat hearts.	mPTP inhibition	The inhibitory effects of calcinuerin were shown not to play a role.
Argaud et al 2005[287]	Male NZW rabbits. CsA vs NIM811.	mPTP inhibition	The inhibitory effects of calcinuerin were shown not to play a role.
Lim et al 2007[288]	Looking for role of mPTP in cardioprotection. Male/female wide type or CyP-D knockout mice; Diazoxide; CsA; IPC; SfA; bradykinin.	Inhibition of mPTP.	No cardioprotection in CyP-D knockout mice indicating that mPTP crucial for cardioprotection.
J.E. Xie et al 2007[289]	Male Sprague-Dawley rats (in vivo); CsA 10mg/kg.	Inhibition of mPTP.	Reduction in infarct size.
Fang et al 2008[290]	Is Post(con) protective against IRI; Sprague-Dawley rats; Post(con), IPC, CsA.	mPTP inhibition	All show Cardioprotection resulting in smaller infarct size.
Ovize et al 2008[291]	Patients presenting with STEMI undergoing PPCI up to 12hrs after onset of chest pain; CsA 2.5mg/kg.	mPTP inhibition	Significant reduction in CK-MB and infarct size.

Key: LVDP- left ventricular diastolic pressure, EDP- end-diastolic pressure,FK506- tarcrolimus, MPO-myeloperoxidase, CPK- creatine phosphokinase, DNP, HSP-70- heat shock protein-70, iNOS- induced nitric-oxide synthase, mitoK_{ATP}, IPC- ischaemic pre-conditioning, Post(con)- ischaemic post-conditioning, CK-MB- Creatinine kinase-MB.

1.6.2. Protecting major organs from ischaemia reperfusion injury using CsA

Liver transplantation remains the definitive mode of treatment for fulminant hepatic failure and end-stage liver disease[292]. This form of treatment however, is unpredictably predisposed to hepatic ischaemia which can ultimately result in tissue injury and graft dysfunction- an important cause of death in liver transplantation[293]. The process of liver donor storage (cold) followed by warm reperfusion can itself result in hepatic ischaemia and the protective effects of CsA have been thoroughly investigated within this setting with beneficial results[294]. The mechanism for hepatic injury rests on the production of oxygen free-radicals namely hydrogen peroxide and superoxides[295]. These tend to accumulate during the following conditions:

- Ischaemia- as aerobic metabolism is lost[296].
- Neutrophil infiltration- production of myeloperoxidase[297].
- Mitochondrial dysfunction- influx of calcium and reactive oxygen species production[298].

Oxygen free radicals cause cellular damage through the destruction of polyunsaturated fatty acids located within cell membranes via a process known as lipid peroxidation[299;300]. As a result, cellular disintegration ensues resulting in tissue injury and organ failure.

It is now accepted that the effects of IRI are widespread, affecting all the major systems, resulting in irreversible damage to tissues and ultimately organ dysfunction.

The concept of 'no flow' phenomenon[301] with reference to skin reconstructive surgery has been widely debated. It is thought that the slowing of blood flow during periods of ischaemia results

in ‘no flow’ within the microcirculation[302]. Although a complex phenomenon, prostaglandins and oxygen free radicals have been identified as playing a major role along with other inflammatory mediators, their effects accounting for significant morbidity after microvascular flap and reconstructive surgery[303].

Kucukcelebi and Ozcan designed a study to determine whether CsA could limit the detrimental effects of ischaemia-reperfusion injury in island skin flap in rats[304]. They found that 10mg/kg of CsA diluted in normal saline significantly improved the survival of skin flaps after being subjected to ischaemia[305]. This study was the first to demonstrate the protective effects of CsA against ‘no flow’ phenomenon and led the way for its administration in other forms of replantation surgery[306].

The effects of gut ischaemia have been implicated in the severe and life-threatening condition known as Necrotising Enterocolitis[307]. This debilitating gastrointestinal syndrome affects severely premature infants leading to sepsis, DIC, intestinal perforation, peritonitis and even death[308]. Puglisi et al sought to attenuate the inflammatory cellular changes associated with gut ischaemia using CsA and aimed to quantify their results using computerised morphometry[309]. They successfully showed that CsA played a role in reducing IRI in the gut with the preservation of mucosal cell function and a reduction in villous destruction[310].

The effects of IRI on the kidneys can manifest itself as acute renal failure or allograft failure in the case of renal transplantation[311]. Singh et al sought to determine whether CsA could offer any protection within this setting. After kidneys had been subjected to IRI they were able to show that at a dose of 3mg/kg, CsA could significantly improve renal function (improved urea

and creatinine clearance) and preserve kidney morphology (with a normal glomeruli and only slight oedema of tubular cells)[312].

The first report to demonstrate the protective effects of CsA against ischaemic reperfusion injury in the brain came in 1992. Shiga et al administered Male Wistar rats with CsA 15mg/kg/day orally for 5 days preceding the ischaemic insult which took the form of occlusion of the middle cerebral artery[313]. They were able to show a significant reduction in brain oedema in the CsA treated group along with a significant reduction in infarct size- with the effect more pronounced in the cerebral cortex than in the basal ganglia[314]. The mechanism proposed was that of immuno-suppression- the binding of CsA to cyclophilin A leading ultimately to the inhibition of T helper cells[315]. Since then other mechanisms have been proposed, including the inhibition of mPTP opening[316], the reduction of the efflux of free fatty acids[317] and the possible role of the activation of the *mitoK*$_{ATP}$ channel[318].

Table 1.02: *A summary of studies investigating the protective effects of CsA on other organs.*

Organ	Study	Aim	Intervention	Proposed mechanism	Outcome
Liver	**Yamanoi et al 1991[319].**	Adult mongrel dogs- to protect against IRI in liver.	CsA 10mg/ml; effects of allopurinol and methylprednisolone were also investigated.	Possibly by stimulating the proliferation of hepatocytes. Mechanism unclear.	Better survival rates and reduction in hepatic injury with CsA.
	Kim et al 1991[320].	Male pigs	10mg/kg CsA oral for 4 consecutive days	Inhibition of macrophages and prevention of intravascular coagulation.	Reduction of hepatic injury; improved survival.
	Goto et al 1990[321].	Male Wistar rats- to protect against IRI- rat liver transplant model used.	10mg/kg CsA for 3 days.	Inhibition of lipid peroxidation by free radicals and suppression of endothelial injury.	Improved survival.
	Kurokawa et al 1992[322].	Male Wistar rats-	Pre-treatment with 10mg/kg CsA iv;	Membrane stabiliser.	Improved recovery of mitochondrial function;
	Suzuki et al 1993[323].	Male Sprague-Dawley rats- protect against IRI in liver.	5mg/kg CsA; 0.3mg/kg FK506 also used.	Inhibition of neutrophil infiltration.	Reduction in liver injury and cell membrane damage (MDA levels). Improved survival.
	Konukoglu et al 1998[324].	Male Sprague-Dawley rats- to protect against IRI in liver.	CsA 25mg/kg; effect of ibuprofen also investigated.	Inhibition of cytokine production, mitochondrial dysfunction and neutrophil adhesion.	Improved survival.
	Travis et al 1998[325].	Mongrel pigs- protect against normothermic IRI in liver.	20mg/kg CsA	Anti-inflammatory- inhibit lymphocyte proliferation and decrease neutrophil migration.	Prevention of hepatic injury and speeding recovery.
	Mizuta et al 1999[326].	Male Wistar rats- protection against warm-ischaemic	CsA dissolved 5mg/mL oral; FTY720 was also investigated.	Inhibiting free radicals via suppression of neutrophil	Decrease in circulatory lymphocytes and PMNs; reduction in

		reperfusion.		migration.	hepatic damage;
	Ishii et al 1999[327].	Female Sprague-Dawley rats	10mg/kg CsA oral for 4 consecutive days	Modulation of TNF production.	Improved survival.
	Leducq et al 2000[328].	Perfused rat livers.	2µM/L CsA	Prevention of mPTP opening.	Prevention of mitochondrial dysfunction.
	Saxton et al 2002[329].	Male Sprague-Dawley rats	30mg/kg CsA oral	Reduction of neutrophil infiltration. Inhibiting the upregulation of Fas gene expression.	Decrease in necrosis; inhibition of apoptosis; decrease neutrophil infiltration.
	Plin et al 2004[330].	Male Wistar rats- to protect against CPWR.	1µMCsA	Inhibition of mPTP opening.	Partial protection against CPWR.
	Theruvath et al 2008[331].	To protect against IRI in rat liver transplantation	10mg/kg of NIM811; minocycline	Inhibition of mPTP opening.	Attenuation of graft injury and improvement in graft survival with both agents.
Brain	**Shiga et al 1992[332].**	Male Wistar rats- to prevent IRI by MCAO.	CsA 15mg/kg/day for 5days.	Immuno-suppression.	Reduction in brain oedema and infarct size.
	Friberg et al 1998[333].	Male Wistar rats- to prevent hypoglycaemic induced brain damage.	CsA 50mg/kg iv; FK506 2mg/kg iv.	Inhibition of mPTP opening.	Decreased hypoglycaemic brain damage.
	Li et al 2000[334].	Male Wistar rats- to prevent IRI in rat brain.	CsA 10mg/kg via carotid artery.	Immuno-suppression and mPTP inhibition.	Decreased cerebral damage and improved survival.
	Phillis et al 2002[335].	Sprague-Dawley rats- to prevent IRI in rat brain.	CsA 5µM (other immune-suppressants were also investigated).	Immuno-suppression and inhibition of mPTP opening led to reduction in efflux of FFAs.	Prevent neuronal injury.
	Santos et al 2003[336].	Adult male C57BL/6 and FVB/N mice- to prevent neuronal damage	CsA 5, 10, and 20mg/kg; FK506 0.5mg/kg.	Maintaining mitochondrial integrity	Protection of hippocampal neurons against excitotoxin cell death.

	Korde et al 2007[337].	Male Sprague-Dawley rats- IRI via CCA and MCA occlusion	NIM811 50mg/kg	Inhibition of mPTP opening.	40% protection against transient focal cerebral ischaemia
	Wu et al 2006[338].	Male Sprague-Dawley rats- to prevent IRI by MCAO.	CsA 0.5/1µmol/L; other agents were also used.	Inhibition of mPTP opening and possibly activation of mitoK ATP channel.	Reduction in brain damage and improved neurological score.
Skin	**Kucukcelebi et al 1992[339].**	Skin flap survival- Male Sprague-Dawley rats.	10mg/kg CsA.	Protection against no flow.	Improved survival of skin flaps.
	Askar et al 2002[340].	Cremaster muscle of male Sprague-Dawley rats.	CsA.	Inhibition of leukocyte infiltration.	Decrease in neutrophil infiltration; preservation of capillaries.
Gut	**Puglisi et al 1996[341].**	Sprague-Dawley rats- gut.	5mg/kg subcutaneous CsA.	Anti-inflammatory.	Preservation of mucosal structure and function.
	Puglisi et al 1996[342].	Sprague-Dawley rats- small bowel.	5mg/kg CsA and 2mg/kg rifampicin.	Anti-inflammatory.	Reduction of xanthine oxidase free radical leading to decrease cell membrane damage.
Kidney	**Zhu et al 2002[343].**	Sprague-Dawley rats- to prevent IRI.	CsA 1.5mg/kg stat followed by 1.5mg/kg/day for 7 days.	Inhibition of neutrophil infiltration and mPTP opening.	Renal protection.
	Singh et al 2005[344].	Male Wistar rats- to prevent IRI in vivo kidneys.	3mg/kg CsA.	Increased expression of HSP and inhibition of apoptotic pathway.	Renal protection.

Key: PMN- polymorphonuclear leukocytes, TNF- tumour necrosis factor, CPWR- cold-preservation warm reperfusion , IRI- ischaemia reperfusion injury, MCAO- middle cerebral artery occlusion, FFA- free fatty acids, CCA- common carotid artery, MCA- middle cerebral artery, HSP- heat shock protein.

1.6.3. The clinical limitations of cyclosporin A

Despite the existence of substantial evidence supporting the protective effects of CsA both in animal models and more recently in the clinical setting, its use is not without reservation. Studies have shown that CsA may either directly or indirectly cause cardiac toxicity. Owunwanne et al showed that after the subcutaneous injection of CsA in rats, there were histological evidence suggesting direct cellular injury to myocytes[345]. Hutcheson et al suggested that CsA induced cardiotoxicity indirectly through its inhibition of Ca^{2+} ATPase (a calmodulin-dependent enzyme) and nitric oxide synthase (NOS) in the rat myocardium. They went on to show that these effects could be reversed by fructose-1,6-diphosphate[346]. Some of the other undesirable effects associated with cyclosporin A including nephrotoxicity, anaphylaxis, immuno-suppression and hypertension, have been attributed to the vehicle[347] rather than the agent itself, and also to its affinity for other molecular targets including calcineurin.

These potential limitations have created a conundrum prompting researchers to develop an agent that could be more specific in its targeting action.

Crompton recently showed that the limitations of CsA are largely due to its interactions with the other cyclophilins. He confirmed that CyP-D played a major role in the energy failure of the cell and that by specifically targeting the mitochondria, the protective effects of CsA could be enhanced[348].

By allowing CsA to bind with triphenylphosphonium (TPP+), the complex was able to enter electrophoretically into the negatively charged inner mitochondrial membrane where it could

interact only with CyP-D[348]. Using hippocampal neurons from Sprague-Dawley rats, the team induced IRI through oxygen and glucose deprivation. They showed a 50% reduction in mortality using 0.8μM of mtCsA (CsA-TPP+). CsA in comparison was less effective than mtCsA as larger concentrations tended to obliterate protection[348].

Crompton concluded that the protective effects of CsA could only be fully understood by specifically targeting the mitochondria using mitochondrial-specific CsA (mtCsA)[348]. This concept is in part supported by previous studies using CsA analogues including NIM 811, sanglifehrin A, Debio 025 and SMB2-CsA all of which avoid the effects of calcineurin.

1.7. Cyclosporin A in cardiac bypass surgery

It has been my hope to convince the reader that CsA, an agent known for its profound immunosuppressive effects, may also harbour cardioprotective benefits by its action on the mitochondrial permeability transition pore through its inhibition of cyclophilin D. Numerous studies have demonstrated using animal models, its effect in attenuation myocardial injury, while others have shown similar protective effects in other major organs. The landmark study by Piot and colleagues was the first to demonstrate the presence of mPTP in humans as well as the potential of inhibiting the pore and bringing about protection[349]. Future efforts are directed at the development of agents that can target the pore specifically thereby avoiding unwanted side-effects. So far, it remains undetermined whether mPTP inhibition can be a target in patients undergoing cardiac bypass surgery and as such, this question formed the basis of my research hypothesis.

1.8. 'Conditioning' in cardiac bypass surgery

Despite advances in surgical techniques and anaesthetic management, CABG surgery continues to be associated with significant morbidity and mortality particularly in high risk patients [350]. In chapter 5, we explore the cardioprotective potential of ischaemic preconditioning (IPC), a potent endogenous strategy that starts with the activation of membrane receptors that subsequently stimulate a protective protein kinase cascade, ultimately finishing with the inhibition of the mPTP downstream.

The beneficial effects of ischaemic preconditioning in the setting of cardiac surgery have been substantiated in numerous proof-of-concept studies. Its effect on robust outcomes such as mortality has not been verified by larger studies for a number of reasons. Firstly, cardiac surgeons have been reluctant to apply this invasive cardioprotective strategy due to the inevitable prolongation of the duration of surgery. In addition, the number of cycles required to establish protection has never been clearly defined. Thirdly, the process of aortic clamping and de-clamping holds a substantial thrombo-embolic risk particularly in the elderly. Therefore, emergence of a concept that implements sub-lethal episodes of ischaemia to one vascular bed resulting in a protective benefit at a distant vascular bed was welcomed by many in the field.

1.8.1. Remote ischaemic conditioning

In 1993, Przyklenk and colleagues were the first to demonstrate the concept of remote ischaemic preconditioning in a proof-of-concept study using a canine model[351]. A preconditioning protocol of ischaemia and reperfusion was applied to the left circumflex artery 5 minutes prior to the occlusion of the left anterior descending artery (LAD) for one hour. They

showed a 35% reduction of infarct size in the LAD territory in the preconditioned group compared with the control[352].

This landmark study did not only re-ignite the field of cardioprotection but contributed to the underlying mechanism of preconditioning by excluding the role of collateral perfusion albeit a major determinant of infarct size[353].

Cardioprotection induced from distant organs has since been demonstrated with the preconditioning protocol applied from the kidney, the intestine, and the limb[354](Reviewed by Lim et al[355]). Birnbaum et al showed that transient limb ischaemia in rabbits brought about preconditioning of the heart[356]. They were able to demonstrate a 65% reduction of infarct size expressed as a ratio of area at risk ($p=0.0006$)[357]. The use of transient ischaemia of skeletal muscle to implement RIPC is now regarded as a potent cardioprotective strategy. Kharbanda and colleagues demonstrated a significant protective benefit in a porcine model against CPB-induced tissue injury[358]. The animals were subjected to 180 minutes of CPB, including 120 minutes of aortic cross-clamping, followed by reperfusion. RIPC was induced by 4 cycles of 5 minute ischaemia/ reperfusion prior to the institution of CPB. The results demonstrated a significant attenuation in experimental myocardial injury as well as a reduction in endothelial dysfunction in humans[359].

Schmidt and colleagues defined the concept of *remote perconditioning* after demonstrating using brief intermittent limb ischaemia, significant protection during an evolving myocardial infarction. They identified the role of ATP-dependent potassium channels as part of the underlying mechanism[360].

Andreka et al demonstrated that the use transient limb ischaemia applied after the induction of myocardial infarction can be protective. Defined as *remote ischaemic postconditioning*, this strategy has been shown to protect against reperfusion-induced persistent ventricular fibrillation[361].

With the classification of these protective strategies dependent on the timing of the brief ischaemic protocol, it is likely that a synergistic effect is possible if these modalities were used in combination with one another; perhaps an objective for future studies.

The underlying mechanism that underpins remote ischaemic preconditioning still remains unclear. Many have suggested that similarities are shared with IPC in terms of the role of autocoids such as adenosine[362] and nitric oxide[363], signal membrane receptors, proteins kinase cascades, ending with the involvement of the mitochondria via the mPTP and mitoK_{ATP} channels[364]. It still remains unanswered as to how the cardioprotective signal is conveyed from the preconditioned organ or tissue to the target organ. Recently, our institution demonstrated that both humoral and neural pathways were integral to this step in the mechanism[365]. Using anaesthetised C57BL/6 mice, the coronary ischaemia was applied by the ligation of the LAD followed by 120 minutes of reperfusion. RIPC was induced by 3 cycles of 5 minutes femoral artery occlusion/ reperfusion. Some study groups were subjected to femoral vein occlusion (humoral pathway), while other groups were subjected to femoral nerve resection and/ or sciatic nerve resection (neural pathway). While RIPC resulted in a reduction of infarct size compared with the control group, occlusion of the femoral vein completely abolished the effect of RIPC. Although the resection of both the femoral and sciatic nerves also completely

abolished the cardioprotective effects of RIPC, isolated resection of either the femoral nerve or the sciatic nerve only partially abolished the cardioprotective effect. This study confirmed that both pathways were required to limit infarct size, however the way in which these two pathways interplay still remains unclear[366]. We can be reasonably certain that the occlusion of the femoral vein acted to prevent washout of cardioprotective factors from the preconditioned limb. The blood-borne carriage of cardioprotective factors is supported by numerous studies. In one example, Dickson et al showed that blood from a preconditioned rabbit can reduce infarct size in a non-treated rabbit[367]. Most recently, Konstantinov et al showed that RIPC induced in a recipient pig could reduce the infarct size of the denervated donor heart[368]. Furthermore, a study by Shimizu et al identified the humoral factors to be hydrophilic and less than 15kDa[369].

With regards to the neurogenic pathway, we know that RIPC can be abolished by pre-treatment with the ganglionic blocker hexamethonium[370]. It seems that adenosine[371] or bradykinin[372] activate a local neural pathway within the remote organ or tissue, with the need for an intact peripheral nervous system being imperative for the delivery of complete cardioprotection.

Three inter-related events have subsequently been identified:

1. The generation of endogenous autocoids at the remote organ triggered by the RIPC stimuli.
2. Conveying the blood-borne cardioprotection signal from the remote organ to the heart with the subsequent activation of neural pathway(s) mediating the cardioprotective effect.

3. A series of events occurring at the target organ to confer protection.

Despite numerous attempts in clarifying the mechanism underlying RIPC, what can be stated with some confidence is that gene expression, leukocyte activation and K_{ATP} channels have a crucial role in the protection induced by brief ischaemia (Reviewed by Saxena et al[373]).

The process of preconditioning the heart using brief ischaemia and reperfusion in the limb was characterised as a non-invasive procedure in human volunteers by MacAllister's research group[374]. Since then, preliminary studies using RIPC have been encouraging albeit with rather small sample sizes. The first of such trials, studied eight male patients undergoing CABG[375]. With the treatment group receiving brief episodes of right upper limb ischaemia, they found inconclusive results with respect to cardiac enzymes, which is likely due to the small sample size[376].

The first randomised controlled trial carried out by Cheung and colleagues studied 37 infants who underwent repair of various congenital defects. With a longer cross-clamp time compared with the previous study, they demonstrated a significant reduction in troponin I and a reduction in inotrope requirement[377]. The following year, using three cycles of 5 minutes ischaemia and 5min reperfusion, our group was able to show that the adult myocardium was also amenable to protection. A 43% reduction in troponin T was observed in the RIPC group after intermittent cross-clamp fibrillation[378]. Following this, we showed that a similar level of protection was possible after cold/blood cardioplegia with the same protocol[379]. Our group is currently looking at diabetic patients undergoing cardiac surgery to assess their predilection for cardioprotection after the application of RIPC.

Other organ systems have been studied and results in favour of protection have been consistent. Using a more invasive protocol of clamping the common iliac artery, Ali and colleagues were able to demonstrate a 27% reduction in myocardial injury in the setting of abdominal aortic aneurysm[380]. So far, the potential of RIPC seems vast with ongoing trials looking at its reno-protective and neuro-protective effects.

Most recently, Thielman et al provided more evidence in support of RIPC in patients undergoing cardiac surgery in the setting of crystalloid cardioplegia[381]. Some may argue that this was to be expected considering that crystalloid cardioplegia has been shown to be less effective as a cardioplegic agent compare with blood due to its deficient oxygen-carrying capability. However, this was disputed by Rahman and colleagues who found RIPC induced by 3 cycles of 5 minute I/R to be of no benefit in patients who underwent elective coronary artery bypass surgery via cold-blood cardioplegia[382].

What is ultimately required however is a large randomised-controlled trial looking at harder end-points such as post-operative death and peri-operative myocardial infarction. Our institution currently embarking on an EME approved multi-centre double-blind randomised controlled clinical trial, investigating the effect of RIPC on clinical outcomes in high-risk patients undergoing CABG surgery. The study titled: 'The Effect of Remote Ischaemic Conditioning on clinical outcomes in Coronary Artery bypass graft Surgery' or ERICCA study aims to determine whether RIPC, a virtually cost-free, non-invasive, non-pharmacological strategy, could improve health outcomes in high-risk patients undergoing CABG +/- concomitant valve surgery. 1610 high-risk patients (additive Euro-SCORE of 6 or more) undergoing CABG +/- valve surgery will be

recruited from 12 UK hospitals and randomly allocated to receive either RIPC or control. It is proposed that major adverse cardiac and cerebral events at one year (MACCE-death, MI, revascularisation, stroke) would be the primary outcome measured. The secondary outcomes measured would include: peri-operative myocardial and renal injury; length of ITU/ hospital stay and inotrope score; 6 minute walk test; quality of life analysis and cost-effectiveness analysis. A sub-study analysis of LV systolic function would be carried out using echocardiography.

Table 1.03*: A summary of studies investigating the cardioprotective effects of RIPC in human cardiac surgery.*

Study	**No.**	**Intervention (RIPC)**	**Myocardial preservation**	**Mean aortic cross-clamp time (RIPC/control) in minutes**	**Outcomes**
Gunaydin et al 2000[383]	8 male patients	Two cycles (3min ischaemia, 2min reperfusion)	CABG	37.8/28.5	Reduction in LDH levels.
Cheung et al 2006[384]-first application in humans	37 infants	Four 5min cycles of upper limb ischaemia	Repair of congenital defects- blood cardioplegia	55/59	Significant reduction in troponin I; less inotrope requirement and significantly less airway resistance.
Hausenloy et al 2007[385]	57 patients	Three episodes (5min ischaemia, 5min reperfusion)	CABG- ICCF	Control 45; RIPC 36	cTnT reduction of 43%.
Ali et al 2007[386]	82 patients	Two cycles (10min ischaemia, 10min reperfusion)- clamping of common iliac artery.	AAA		27% reduction in myocardial injury.

Venugopal et al 2009[387]	45 patients	Three episodes (5min ischaemia, 5min reperfusion)	CABG- cold blood cardioplegia	Control 65; RIPC 53	Absolute cTnT reduction by 42%.
Zhou et al 2010[388]	60 infants	Two episodes (24hr and 1hr preop)- three 5min cycles of L upper limb- for early and late phase protection.	VSD repair- cardioplegia	24.13/24.17	Significantly lower cardiac enzyme release; no difference in EF; less inotrope need
Thielmann et al 2010[389]	53 patients	3 x 5min cycles of L upper arm ischaemia.	CABG- standard crystalloid cardioplegia		Peak post-op cTnI concentrations significantly reduced.
Rahman et al 2010[390]	162 patients	3 x 5min cycles of L upper arm ischaemia.	CABG- blood cardioplegia	80 RIPC/ 82 control	RIPC did not reduce troponin release, improve haemodynamics, or enhance lung or renal protection.

Key: CABG- coronary artery bypass graft surgery, LDH- lactate dehydrogenase, cTnT- cardiac troponin T, ICCF- intermittent cross-clamp fibrillation, AAA- abdominal aortic aneurysm, RIPC- remote ischaemic preconditioning, VSD- ventricular septal defect.

Over the last four decades cardioprotection in the setting of cardiac surgery has steadily progressed particularly with the improvement of general anaesthesia. However, as society evolves and demographics change, cardiac surgery is destined to become even more challenging with an inevitable increase in peri-operative morbidity and mortality despite pre-existing strategies of cardioprotection.

It is likely that the future of cardioprotection will lie not only in our understanding of cellular and mitochondrial pathways of protection but also in the discovery of viable pharmacological targets which present minimum risk to patient safety peri-operatively. This along with the

support of large randomised controlled trials is likely to revolutionise cardioprotection for the 21st century.

1.9. Indicators of myocardial injury in cardiac surgery

Since its conception in 1997, troponins have enjoyed widespread use as detectors of myocardial injury and infarction in the setting of acute coronary syndromes (ACS). There higher specificity and sensitivity for myocardial injury compared with their pre-existing counterparts myoglobin and creatine kinase MB, have more recently highlighted the seriousness of this injury in the setting of cardiac surgery. However, it is the correlation of elevated troponins with mortality that has geared our research team into studying novel ways and strategies in which this injury could be attenuated.

Assessing the effectiveness of cardioprotective strategies deployed in cardiac surgery requires accurate and reliable documentation of myocardial injury. Clearly the gold standard for measuring outcomes is the documentation of mortality and/ or the occurrence of peri-operative myocardial infarction. The incidence of peri-operative mortality in CABG surgery has been documented at 1.9% rising to between 5-7% in complex cardiac surgery (CABG+ valve or redo CABG)[391]. The incidence of post-operative myocardial infarction has been recorded at about 2% in most studies. These variables being relatively low would therefore require large studies that would supply sufficient power for reliable comparisons to be made and conclusions drawn.

Haemodynamic parameters for assessing myocardial function such as pulmonary artery balloon catheterisation and nuclear vetriculography do give reproducible results. However the invasive

nature of the procedure carries a morbidity risk which renders it unusable in the clinical or research setting[392].

1.9.1. Serum biochemical markers

The serum biochemical markers can be divided into 3 groups: enzymatic, non-enzymatic cytoplasmic, non-enzymatic non-cytoplasmic[393]. The main limitation with regards to serum biomarkers lie with their non-specific release from both skeletal muscle and the myocardium during cardiac surgery[394].

i) Myoglobin

Myoglobins are low molecular weight cytoplasmic proteins present in cardiac and skeletal muscle. They show a rapid rise in plasma concentrations during myocardial injury as a result of cellular membrane disruption and so give valuable information on the timing of injury. Its specificity is improved when measured within the coronary sinus and when simultaneously measured with carbonic anhydrase III[395]. While skeletal muscle injury shows a rise in both proteins, myocardial injury is associated predominantly with myoglobin release[396]**.** The measurement of myoglobin in the setting of cardiac surgery is usually made in conjunction with other cardiac biomarkers when detecting myocardial injury.

ii) Fatty-acid-binding protein

Fatty-acid-binding proteins (FABPs) are early markers of myocardial injury and serum and urine levels tend to be elevated in myocardial infarction. They are rapidly excreted and therefore require frequent early sampling and rapid assays[397].

iii) Creatinine kinase (CK)

Creatinine kinase plays a role in transferring high energy phosphate from ATP to form creatine phosphate[398]. It is not excreted in the urine and therefore not affected by renal impairment. Its isoenzymes are defined by the presence of subunits M and B; MM found predominantly in striated muscle, BB in the brain and MB in the heart[399]. The isoforms MB1 and MB2 have been identified and seem to offer a more precise diagnosis of myocardial injury[400]. However due to the technical difficulties involved in the electrophoretic analysis, their use is limited in the research and clinical setting.

iv) Troponin

Troponins are regulatory proteins that are located within striated muscle. Within the healthy cardiac muscle, they are bound to the contractile apparatus while some exist within the cytoplasm, allowing for very low plasma concentrations. In injury, Troponins are released in a biphasic manner with cytoplasmic release occurring after 3-5 hours due to loss of membrane integrity. This is then followed by a later phase after 5 days corresponding to the destruction of the contractile apparatus and cell death[401].

Of all the cardiac enzymes, Troponins are the most sensitive and although some cross-reacting exists between myocardial and skeletal Troponin T, modern monoclonal assays are able to reduce this to less that 1%. Troponin I has been shown to be more sensitive that Troponin T with the added advantage of maintaining its levels in renal impairment[402].

(For more on troponin see *Section 1.10 Troponin release as a predictor of mortality*).

v) Glycogen-6-phosphorylase

Glycogen-6-phosphorylase is crucial for glycogenolysis and exists as 3 isoenzymes BB, LL, and MM. During myocardial ischaemia, glycogenolysis increases significantly, resulting in the release of G6P-BB into the circulation[403]. Early studies by Muir and colleagues demonstrated its sensitivity for myocardial injury but like Troponin T, it is less reliable in the setting of renal impairment or cerebral injury[404].

1.9.2. Imaging findings

i) Echocardiography

The advancement of imaging techniques used to assess cardiac structure and physiology has led to the discovery of diagnostic modalities that have proven to be both sensitive and specific for myocardial injury.

Tissue Doppler imaging measures the Doppler shift frequencies produced by the contraction and relaxation of longitudinal muscle fibres running from the atrio-ventricular annuli to the apex[405]. This allows for the quantification of both systolic and diastolic function. Vassalos and co-workers were able to demonstrate in paediatric cardiac surgery that a reduction in post-operative right ventricular velocities was associated with myocardial injury defined by an increase in troponin I release[406]. A reduction in pre- and post-operative left ventricular velocities was associated with longer ventilation times and longer hospital stays. Incidentally, they were able to show that because the damage due to trauma was minimal, the main cause

for troponin release had to be ischaemia-reperfusion injury due to prolonged aortic cross-clamp time. They concluded that ischaemia-reperfusion injury was the main contributor to reduced contractility with the right ventricle being the most vulnerable anatomically[407].

ii) Cardiac MRI

It is widely acknowledged that off-pump cardiac surgery offers less myocardial injury compared with cardioplegic cardiac surgery[408]. However Selvanayagam and colleagues aimed to show that this reduction in injury, previously show using biochemical markers, also translated to a reduction in myocardial stunning and irreversible myocardial damage[409]. Using cine MRI to measure LV function and contrast-enhanced MRI to identify irreversible myocardial injury, they were able to show a 6% reduction in ejection fraction after cardioplegic surgery compared with off-pump surgery. They postulated that the lower end-systolic volumes seen in off-pump surgery resulted in an improvement in left-ventricular function[410]. Contrast-enhanced MRI showed no difference between the two groups with respect to myocardial injury. They also showed proportionality between aortic cross-clamp times and changes in LV volumes suggesting that longer cross-clamp times resulted in an increase in myocardial stunning[411].

1.10. Troponin release as a predictor of mortality

The consequences of peri-operative myocardial infarction (PMI) have been widely documented due to the undeniable impact on morbidity and mortality[412]. Despite its high incidence (6.4% was recorded from the Coronary Artery Surgery trial[413]) PMI still presents a diagnostic challenge to surgeons and clinicians alike. The ECG and echocardiogram carry limited usefulness in the clinical setting as they invariably fail to identify subtle degrees of myocardial injury which may

indeed carry prognostic significance. The emergence of cardiac biomarkers made considerable in-roads in addressing this conundrum. Having previously explored the mechanisms of myocardial injury during cardiac surgery, we can now appreciate that irrespective of the underlying mechanism, elevations in cardiac biomarker release hold strong prognostic value in predicting length of hospital stays[414] as well as short and long-term adverse outcomes[415].

Myocardial damage is a continuous phenomenon that occurs universally in all cardiac surgery[416]. A threshold therefore is needed above which an elevation in cardiac biomarkers can be said to indicate prognostically significant damage. Using a cohort of 3,812 patients undergoing CABG, Brener and colleagues showed that an elevation of CK-MB greater than 10 times the upper limit of laboratory normal (ULN), was a positive predictor of mortality[417]. This result substantiated the outcomes of preceding data from the GUARDIAN[418] and ARTS[419] studied which had shown a similar correlation between elevations of CK-MB and both 6-month and one-year mortality respectively.

The past decade saw the development of commercially available assays for detecting cardiac troponin which has since revolutionised cardiovascular diagnostics. Cardiac troponin isoforms (cTnI, cTnT, TnC) are proteins belonging to the thin filament regulatory system of the contractile complex. Troponin T and I are highly sensitive and specific for cardiac muscle (never expressed in skeletal muscle) making them appropriate markers for the detection of myocardial injury. Troponin C is less useful clinically as it is also expressed in smooth muscle cells (reviewed by Baker et al[420]). In 2007, the ESC/ACC Joint Task Force redefined PMI, suggesting a cut-off at five times the upper limit for laboratory normal (ULN) of biomarkers (preferably troponin) within

the first 72 hours following CABG, when associated with the appearance of new pathological Q-waves or new left bundle branch block (LBBB), or angiographically documented new graft or native coronary artery occlusion, or image evidence of new loss of viable myocardium was sufficient to make a diagnosis (type 5 MI)[421]. This new definition was not without its objections, as it remained unproven and lacked validation in the cardiac surgical population. Nonetheless, what is gaining wide consensus is that elevations in cardiac troponin strongly predict poor outcomes after cardiac surgery.

Eigel and colleagues were the first to look at cardiac troponin as a prognostic marker of adverse outcomes. In a study of 540 adult patients, they were able to show that a cut-off at cTnI > 0.495ng/L was a strong predictor of adverse outcomes with measurements taken after CPB and prior to sternum closure[422].

Troponins are preferred over CK-MB for the clinical detection of myocardial injury peri-operatively as they offer greater accuracy and a higher sensitivity. This was verified most recently by Muehlschlegel and colleagues after the retrospective analysis of data collected on 545 adult patients who underwent CABG surgery. They were able to show that cTnI was indeed the strongest predictor of 5-year mortality when compared with other diagnostic modalities including the ECG and CK-MB[423].

Over the past decade, researchers have consistently demonstrated an association with elevated troponins I[424-427] and T[428;429] with poor in-hospital outcomes. Many have carried out medium to long-term follow-up studies showing that the predictive value of troponin was still validated[430-

[432]. However, others have contested these findings, suggesting that CK-MB may be much better at predicting long-term outcomes[433].

In paediatric cardiac surgery, Mildh and colleagues were the first to demonstrate a relationship between cardiac troponin T and patient survival. In a study of 1001 children undergoing corrective congenital cardiac surgery, they showed that a 24-hour post-operative troponin T > 5.9µg/L was a powerful predictor of mortality[434].

The emergence of a highly sensitive assay for detecting cardiac troponin is set to lead to changes in our understanding of the pathophysiology of coronary artery disease and how we interpret elevations in the clinical setting. The highly sensitive assays are 1000-10,000-fold more sensitive than the original first generation assays (Singulex high sensitivity cTnI) and hold the potential of improving diagnostic certainty of acute coronary syndromes at time points earlier than 10-12hrs. In addition, they allow for early testing, thereby expediting treatment in NSTEMI or discharge from 'Medical Admissions Units' due to their high negative predictive value[435].

In a recent sub-study of patients taken from the PEACE trial, Omland and colleagues showed that elevated levels of highly sensitive cardiac troponin T (hsTnT) were detectable in patients with stable coronary artery disease and preserved LV systolic function[436]. This elevation was shown to have prognostic significance as it was associated with an increase risk of heart failure and cardiovascular death. A rise in troponin level is thought to occur as a result of clinically silent ischaemic episodes and small vessel occlusions; inflammatory processes; cardiomyocyte

apoptosis; reduced renal clearance; and increased myocardial strain due to pressure or volume overload[437].

Despite its promising benefits in acute coronary syndromes, uncertainties remain regarding the use of high sensitivity assays in cardiac surgery. The use of a 99th percentile healthy population cut-off as the ULN reduces its specificity for MI diagnosis but does increase the sensitivity of myocardial necrosis through which future research in myocardial protection will certainly benefit.

Table 1.04*: A summary of studies investigating the prognostic value of cardiac biomarkers in the setting of cardiac surgery.*

Study	Patient number	Surgery	Cardiac enzyme	Outcome
Brener et al 2002[438]	3812 adult patients	CABG	CK-MB	CK-MB elevation x10 ULN was independent predictor of mortality.
Eigel et al 2001[439]	540 adult patients	CABG	Troponin I (after CPB and before sternum closure)	cTnI > 0.495ng/L was optimal cut-off for predicting adverse outcomes
Lasocki et al 2002[424]	502 adult patients	CABG or valve	Troponin I	cTn I >13ng/ml independent predictor of in-hospital mortality.
Fellahi et al 2003[430]	202 adult patients	CABG	Troponin I (peak post-op value)	Trop I >13ng/ml associated with increased risk of 2-year mortality.
Katherisan S. et al 2004[440]	136 adult patients	CABG	Troponin T (18-24hr post-op)	TnT > 1.58ng/ml strong predictor of 1-year mortality rate
Lehrke et al 2004[441]	204 adult patients	CABG	Troponin T	Cardiac troponin T >/=0.46ug/L at 48hrs is associated with 4.9-fold increase risk of mortality.
Paparella et al 2005[442]	230 adult patients	CABG	Troponin I (peak post-op value)	Tn I > 13ng/l independent predictor of in-hospital mortality. Tn I did not predict outcome at 2 years.
Bottio et al 2006[443]	520 adult patients	Correction of congenital heart disease	Troponin I (peak value)	cTnI >35µg/L lost its prognostic significance at 12 months.
Mildh et al 2006[444]	1001 children	Paediatric cardiac surgery	Troponin T (24-hr post-op)	Tn T > 5.9µg/L was a powerful predictor of death.
Fellahi et al 2008[426]	184 adult patients	CABG or AVR	Troponin I	In measuring serial Tn I release compared with a

				single 24-hr measurement- both equally good at predicting in-hospital outcome.
Buse et al 2009[445]	741 adult patients	CABG	Troponin T (day 1 and day 2 post-op)	Troponin T > 0.1µg/L positive predictor of 12-month mortality.
Nesher et al 2008[446]	1918 adult patients	CABG, valve, CABG+valve	Troponin T (peak levels in 24hr)	cTn T >0.8µg/L associated with increased MACE.
Muehlschlegel et al 2009 (retrospective analysis from prospectively collected data)[447]**.**	545 adult patients	CABG	Troponin I	Compared with ECG and CK-MB, cTnI was strongest predictor of 5 year mortality.
Mohammed et al 2009[448]	847 adult patients		Troponin T (in 24hr post-op)	A linear association demonstrated between cTnT and length of stay and ventilator hours; cTnT independently prognostic for death, death or HF, death or need for vasopressor and the composite of all 3.
Van Geene et al 2010[449]	938 adult patients	CABG or valve	Troponin I (at 1 hour)	cTn I > 4.25nl/L as optimal cut-off for predicting in-hospital mortality.

Despite the benefits gained from cardiac bypass surgery, post-operative mortality rates remain high particularly in high-risk patients undergoing more complex procedures. Therefore, the need for novel cardioprotective measures has never been more paramount in the clinical setting. In the next chapter, I set out the hypothesis and objectives which form the basis of this thesis were I look at the role of mPTP inhibition as well as the benefits of targeting the RISK pathway through remote ischaemic preconditioning.

3.6. Risk stratification in cardiac surgery

3.6.1. EuroSCORE

European system for cardiac operative risk evaluation (EuroSCORE) was developed in 1995 and first published in 1999 to provide a simple additive risk model to cardiac surgery[450]. Its use has since evolved into a decision-making tool to identify 'high-risk patients' and determine mortality. It has subsequently gained wide acceptance across Europe, Asia and Africa.

Two models exist- the additive (initial model now thought to underestimate risk) and the logistic (based on logistic regression) risk stratification models[451]. It is the logistic model that is increasingly used to justify therapeutic decision-making. However because of the methodological limitations of this model, its statistical accuracy has been brought into question when used for individual predications.

Some studies have suggested that the logistic EuroSCORE overestimates surgical risk[452-454] and should therefore be used with caution in the decision-making process.

The score focuses on the complexity of the procedure, the patient's age, and their co-morbidities. It does not take into account pre-operative medical management, improved surgical tools, advancements in peri-operative and post-operative care as the score is built on retrospective data.

It has therefore been suggested that the clinical status of the patient must still form the crucial basis for medical decision-making.

Table 3.01: *EuroSCORE. Reproduced from Nashef et al*[455]

CHAPTER 2

2. Targeting mechanistic pathways for improving cardioprotection in cardiac surgery- Hypothesis, aims and objectives

2.1. Introduction

Coronary artery bypass surgery provides an important option for revascularisation therapy particularly for the severest form of coronary artery disease. This however, is not without subjecting the heart to a considerable amount of injury, particularly during more complex procedures where the aortic-cross clamp times are prolonged.

Cyclosporin A is a pharmacological agent that acts directly to inhibit the mitochondrial permeability transition pore. Researchers have investigated the cardioprotective effects of CsA in the setting of ST elevation myocardial infarction with promising results albeit in a small sample size[456]. Cardiac surgery is an ideal model of ischaemia-reperfusion injury and provides a setting in which the cardioprotective potential of CsA has yet to be studied.

Remote ischaemic preconditioning triggers a powerful endogenous cardioprotective cascade and its potential has already been demonstrated clinically by a number of research institutions including our own. However, the majority of the evidence is extrapolated from proof-of-concept studies and a larger recent study by Rahman and colleagues, failed to show any statistically significant cardioprotection[457]. It is also worth stating at this point that the number of cycles and cycle-duration for the optimum preconditioning stimulus protocol has yet to be confirmed. In addition, whether the cardioprotective benefits are more pronounced in more complex cardiac surgeries has yet to be demonstrated.

2.2. Hypothesis (study 1)

CsA reduces myocardial injury and hastens post-operative recovery in patients undergoing cardiac surgery by inhibiting the formation of the mitochondrial permeability transition pore.

2.2.1. Overall aim (study 1)

- To study the effect of cyclosporin A on myocardial injury in adult patients undergoing coronary artery bypass surgery with or without concomitant valve repair or replacement (with blood cardioplegia).
- To investigate the effect of cyclosporin A on the immediate recovery of other major organs post-operatively.

2.2.2. Objectives (study 1)

- To administer CsA to randomised patients undergoing elective coronary artery bypass graft surgery with or without valve repair or replacement and study its effect on myocardial injury.

- To study the effect of CsA on outcomes after elective cardiac surgery-

  Short term outcomes:

- Duration of ventilation support
- Duration of ITU stay
- Inotrope score

- Serum creatinine over the first three postoperative days
- Incidence of atrial fibrillation.

2.3. Hypothesis (study 2)

Remote Ischaemic Preconditioning reduces myocardial injury in patients undergoing complex cardiac surgery (redo CABG or CABG+ aortic valve surgery).

2.3.1 Overall aim (study 2)

- To study the effect of Remote Ischaemic Preconditioning on myocardial injury in patients undergoing complex cardiac surgery (redo CABG or CABG + aortic valve surgery).

2.3.2 Objectives (study 2)

- To study the effect of remote ischaemic preconditioning on myocardial injury in patients undergoing elective complex cardiac surgery using intermittent cold blood cardioplegia as the technique for myocardial preservation.

CHAPTER 3

3. Enhancing Cardioprotection in the setting of Cardiac Surgery- Study methodology

To elucidate the cardioprotective effects of CsA and RIPC within the clinical setting of cardiac surgery, it was thought that a multi-centre randomised-controlled, single-blinded trial would be the most appropriate and logistically feasible study-design that would best answer the research question.

During the process of carrying out this research project, numerous departmental restrictions and logistical obstacles had to be overcome in the quest to acquire sufficient and tangiable data that could subsequently be analysed.

This chapter highlights the study methology as well as some of the logistical and practical challenges encountered. The process of obtaining the sample size and power calculation is also discussed here.

3.2. Ethical approval and informed consent

The research project was constructed in accordance with the International Conference on Harmonisation- Good Clinical Practice (ICH-GCP) guidance. Research ethics was sought from, and successfully approved by the joint University College London (UCL) / University College London Hospitals (UCLH) Committees (now known as London Bentham based at Royal Free Hospital). The application form for ethical approval was initially provided by NHS COREC (Central Office for the Research Ethics Committees) which has now integrated with the National Research Ethics Service (NRES), affiliated to the National Patient Safety Agency. Since the initial

protocol was written the format for ethical approval application is now entirely done on-line on the Integrated Research Approval System form and this method was used for recruiting other centres as the study expanded into a multi-centre trial. In addition to the study protocol, the patient information sheet, consent form and letter to the patient's general practitioner were also subject to approval by the ethical committee.

After obtaining ethical approval, a separate application was made to the research and development department within UCLH who also acted as a sponsor for the study. During the research period, amendments to the protocol, consent form and information sheet were made by myself. The new versions were clearly numbered and dated. The documents were then annotated and categorised as major or minor, and subsequently submitted to the ethical committee.

3.3. Patient selection

There were two approaches through which patients were recruited for the research project. Some patients were recruited during the pre-admissions clinic. Patients would be requested via letter to come to the clinic two weeks prior to the date of their elective surgery. They would then be shown a short video of the final steps leading to surgery as well as the processes involved during post-operative recovery. After the video, I delivered a short presentation of my research project and supplemented this by handing out a shortened version of the information sheet containing details of the study. Patients were then taken on a tour around the hospital's intensive care unit to allow them to familiarise themselves with the hospital's surroundings. On their return, those who wished to participate in the study and deemed eligible, were given the

full version of the information sheet. Having read the full version, those still happy to proceed were consented for the research study. They received a copy of the signed consent form with a further copy attached to their clinical notes. The original copy was kept for our records. On the morning of the surgery, patients who had been recruited were approach again to ensure that they were still happy to participate in the study.

The majority of patients were recruited twenty-four hours prior to their elective operation at the time of their admission into hospital. After scrutinising their clinical notes, those deemed eligible were approached and given a relatively detailed account of what the study involved including the benefits of participating as well as the potential risks involved. They were then given as much time as needed to consider their inclusion into the study. Those willing to participate in the research study were then consented in the appropriate manner.

3.4. Anaesthetic procedure

The pre-anaesthetic protocol was standardised across the two study centres. Patients were pre-medicated with temazepam (10-20mg) one hour prior to surgery. They would then receive midazolam intravenously while in the anaesthetic room. This was then followed by the insertion of an arterial cannula for invasive BP monitoring. A large-bore cannula was also inserted for the infusion of normal saline solution.

Anaesthesia was then induced with Fentanyl (5-15 μg/kg), followed by Etomidate (causes less haemodynamic instability) or Propofol and this was consistent at both centres. It is important to emphasise at this stage that the type of muscle relaxant used at the two centres were different. The choice of muscle relaxant adopted within the anaesthetic protocol was left to

the anaesthetist's discretion. The anaesthetists at the Heart Hospital used rocuronium as their preferred muscle relaxant while the anaesthetic team at King's College Hospital chose atracurinum as their preferred agent- the differences between the two agents is further expanded upon in chapter 4.

At both centres, the trachea was then intubated and mechanical ventilation started with oxygen +/- air. Anaesthesia was maintained using either a halogenated anaesthetic such as Isoflurane or Sevoflurane or with infusion of Propofol administered by target controlled infusion to achieve a target plasma concentration of 3 to 8 μg/ml. Midazolam, Fentanyl and a neuromuscular blocking agent were given as required. Arterial BP, central venous pressure, ECG and nasopharyngeal temperature were recorded at set intervals. The trans-oesophageal echocardiogram was used particularly in the setting of valve surgery to ensure a well-seated prosthetic valve and a gross assessment of LV systolic function.

3.5. Surgical procedure

3.5.1. CABG

The patients selected for CABG surgery tended to have one of the following: left main stem disease, multi-vessel disease, double-vessel disease with proximal left anterior descending artery involvement with or without angina, myocardial infarction and LV dysfunction.

Coronary arteries with greater than 70% stenosis were usually bypassed as graft patency is compromised if native vessels compete for flow. The angiographic criteria play a significant role in decision-making and surgery remains the treatment of choice particularly when success from percutaneous coronary intervention (PCI) is difficult.

A short median sternotomy exposes the ascending aorta and aortic valve following which standard cannulation techniques are commenced.

The proximal anastomosis of the free bypass conduits was performed at the ascending aorta before or after the distal anastomosis while the aorta was cross-clamped. A partial occlusion clamp was most commonly used but a single clamp technique avoids additional aortic manipulation and the risk of neurological injury. However this technique does pose an ischaemic injury risk due to a longer ischaemic time.

Prior to commencing the distal anastomosis, visual inspection of the epicardium was performed were the target native arteries were examined and a strategy for the sequence of anastomosis was formulated in the hope of reducing cross-clamp time.

Overall UK mortality of isolated CABG is 1.5%[458]. Usage of bilateral internal thoracic artery is associated with significantly better survival than single internal thoracic artery, with a lesser need for re-operation or angioplasty.

3.5.2. Mitral valve replacement

Mitral valve replacement was indicated in the event of symptoms, thrombo-embolic episodes, endocarditis, poor pulmonary haemodynamics or a depression in myocardial function. Mitral valve replacement is not generally as satisfactory as a good repair procedure and the possibility of a repair is generally considered.

Anaesthetic technique and monitoring was as standard for all cardiac surgery.

As previously mentioned, the trans-oesophageal echocardiogram (TOE) is an important adjunct to mitral valve surgery. It is used to guide repair procedures, confirms unsuitability for repair (in cases of calcific masses or severe chordal fusion), and confirm normal function after replacement.

A vertical sternotomy was the commonest approach used as it allowed optical access to the aorta for de-airing and defibrillation to take place.

The cannulation for cardiopulmonary bypass (CPB) occurred before the manipulation of the heart to avoid cardiac instability and dislodging of an atrial thrombus. Separate cannulae were placed in the inferior and superior vena cava to allow access to the left atrium. CPB was established with a moderate hypothermia of 32^0C.

Following cross-clamping of the ascending aorta, cold blood cardioplegia was delivered via a Medicut cannula into the aorta.

The left atrium was then opened (by an incision close to the right superior pulmonary vein) at the start of cardioplegic administration. The incision continued superiorly toward the left atrial roof, and inferiorly in front of the inferior pulmonary vein and behind the inferior vena cava.

The mitral valve was then exposed using a Cosgrove/ Cooley retractor. It is at this point that the possibility of a mitral repair is considered.

Resection of the valve was made at the junction of the anterior leaflet and atrial floor; the posterior leaflet was left in place and any calcified or fused chordate was removed. Any excess tissue was resected along with any loose chordate which could interfere with the mechanical

valve. Resection of the chordate was done just above the insertion into the papillary muscle avoiding transection of muscle itself.

Anchoring and placement of prosthesis was achieved following a series of complex suturing techniques after which the atrium was closed. De-airing of the heart was aided by ventilating the lungs which encouraged left atrial venous return. Once de-airing was complete the aortic cross-clamp was released and regular left ventricular contractions were established.

3.5.3. Aortic valve replacement

After cannulation of the ascending aorta and the right atrium, CPB was instituted and the patient cooled to 30^0C. After achieving electro-chemical standstill with cold blood cardioplegia, the aorta was opened. The stenotic or degenerative valve was then removed and decalcification of the annulus was performed. A surgical sponge was placed within the ventricle to capture debris which is unavoidable at this stage.

Careful and diligent work is done to prevent detachment of the aorta from the fibrous skeleton of the heart. Calcification which can extend to the outflow tract and the anterior mitral leaflet was removed while avoiding penetration of the Bundle of His.

Sizing of the annulus was then undertaken followed by complex suturing and implantation of prosthesis.

After the prosthesis was securely implanted, warm retrograde cardioplegia (hot shot) was started, and the aortotomy was closed.

With the heart de-aired and ejecting, the function of the prosthesis was checked by trans-oesophageal echocardiography (TOE) to detect any technical problems including paravalvular leaks.

3.7. Serum Troponin-T measurement

Blood samples for the measurement of troponin-T were taken pre-operatively and at 6, 12, 24, 48 and 72 hours following surgery. Troponin-T was measured quantitatively by a one-step enzyme immunoassay based on electrochemiluminescence technology (Elecsys 2010; Roche). The lower detection limit of this assay was 0.01µg/L with a recommended diagnostic range of 0.03-0.09µg/L indicating possible myocardial injury and a threshold of >0.1µg/L indicating myocardial injury suggestive of myocardial infarction.

The Elecsys TnT assay employs two monoclonal antibodies specifically directed against human cardiac troponin T. The antibodies specifically recognise two epitopes (amino acid position 125-131 and 136-147) located in the central part of the cardiac troponin T protein, which consists of 288 amino acids. The quatitative measurement of TnT is done using the 'sandwich principle'. In the first incubation phase, 50 µL of blood sample together with a biotinylated monoclonal cTnT-specific antibody and a monoclonal cTnT-specific antibody labelled with a ruthenium complex react to form a sandwich complex. Streptavidin-coated microparticles are then added to the complex during the second incubation phase. The reaction mixture is then aspirated into a measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. A voltage is then applied to

the electrode to induce a chemiluminescent emission which is measured by a photomultiplier. Results are then determined using a calibration curve provided via the reagent barcode.

The area under the curve indicative of absolute troponin release over 72 hours was calculated as follows:

$$AUC_{t1\text{-}t2} = [(cTnT \text{ at } t1 \text{ hours} + cTnT \text{ at } t2 \text{ hours})/2] \times (t2\text{-}t1)$$

$$AUC_{72\ hours} = AUC_{0\text{-}6} + AUC_{6\text{-}12} + AUC_{12\text{-}24} + AUC_{24\text{-}48} + AUC_{48\text{-}72}$$

Troponin-T analysis could not be carried out at King's College Hospital as their biochemistry laboratory only performed Troponin-I. An arrangement was set up with the biochemistry laboratory at St Thomas' Hospital, London for blood samples to be centrifuged and the serum separated for CK-MB analysis at King's laboratory and a separate batch frozen (-80^{0}C) and transported to the biochemistry department at St Thomas' Hospital for Troponin-T analysis.

3.8. CK-MB measurement

CK-MB was measured using the VITROS ECi/ECiQ Immunodiagnostic Systems and this technology was available at both centres. An immunometric immunoassay technique was used, which involves the simultaneous reaction of CK-MB present in a 40 µL blood sample with a biotinylated antibody and a horseradish peroxidise (HRP)-labelled antibody conjugate. The antigen-antibody complex is captured by streptavidin (a protein purified from the bacterium Streptomyces avidinii) and unbound materials are removed by washing.

The bound HRP conjugate is measured by a luminescent reaction. A reagent containing luminogenic substrates and an electron transfer agent are then added. The HRP in the bound conjugate catalyses the oxidation of the luminal derivative, producing light. The electron transfer agent increases the level of light produced and prolongs its emission. The light signals are interpreted by the system. The amount of HRP conjugate bound is directly proportional to the concentration of the CK-MB present.

The normal range of the assay was 0-3 ng/ml.

3.9. Statistical analysis

Standard statistical methods were used for analysis. The data was assessed in both groups to check for normal distribution. Categorical variables were assessed for differences using the Chi-squared test. The significance was interpreted at the 95% confidence interval prior to which any inequality of variance between groups was corrected for. The data was analysed using the SPSS statistical software (PASW) version 18.

4.2. Power and sample size calculation

This clinical study was designed such that our primary end-point (Troponin T AUC over 72 hours) would be acquired after comparing independent control and experimental subjects with approximately 1 control per experimental subject. In previous studies conducted within our institution, Troponin T AUC over 72 hours was normally distributed with standard deviation 25µg/l[459]. As the true difference in total serum troponin T release over 72 hours between the experimental and control means was 15µg/l, we calculated that we will need to study 45

experimental subjects and 45 control subjects to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of the null hypothesis is 0.05. The study design has been graphically represented in the figure below.

***Figure 4.01**: The reciprocal relationship between the experimental sample size and the difference in population means is shown by the graph below.*

5.3. Power and sample size calculation

The study was designed such that the continuous response variable (Troponin T AUC over 72 hours) would be acquired from the analysis of an independent control and experimental subjects with approximately 1 control per experimental subject. In a previous study the response within each subject group was normally distributed with standard deviation 25µg/l. If

the true difference in total serum troponin T release over 72 hours between the experimental and control means is 15µg/l, we will need to study 45 experimental subjects and 45 control subjects to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of the null hypothesis is 0.05. The study design has been graphically represented in the figure below.

***Figure 5.02**: The reciprocal relationship between the experimental sample size required and the difference in population means is shown by the graph below.*

CHAPTER 4

4. Cyclosporin A reduces Myocardial Injury in Patients undergoing Coronary Artery Bypass Surgery and Valve Replacement Surgery

4.1. Introduction

Coronary artery disease (CAD) is estimated to cost the UK economy £ 7.9 billion a year through direct health care costs, public education and productivity loses. Despite this, the yearly mortality stands at 94,000 making CAD the most common cause of death in the UK[460].

We already know that infarct size is the most common cause of mortality and so it seems logical that limiting infarct size should form the basis of strategies directed at improving outcomes. Currently the mainstay of treatment for acute myocardial infarction is to achieve reperfusion through coronary angioplasty or thrombolytic therapy, with CABG surgery reserved for treating the severest form of the disease. However, the evidence for the detrimental effects of reperfusion including lethal reperfusion injury is now extensive, with the opening of the mitochondrial permeability transition pore playing a major role. It is this that prompted Piot and colleagues to look at whether the administration of cyclosporin A at the onset of reperfusion reduced infarct size in patients with ongoing acute myocardial infarction[461]. Their study was the first of its kind to look at the cardioprotective effects of CsA in the clinical setting of primary percutaneous coronary intervention.

In this study, we aimed to determine whether CsA could induce similar cardioprotective effects on adult patients undergoing elective coronary artery bypass surgery with or without concomitant valve replacement or repair.

4.3. Overview of methods

During the initial stages of the study, the rate of recruitment was slow and so it was decided to expand the project into a multi-centre study. It was felt that this expansion would allow us to reach the sample size target within the time allocated allowing us to make statistical inferences when analysing the data.

Discussions were held with the research team at Kings College Hospital NHS Foundation Trust, another leading cardiac centre that was in the process of embarking on a similar study. Upon agreement on a multi-centre randomised controlled trial, I began the process of gaining ethical approval, adding another research centre and drafting a major amendment request.

After successfully gaining ethical approval with UCL acting as sponsor, attention was focused on the logistics of managing a multi-centre study.

4.3.1. Logistics of multi-centre study

Patient recruitment was conducted via a similar approach as previously mentioned.

(See chapter 3). Patients were either consented two weeks prior to their elective cardiac surgery in the pre-admissions clinic or consented twenty-four hours prior to their surgery. Patients who were deemed eligible for the study were approached by the principal investigator at Kings College Hospital on the day before their surgery. I was then informed by email of the eligible patients who had formally consented to participate in the study along with their operation times.

4.3.2. Patient recruitment

Consecutive adult patients admitted for elective cardiac surgery with or without concomitant valve replacement or repair, were recruited from October 2008 to September 2010. Recruitment was carried out simultaneously at the Heart Hospital (UCL) and Kings College Hospital, two major cardiac centres in London as part of a multi-centre project.

Patients with moderate to severe renal impairment were excluded because CsA has been known to worsen renal function. It is also known that the excretion of Troponins is impaired in these patients potentially distorting the accuracy of the results.

Patients who had suffered an acute myocardial infarction (MI) were excluded due to the elevated Troponins seen for up to two weeks post MI. It would be difficult to ascertain whether an elevation in Troponins was due to the MI or as a result of the injury caused by the surgery itself.

It has been documented that the cardioprotective effects of ischaemic preconditioning is not seen in the elderly but no evidence exists suggesting that mPTP inhibition does not occur in this patient group. It was therefore decided that all patients over 18 years and considered fit for cardiac surgery were to be recruited. Ischaemic heart disease affects a predominantly elderly population and it is this group that could potentially benefits from a pharmacological cardioprotective agent.

i) Inclusion criteria for cyclosporin A

- All adult cardiac surgery- CABG, valve surgery, redo operations.

- Male and female.
- All ages provided medically fit for cardiac surgery.
- Informed consent.

ii) Exclusion criteria for cyclosporin A

- Moderate to severe renal impairment- eGFR< 45 ml/min/1.73sqm.
- Cirrhotic liver disease.
- Immuno-compromised conditions eg. Retroviral disease, malignancy, chemotherapy.
- Recent infective endocarditis (surgery < 4weeks of diagnosis).
- Uncontrolled hypertension (systolic >160mmHg).
- Recent myocardial infarction (STEMI or NSTEMI within four weeks before the surgery).
- Angina within 3 days of elective surgery.

4.3.3. Anaesthetic protocol

The anaesthetic protocols used at the Heart Hospital and at Kings College Hospital were similar as that previously describe in chapter 3. The main difference was in the type of muscle relaxing agent used in the two centres.

Rocuronium is the muscle-relaxing agent of choice used at the Heart Hospital. It has been widely available in Europe since 1994 and continues to be a popular amino-steroidal non-depolarising neuromuscular blocking agent[462]. It popularity stems from its rapid onset time,

minimal cardiovascular effects with no significant histamine release making bronchospasm an extremely uncommon occurrence. This has led many anaesthetists to prefer rocuronium compared to other neuromuscular blocking agents[463]. However, there are a number of case reports and articles that have highlighted an increase in incidence of both anaphylaxis and anaphylactoid reactions after the use of rocuronium; some suggesting that the rise is in proportion to the use of the drug. What remained unclear was how a potential adverse event could impact on the research project.

Atracurium was the main neuromuscular blocking agent used at Kings College Hospital. It is a short-acting relaxant which is rapidly metabolized by the body. It produces few direct circulatory effects, but the absence of vagal blocking activity exposes patients to bradycardias during anaesthesia[464]. Histamine release is triggered at higher doses but rarely causes bronchospasm at standard doses[465].

4.3.4. Administration of CsA

Cyclosporin A was administered at a dose of 2.5mg/kg less than 10 minutes before direct stenting in Piot's study[466]. This dose was chosen after observations from experimental data demonstrated that 2.5mg/kg of CsA was able to reduce infarct size and did not result in any significant haemodynamic effect[467]. Being the first and only clinical trial to use CsA in the setting of myocardial injury, Piot's study served as an important source of evidence in allowing us to gain approval from our hospital pharmacy before placing orders for the drug.

CsA has been used for many years as an immuno-suppressive agent. Its long term use is associated with potentially adverse effects including renal and hepatic toxicity, predisposition to infections and malignancy. Acute administration has been linked with anaphylactic reactions but these events are deemed very rare, occurring at a rate of 1 in 1900.

On the morning of the operation, CsA was prepared in the treatment room according to the patient's weight. A dose of 2.5mg/kg was diluted in 100mls of normal saline fifteen minutes prior to induction and stored in the fridge away from direct light.

Patients were randomised at this point using a computerised sequence generator with the control group labelled (A) and the treatment group labelled (B). This information was known only to the investigator (the author) as the surgical and anaesthetic teams remained blind to the allocation protocol.

The bag containing the treatment drug was labelled (B) and the bag containing 0.9% normal saline (control) was labelled (A). No one else was made aware of this labelling structure. Immediately after induction, the pre-prepared agent was attached to a drip in the patient's left forearm and allowed to infuse over 30 minutes. During the infusion, the patient's vital signs were carefully monitored along with any skin changes which may indicate an adverse reaction.

After the infusion was complete, the line was flushed with normal saline and the patient was transferred into theatre to be prepped for surgery.

4.3.5. Summary of administration protocol for study

- The control group (A) received 100mls of normal saline which was also infused over 30 minutes.
- The treatment group (B) received 2.5mg/kg of CsA diluted in 100mls of normal saline. The prepared drug was infused immediately after induction over 30 minutes.

4.3.6. Secondary end-points

The protective effects of CsA have been shown to extend beyond the myocardium to include other major organs such as the brain[468], liver[327;469-471], kidneys[472;473] and gut[474;475], albeit in animal studies. Other studies have demonstrated the direct pleiotropic protection exerted by CsA on isolated cardiomyocytes[476] which along with mPTP inhibition could serve to reduce inadequate preload, impaired ventricular function and the subsequent excessive use of inotropes.

The trauma of cardiac surgery predisposes the heart to both atrial and ventricular arrhythmias. Atrial fibrillation (AF) can affect normal atrio-ventricular synchrony and result in a 15-20% reduction in cardiac output post-operatively. It is possible that the cardioprotective effects of CsA could reduce the incidence of post-operative AF by limiting the inflammatory response and subsequent necrosis and scar formation.

Acute renal failure occurs in up to 30% of patients post cardiac surgery, when defined as a 50% increase in serum creatinine concentration above baseline[477]. One to five percent of patients require dialysis. A prospective cohort study documented a 30-day mortality of 64% compared

to 4% in patients with normal renal function[478]. Given the reno-protective effects of CsA in previous animal studies it is possible that CsA could have an impact on our treated cohort.

A series of parameters that are routinely monitored post-operatively were diligently recorded on day 0, day 1, day 2, and day 3:

- Inotrope use (the duration, drugs and dosages were recorded).
- Atrial fibrillation (paroxysmal or persistent).
- Hourly urine output during post-operative day one.
- Daily serum Creatinine measurements over the first 72 hours.
- Duration of stay in the intensive therapy unit- ITU (defined as the interval from the time of admission of the patient from the operating theatre to the ITU up to the time a decision was made by the supervising intensivist or surgical team to transfer the patient to the high dependency unit or the ward).
- Duration of ventilation.

Secondary outcomes measurements were carried out prospectively. As mentioned earlier, patients who developed post-operative renal impairment were excluded from the analysis of myocardial injury as troponin T release is overestimated in these patients due to impaired excretion of the protein. However these patients were included for the purpose of measuring secondary outcomes.

i) Calculation of inotrope score

The inotrope score provides an objective measurement of the requirement of inotropes in the immediate post-operative period. This was adapted from Ko et al[479] and was calculated using the maximum inotropic dose administered during the first post-operative day.

Inotrope score = Dosages *(in µg/kg/min)* of Dopamine + Dobutamine + [(Adrenaline + Noradrenaline + Isoproterenol) x 100] + [Enoxinome x 15]

ii) Measurement of Acute Kidney Injury (AKI)

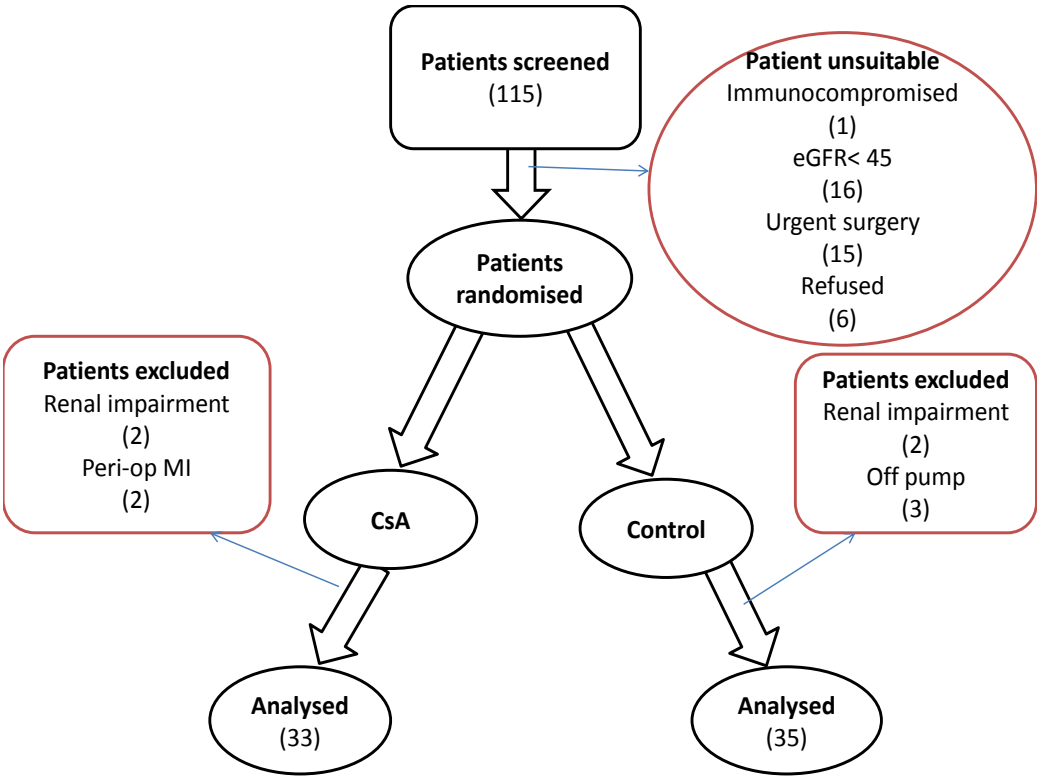
The Acute Kidney Injury Network (AKIN) recently proposed a new definition and classification of renal injury which could be used as a uniform standard for patient management as well as for clinical and translational research[480]. This has been validated in the intensive care setting and correlated with outcomes in patients admitted to the intensive care unit[481;482]. In our patients, the AKIN criteria[480] were used to define peri-operative AKI 1, 2 and 3 over the first 3 post-operative days (with the first 24-48 hours in ITU and the following 24 hours in the High Dependency Unit- HDU).

4.4. Results

A total of 115 patients admitted for elective adult cardiac surgery at both sites were assessed for inclusion into the study. Fifteen patients required urgent coronary artery bypass surgery after suffering an acute myocardial infarction; 16 patients had renal impairment with an eGFR<45 on admission- as a result, all were unsuitable for recruitment. One patient had a retroviral illness in her past medical history and as such, was deemed unsuitable for consent. Six patients refused to take part in the study.

Of the 77 patients randomised, 4 suffered from renal impairment after the operation; three patients were done off-pump and 2 patients met the criteria for peri-operative myocardial infarction. A total of 68 patients went through to the end of the study and a schematic representation can be seen below.

Figure 4.02: *Schematic of patient screening and recruitment* ***change diagram 72 pts***



Key: eGFR- estimated glomerular filtration rate, MI- myocardial infarction, CsA- cyclosporin A.

Table 4.01: *Baseline characteristics and patient profile.*

DEMOGRAPICS	**Control**	**Cyclosporin A**
Age	67+/- 11.1	70+/- 8.1
Male **Female**	24 (34.8%) 11 (15.9%)	27 (39.1%) 7 (10.1%)
Hypercholesterolemia	19 (27.5%)	18 (26.1%)
Hypertension	23 (33.3%)	20 (29.0%)
Diabetes mellitus	7 (10.1%)	7 (10.1%)
Previous MI	10 (14.5%)	11 (15.9%)
Previous stroke	4 (5.8%)	4 (5.8%)
Peripheral vascular disease	2 (2.9%)	0
Smoking history ***Current smoker*** ***Ex smokers*** ***Never smoked***	 2 (2.9%) 18 (26.1%) 15 (21.7%)	 2 (2.9%) 14 (20.3%) 18 (26.1%)
Family history of IHD	9 (13.0%)	13 (18.8%)
Euro SCORE	2.3	2.8

Drug History	Control	Cyclosporin A
Anti-platelet	25 (36.2%)	25 (36.2%)
Anticoagulants	2 (2.9%)	5 (7.2%)
Beta-blockers	21 (30.4%)	18 (26.1%)
Ca-channel blockers	10 (14.5%)	4 (5.8%)
ACE inhibitor/ATIIRBs	22 (31.9%)	23 (33.3%)
Diuretics	10 (14.5%)	5 (7.2%)
Nitrates LA	5 (7.2%)	4 (5.8%)
Amiodarone	1 (1.4%)	2 (2.9%)
Statins	29 (42.0%)	26 (37.7%)
Oral hypogylcaemics	3 (4.3%)	4 (5.8%)
Insulin	3 (4.3%)	2 (2.9%)

Table 4.01 *continued* **Key:** MI- myocardial infarction, IHD- ischaemic heart disease, ACE- angiotensin converting enzyme, ATIIRB- angiotensin 2 receptor blocker, LA- long-acting.

4.4.1. Baseline characteristics- patient profile

The baseline characteristics of the patients are summarised in table 4.01 above. There were no significant differences between the control group and the CsA treated group. The mean EuroSCORE of patients in the CsA sub-study was 2.3 in the control group and 2.8 in the CsA group which puts the patients in a low risk category.

4.4.2. Baseline characteristics- intra-operative variables

The intra-operative variables of the two groups are summarised in table 4.02. The average cross-clamp time in the CsA treated group is 7 minutes longer than that in the control group (66+/-30 vs 59+/-22) but this difference was not significant. This is likely to be due to one patient in the CsA treated group undergoing CABG with aortic and mitral valve replacements.

Table 4.02: *Baseline characteristics- intra-operative variables*

	Control	**Cyclosporin A**	**p value**
Aortic Cross clamp time (min)- mean +/- SD	59 +/- 22	66 +/- 30	
Bypass time (min) Mean +/- SD	88 +/- 44	99 +/- 34	
Type of surgery			0.599
CABG	19 (54.3%)	16 (47.1%)	
AVR	5 (14.3%)	9 (26.5%)	
CABG+AVR	4 (11.4%)	5 (14.7%)	
REDO OP	1 (2.9%)	1 (2.9%)	
MVR	3 (8.6%)	1 (2.9%)	
CABG+MVR	0 (0%)	0 (0%)	
AVR+MVR	1 (2.9%)	1 (2.9%)	
CABG+AVR+MVR	0 (0%)	1 (2.9%)	

Key: SD- standard deviation, AVR- aortic valve replacement, REDO OP- redo-operation, MVR- mitral valve replacement.

Almost half of the patients in both groups underwent CABG surgery alone, with the second most common surgery being isolated aortic valve replacement. Complex cardiac operations tended to be performed with much less frequency (11.4% in control vs 14.7% in CsA treated

group), an important observation as this is the group that could potentially benefit from cardioprotective interventions.

4.4.3. Serum concentration of CsA

In Piot's study measurements of blood concentrations of CsA revealed circulating levels above values recommended in transplant patients as early as 1 minute after reflow and for at least 3hr after reperfusion[483].

Figure 4.03: *High blood concentrations of CsA at the time of reperfusion which will most certainly have guaranteed a well saturated myocardium. (The graph below taken from Piot's study[484]).*

We measured serum CsA at the time of aortic cross-clamp removal in three patients with the following results:

Table 4.03: *The serum CsA level after cross-clamp removal.*

Case number	Serum CsA level (µg/L)
1	220
2	582
3	664

Normal therapeutic range for CsA 100-400 (µg/L)

We were only able to measure the CsA serum levels of 3 patients because the laboratory test was deemed too expensive to carry out on all the patients recruited. In addition, securing funding prior to starting the recruitment process presented its own challenges that threatened to delay the study start date and so this option was abandoned. Ideally, we would have preferred to measure the serum CsA levels in all cases, comparing the pharmacokinetic treads with that of Piot's study. Despite this, important information was revealed from these three cases through which inferences could be made.

Firstly, it is likely that the levels required to inhibit the mPTP are considerably higher than that reached in our study. Very high levels of CsA were reached immediately after transfusion and at the time of reperfusion in Piot's study, and it is likely that this may have had an important influence on their findings.

Secondly, it brings into question whether or not the administration of CsA should have taken place at the time at which the aorta was unclamped. These points are further expanded upon in the discussion.

4.4.4. Serum troponin T release

Testing for normality

The data was tested for normality in order to establish the appropriate statistical test to use. The skewness and kurtosis of the data were analysed and the Shapiro-Wilk test was applied to test for normality.

Table 4.04: *Descriptive statistics of study data.*

Descriptive statistics	**Skewness**	**Kurtosis**
CsA	1.043	0.037
Control	2.171	4.714

Key: CsA- cyclosporin A

Table 4.05: *Testing for normality.*

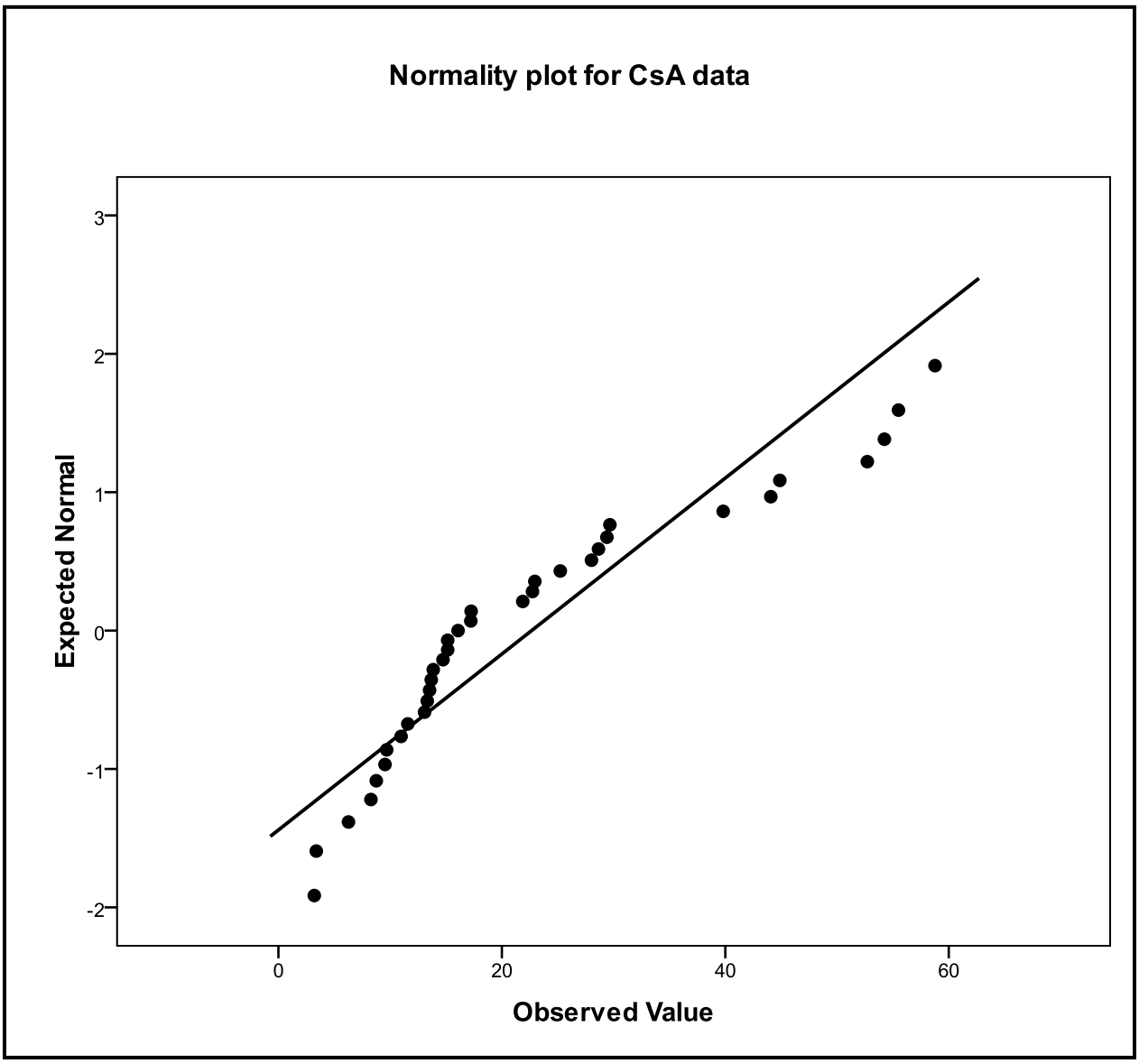
Tests of normality	**Shapiro-Wilk test**
CsA	0.001
Control	0.000

Key: CsA- cyclosporin A

The entire study data is not normally distributed and this is demonstrated by the skewness being greater than 1 and a kurtosis greater than 0. I was also able to demonstrate homogeneity of variance between the two samples with a trimmed mean of 0.811. Please see Tables 4.04 and 4.05 above.

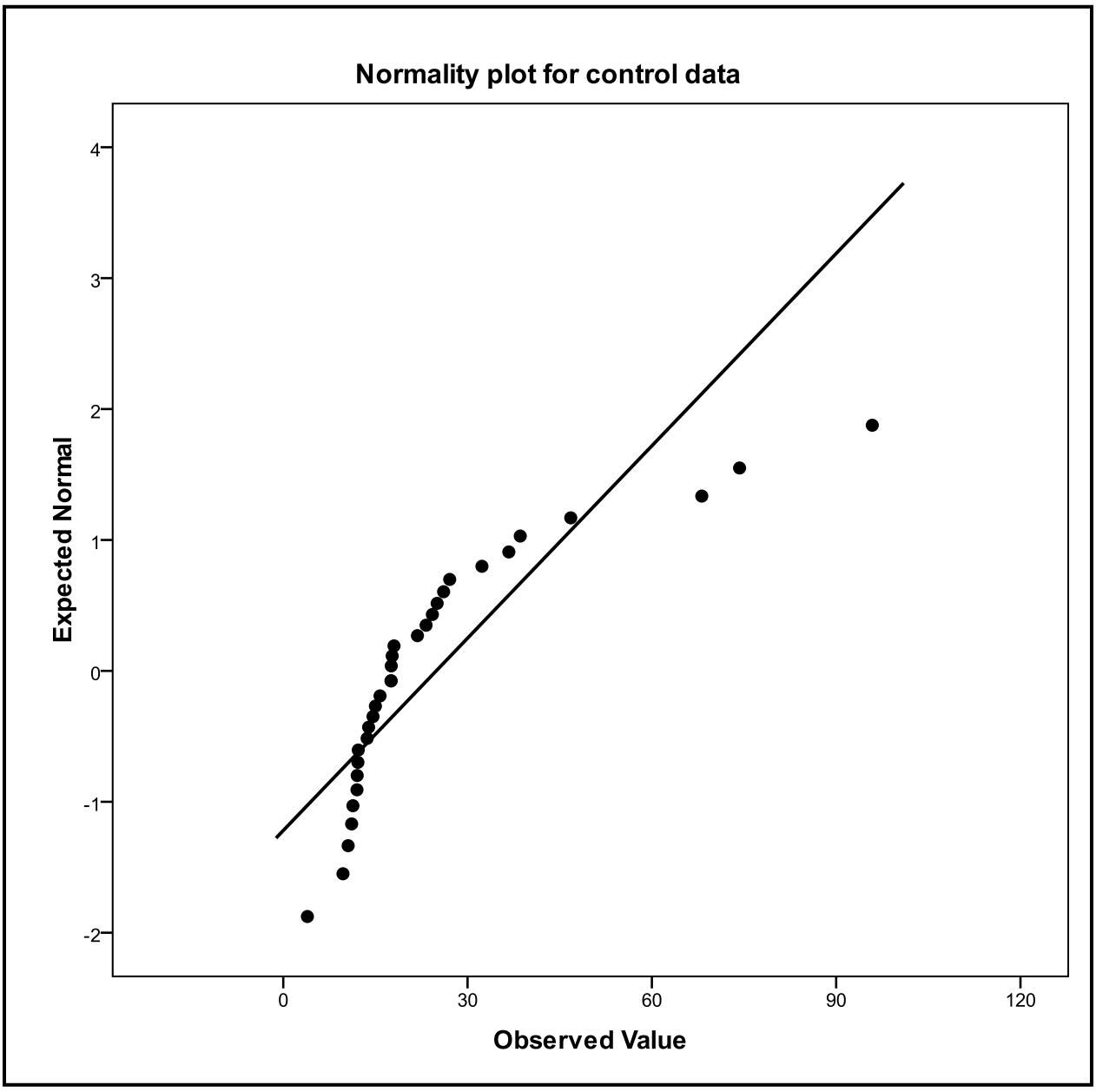
The data from both the CsA group and the control group are displayed on normality plots and summarised on box-plots below.

Figure 4.04: *Displays the normal probability plot for the CsA group.*



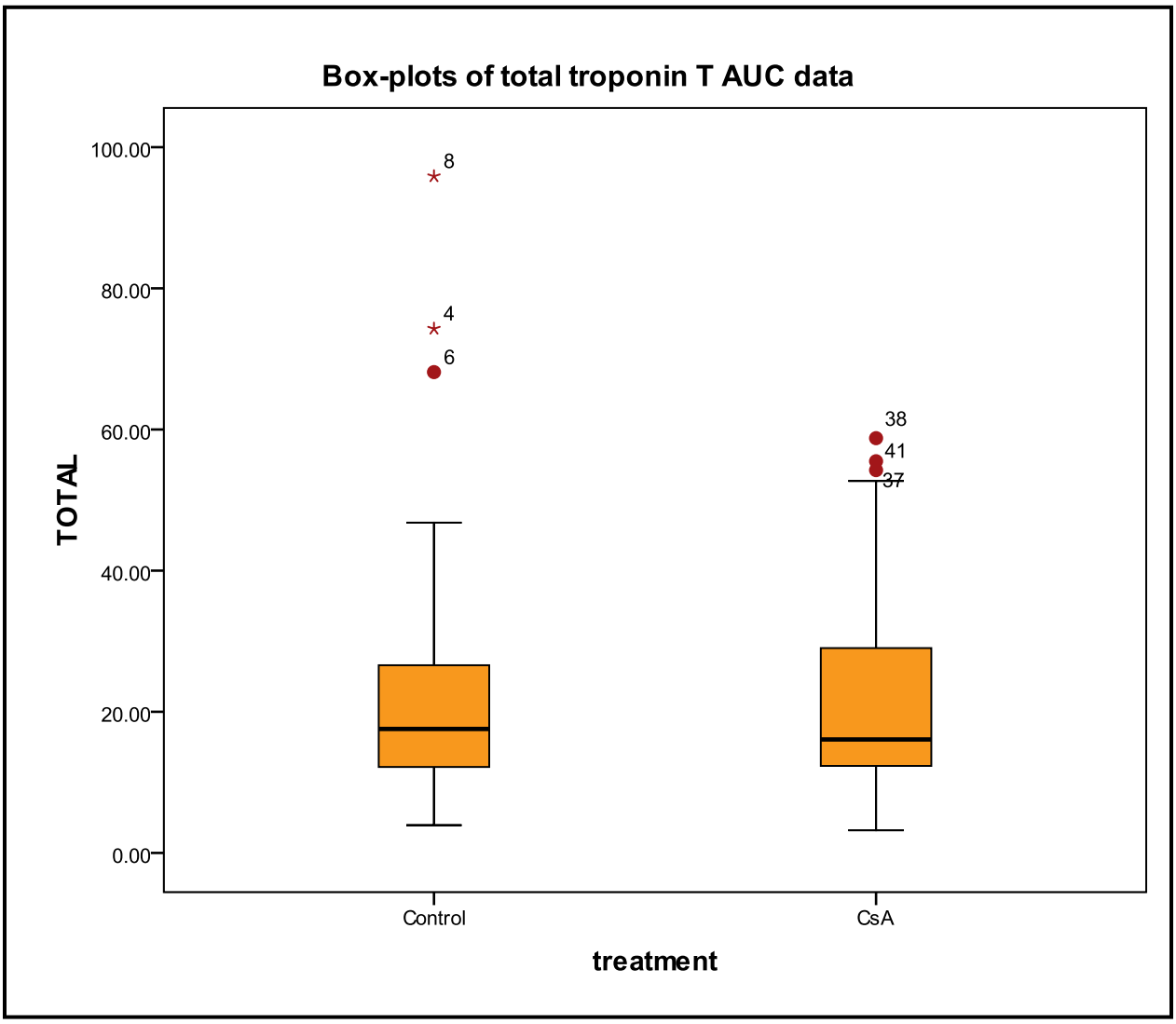
The graph above displays the relationship between the expected normal and the CsA group data. The data seem to spiral around the normality line which does not reflect a normal distribution.

Figure 4.05: *Displays the normal probability plot for the control group data.*



The graph above displays the relationship between the expected normal and the control group. Clearly the data do not lie on the normality line suggesting that the data is not normally distributed.

Figure 4.06: *The box-plots display the data of both the control group and the CsA group.*



The box-plots above summarise the study data. As the data is positively skewed, it was deemed appropriate to perform non-parametric tests as the main tool for comparing the two groups.

Table 4.06: *Application of non-parametric tests for total AUC troponin T over 72 hours (mcg/L.72hr) .*

Study group	n	Mean rank	Median	Sum of ranks	Mann-Whitney U	Z-statistic	Asymptotic Sig. (2-tailed)
CsA	34	37.37	17.58	1308.00	617.000	-0.344	0.731
Control	37	35.68	17.22	1320.00			

Key: CsA- cyclosporin A

Seventy-one patients were analysed all of whom had a complete data set. The 34 patients in the CsA group had a mean rank of 37.37 and a median total troponin T AUC over 72 hours of 17.58mcg/L.72hr. The 37 patients in the control arm had a mean rank of 35.68 and a median total troponin T AUC over 72 hours of 17.22mcg/L.72hr. No significant difference was demonstrated between the two groups confirmed by a 2-tailed asymptotic significance of 0.731.

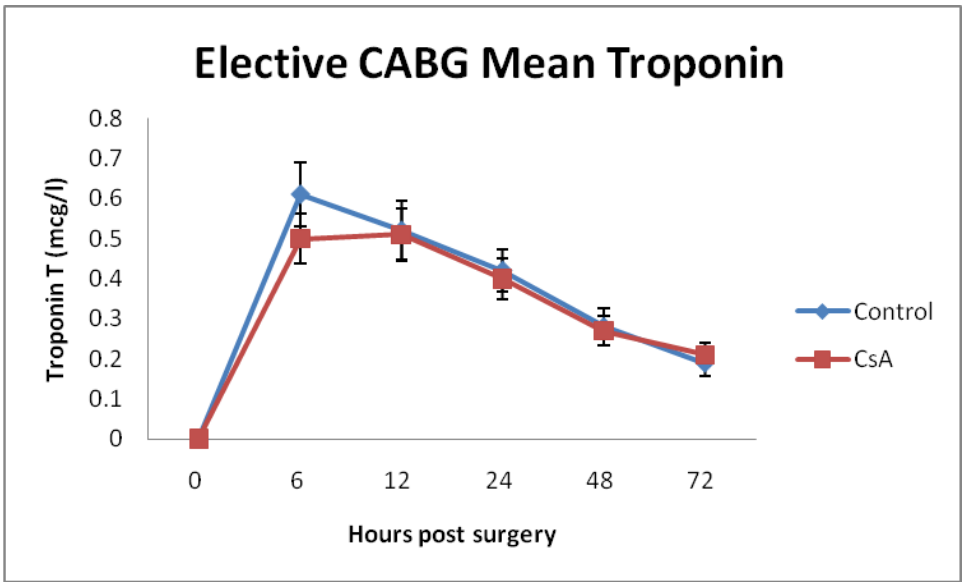
Cardiac troponin-T levels were measured preoperatively (baseline) and then at 6, 12, 24, 48 and 72 hours post-operatively. The mean difference, confidence intervals and p-values are shown in table 4.3 below. The largest difference can be seen at 6 hours where a value of 0.61 is seen in the control group compared with 0.50 in the CsA group. This suggests that if any benefit is to be detected, this would be seen in the first 6 hours post-operatively. At 12 hours and beyond, there seems to be little difference separating the two groups.

Table 4.07: *Serum troponin T levels over 72 hours following cardiac surgery*

Time (hours)	Control (mcg/L)	CsA (mcg/L)	Mean difference	Confidence interval	P value
6	0.61	0.50	0.11	-0.096 to 0.315	0.294
12	0.52	0.51	0.005	-0.190 to 0.199	0.962
24	0.42	0.40	0.013	-0.133 to 0.159	0.860
48	0.28	0.27	0.013	-0.104 to 0.131	0.820
72	0.19	0.21	-0.02	-0.106 to 0.066	0.646

Key: CsA- cyclosporin A

Figure 4.07: *Troponin T release over 72 hours postoperatively (mean +/-SEM) all cardiac surgery.*



Over the 72 hour period, the cardiac troponin-T levels were slightly lower in the CsA treated group compared with the control group, but this was not found to be statistically significant.

4.4.6. Correlation and regression analysis

As expected, there was a positive correlation between the total mean Troponin T AUC over 72 hours and the aortic cross-clamp time. These findings suggest that as the cross-clamp duration increases, the myocardial injury sustained during cardiac surgery increases. The regression lines of the control data and the CsA treated data seem to intersect just before cross-clamp duration of sixty minutes (see figure 4.07). It therefore seems reasonable to postulate that the effect of cardioprotection is likely to be more apparent in complex operations particularly those with longer cross-clamp durations which also tend to carry a higher peri-operative risk.

However as previously mentioned, complex operations involving CABG and valve replacement occur less commonly compared with CABG only or isolated valve replacement. Therefore to test this hypothesis, a large, multi-centre randomised controlled trial would be required.

Figure 4.08: *The correlation between the mean total troponin T AUC over 72 hours and the aortic cross-clamp time (minutes) is displayed in the scatter diagrams below.*

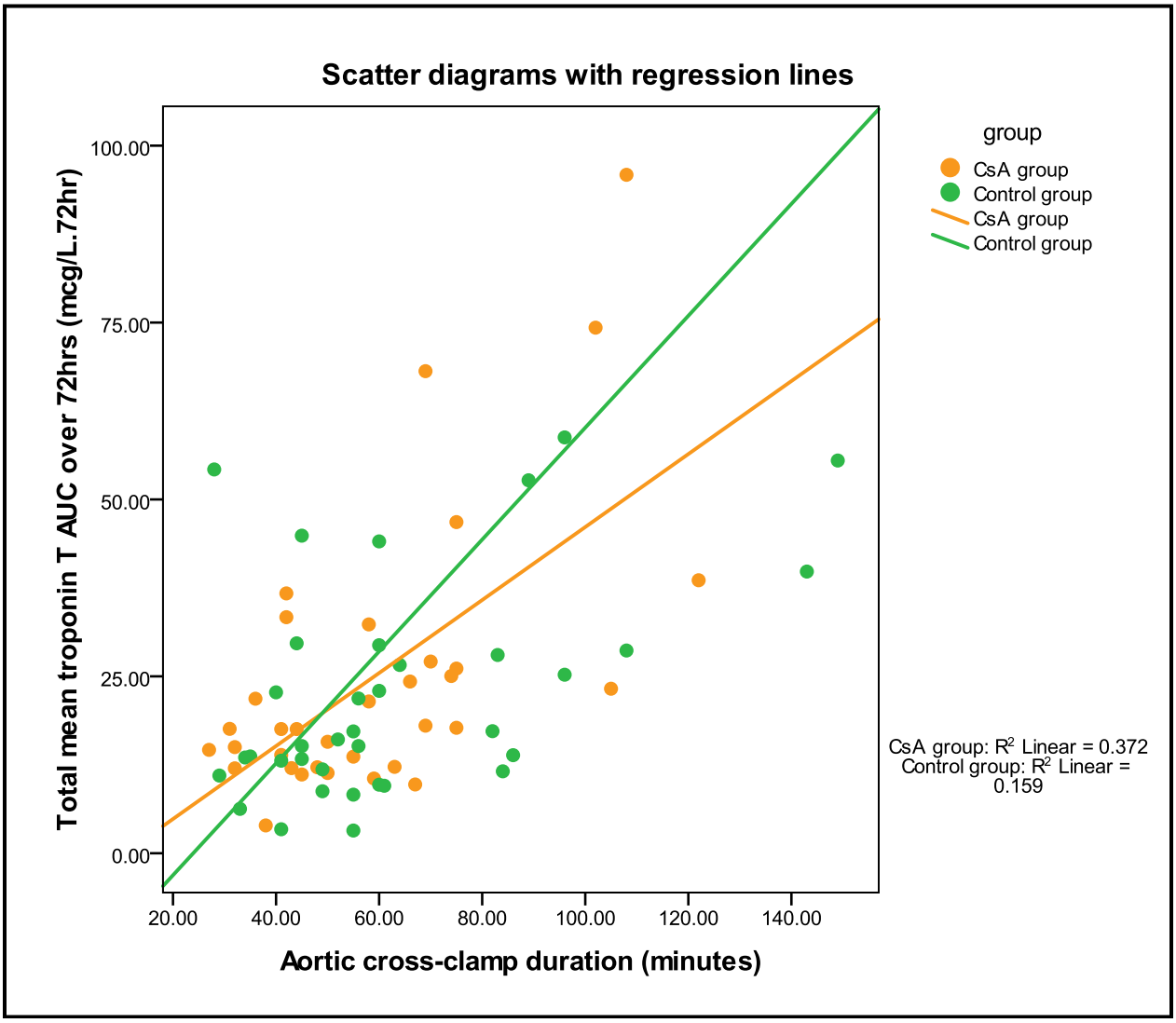


Table 4.08: *Displays Pearson's correlation of regression lines with p-values.*

Study group	n	Pearson's correlation	p-value
CsA	34	0.610	<0.001
Control	37	0.399	0.014

Key: CsA- cyclosporin A

The CsA group demonstrates a moderate correlation (r=0.610) between troponin T release and aortic cross-clamp duration with a p value of <0.001. In comparison, the correlation between

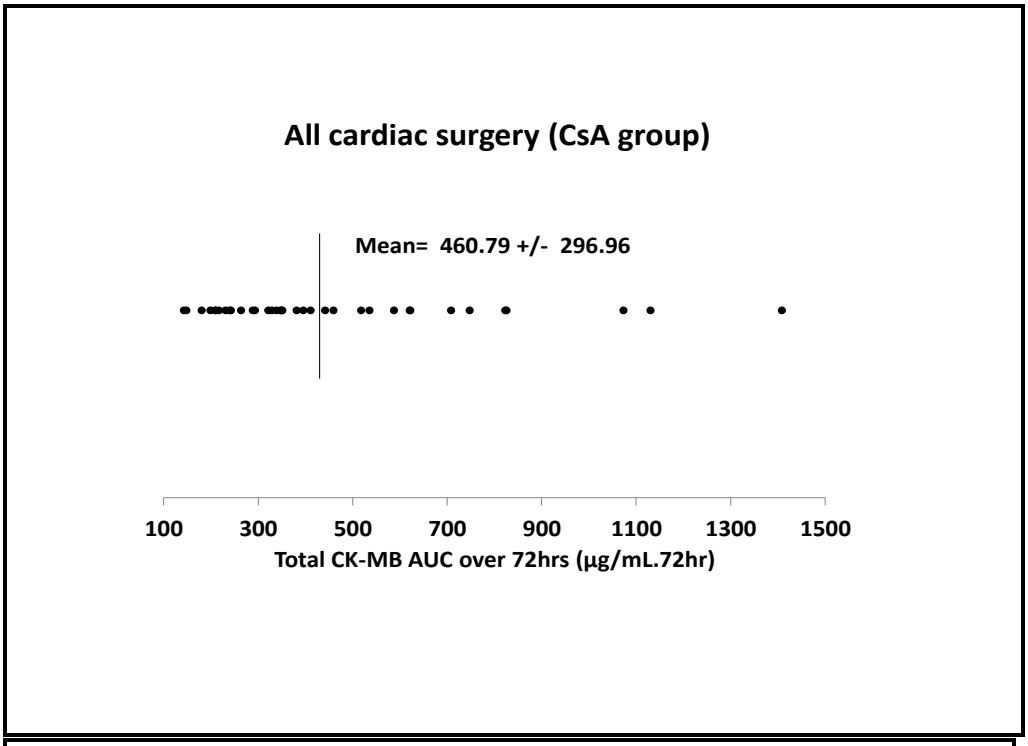
troponin T and aortic cross-clamp duration in the control group is weaker but still within the moderate range at r=0.399.

The scatter diagram also seems to imply that for a given increase in aortic cross-clamp duration, the increase in total mean troponin T AUC over 72 hours was less in the CsA group compared with the control group. This suggests that as the aortic cross-clamp duration increase, contributing to an increase in myocardial damage, the cardioprotective effects of CsA is likely to be more pronounced. Clearly a large randomised control trial is needed to confirm this potential observation.

The positive correlation between cardiac enzyme release and aortic cross-clamp time in the scatter diagrams is consistent with previous studies that identified the aortic cross-clamp time as an independent predictor for myocardial injury.

4.4.8. Serum CK-MB release

Figure 4.09: *Dot-plot diagrams displaying total mean CK-MB AUC over 72hrs in study groups.*



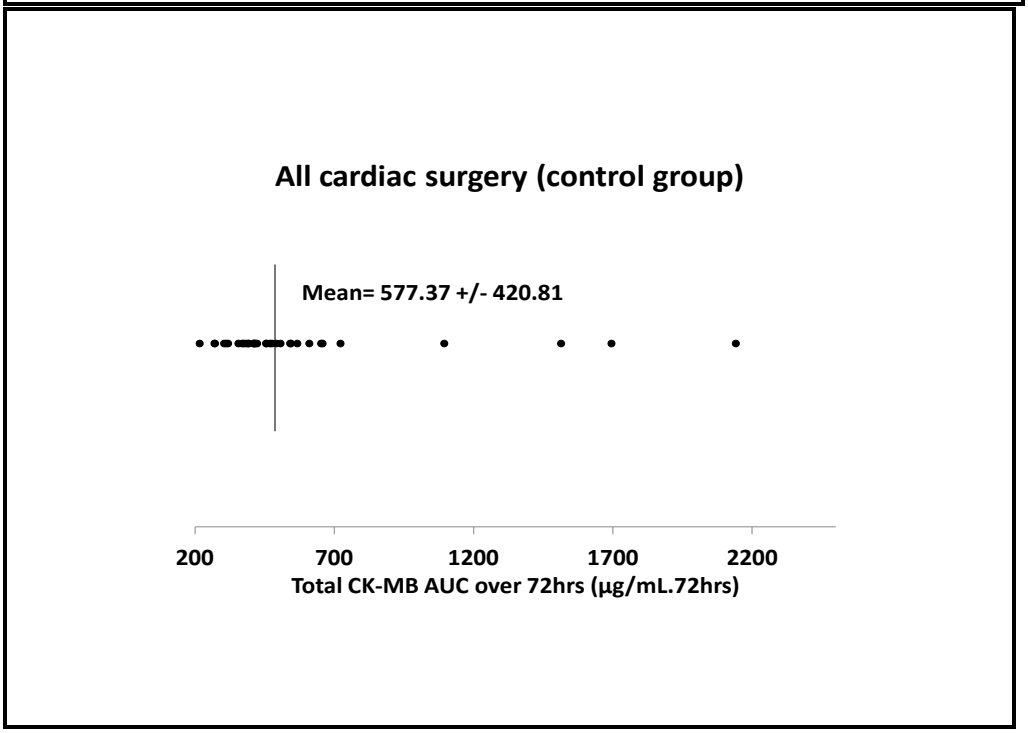


Table 4.09: *Application of non-parametric tests for total AUC CK-MB over 72 hours (µg/ml.72hr)*
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Study group	n	Mean rank	Median	Sum of ranks	Mann-Whitney U	Z-statistic	Asymptotic Sig. (2-tailed)
CsA	36	31.11	349.50	1120.00	454.00	-1.857	0.063
Control	34	40.15	438.90	1365.00			

Key: CsA- cyclosporin A

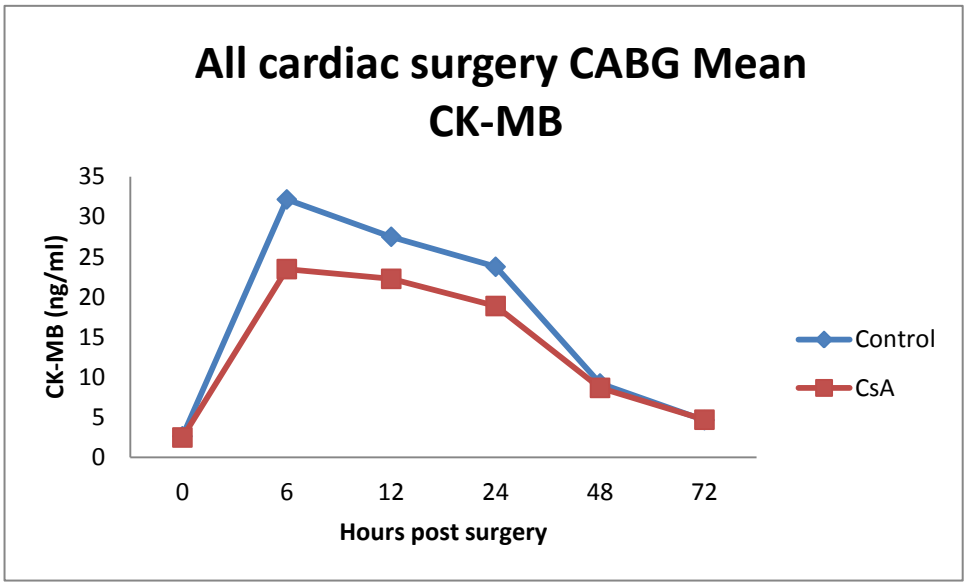
The mean and standard deviation of the total mean CK-MB over 72 hours are displayed in the dot-plot diagrams above (see figure 4.09). The CsA group had a lower mean of 460.79µg/mL.72hr compared with a mean of 577.37µg/mL.72hr in the control group. This correlates with the median which is lower in the CsA group at 349.50µg/mL.72hr compared with 438.90µg/mL.72hr in the control group. According to the Mann-Whitney U test, this difference is not significant but is clearly demonstrating a tendency towards significance with a p-value = 0.063. With a larger sample size, it is likely that a significant difference would be seem between the two groups.

Table 4.10: *Shows the mean total CK-MB AUC over 72 hours (ng/mL.72hr) within the specified time intervals.*

Time (Hours)	Control	CsA	Mean difference	CI	P value
0-6	104.31	77.81	26.49	-6.27 to 59.27	0.111
6-12	178.98	137.49	41.49	-17.70 to 100.68	0.166
12-24	153.78	124.32	29.56	-20.73 to 79.64	0.246
24-48	98.87	83.60	15.27	-15.19 to 45.73	0.321
48-72	41.44	40.70	0.74	-12.17 to 13.64	0.910

Key: AUC- area under the curve, CI- confidence interval

Figure 4.10: *The total CK-MB AUC over 72 hours of all cardiac surgery patients.*



The table above shows a clear difference in the magnitude of CK-MB release in the CsA group compared with the control group, however this difference was not significant at 0.05 level. This difference can be seen within the first 24 hours but in particular in the 6-12 hour interval revealing a mean difference of 41.49ng/ml. The findings also seem to point towards the

maximum benefit occurring within the first 6-24 hours post-operatively. This could potentially have significant clinical implications in the optimization of patient care post-operatively and therefore improving intermediate to long-term clinical outcomes.

Figure 4.11: *The correlation between the CK-MB AUC over 72 hours and the aortic cross-clamp time (minutes) are displayed in the scatter diagrams below.*

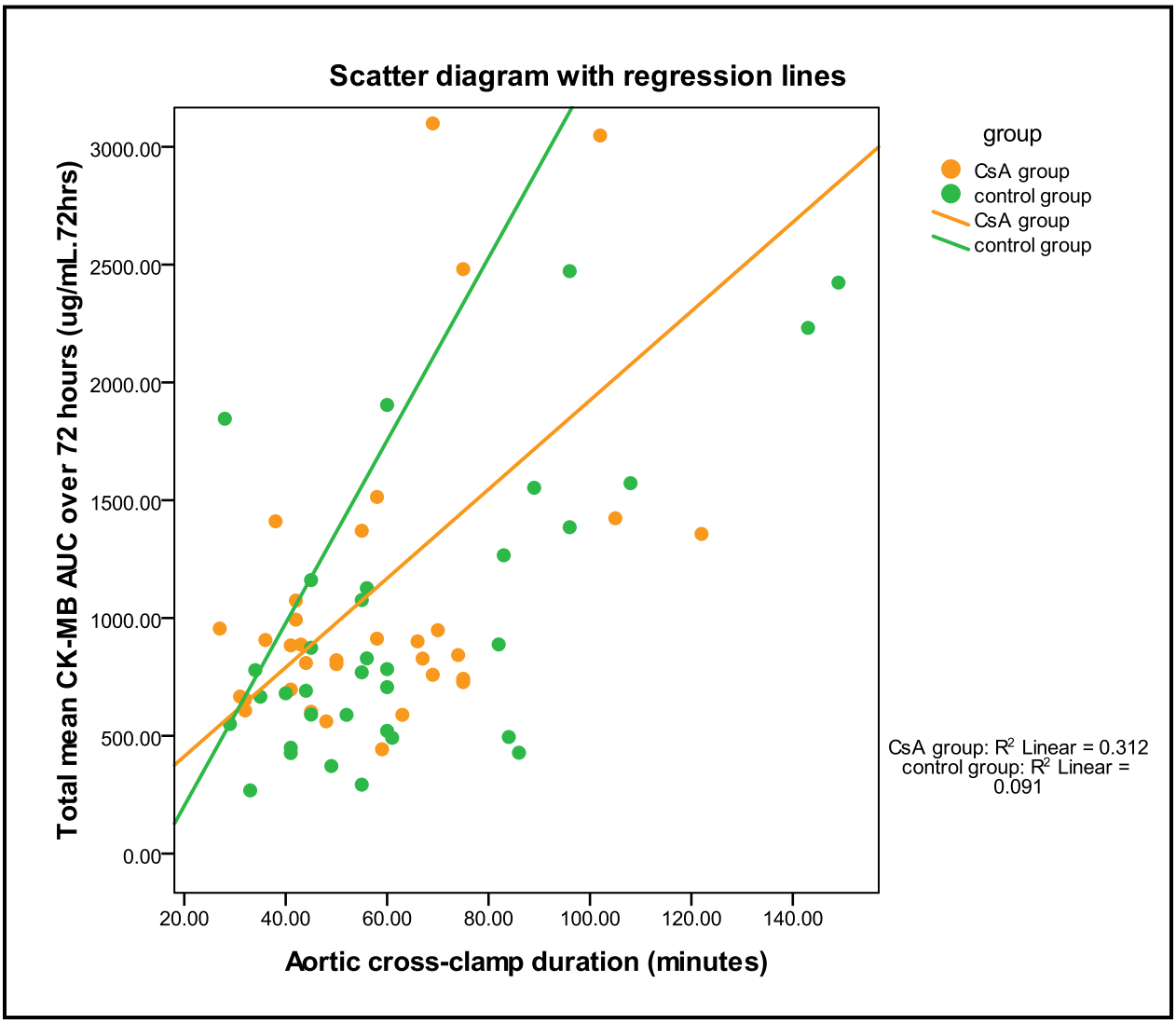


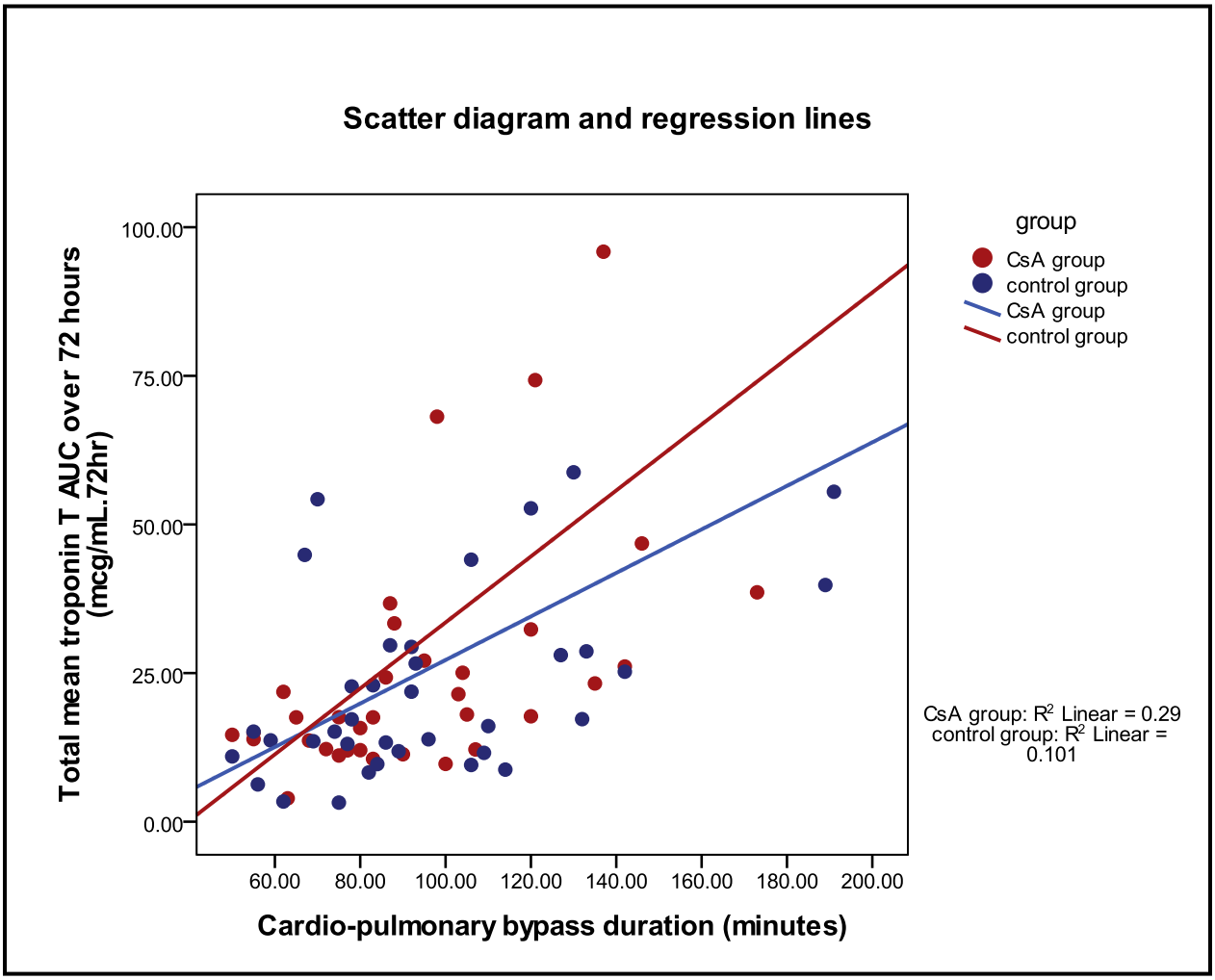
Table 4.11: *Displays the correlation coefficient and the p-values in the study groups.*

Study group	**n**	**Pearson's correlation**	**p-value**
CsA	34	0.558	0.001
Control	37	0.301	0.07

The scatter diagram above displays the correlation between the total mean CK-MB AUC over 72 hours and the aortic cross clamp time. Moderate correlation can be seen in both the CsA group and the control group with a stronger correlation in the CsA group (Pearson's= 0.558 vs 0.301). The regression lines diverge just before 40 minutes of aortic cross-clamp time. The scatter diagram seems to demonstrate that for a given aortic cross-clamp duration, a lower CK-MB release is seen in the CsA group when compared with the control group.

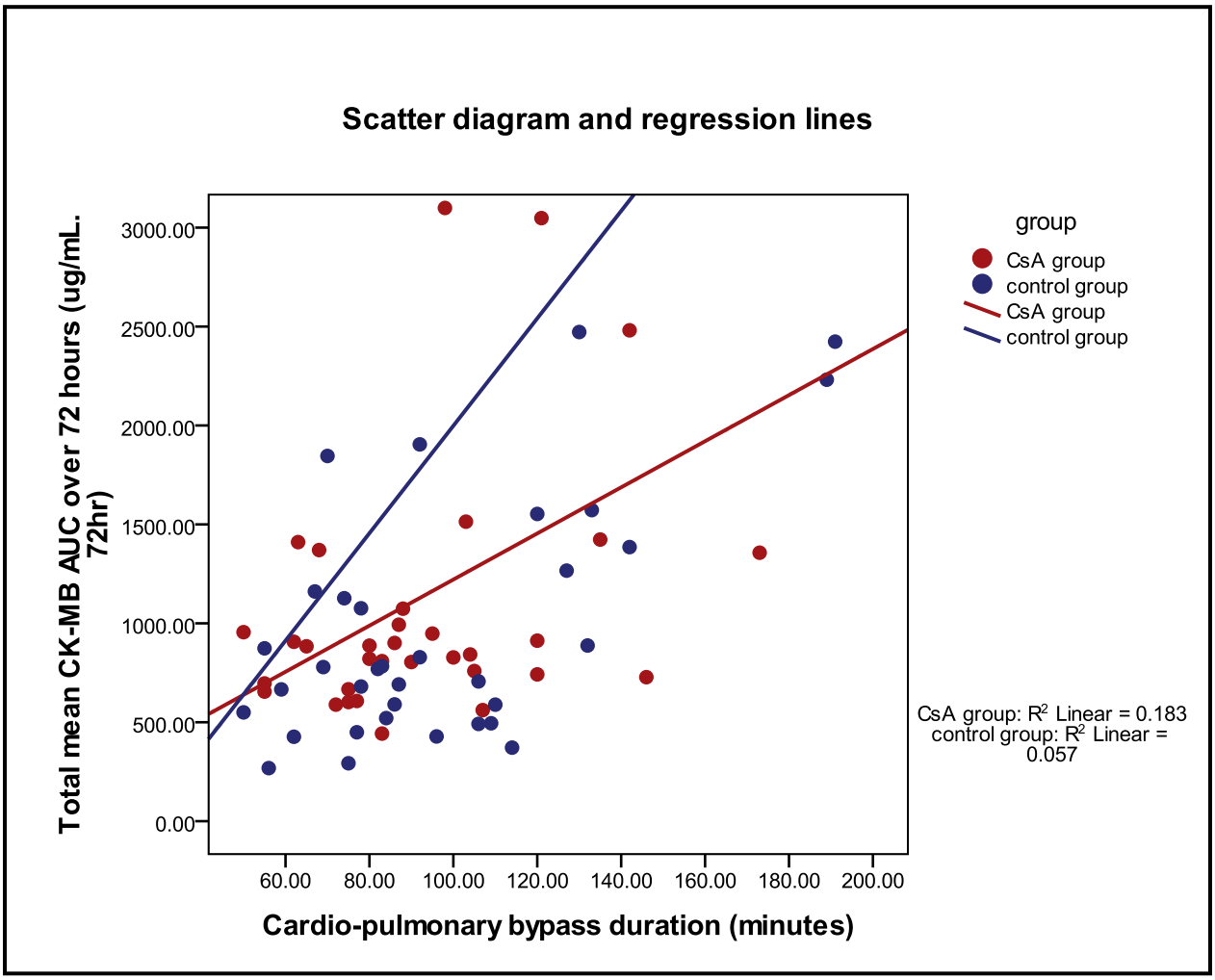
The scatter diagram below demonstrates a moderate correlation between the total mean troponin T AUC over 72 hours and the cardio-pulmonary bypass duration in both the CsA group and the control group. It is also clear that for a given CPB duration there is a lower troponin T release in the CsA group compared with that in the control group. The p values of the regression lines are shown in table 4.12 below.

Figure 4.12: *The correlation between the total mean troponin T AUC over 72 hours and the cardiopulmonary bypass time (minutes) is displayed in the scatter diagram below.*



Study group	n	Pearson's correlation	p-value
CsA	34	0.539	0.001
Control	37	0.317	0.056

Figure 4.13: *The correlation between the total mean troponin T AUC over 72 hours and the cardiopulmonary bypass time (minutes) is displayed in the scatter diagram below.*



Study group	n	Pearson's correlation	p-value
CsA	34	0.428	0.012
Control	37	0.239	0.155

Once again we see a positive correlation between the total mean CK-MB AUC over 72 hours and the CPB duration. The relationship is stronger in the CsA group compared with the control

group with a p-value of 0.012 and 0.155 respectively. The regression lines seem to diverge just before 60 minutes of CPB duration. We can also see that for a given CPB duration the CK-MB release is lower in the CsA group compared with the control group.

4.5. Cyclosporin A and the risk of anaphylaxis

While conducting the first sub-study looking at the cardioprotective effects of cyclosporin A, two events occurred that were deemed moderate in severity. In both cases, soon after the administration of Propofol, Fentanyl, Rocuronium (muscle relaxant) and cyclosporin A, the patients became hypotensive and developed a generalised macular rash. Their blood pressures failed to respond to fluid resuscitation or ephedrine and subsequently required boluses of epinephrine to maintain haemodynamic stability. It was possible that cyclosporin A was the culprit agent responsible for what was confirmed as an anaphylactic reaction, but it was equally as likely that the other pre-anaesthetic agents could have caused the episode. Nonetheless, both events were reported to the research and development department by filling in a serious adverse events form. In both cases it was concluded that the risk to patient safety posed by cyclosporin A was negligible and so the study was allowed to continue.

Patients admitted for elective cardiac surgery were approached on the evening of their admission. Their notes were scrutinised and their clinical letters check for eligibility. Informed consent was then obtained using the latest revised versions of the documents along with the patient's signature. A copy of the signed consent form was given to the patient, another copy

was file in the patient's notes and the original copy was file in a locked room within the Hatter Institute.

Hypersensitivity reactions to cyclosporin A are rare, with the latest data suggesting an occurrence of 1 in 1900; as such the mechanism of the reaction has yet to be clearly defined.

Of all the documented cases in the literature (about 30 cases in MEDLINE), it is the intravenous preparation of CsA that is most associated with adverse reactions. It has been suggested that it is the polyoxyethylated castor oil (Cremaphor E) solubilizer used in the injective formulation of cyclosporin A that triggers a hypersensitivity reaction.

The mechanism of the reaction is not anaphylactic or IgE-mediated as skin tests conducted on patients who had suffered from hypersensitivity reactions after treatment with intravenous CsA have been largely negative for the antigen cremophor E. It seems more likely that the reaction is anaphylactoid in nature resulting from direct mast cell degranulation and complement activation by cremophor E.

4.5.1. Clinical presentation and diagnosis

Adverse reactions after intravenous cyclosporin A tend to occur after just one administration, further supporting the mechanism of an anaphylactoid reaction. It is highly recommended that care is taken during the preparation of intravenous CsA to avoid high concentrations of the solubilising agent being injected during administration as this increases the risk of hypersensitivity.

Hypersensitivity reactions are usually seen within minutes of administration of CsA, with patients developing a generalised erythematic rash and may complain of pruritus. Clinical signs include tachycardia and hypotension due to an extensive capillary leakage leading to circulatory collapse and a depression in cardiac output. A trans-oesophageal echocardiogram might reveal an empty ventricle. In patients who are ventilated, an increase in airway resistance is noted.

4.5.2. Management

Immediate management involves a rapid assessment of airway, breathing and circulation. The infusion should be stopped immediately. In the event of stable haemodynamics, symptoms can be controlled with corticosteroids. However in severe cases of acute capillary leak syndrome, the use of adrenaline and intravenous fluids are required to treat the circulatory collapse.

4.5.3. Medical and clinical research implications

The medical implications of cyclosporin A hypersensitivity is clearly distressing for the patient as well as the medical team present at the time. From a management perspective, provided that the indications for its use and the method of administration were in accordance with the set institutional guidelines, further issues need not arise. However, in the setting of this clinical research trial, the implications were more complicated with the development of further repercussions.

One of the main issues that arose after one of the patients suffered what could have been a hypersensitivity reaction to CsA was whether or not to continue with their elective operation. Of the two patients who suffered hypersensitivity reactions and subsequent acute capillary leak

syndrome, one had their surgery cancelled and postponed. The patient was subsequently awoken from his anaesthesia and informed along with his family of the event that had occurred.

From a clinical governance perspective, the cancellation of surgical procedures result in issues of cost-effectiveness, and reassurances must be made to the clinical team as well as the patient and their families with regards to patient safety.

An extensive 'severe adverse event' form was completed along with a 'clinical incident form' clearly outlining the sequence of events and the measures taken to ensure the upmost care to the patient according to Good Clinical Practice.

Another point considered was the continuation or termination of the study and the impact on other centres conducting the study using a similar protocol. In this regard, the Research and Development department favoured the continuation of the study concluding that the risk posed by cyclosporin A was small.

4.6. The effect of cyclosporin A on secondary outcomes

So far we have studied the effects of CsA on the myocardium during cardiac surgery and I intend to conclude this thesis by discussing the possible reasons for our findings. Previous studies have looked at the protective effects of CsA in other organs and have demonstrated protective effects. I intended to look at the effect of CsA on secondary outcomes despite the study not being adequately powered for this purpose. With regards to major cardiovascular events and mortality, one would need a very large population study as the operative risk in the patient group studied is very small, between 1-3%[485].

Table 4.09: *The classification of AKI.*

AKI Grade	Serum Creatinine criteria	Urine output criteria
1	A rise of >26.4µmol/L or 150-200% of baseline	<0.5ml/kg/hr for >6 hours.
2	An increase of 200-300% of baseline	<0.5ml/kg/hr for >12 hours.
3	An increase of >300%; or serum Creatinine >354µmol/L with an acute rise of at least 44µmol/L.	<0.3ml/kg/hr for >24 hours or anuria for 12 hours

Key: AKI- acute kidney injury.

Only one criterion was required for each grade. If the patient was classified into different grades by the separate criteria the higher grade was used. All patients who needed renal replacement therapy were classified as AKI3 irrespective of other criteria.

The table below shows the mean and standard deviation of the secondary outcomes measured during the CsA study. The results demonstrate that patients treated with CsA had a significantly shorter stay in ITU compared with control with a mean period of 30.1 +/- 13.9 hours vs 40.6 +/-

29.4 hours respectively. This difference did not translate to ventilation time as no difference was demonstrated between the two groups in this regard. The control group mean ventilation duration was 6.61 +/- 3.00 hours vs 7.91 +/- 3.54 hours in the CsA group. At this point I would like to stress that no formal criteria was implemented to determine when patients were suitable for discharge. In general, a number of factors are taken into consideration before a patient is deemed suitable for 'step-down' from the cardiac intensive care unit. These include their:

- POTTS score
- other criteria- look up internet

Establishing a formal criteria for the study and implementing it to the two study centres proved a challenging task as it risked interfering with local clinical practice. At the same time we appreciate that this does pose a limitation to the study and caution should be taken when interpreting the effects of CsA on ITU stay.

Table 4.10: *Descriptive statistics of secondary outcomes with a display of the mean and standard deviation.*

Outcome	**Control (38)**	**CsA (43)**	**p-value**
ITU stay (Hours)	40.6+/- 29.4	30.1+/- 13.9	0.04
Ventilation (Hours)	6.61+/- 3.00	7.91+/- 3.54	0.08
Serum Creatinine (µmol/L)			
Baseline	84.52 +/- 14.51	88.15 +/- 15.75	0.381
Day 1	82.30 +/- 20.09	87.04 +/- 23.26	0.430
Day 2	86.74 +/- 36.35	96.85 +/- 30.53	0.279

Day 3	81.52 +/- 28.38	86.31 +/- 24.36	0.513
Urine output day 1 (mls)	2211.07 +/- 657.67	2159.08 +/- 895.66	0.810
AKI 1	1	1	
AKI 2	1	0	
AKI 3	0	0	
Dialysis	0	0	
AF	8 (9.9%)	16 (19.8%)	0.161
Stroke	0	0	
Inotrope use	14 (17.3%)	16 (19.8%)	0.973
Inotropic score	15.02 +/- 64.95	5.96 +/- 15.80	0.378

Key: ITU- intensive care unit, AKI- acute kidney injury, AF- atrial fibrillation

There were no deaths, myocardial infarctions or revascularisations during the period of hospital stay. There were no documented occurrences of cerebrovascular events suffered by patients in both group during the period of study.

There were no significant differences in the baseline Creatinine of the CsA group and the control group and this remained the case over the 72 hours through which patients were monitored. The urine output was measured hourly over post-operative day 1 with the control group averaging 2211.07 +/- 657.67mls and the CsA group 2159.08 +/- 895.66mls. Both groups had one patient who was classified as having grade one acute kidney injury and one patient on the control group was classified as having grade two AKI. No patient in either group required dialysis post-operatively.

There were twice as many patients who suffered from atrial fibrillation post-operatively in the CsA group compared with the control group but this was not found to be statistically significant as the overall number of patients suffering from post-operative AF was low.

There were no differences in inotropic use in both groups with 14 (17.3%) in the control and 16 (19.8%) in the CsA group. However, when inotropes were used, three times as much was needed in the control group compared with the CsA group. The average inotrope score in the control group was 15.02 +/- 64.95 compared with a score of 5.96 +/- 15.80 in the CsA group.

DOES ANOVA HAVE A ROLE HERE???

4.7. Discussion

Recent decades have seen great advances in the treatment of ischaemic heart disease, yet this condition remains the leading cause of death and morbidity within the western world. The severest form of this condition, treated by coronary artery bypass surgery is not without its risks. The clamping of the aorta and the application of cold blood cardioplegia during the cardiopulmonary bypass process renders the myocardium ischaemic. The subsequent reperfusion of the myocardium through unclamping the aorta in addition to graft anastomosis is paradoxically associated with the death of cardiomyocytes, a phenomenon termed lethal ischaemia reperfusion injury. Despite the use of cardioprotection mainly in the form of cardioplegia frequently implemented during CABG surgery, myocardial injury is still a significant problem, manifesting itself in the form of low cardiac output syndrome and arrhythmias in the short term and a prolonged length of recovery and increased mortality in the intermediate to long-term.

It is without question therefore that novel therapeutic strategies are needed particularly during the peri-operative period to protect against the effects of lethal ischaemia reperfusion injury, thereby improving cardiac function and augmenting post-operative recovery.

The crucial observation by Fournier and colleagues, that CsA could inhibit the influx of calcium into the mitochondrion[486], followed by the discovery that CsA could inhibit the mPTP[192;487], has led to a surge in basic science research into the mechanism of the CsA-mPTP interaction.

In this chapter, the mitochondrial permeability transition pore was investigated as a target for cardioprotection on the basis of previous studies that have identified its critical role in cell death.

Of the 115 patients who were screened for eligibility, 68 patients were successfully recruited. Of those successfully recruited, there were no significant differences in the demographics, drug treatment or type of surgery between the treatment group and the control. Interestingly, the mean EuroSCORE in both groups was less than 3 which is considered a low risk. As previously discussed (see chapter 1), this score is based on a number of pre-operative factors that include the level of complexity of the surgery. The results from this study suggest that a significant cardioprotective benefit cannot be achieved in operations deemed to be low risk. Future studies in cardioprotection in the setting of cardiac surgery should focus on patients with a EuroSCORE greater than 6 with particular attention paid to the complexity of the operation. Higher risk operations result in a higher level of myocardial injury as they often require prolonged aortic cross-clamp times.

In Piot's study which looked at the cardioprotective effects of CsA in the setting of STEMI, the administration of intravenous CsA at 2.5mg/kg (without dilution) occurred less than 10 minutes before direct stenting of the culprit artery. In our study, the administration of CsA occurred between 2-3 hours prior to the removal of the aortic cross-clamp. We took blood samples at the time of the cross-clamp removal to measure the level of CsA within the circulation. We found that the level of CsA within the bloodstream remained within therapeutic levels at the time the cross-clamp was removed. What remains uncertain however is whether the dose

required for pore inhibition falls at a much higher level. Piot's study demonstrated that very high levels of CsA were available at the time of coronary reperfusion- the time in which it has been shown for the mitochondrial permeability transition pore formation to occur. It is likely that the timely administration of CsA coupled with the much higher levels of myocardial injury seen in STEMI in the absence of additional elements of cardioprotection may explain why a cardioprotective benefit was seen in Piot's study and not in our own. It may be such that the differences in the two clinical settings may account for the disparity in results.

A number of ways for achieving better CsA delivery and therefore better mPTP inhibition could be explored in future studies. They include:

- The intravenous administration of CsA at the time of the removal of the aortic cross-clamp.
- Increasing the administration dose of CsA from 2.5mg/kg to 5mg/kg.
- Adding the study dose of CsA to the cardioplegic solution.
- Administering the study dose of CsA without dilution.

The analysis of the primary end-point (troponin T AUC over 72 hours) showed no significant statistical difference between the treatment group and the control group. Some of the reasons for this result may lie in the nature of the clinical setting; where cardioplegia and advances in anaesthesia may provide a level of protection leaving only a small amount of injury to protect against. A further reason for this result may lie in the mode of administration of CsA previously discussed.

An interesting finding that became apparent during the analysis of the results was the effect of mitral valve surgery on the data. As described earlier, the level of direct myocardial trauma is particularly high in this type of surgery as it involves the opening of the left atrium. The amount of troponin release was particularly high when compared with the other types of surgery and this subsequently resulted in some skewing of the data. It is for this reason that further studies should exclude mitral valve replacements from the trial cohort.

We assessed the mean level of CK-MB release at specified time points over the 72 hour period. Although the graphs do separate particularly within the first 6-24 hours, this separation was not statistically significant. The mean total CK-MB AUC over 72 hours was not significantly different between the treatment group and the control group. Although this result is consistent with our primary end-point, interestingly, it differs from Piot's study which albeit in the setting of STEMI, found a 40% reduction in CK-MB release in the CsA group. The reason for this disparity in Piot's study is not clear; however the emergence of highly sensitive troponins may have a role in future studies in addressing this disparity.

The scatter diagrams (A) and (B) display a positive correlation between the total troponin T AUC over 72 hours and the aortic cross-clamp time (XCT), a finding that is consistent with previous studies that have implicated XCT and cardiopulmonary bypass time (CPBT) as positive predictors of long-term morbidity and mortality[488]. Scatter diagram (A) which displays data for all cardiac surgery shows a divergence of the lines of best fit at durations between 40 and 60 minutes. This is suggestive of a possible cardioprotective benefit of CsA in cardiac surgeries with more prolonged durations of XCT. Interestingly, after the removal of surgeries involving

the mitral valve, this benefit was no longer present. While searching for a plausible explanation for this, it is important to remember that the correlation between the variables of XCT and cardiopulmonary bypass time (CPBT) is not straight-forward. It is very difficult to separate the negative impact of these variables from the effect of the patient's own individual risk, the expertise of the surgeon, the anaesthetist and the influence of post-operative care.

The scatter diagrams C and D reflect the expected observation of a positive correlation between the total CK-MB AUC over 72 hours and the XCT. Scatter diagram C shows an early divergence of the lines of best fit, alluding to a cardioprotective benefit of CsA. However, as with troponin T, after the removal of mitral valve surgeries, this protection is no longer seen.

The positive correlation seen in the scatter diagrams (E) and (F) reflect the findings by Nissinen and colleagues who showed that in fact it is the CPBT that has more of a significant impact on long-terms outcome when compared with XCT.

The effects of lethal ischaemia-reperfusion injury extends beyond the myocardium, affecting other major organs including the kidneys, liver, brain and gut (see introductory chapter). It contributes significantly to acute kidney injury which affects 30% of patients undergoing cardiac surgery, with up to 2% of patients requiring renal replacement therapy[489]. In addition, changes in serum creatinine level >0.5mg/dL after cardiac surgery can contribute to an increase in 30-day post-operative mortality. We looked for an indication of a reno-protective effect of CsA through measurements of serum creatinine level and urine output post-operatively. Our results did not show any evidence of reno-protection. One patient in the control group

suffered grade 2 AKI while there were no patients in the treatment group that suffered AKI > grade 1.

There was a significant reduction of ITU stay in the CsA group when compared to the treatment group. It is difficult to discern whether this difference can be attributed to an organ-protective effect as there are other factors that impact on ITU duration including experience of post-operative care nursing staff and bed availability.

In summary, this study did not show a cardioprotective benefit of CsA in the setting of cardiac surgery. However, it exposed the challenges involved in setting up a multi-centre randomised control trial and went some way to address future considerations in the administration of CsA in the surgical setting. It begs the question as to whether the therapeutic level of CsA can be considered adequate enough for mPTP inhibition in this setting. This study was limited by its sample size and so it cannot be categorically stated that CsA is ineffective in cardioprotection in the setting of cardiac surgery. As we continue to study this heterogenic group, we are now more informed from this preliminary analysis about the challenges involved in implementing protective strategies over-and-above pre-existing measures.

4.7.1. Limitations of the study

The anaesthetist and the surgeon were blinded to the study protocol; however, for safety, the administrator was aware of which patients were receiving the control sample or the treatment sample in order to anticipate and potentially initiate early treatment for anaphylaxis.

The doses of volatile gases were not strictly controlled and the choice of volatile gas was left for the anaesthetist's discretion. In most cases, isoflurane was avoided as it is a known preconditioning agent.

This study remains limited due to its sample size and as such, recruitment is still ongoing.

CHAPTER 5

5. Remote Ischaemic Preconditioning in the Setting of Complex Cardiac Surgery

5.1. Introduction

After successfully demonstrating the protective effects of remote ischaemic preconditioning in the setting of CABG surgery[490], the next logical step was to investigate whether a similar level of protection could be seen in a higher risk patient group where the level of myocardial injury is likely to be increased. In the previous chapter, we investigated the cardioprotective effects of cyclosporin A in all adult cardiac surgery and a positive linear correlation was shown between the total troponin T AUC and the aortic cross-clamp time. Following this, it seemed highly suggestive that the maximum cardioprotective effects of RIPC, which targets the entire RISK pathway as well as the mPTP, would be best seen in cardiac surgeries with prolonged aortic cross clamp times. To date, studies that have looked for the cardioprotective effects of RIPC in the setting of CABG surgery, in general, tended to have relatively shorter aortic cross-clamp times. Some authors have since emphasised the need for researchers to focus mainly on complex cardiac surgery as it is these high risk patients that are likely to benefit from novel cardioprotective strategies. The advancement of routine cardioprotective strategies along with a focus on a lower risk patient group may go some way to explaining the inconsistencies in results that has so far been seen in recent proof-of-concept studies.

The aim of this sub-study was to investigate whether patients who underwent complex cardiac surgeries with aortic cross clamp times greater than sixty minutes were more amenable to protection with RIPC.

5.2. RIPC procedure

Remote ischaemic preconditioning was induced by transient upper limb ischaemia in patients randomised to receive the intervention. This was achieved by inflating the blood pressure cuff to 200mmHg in the upper arm for five minutes and then deflating the cuff for five minutes. This cycle was repeated to achieve a total of three cycles.

The protocol was applied to the left arm whenever possible. However if the arterial cannula that provides a more accurate measurement of the blood pressure was placed in the left arm, then the RIPC protocol was applied to the right arm.

After induction, a 9cm blood pressure cuff was placed around the left upper arm. At this point an envelope containing a random computer generated number was opened to determine the allocation of the patient. The allocation determined whether the patient underwent the cycles of cuff inflation and deflation.

In the control group, the blood pressure cuff was placed around the left upper arm and remained deflated throughout the period allocated for the stimulus application. The operating surgeon remained blinded to the patient selection process thoughout the duration of the study but the anaesthetist was unblinded. Due to time constraints we were unable to modify the study protocol to address this issue but we recognise it as a limitiation to the study.

The decision for administration of inotropic support was made between the anaesthetist and the surgeon. Introduction of support was indicated if the operating surgeon identified poor contractility at separation of CPB or if haemodynamics were deemed to be unacceptable.

Efforts were made to standardise the criteria for extubation, ICU and hospital discharge critieria and post-op AF care as much as possible within the constraints of local hospital practice.

Figure 5.01: *Stills from a short film titled "The Good Heart Attack" produced by Uli Hesse and Dr Sean Davidson (of the Hatter Institute) as part of the Science on Film initiative run by the Wellcome Trust.* *http://www.wellcome.ac.uk/Achievements-and-Impact/Initiatives/Public-engagement/Broadcast-media-strategy/Science-on-fim/index.htm.*

5.2.1. Inclusion criteria for RIPC in complex surgery

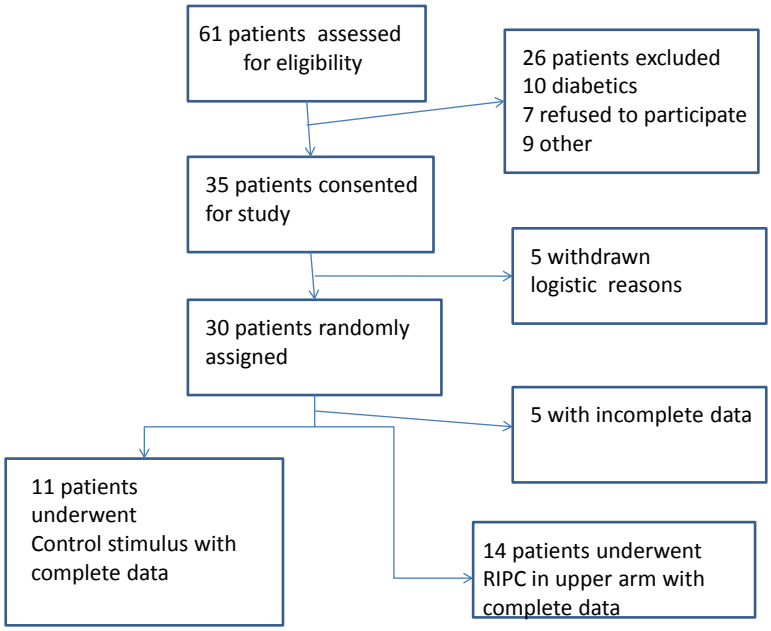
- Consecutive patients undergoing complex cardiac surgery (CABG + aortic valve replacement).
- Age 18-80 years.
- Informed consent.

5.2.2. Exclusion criteria for RIPC in complex surgery

- Patients older than 80 years.
- Moderate to severe renal impairment- eGFR< 45 ml/min/1.73sqm.
- Peripheral vascular disease.
- Diabetic patients on glibenclamide.
- Recent myocardial infarction (STEMI or NSTEMI within four weeks before the surgery).
- Angina within 3 days of elective surgery and patients on nicorandil.

5.3.1. Patient recruitment chart

Figure 5.02: *The patient recruitment strategy adopted for this sub-study.*



Twenty-six patients were excluded after initial assessment- 10 were diabetic, 7 refused to participate primarily due to bad experiences in previous trials they had been involved in; the remainder met the exclusion criteria.

5.4. Overview of methods

This was a single-centre, single-blinded, prospective, randomised placebo-controlled trial in patients undergoing complex cardiac surgery. Subjects were randomly assigned on a 1:1 basis.

Adult patients who were admitted for planned CABG surgery with concomitant valve surgery between January 2010 and September 2010.

Complex cardiac surgery was defined as coronary artery bypass graft surgery with concomitant aortic valve surgery with XCT > 60 minutes or redo cardiac surgery with XCT > 60 minutes.

Mitral valve surgery releases disproportionately high troponin T levels compared to those who undergo aortic valve surgery. This is largely due to the direct effect of surgical trauma when an incision is made in the left atrium to expose the diseased mitral valve. The results displayed below are of all patients who were subsequently deemed to be eligible for recruitment.

5.4.1. Study protocol

The protocols adopted for premedication, anaesthesia, perfusion, cardioplegia were all standardised (refer to chapter 3).

An ECG was performed prior to surgery and on post-operative day 1 and 4. This was done primarily to identify and potentially exclude patients who suffered peri-operative myocardial infarction. Peri-operative myocardial infarction was defined as any new left bundle branch block or new Q waves of 2mm in depth in two contiguous leads by day 3.

Continuous telemetry (ECG) monitoring was carried out six hours prior to surgery and continued up to 48 hours after surgery.

5.5. Results

Table 5.01: *Baseline characteristics and patient profile.*

DEMOGRAPICS	**Control**	**RIPC**
Age	68 (+/- 12.5)	68 (+/- 11.4)
Male **Female**	8 (32%) 6 (24%)	11 (44%) 0
Hypercholesterolemia	10 (38.5%)	7 (26.9%)
Hypertension	11 (42.3%)	9 (34.6%)
Diabetes mellitus	0	0
Previous MI	2 (7.7%)	1 (3.8%)
Previous stroke	3 (11.5%)	1 (3.8%)
Smoking history ***Current smoker*** ***Ex smokers*** ***Never smoked***	 5 (19.2%) 3 (11.5%) 6(23.1%)	 2(7.7%) 3 (11.5%) 6 (23.1%)
Family history of IHD	2 (7.7%)	1 (3.8%)
EuroSCORE	3.2	3.6

Key: RIPC- remote ischaemic pre-conditioning, MI- myocardial infarction, IHD- ischaemic heart disease

This sub-study is currently in its preliminary stages and during the writing of this thesis, recruitment was still ongoing. There was no difference in the average age of patients in both the treatment and the control groups. There were eleven male patients in the RIPC group compared with eight in the control group. There were obvious differences within the female patients with six in the control group compared with none in the RIPC group. There were five current smokers in the control group compared with two in the RIPC group. Both groups had an equal number of ex-smokers and those who had never smoked.

Table 5.01 continued: *Baseline characteristics and patient profile.*

Drug History	**Control**	**RIPC**
Anti-platelet	10 (38.5%)	10 (38.5%)
Anticoagulants	0	0
Beta-blockers	6 (23.1%)	7 (26.9%)
Ca^{2+}channel blockers	3 (11.5%)	3 (11.5%)
ACE inhibitors/ATiiRBs	10 (38.5%)	9 (34.6%)
Diuretics	0	0
Nitrates LA	0	1(3.8%)
Amiodarone	0	0
Statins	10 (36.8%)	8 (31.6%)
Oral hypogylcaemics	0	0
Insulin	0	0

Key: ACE- angiotensin converting enzyme, ATIIRB- angiotensin 2 receptor blocker, LA- long-acting.

There were no significant differences in the drug history of patients in the RIPC group compared with patients in the control group.

5.5.1. Baseline characteristics- intra-operative variables

Table 5.02: *Summary of the intra-operative variables in the RIPC sub-study.*

	Control	**RIPC**	**p value**
Aortic Cross clamp time (min)- mean +/- SD	78.30+/- 27.4	74.89+/-29.7	0.798
Bypass time (min) Mean +/- SD	110.90+/-33.8	102.4+/-34.7	0.598

Key: SD- standard deviation, RIPC- remote ischaemic pre-conditioning.

There was no difference in the mean aortic cross-clamp times between the control group (78.3+/- 27.4min) and the RIPC group (74.9+/- 29.7min) which was reassuring considering the small sample size. No difference could be seen in the mean bypass times between the two groups (110.9+/-33.8min vs 102.4+/- 34.7min respectively). When looking at the breakdown of the types of surgery performed, some heterogeneity could be seen which was largely due to the small sample size.

Table 5.03: *Summary of type of cardiac surgery performed within the two groups.*

Type of surgery	RIPC	Control	*P-value*
CABG	5 (45.5%)	6 (54.5%)	*0.539*
MVR	1 (100%)	0 (0%)	
AVR+MVR	1 (100%)	0 (0%)	
CABG+valve	5 (71.4%)	2 (28.6%)	
CABG+AVR+MVR	2 (50%)	2 (50%)	
Redo-surgery	0 (0%)	1 (100%)	

Key: RIPC-remote ischaemic pre-conditioning, CABG-, AVR- aortic valve replacement, MVR- mitral valve replacement.

5.5.2. Serum troponin T release

Testing for normality

The data was tested for normality in order to establish the appropriate statistical test to use. The study is still ongoing and the data collected so far is in its preliminary stages. It is anticipated therefore that the data would not be normally distributed and so non-parametric tests would have to be applied to compare the two study groups.

Table 5.04: *Descriptive statistics of study data.*

Descriptive statistics	Skewness	Kurtosis
RIPC	0.550	-0.249
Control	2.179	6.076

Key: RIPC- remote ischaemic preconditioning

Table 5.05: *Normality test.*

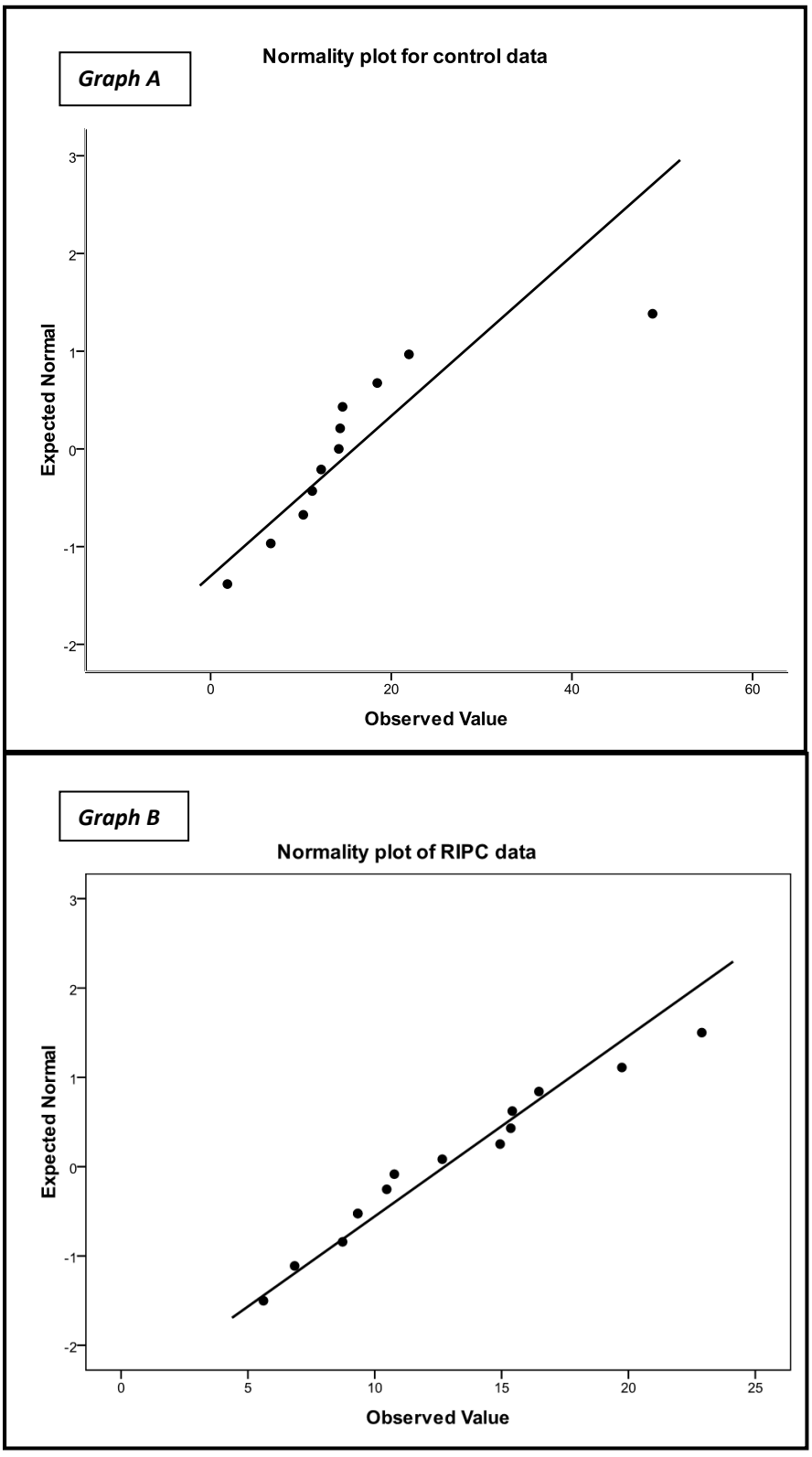
Study group	Shapiro-Wilk test
RIPC	0.685
Control	0.004

Key: RIPC- remote ischaemic preconditioning

Interestingly the RIPC data is normally distributed and this is demonstrated by a skewness of 0.550 (less than 1) and a kurtosis of -0.249 (less than 0). The control data was not normally distributed and as a result, non-parametric statistical tests were used to compare the two groups. I was also able to demonstrate homogeneity of variance between the two samples with a trimmed mean of 0.269. Please see Tables 5.04 and 5.05 above.

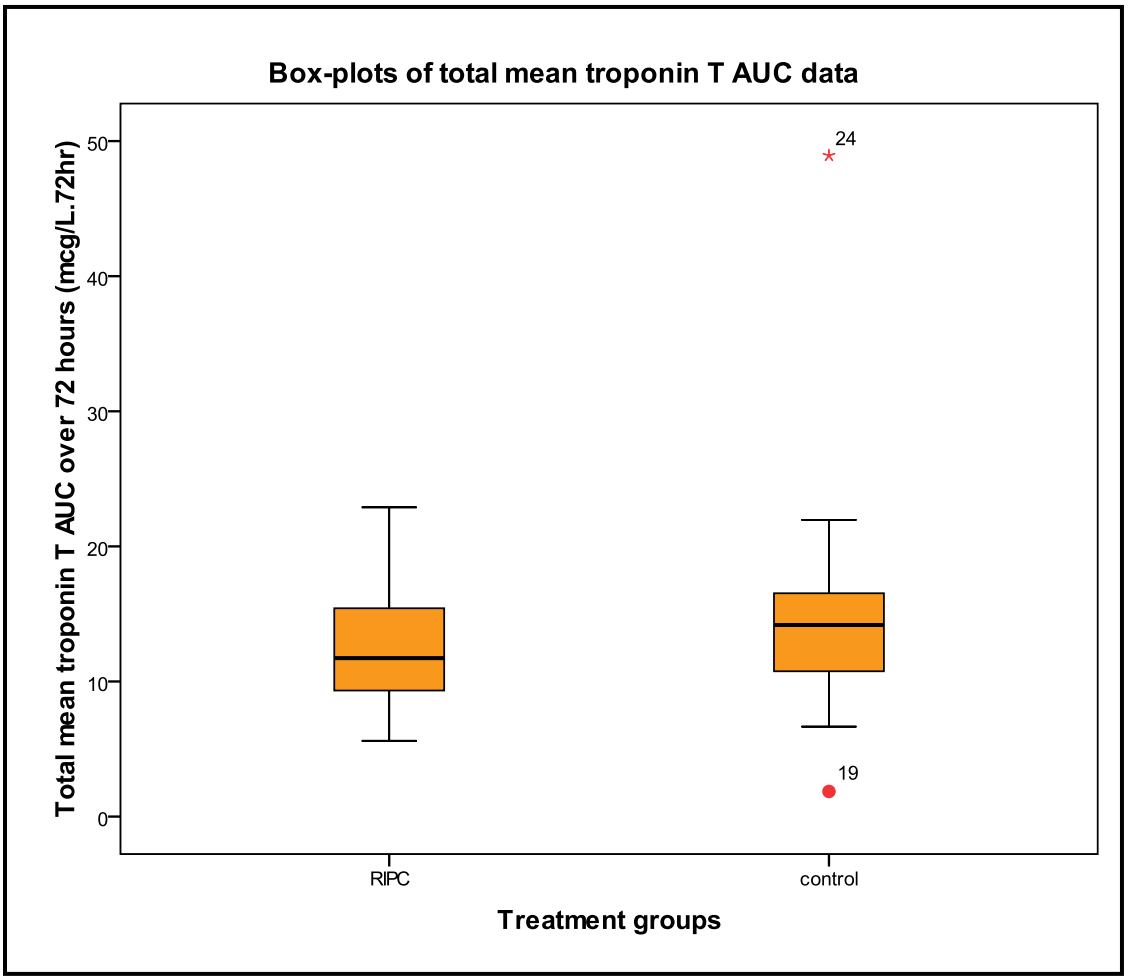
The data from both the RIPC group and the control group are displayed on normality plots and summarised on box-plots below.

Figure 5.03: *Normality plot for RIPC data.*



The graphs A and B above display the relationship between the expected normal and the RIPC and control data respectively. In graph B, a close relationship is seen suggestive of normality while in graph A, the relationship is less suggestive of normality as the plots do not lie as closely to the normality line.

Figure 5.05: *Box-plots of study data*



The box-plots above summarise the study data. There is some suggestion of a positively skewed RIPC data and a negatively skewed control data. Based on this, it was deemed appropriate to perform non-parametric tests as the main tool for comparing the two groups.

Figure 5.06: *The dot-plot diagrams A and B display the mean and standard deviation of the total troponin AUC over 72hrs in the RIPC group and the control group***.**

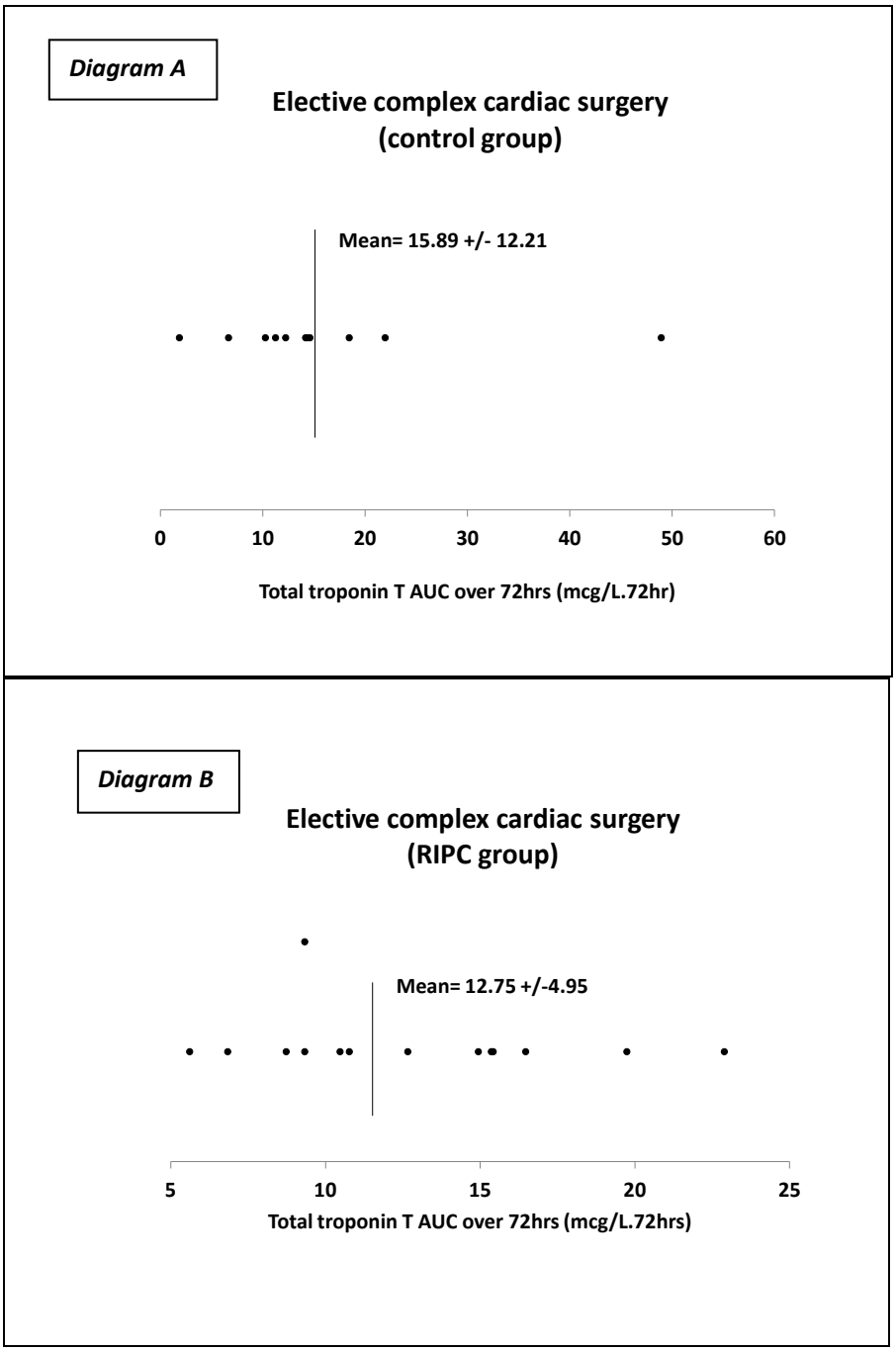


Table 5.06: *Application of non-parametric tests for total AUC troponin T over 72 hours (mcg/L.72hr) .*

Study group	n	Mean rank	Median	Sum of ranks	Mann-Whitney U	Z-statistic	Exact Sig. (2-tailed)
RIPC	14	12.57	11.72	176.00	71.00	-0.329	0.757
Control	11	13.55	14.19	149.00			

Key: RIPC- remote ischaemic pre-conditioning

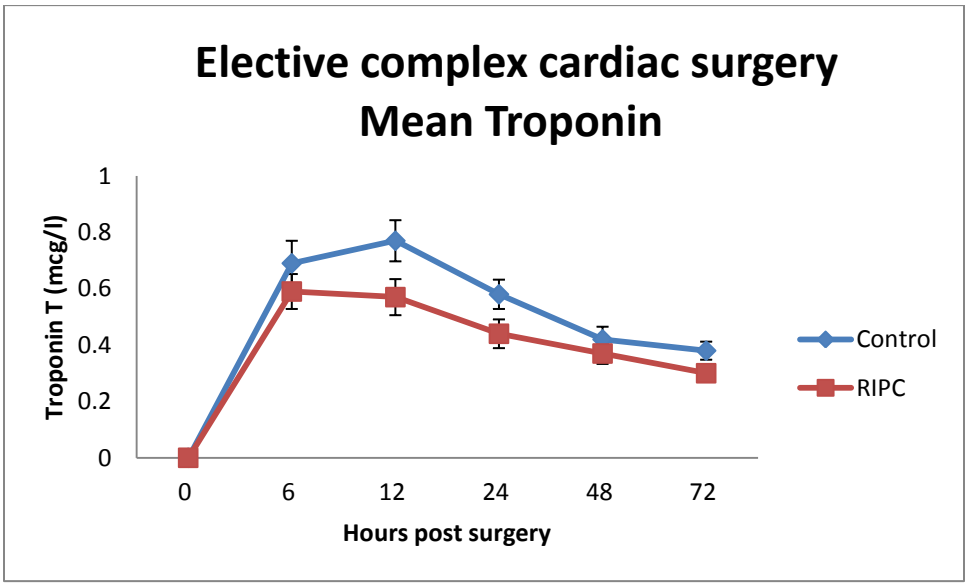
Non-parametric analysis of the study groups revealed a mean rank of 12.57 in the RIPC group compared with a mean rank of 13.55 in the control group. These values are much lower than would be expected for complex cardiac surgery with more prolonged aortic cross-clamped times contributing to an increase in myocardial injury. However as these results are preliminary, conclusions remain difficult to draw at this stage. The median in the RIPC group was 11.72mc/L.72hr which was less than that in the control group of 14.19mcg/L.72hr. The Mann-Whitney U value was 71.00 with a Z-statistic of -0.329. The 2-tailed exact significance value was taken as the sample size was less than 61 giving a value of 0.757 suggesting no significant difference at this stage between the 2 groups.

Table 5.07: *Serum troponin T levels at set intervals over 72 hours following cardiac surgery.*

Time (Hours)	Control	RIPC	Mean difference	CI	P value
0-6	0.69	0.59	0.10	-0.415 to 0.211	0.507
6-12	0.77	0.57	0.20	-0.667 to 0.261	0.376
12-24	0.58	0.44	0.14	-0.405 to 0.132	0.304
24-48	0.42	0.37	0.05	-0.254 to 0.159	0.641
48-72	0.38	0.30	0.07	-0.279 to 0.131	0.464

Key: CI- confidence interval.

Figure 5.07: *The mean total troponin T (AUC) over 72 hours.*



Furthermore, the mean troponin T at each time interval was compared between the group to identify whether there was a particular point post-operatively were protection was optimal. Although the the control group demonstrated a higher mean troponin T release over the entire 72 hour period post-operatively, there was no interval in which this difference was significant.

Serum CK-MB release

Figure 5.08: *The dot-plot diagrams display the mean and standard deviation of the total CK-MB AUC over 72hrs in the RIPC group and the control group***.**

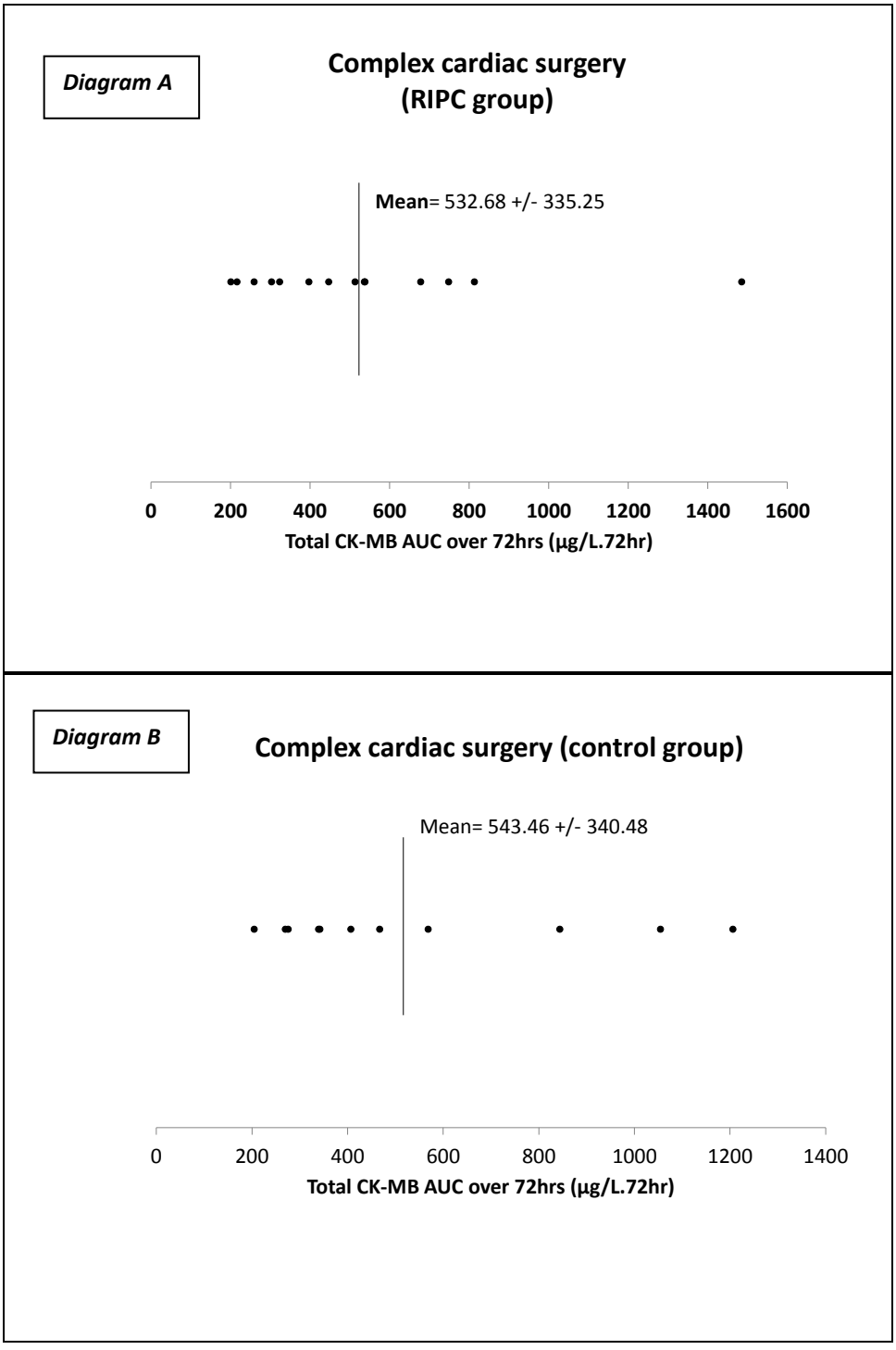


Table 5.08: *Application of non-parametric tests for total AUC CK-MB over 72 hours (µg/ml.72hr)*

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Study group	n	Mean rank	Median	Sum of ranks	Mann-Whitney U	Z-statistic	Exact Sig. (2-tailed)
RIPC	14	12.85	479.85	180.00	75.000	-0.109	0.936
Control	11	13.18	406.80	145.00			

Key: RIPC- remote ischaemic pre-conditioning

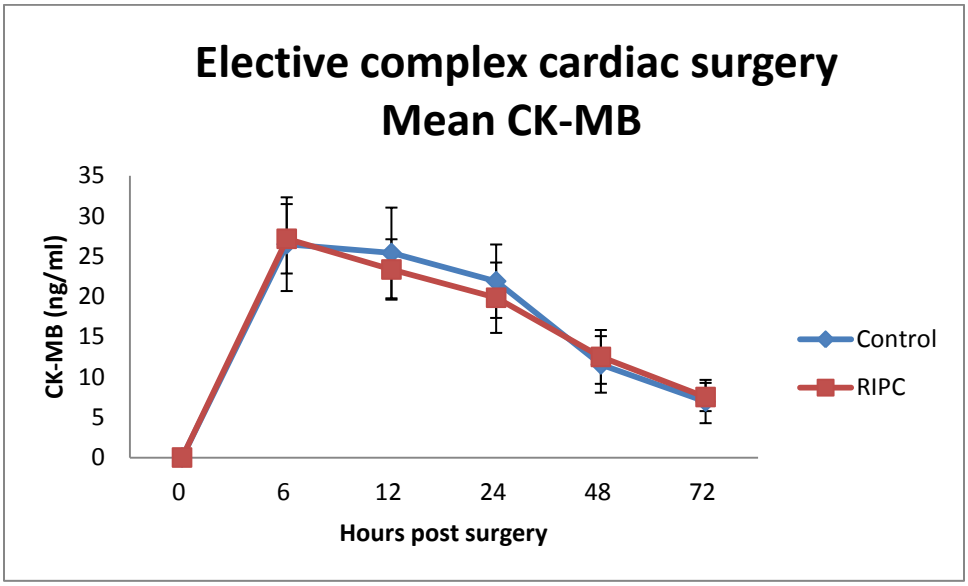
Comparatively, the dot-plot diagrams display a lower mean total AUC CK-MB of 532.68µg/ml.72hr in the RIPC group in contrast to 543.46µg/ml.72hr in the control group. However the RIPC group had a median of 479.85µg/ml.72hr compared with 406.80µg/ml.72hr in the control group. Attention must be drawn to the Mann-Whitney value of 75.000 and a Z-statistic of -0.109. Once again, as the sample size was less than 61, I adopted to interpret the data using the 2-tailed exact significance which revealed a value of 0.936. This suggests accepting the null hypothesis, so based on these preliminary results there is no difference between the two groups with regards to CK-MB release. Once again these results should be reviewed with caution as the study is still ongoing.

Table 5.09: *The mean serum CK-MB levels at set intervals over 72 hours following cardiac surgery.*

Time (Hours)	Control	RIPC	Mean difference	CI	P value
0-6	26.49	27.16	-0.67	-13.984 to 15.316	0.9
6-12	25.40	23.35	2.05	-15.555 to 11.455	0.756
12-24	21.89	19.84	2.05	-15.236 to 11.140	0.751
24-48	11.56	12.49	0.93	-9.191 to 11.053	0.851
48-72	6.95	7.51	0.56	-5.830 to 6.942	0.859

The mean CK-MB levels were calculated at set intervals over the 72 hour period post-operatively. There was no significant difference between the two groups, the results of which are graphically represented in the figure below.

Figure 5.09: *The level of CK-MB release at specific time points over a 72 hour period is shown in the graph below. These early results do not show any significant difference between the two groups.*



Post-operative secondary outcomes

Table 5.10: *Post-operative secondary outcomes.*

	Control (n=14)	RIPC (n=11)	P value
Treated AF	5(35.7%)	4(36.4%)	
New onset LBBB	(0%)	(0%)	
New onset Q waves	(0%)	(0%)	
Postoperative IABP	0(0%)	0(0%)	
12-hours LCO syndrome	2(14.3%)	0(0%)	
Inotrope usage	2(14.3%)	0(0%)	
Vasoconstrictor usage	1(7.1%)	2(18.2%)	
Δ creatinine (day 0-3)	2.2 (-19.9, 24.3)	-4.1 (-39.3, 31.1)	0.589
ITU stay (days)	3 (2.1, 3.9)	2.8 (2, 3.6)	0.582
Ventilation (Hours)	5.7 (5, 6.4)	6.1 (5.6, 6.6)	0.165

Key: AF- atrial fibrillation, LBBB- left bundle branch block, IABP- intra-aortic balloon pump, LOC- low cardiac output, ITU- intensive care unit.

The electrocardiograms of patients in both groups were analysed 6 hours prior to surgery and for 48 hours over the post-operative period. There were no incidences of new-onset LBBB or Q-waves suggestive of peri-operative myocardial infarction. In the control group, 5 patients developed peri-operative atrial fibrillation requiring treatment with intravenous Amiodarone while this was noted in 4 patients in the RIPC group. Two patients developed low cardiac output syndrome in the control group compared with none in the RIPC group. The diagnosis was based on clinical findings supported by haemodynamic parameters. There were 2 patients in the control group who required inotropic support compared with none in the treatment

group. Vasoconstrictor usage was required in one patient in the control group compared with two patients in the RIPC group.

There were no significant differences between the two groups in the change in creatinine pre-operatively to day 3 post-operatively. No significant differences could be identified in the ventilation times and in the ITU stay in both groups. Once again, one must stress that no formal criteria was implemented to determine when patients were suitable for discharge. The results for the effects of RIPC on ITU stay should therefore be viewed with caution.

5.6. Discussion

Coronary artery disease accounts for fifty percent of all cardiovascular events[491]. It is the leading cause of disability and mortality worldwide and its impact on global economics through loss of productivity is staggering. Revascularisation therapy through coronary artery bypass surgery remains the mainstay of treating the severest form of this disease; however establishing reperfusion in an occluded artery inevitably gives rise to lethal ischaemia reperfusion injury (IRI), a phenomenon that results in further myocardial injury and cardiomyocyte death.

Reducing the burden of IRI in the setting of cardiac surgery has been the focus of this research chapter.

The idea of inducing sub-lethal doses of ischaemia in one vascular bed, and subsequently incurring protection in another vascular bed generated much excitement to researchers, as it avoids direct manipulation of the organ in question. First described in 1993 by Przyklenk and

colleagues, remote ischaemic preconditioning (RIPC) is favoured amongst surgeons when compared with IPC, mainly because the ischaemic protocol can be applied non-invasively to an organ or tissue distant from the organ being protected with minimal interruption to the operation. Przyklenk et al discovered that the size of the infarct that occurred as a result of occluding the left anterior descending artery for one hour in canine hearts was substantially reduced by the application of sub-lethal levels of ischaemia and reperfusion to the left circumflex artery 5 minutes prior to the index ischaemic insult.

Following this, researchers have shown using animal models that distant organs including the kidneys, intestine and the limb can indeed bring about cardioprotection with as high as a 65% reduction in infarct size.

Reassuringly, the magnitude of the protective effect of RIPC is thought to be similar to that of IPC. Furthermore, based on what we understand so far, the recruitment of collateral arterioles is not part of the mechanism of RIPC. Rather, a similar mechanism to IPC has been postulated, involving the role of membrane signalling receptors, pro-survival protein kinases and mitochondrial involvement with the mPTP and mitoK_{ATP} (see introduction for mechanism of RIPC).

Some of the most important proof-of-concept studies in this field have shown that RIPC can be effective in reducing the level of myocardial injury incurred during cardiac surgery[492-495]. Researchers have demonstrated cardioprotective benefits in children undergoing congenital heart defect repair using CPB with a reduction in troponin release and inotropic requirement after the implementation of RIPC to the lower limb[496]. In a more invasive approach to RIPC,

unilateral iliac artery clamping brought about a significant reduction in troponin release and renal injury in the setting of AAA surgery[497].

The significance of troponin release in the setting of cardiac surgery has been widely debated. Some have suggested that it does not represent myocardial injury- rather that this 'transient' rise in cardiac enzymes is a consequence of breaching of the sarcolemmal membrane with an increase in its permeability[498]. However the occurrence of low cardiac output syndrome post-operatively and the subsequent impact on intermediate to long-term post-operative outcomes has led some researchers to suggest that cardiac troponins an important predictor of mortality[499-501].

The aim of this study was to determine the effectiveness of RIPC in promoting protection in complex cardiac surgery. We also wanted to see if these benefits were reproducible.

Clearly, at this preliminary stage in the study, it is difficult to draw definitive conclusions based on the results. The prolonged aortic cross-clamp times resulted in a higher mean troponin AUC which is consistent with our hypothesis. However no significant difference could be seen between the two groups. Further analysis of myocardial injury with a comparison of the total CK-MB AUC over 72 hours did not show any significant difference between the two groups.

This study is still ongoing; however analysis at this interim phase has revealed some considerations as well as limitations that are worthy of mention.

Some researchers have suggested that troponin release reflects myofibrillar damage or myocyte necrosis or increased sarcolemmal permeability with leakage of cytosolic pools. It may

be that RIPC exerts its effect on necrotic damage but has a lesser effect on sarcolemmal leakage. Myocardial stunning post-CABG has also been implicated as a possible cause for a 'transient' period of myocardial injury that is largely corrected through the optimisation of haemodynamics with the use of inotropic support. What we know from studies looking at RIPC in the setting of PCI is that post-ischaemic stunning cannot be attenuated through 'conditioning'.

Previous studies have reported that classic ischaemic preconditioning reduced post-operative tachy-arrhythmias which occurred as a result of scarring, oedema or reperfusion. We did not find any significant differences between the treated group and the control group with regards to the incidence of treated atrial fibrillation in the post-operative 48 hour period. Further recruitment is required to delineate the true impact of RIPC on reperfusion fibrillation.

At this stage it is reasonable to consider whether a stronger stimulus is required to bring about protection. Ali et al subjected a larger muscle mass to the RIPC protocol while others have applied the cuff to the lower limb with good result[502]. Future studies should consider longer ischaemic durations applied to a larger muscle mass in the hope of optimising the cardioprotective effects of RIPC.

5.6.1. Limitations of the study

Logistically, we were unable to remove inadvertent bias from the anaesthetist's perspective. The implications of this limitation is indeed siginificant as the anaesthetist has an important role in affecting which cardioprotective modality is implemented through the choice of anaesthesia given. With the anaesthetist remaining unblended, he/she could choose to affect the results by adopting various anaesthetic protocols that may in turn have variable cardioprotective effects. However while trying to maintain the integrity of the study, this at times had to be balanced with the need to minimise the disruption to local practice and anaesthetic preference. Ideally the anaesthetist and the researcher should have been blinded to achieve true double-blind status. The single-blind status was maintained by having the protocol conducted within the anaesthetic room so the surgeon was blinded to the protocol.

Regarding discharges from ITU, a standardised criteria was needed to thoroughly assess whether or not this particular outcome measured was affected by the cardioprotective strategy. Once again, being conscious of the affect that the introduction of a new criteria would have on the working practices of health professionals, it was decided that the standardisation of ITU and HDU discharges would not be strictly adhered to and therefore best not implemented. The limitation to the study as a result of this decision is that the results must be reviewed with some caution.

The doses of volatile gases were not strictly controlled and the choice of volatile gas was left to the anaesthetist's discretion. In most cases, isoflurane was avoided as it is a known

preconditioning agent. This study is still at its preliminary stage and as such is significantly limited due to its sample size.

Despite the study limitations, much of this work holds value at least as a hypothesis generating study which can be used to inform the design of larger randomised control trials attempting to delineate the effects of RIPC in this patient group.

CHAPTER 6

6.1. The Challenges of Translation in the Pursuit of Clinical Cardioprotection

The burden of ischaemic heart disease cannot be overstated, with one in five deaths worldwide occurring as a result of cardiovascular disease[503]. The past five decades have witnessed an evolution in cardiac surgery, with the improvements of CPB and cardioplegia resulting in marked reductions in morbidity and mortality. Despite this however, peri-operative damage to the myocardium in the form of ischaemia-reperfusion injury still poses prognostic significance, and has been demonstrated through the elevation of cardiac enzymes and functional imaging modalities including the cardiac MRI.

This thesis explored two distinct strategies for protecting the heart against ischaemia reperfusion injury. The first strategy involved directly inhibiting the formation of the mitochondrial permeability transition pore by inhibiting cyclophilin D using cyclosporin A. We failed to produce a significant difference between the treated group and the control group with regards to a reduction in myocardial injury- however it must be noted that due to time constraints the study was under-powered. We have since applied for ethical approval for a substantial amendment which will allow for the increase in the number of patients recruited at King's College Hospital. As the study continues, it is our hope to definitively establish the cardioprotective efficacy of CsA in the setting of cardiac surgery.

The second strategy directly targeted the RISK pathway using the potent endogenous technique of remote ischaemic preconditioning. Prior to the conception of RIPC by Przyklenk et al[504] and the subsequent demonstration of Schmidt et al[505] that short episodes of limb ischaemia can

indeed bring about cardioprotection, the concept of 'conditioning' had been extensively researched with proof-of-concept studies predominantly focused on animal models. In this concluding chapter, I wish to explore the reasons for the failure to translate some of the positive results seen at the bench to the bedside. I will also take a brief look at some of the evidence for pharmacological pre-conditioning and conclude with a look at what lies ahead for these novel therapeutic applications.

It has been widely reported by researchers that the application of short episodes of non-lethal ischaemia and reperfusion to the heart itself or to an organ or tissue away from it can bring about a reduction of infracted tissue by 40-50%. The cardioprotective benefits have been present when the conditioning stimulus has been applied prior to, at or after the lethal ischaemic event (reviewed by Levi)[506].

Isolated, in vitro, buffer-perfused animal hearts are the current models used by most researchers in the field. The hearts are subjected to regional and global ischaemia following which, cardiac enzymes, cardiac function and infarct size can be measured[507]. The benefits of the in vitro model are that it is largely reproducible, robust and allows researchers to have more control over the application of treatment strategies. In contrast, the 'in vivo' model is certainly more technically demanding. However it holds an advantage in its utilisation of an intact nervous and circulatory system[508]. These models provide some insight into the mechanism of IRI but they do not compare to the complexities of the human biology of disease seen in cardiac surgery or AMI.

Infarction in the animal model is usually induced by external compression of an otherwise healthy artery while in a patient with AMI, the rupture of an unstable atherosclerotic plaque

followed by platelet activation and aggregation and thrombus formation is responsible for coronary occlusion. It is also worth mentioning that the inflammatory response that follows cannot be easily emulated in experimental models despite various attempts at doing so.
The long-term effects of cardiac remodelling, infarct size reduction and mortality remain undetermined in the experimental setting. However, imaging modalities such as the echocardiogram and the cardiac MRI can provide information on cardiac indices that may be of clinical relevance.
One of the ways in which researchers have tried to mimic human pathophysiology has been through the promotion of thrombus formation directly within the coronary artery. Ferric chloride, an endothelial irritant has been used to activate the clotting cascade[509]. Other methods have included: Rose Bengal, green light laser agitation, a high cholesterol diet and partial surgical ligation. These methods are challenging to perform and the best among them still fail to mirror the human scenario[510].

6.2. The impact of cardiovascular risk factors on cardioprotection

The typical patient with established coronary artery disease is usually an elderly male with one or more cardiovascular risk factors. Ageing is associated with a decrease in the protective effects of ischaemic conditioning in animals as well as humans. The effects of RIPC and pharmacological conditioning have also been shown to decline with age[511]. Other studies have suggested that the protection is preserved[512]. What remains certain is that ageing is an unavoidable variable which is likely to impact on the effectiveness of 'conditioning' in adults with IHD. It is likely that the threshold for protection is increased in this group of patients-

therefore protocols providing a stronger stimulus, for example the use of the lower limb, is one way for future studies to address this issue.

Diabetic patients seem to show a reduction in the cardioprotective effects induced by conditioning. This is thought to be related to the effect of a hyperglycaemic state on the K_{ATP} channels, acting effectively, along with oral hypoglycaemic agents to abolish cardioprotection[513].

While non-smokers display a significant increase in forearm blood flow and acetylcholine release after preconditioning, this response is absent in smokers possibly due to the presence of endothelial dysfunction[514]. Similarly, the attenuation of cardioprotection by preconditioning has also been shown to occur in the presence of hypertension[515] and hyperlipidaemia, with the later suppressing the opening of mitoK_{ATP} channels [516]. With the majority of patients with IHD who could benefit from cardioprotective strategies also presenting with metabolic syndromes that attenuate these protective effects, it is clear that a stronger stimulus must be implemented in adult patients. In fact, our institution is currently looking at applying remote ischaemia preconditioning in the upper and lower limbs using x2 10 minute cycles.

6.3. Evidence of pharmacological preconditioning in ischaemia-reperfusion injury

The use of pharmacological conditioning has so far not been addressed in this thesis. However, its role in preconditioning is worthy of a mention as it remains an area of important ongoing research.

Calcium channel blockers, nitroprusside and adenosine are all used in the treatment of 'no-flow' phenomenon. Studies have shown calcium channel blockers to improve blood flow in both the animal model[517]and in humans[518]. In a small study looking at 98 patients presenting with STEMI, nitroprusside showed no significant benefit in its primary end point of >70% resolution if ST-segment resolution when compared with placebo but showed a significant difference in six month mortality[519].

Adenosine has experienced widespread use in the setting of 'no-flow' phenomenon. Due to its vasodilatory effects on the microcirculation, it improves myocardial blood flow and preserves endothelial integrity[520]. Adenosine receptors are involved in the conditioning of the heart (see chapter 1). The receptors are sensitised during the preceding ischaemia and this in turn facilitates their activation by agonists released during conditioning. Adenosine has both anti-platelet and anti-inflammatory effects and brings about cardioprotection through the opening of ATP-sensitive potassium channels. It has also been shown to replenish high energy phosphate stores in the endothelial and myocardial cells; promote preconditioning; improve microvascular function and inhibit ROS formation. The AMISTAD-2 trial looked at 2118 patients presenting with anterior STEMI treated with either PPCI or thrombolytic therapy. The treated group demonstrated a reduction in infarct size at high doses of adenosine compared with placebo. However there was no difference in the primary end point of heart-failure and death[521]. The overall data is more in favour of ischaemic preconditioning when compared with pharmacological preconditioning, a finding likely to be due to the action of multiple mechanistic pathways acting synergistically.

6.4. What lies ahead for therapeutic applications

The landmark proof-of-concept study by Piot and co-workers[522] is the first to demonstrate the cardioprotective effects of CsA in STEMI patients. It highlighted the role of the mPTP as a critical mediator in IRI and confirmed the existence of this injury in man. As a result of this study, a large multi-centre randomised control trial is now underway to determine whether CsA, administered as adjuvant therapy with PPCI can improve clinical outcomes in patients with IHD presenting with STEMI. Furthermore, the role of CsA as an mPTP inhibitor could be investigated in the settings of elective and urgent PCI to determine its effect against peri-procedural injury. The immunosuppressive effect of CsA is readily utilised in cardiac transplantation but its effect against IRI presents a further setting for investigation.

The question still remains as to how best to maximise the preconditioning stimulus in order to overcome cardiovascular risk factors which tend to attenuate cardioprotection. Possible directions of study include: the combination of IPC and pharmacological preconditioning; the combination of per-conditioning with preconditioning and increasing the ischaemic burden in RIPC by using both the upper and lower limbs to increase the preconditioning stimulus. Our institution is currently conducting a multi-centre randomised control trial to determine whether RIPC reduces the damage to the myocardium during cardiac surgery. It is anticipated that this study would address the pertinent question as to whether RIPC could improve health outcomes in terms of better patient survival and reduction in cerebrovascular morbidity.

With regards to the future of mPTP inhibition, the use of CsA is not without its potential adverse effects. The future would seem brighter after the development of safer, more selective mPTP inhibitors that could potentially benefit patients with IHD.

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