Trauma-Induced Secondary Cardiac Injury

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Abstract

Trauma-induced secondary cardiac injury (TISCI) represents an under recognised complication of severe injury with haemorrhage. A limited number of clinical studies have supported the development of adverse cardiac events, such as arrhythmia, in association with biomarker proven TISCI. Pre-clinical studies using small animal models have provided insights into potential mechanisms and key effector molecules involved in the development of TISCI, but there remains a general lack of understanding regarding the *in vivo* functional implications of this indirect cardiac injury resulting from trauma-haemorrhage.

This project aimed to investigate the implications of cardiac injury on myocardial systolic function. A robust, translatable model of TISCI was developed, which reflected the cardiac biomarker profile identified in clinical studies and, for the first time, demonstrated a significant, dose-dependent rise in Heart-type Fatty Acid Binding Protein (H-FABP) in response to trauma-haemorrhage. Non-invasive echocardiography was used to determine the acute myocardial response to injury and haemorrhage and also to assess the response of the left ventricle to resuscitation after an antecedent 60-minute period of trauma-haemorrhage.

The functional studies presented here have enabled real time visualisation of the impact of trauma-haemorrhage upon systolic left ventricular function over 1 to 6 hours, both with and without resuscitation. Having established the trends in *in vivo* systolic function over time, further studies were then conducted to test the combination of adenosine, lidocaine and magnesium (ALM) as a cardiovascular rescue agent in TISCI. ALM, as an adjunct to fluid resuscitation, has shown great promise as a therapeutic agent which improves haemodynamic outcomes, reduces the volume of resuscitation fluid required and favours survival in the murine model of TISCI.

Declaration

I hereby certify that the work described in this thesis is the result of my own independent investigation, except where otherwise stated. Any assistance received has been acknowledged in the text.

Publications and Presentations

Publications

Wilson, N., Wall, J., Naganathar, S., De'Ath, H., Brohi, K. (2017). Mechanisms involved in secondary cardiac dysfunction in animal models of trauma and haemorrhagic shock. Shock, 48(4): 401-410.

Naganathar, S., De'Ath, HD., Wall, J., Brohi, K. (2015). Admission biomarkers of trauma-induced secondary cardiac injury predict adverse cardiac events and are associated with plasma catecholamine levels. The Journal of Trauma and Acute Care Surgery, 79(1): 71-77

Presentations (Abstract)

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I am also fortunate to have a supportive family and I will finish by dedicating this thesis to the memory of my late father who passed away unexpectedly while I was carrying out this project. He is greatly missed.

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List of Abbreviations

A	Adenosine
AA	Atrial Arrhythmia
ACCU	Adult Critical Care Unit
ACE	Adverse Cardiac Event
AF	Atrial Fibrillation
ALM	Adenosine, Lidocaine and Magnesium
AMI	Acute Myocardial Infarction
BNP	Brain Natriuretic Peptide
BCI	Blunt Cardiac Injury
BPM	Beats per Minute
C	Celsius
CO	Cardiac Output
CAD	Coronary Artery Disease
CI	Cardiac Index
CSL	Crystalloid
CTL	Control
ECHO	Echocardiography
ECG	Electrocardiogram

EF	Ejection Fraction
ELISA	Enzyme-Linked Immunosorbent Assay
FS	Fractional Shortening
g	Gram
H	Haemorrhage
H-FABP	Heart-type Fatty Acid Binding Protein
Hr	Hour
HR	Heart Rate
ICU	Intensive Care Unit
IL	Interleukin
IR	Ischaemia-Reperfusion
Iso	Isoflurane
JV	Jugular Vein
ISS	Injury Severity Score
Kg	Kilogram
L	Lidocaine
LV	Left Ventricle
LVEDV	Left Ventricle End Diastolic Function
LVSF	Left Ventricle Systolic Function

M	Magnesium
MAP	Mean Arterial Pressure
MABP	Mean Arterial Blood Pressure
MI	Myocardial Infarction
Mins	Minutes
mL	Milliliters
mmoL	Millimoles
nmoL	Nanomole
mmHg	Millimeters of mercury
MODS	Multiple Organ Dysfunction Syndrome
MOF	Multiple Organ Failure
N	Number
NT-proBNP	N-terminal pro-Brain Natriuretic Peptide
Pg	Picogram
Resus	Resuscitation
Rpm	Revolutions per Minute
S	Sham
SB	Shed Blood
SD	Standard Deviation

SIRS	Systemic Inflammatory Response Syndrome
SOFA	Sequential Organ Failure Assessment
SV	Stroke Volume
SVT	Supraventricular Tachycardia
T	Trauma
TH	Trauma Haemorrhage
THS	Trauma Haemorrhage Shock
TISCI	Trauma Induced Secondary Cardiac Injury
TNF-α	Tumour Necrosis factor alpha
TnI	Troponin I
µL	Microlitre
vs	Versus

Chapter One

Introduction

Chapter One

1 Introduction

1.1 Trauma: The Nature of the Disease

Trauma is a globally prevalent, common disease with a wide severity spectrum, which typically, but not exclusively, afflicts the young. Injury is the predominant cause of death in adolescence to middle age with trauma being the leading cause of death in the 15 to 44 year old age group. Given the disease's propensity to affect the young, trauma is the leading cause of life years lost worldwide (WHO 2017).

Physical trauma results from penetrating or blunt injury sustained due to inter-personal violence, such as assault and stabbing, falls and more commonly, road traffic collisions (RTC). Major (sometimes referred to as complex) trauma, can be defined as serious injuries, affecting a number of body systems, which pose a significant risk of death or disability.

As with other common diseases, prevention is preferable to cure; however the nature of traumatic injury means that although there are opportunities to limit severity, such as legislative changes such as the introduction of laws relating to seat-belts and workplace health and safety initiatives, we are unlikely to eradicate the condition.

There are multiple phases in the progression of this disease, commencing at the scene of injury. These phases can be regarded as offering different points of opportunity where appropriate intervention is vital in limiting severity and ensuring improved patient outcomes.

Clinical management of trauma patients aims to restore meaningful life after injury. Trauma research is vital to equip clinicians with the techniques and expertise required to manage

complex patients with life-threatening physiological derangement in order to optimise patient outcomes.

1.1.1 Epidemiology

Trauma is the causative factor in millions of deaths annually internationally and is the sixth leading cause of death worldwide (Soreide K, 2009). In England and Wales, 12,500 people die due to physical injury every year according to data released by TARN in 2016 (Trauma Audit and Research Network, UK). The magnitude of the public health challenge becomes clearer when one considers that for every death due to trauma, there are two survivors left with life-changing disability (NICE 2013).

1.25 million deaths annually worldwide are attributable to RTCs and road traffic injuries are the leading cause of death in the 15-29 year old age group. Males are more likely to be involved and 73% of all RTC related deaths occur in men under the age of 25 (WHO 2017).

Death related to road traffic collisions remains the leading cause of death in the people aged between 15 and 44 years of age in the United States and the fourth leading cause of death overall when all ages are considered (CDC 2015). In 1990, road injuries and interpersonal violence claimed 73,680 lives in the U.S. There has however been a decline in these figures with 12,650 fewer deaths due to these modes of injury (GBD 2013, published in The Lancet).

The trends seen in the U.S may point to improvement, but regardless of these figures, injury still kills significant numbers of people annually and survivors face challenges relating to morbidity. Globally, deaths resulting from injury represent 8.8% of all mortality; a figure which, incidentally, has not changed since 2000. Road traffic collisions are the leading cause of deaths when unintentional injury is considered (WHO 2015).

The WHO reports on the burden of diseases globally. The mortality and morbidity related to disease is quantified in terms of Disability-adjusted life years (DALYs) and RTCs were the leading cause of DALYs lost in 15 to 19 year old males (WHO 2015).

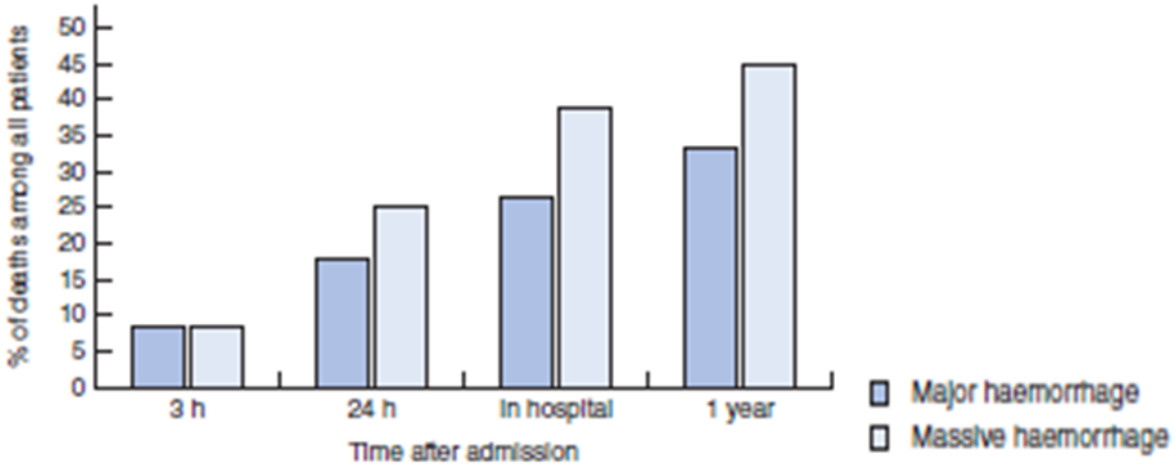
1.1.2 Death due to injury

Death as a result of injury can be defined as an acute, early or late event. Advances in the care of injured patients mean that more patients are surviving to reach hospital. Some deaths, however, remain sadly inevitable. Central nervous system (CNS) injury, such as catastrophic brain or spinal cord injury, obviously lead to rapid, predictable fatality.

Uncontrolled haemorrhage with resultant exsanguination is also a cause of acute and 'early' death (defined as being within 3 to 7 days of sustaining injuries) and CNS injury, not severe enough to cause instant death, along with uncontrolled bleeding, represents another cause of early deaths (Sauaia *et al* 1995 and Kauvar *et al* 2006).

A prospective study carried out by 22 major trauma centres and smaller trauma units in the United Kingdom demonstrated the prevalence and outcomes associated with trauma and major haemorrhage (Stanworth *et al* 2016). Over the duration of the study, 442 patients had injuries severe enough to trigger the activation of a massive haemorrhage protocol. Patient outcomes were poor with high early mortality. Two thirds died within 24-hours and of these, 50% did not survive beyond 4 hours of their arrival to the emergency department. The death rates were highest for those patients requiring a 'massive' blood transfusion (defined as being the administration of 10 or more units of packed red blood cells over the first 24 hours of admission). At 1 year after presentation, one third of patients had died (Fig 1.1).

A



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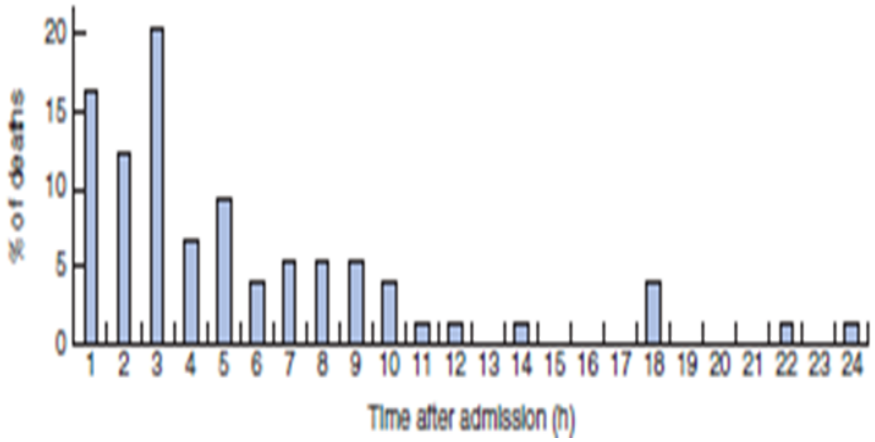

Figure 1.1. Mortality associated with major and massive haemorrhage.

A. Mortality with major and massive haemorrhage at 3 hours, 24 hours, in-hospital and at 1 year. B. Mortality within the first 24 hours of presentation with major haemorrhage. Patients receiving at least 4 units of packed red blood cells (PRBC) were eligible and defined as experiencing 'major' haemorrhage. Massive transfusion was defined as 10 or more units of PRBC being administered within the first 24 hours (Figure adapted from Stanworth *et al*, 2016 and used with permission).

Study data was used to estimate the national incidence of major trauma-haemorrhage and associated mortality. England and Wales were estimated to have 5000 patients with major trauma-haemorrhage annually and one third of these will die as a result of their injuries.

Life-threats change over the hours and days of a patient's admission. Multiple organ failure (MOF) after trauma has been shown to occur in a bimodal distribution in survivors. The first peak is seen in the 1st 3 days after injury with the second being seen at 5-7 days. (Durham *et al* 2003). MOF is the most common cause of late (after 7 days) deaths after trauma (Sauaia *et al* 1995).

A study by Probst *et al* in 2009 analysed patient records spanning up to 3 decades after initial injury. Cardiovascular disease (including arrhythmia) accounted for the majority (37%) of late deaths and trauma survivors had a significantly higher mortality compared to an age and gender matched cohort during the first 12 months after injury. This study gives some insight into the cause of death in trauma patients in the months and years following injury, but this is otherwise a relatively overlooked area of research.

1.1.3 The impact upon the individual

Personal health and well-being may be affected in ways apart from the more obvious outcomes of death and disability. Exposure to violence for example, can increase the risk of smoking, illicit substance abuse, mental illness and suicidality (WHO 2017). The health implications associated with trauma can therefore be wide ranging and chronic. There is also an association with crime and importantly, further acts of violence thereby creating a cycle of violence which generates further victims. In a retrospective study of trauma survivors, 19% of those surviving to be discharged later died due to a second major trauma (Probst *et al* 2009).

Trauma survivors have experienced significant psychological as well as physical upset. They may face significant changes in their social standing and personal relationships due to their loss of 'health'. Scarring and loss of limbs may result in issues surrounding self-image (perhaps particularly relevant in societies which place a great emphasis on physical

appearance). Self-esteem may also be negatively affected if survivors are unable to return to work or follow career paths which had been previously open to them before their injury.

A study of functional outcomes after trauma used standard measures of functional well-being and found that 16% of the trauma survivors questioned reported a reduction in well-being after hospital discharge (Kauvar *et al* 2006).

A study by Probst *et al* in 2009 looked at late deaths after severe trauma (ISS of 16 or more) and reported that 10% patients surviving to be discharged from hospital subsequently went on to commit suicide.

The American Trauma Recovery Project (TRP) was a prospective study which evaluated multiple factors, including functional and psychological outcomes up to 18 months after severe injury. The study employed a standardised quality of life measure (QWB scale) and revealed that at the 12-month follow up point, only 18% of patients questioned reported restoration of normal functional outcomes and when reassessed at 18 months, there had been no significant improvement in their situation. The length of admission to critical care as a consequence of injury was identified, along with post-injury depression and emergence of a post-traumatic stress disorder, as an independent predictor of outcome (Holbrook *et al* 1999).

As survival after injury improves, the physical and psychological sequelae of trauma and its impact upon rehabilitation and restoration of an acceptable quality of life should not be underestimated.

1.1.4 The impact upon society

Caring for injured patients is expensive. RTCs alone are estimated to cost most countries 3% of their gross domestic product (WHO 2017). Lengthy hospital admissions, critical care, surgery and rehabilitation are associated with significant costs to health services.

Trauma is, as discussed, a disease of the young and as such, injury can impact upon the financial contribution that younger citizens are able to make which can be thought of as 'productive years lost'. Family members may have to leave work in order to become carers. Other financial implications to society include costs related to legal considerations such as prosecutions and imprisonment. The Morgan-Jones report (2011) states that complex trauma costs the NHS £0.3 to £0.4m per annum with a long-term cost to the UK economy of in excess of £3.5 billion pounds per annum. European studies published in the 1990s reported that only 42 to 50% of trauma survivors were back in employment at 2 years. (Holbrook 1999 and Vasquez 1996).

The ability to restore meaningful, fulfilling, productive life after injury has important benefits, not just for the individual, but for society as a whole.

1.2 Trauma Research in the United Kingdom

In 1988 a working party chaired by Professor Sir Miles Irving at the Royal College of Surgeons of England concluded that a significant proportion of trauma deaths were preventable and related to inadequate delivery of medical care. A number of recommendations were made based upon this including improvement of pre-hospital care, rapid transfer of patients with the use of air ambulances and the auditing and research of injury and trauma systems.

By 1992, 33 UK hospitals had, by using an agreed methodology, analysed the effectiveness of their trauma facilities (TARN 2016). Results were published in the British Medical Journal, thereby disseminating the information to a wider audience and generating further interest in improving the management of trauma patients.

The Rand report (Morgan-Jones *et al* 2011) identified that trauma was an important but relatively poorly funded area when allocation of research funding in the UK was assessed. Less than 1% of total UK funding of health research was allocated to trauma research.

This relative underfunding by no means reflects the substantial need for improvement in the care of injured patients in the U.K. There is a need to broaden the horizons of trauma research in order to address the current issues in both the pre-hospital and in-patient settings and identify those points within the patient's journey where novel treatments and interventions can improve clinical outcomes.

1.2.1 Recent advances in the care of the injured patient

Improvements in trauma care can be regarded as clinical and organisational and the success of one is dependent upon success of the other. The Rand report (Morgan-Jones *et al* 2011) commissioned by the U.K's Department of Health, stated that there were 'fewer than five, and possibly no more than two, centres in the UK capable of handling major trauma cases'. There was a recommendation that the field was under-resourced, translational research was not occurring quickly enough, and 'clinical gaps' needed review and an assessment of whether current research were addressing these gaps.

The key findings of this report have been, in part, addressed and as of October 2016, the UK has 27 hospitals with major trauma centre status; 4 in London alone and a number specialising in paediatric trauma (www.nhs.uk). Major trauma centres offer 24 hour dedicated trauma teams, led by senior clinicians. An integrated, multidisciplinary approach involving departments as diverse as radiology and the blood transfusion laboratory, offers a co-ordinated approach to the management of the patient.

There is an emphasis on a protocol led approach to trauma patients with triage aimed at ensuring an appropriate response. An example of this is the declaration of a 'code red' in

response to on scene physiological derangement and preparation to transfer the patient to theatre as soon as possible after arrival. Cole *et al* in 2017 reported that the introduction of a co-ordinated trauma system in London had resulted in vast improvements in terms of access to hospital trauma specialists on arrival with higher rates of senior clinician involvement and improved rates of survival in critically ill trauma patients (Cole *et al* 2017).

There are many points on the trauma patient's journey which can be viewed as opportunities to intervene to limit mortality and morbidity. This starts at the scene of the injury. A retrospective analysis at a single centre over the course of 3 decades reported a significant reduction in transfer times of patients from scene to the definitive care centre with the use of helicopters (Probst *et al* 2009). The use of air ambulances such as the Helicopter Emergency Medical Services (HEMS) in the U.K, has led to swifter arrival of specialist teams with trauma-specific expertise with notable successes. The personnel on board the helicopters has not been the only positive factor; the availability of blood on board has allowed for newer strategies of initial fluid resuscitation of bleeding patients to be implemented in the first hour after injury.

The use of tranexamic acid represents another success story in trauma research. The CRASH-2 trial concluded that early administration of tranexamic acid significantly reduced all-cause mortality when given to bleeding trauma patients and tranexamic acid is currently included in code red protocols in the UK.

Transfusion protocols including high-dose fresh frozen plasma for major haemorrhage have been shown to confer survival benefit to patients, even within the first 3 hours of presentation to the emergency department when mortality rates are high (Stanworth *et al* 2016).

Identification of the major barriers to delivering effective trauma care and an appreciation of the need for research have been instrumental in making demonstrable improvements in the U.K's delivery of these services.

1.3 Organ Dysfunction after Trauma

Improvements in the acute management of injured patients have however been associated with a shift in the development of complications and associated mortality and morbidity. Improvements in pre-hospital care for example, mean that more people are surviving injuries which would have previously resulted in swift death at the scene. Such patients however have experienced severe physiological derangement necessitating aggressive treatment strategies. More may be surviving to reach a definitive care centre however, the majority will require prolonged resuscitation, immediate surgery, and critical care.

The patient may have survived the acute phase, but the issues may be best demonstrated with the finding that the incidence of multiple organ failure (MOF) after trauma rose over a period of 3 decades with improvement in acute and early pre-hospital and inpatient care (Probst *et al* 2009). Gains on one hand can be associated with losses on the other in the context of the physiological maelstrom of severe trauma.

Organ dysfunction and failure after trauma is a significant problem and multiple organ failure (MOF) is the leading cause of late deaths (Frohlich *et al* 2014). The incidence of MOF after trauma follows a bimodal pattern with the first peak occurring in the first 3 days, and the second peak in incidence at 5 to 7 days post injury (Kauvar *et al* 2006).

Multiple organ failure (MOF) is currently thought to affect 15 – 40% of severely injured patients, the incidence may vary in part due to the use of different assessment and scoring systems across centres and, perhaps more fundamentally, a lack of a single, accepted definition (Dewar 2014). A study of adult trauma patients admitted to intensive care revealed that 47% developed failure of multiple organ systems (with cardiovascular failure featuring in 85% of cases). Whereas failure of a single organ was associated with low mortality, MOF was shown to be associated with a 6-fold overall increased risk of death (27% of patients admitted who developed MOF died). MOF survivors had a nearly 4 times greater risk for requiring long-term assistance with personal care and daily activities on discharge from hospital (Ulvik *et al* 2007). The importance therefore of early recognition and prompt treatment to prevent and / or limit organ dysfunction and failure cannot be overemphasised.

Multi-organ dysfunction syndrome (MODS) is a newer term which recognises a continuous process of organ dysfunction in which the contribution of each organ can be variable rather than the 'all-or-nothing' model of multi-organ failure described in the 1970s.

It is important to recognise that failure of organs in this way after trauma is not a direct result of injury; acute kidney injury for example does not refer to direct renal injury with resultant loss of function. Visceral dysfunction after injury is not necessarily a consequence of mechanical disruption sustained by the organ but rather due to a culmination of physiology related to injury and the interventions undertaken to resuscitate and stabilise the patient. The precise pathophysiology of organ dysfunction after trauma remains poorly understood. There is evidence for the involvement of pro-inflammatory pathways, initiated at the time of injury to promote survival, becoming self-harming and mediating organ damage in the later stages (Tsukamoto *et al* 2010). The exact contribution of inflammation to organ dysfunction syndromes is a widely researched area and much remains unclear.

It is perhaps not heresy to speculate that trauma survivorship will plateau when the acute and early, preventable deaths are all but prevented but the organ failure and dysfunction experienced whilst on critical care and beyond contribute to later morbidity. There needs to be more focus therefore, on strategies to identify, prevent and treat organ injury occurring indirectly as a result of trauma.

1.3.1 Organ failure scoring systems and measures of cardiac outcome

Similarly to the adoption of scoring systems to grade injury severity (such as the Injury Severity Score or ISS), a number of scoring systems exist in critical care and indeed trauma research to identify and monitor organ outcomes after injury. Scoring systems have been in use for decades to study and predict patient outcomes. The Acute Physiology and Chronic Health Evaluation (APACHE) II and III, the Simplified Acute Physiology Score (SAPS) II have been widely adopted in critical care. The Sequential Organ Failure Assessment (SOFA) is a more recent, validated addition and the Denver Post-injury Multiple Organ Failure Score is also used to grade severity of MOF after trauma.

Different centres employ different scoring systems and each has its strengths and limitations. The basic premise however remains largely the same. Physiological parameters from the different organ systems are used to generate cumulative scores which indicate severity of organ dysfunction and associated mortality. Cardiovascular parameters are scored in each of the systems with heart rate and blood pressure recordings contributing to the scoring. The use of cardiovascular drugs such as noradrenaline also featuring as an indicator of severity of cardiovascular dysfunction and the level of organ support that is required by the patient.

1.4 Direct Cardiac Injury

When the heart is wounded directly, be it with a knife or as a result of an unrestrained driver accelerating forwards into a steering wheel, this is regarded as direct cardiac injury. Incised

wounds to the heart, such as would be experienced with a sharp, penetrating object, carry a high mortality rate and will require immediate surgical intervention in most cases.

1.4.1 Blunt cardiac injury

Direct, blunt cardiac injury (BCI) results from the transfer of energy to the thorax, of a magnitude that will deliver an impact to the myocardium. Such an injury may be 'silent' with no clinically appreciable outcome. In some cases however, the impact is enough to cause myocardial bruising (contusion), and more serious injury however may induce arrhythmia (Guan *et al* 2007, Ismailov 2007) which can lead to sudden cardiac death as in the case of commotio cordis. In some cases, the impact and energy transfer is enough to result in mechanical disruption of the heart with muscle rupture (Maron & Estes 2010).

Blunt cardiac injury has been demonstrated to be associated in a 2.6 fold increase in AMI in trauma patients aged 46 or younger and the development of AMI was independent of pre-existing coronary artery disease (Ismailov *et al* 2005).

In such cases, there is a suggestion that the acceleration-deceleration forces and direct blunt chest trauma experienced leads to damage to the intima of coronary arteries and, even in the absence of atherosclerosis, could precipitate clot formation and resultant myocardial ischaemia.

The treatment of blunt cardiac injury is mainly aimed at the complications which may arise. Mild contusions may instigate serial electrocardiograms (ECGs) and cardiac biomarker assessment with little else and generally are not a cause for immediate concern. Arrhythmia or heart failure arising as a consequence will obviously instigate more active management as they arise.

1.5 Indirect, Secondary Cardiac Injury

There is a relative paucity of clinical research into the trauma-induced secondary cardiac injury (TISCI) that occurs as an indirect consequence of injury. Most of the evidence for this organ injury and dysfunction has been reported from pre-clinical studies. Studies related to TISCI generally speaking investigate how the condition manifests clinically (for example the development of arrhythmia), how the myocardium demonstrates injury with the release of cardiac specific biomarkers or how cardiomyocyte damage is apparent on histological and post-mortem studies.

1.5.1 Autopsy and histology of cardiac injury after trauma

A number of studies have been conducted investigating the clinicopathological correlation between cardiac lesions and shock. Left ventricular abnormalities found at autopsy include sub-endocardial haemorrhages and the term ‘shock lesion’ has been applied to these in the past due to the association with shock states (Sevitt. 1970).

A retrospective study of 15 patients who had been subjected to physical assault and died during their original inpatient admission, was conducted in order to investigate any link between sudden death and the ‘stress’ associated with violence. Myofibrillar degeneration and contraction band necrosis was seen in 11 out of the 15 cases when cardiac histology was performed. This was interpreted as evidence for ‘stress cardiomyopathy’ and no similar histological finding was seen when compared with a cohort of age and cardiac disease matched controls. In 1 case, there was evidence that the patient had experienced arrhythmia during their admission. The researchers concluded that the findings were similar to those seen in animal models and represented catecholamine-mediated cellular changes initiated due to the assault (Cebelin *et al* 1980).

Despite the small size of the study by Cebelin *et al*, more recent work has also reported similar microscopic changes in the myocardium of trauma patients. After demonstrating the presence of calmodulin in myocardial tissue taken from a canine model of regional ischaemia, Yoshida *et al* went on to demonstrate that this early indicator of cardiac ischaemia and necrosis was also present in human tissue after death due to a variety of trauma mechanisms. They also supported the previous work of Cebelin *et al* with the identification of contraction band formation and myofibrillar degeneration, interpreted as being related to trauma rather than pre-existing cardiac disease. The most severe microscopic abnormalities were seen in a patient after RTC who was shocked and required 'intense resuscitation for 2 hours' before death. In this case, subendocardial haemorrhages were also noted (Yoshida *et al*, 1992).

The development of sub-endocardial haemorrhages in association with a number of conditions including trauma and haemorrhage have been recognised for some time. In the 1970s, a paper published in Forensic Science journal reported that particular areas of the heart, such as the sub-endocardium and papillary muscles, appeared to be at particular risk for the development of lesions. A 'general hypoxic cardiovascular injury' was described as occurring as a result of acute blood loss and progressing to cause complications in the longer term (Rajs. 1977).

A more contemporary, prospective post-mortem study has also reported the finding of sub-endocardial haemorrhage in victims of trauma. 125 hearts were assessed grossly and microscopically with the aim of determining the incidence and significance of myocardial lesions resulting from trauma (including burns). Patient survival ranged from 4 to 12 hours, to over 1 month. None of the hearts showed evidence of coronary pathology grossly, however a variety of microscopic abnormalities were noted in 25 of the hearts. These ranged from interstitial oedema and mononuclear cell infiltrates, to fibrosis and hypertrophy. 13.6% of

hearts examined demonstrated microscopic changes consistent with sub-endocardial haemorrhage with the vast majority of these being seen in patients who had experienced 'mechanical trauma' rather than burns. The development of sub-endocardial changes occurred early (nearly half of hearts examined in cases where the patient had died within 12 hours featured these changes) and in almost all of the cases where the heart had myocardial changes, hypotension had been a feature of the patients' clinical presentation (Gawande *et al* 2014).

The development of sub-endocardial haemorrhage, in the absence of direct thoracic trauma, is particularly interesting as it is the sub-endocardium that contains the specialised cardiomyocytes that form the Purkinje fibres of the heart's conduction system. One may speculate that the early onset of histological changes reported in this anatomical region may contribute to the development of later aberrant conduction. Unfortunately, the study had significant limitations, including the absence of data regarding severity of injury grade and incidence of arrhythmia and it is therefore difficult to fully establish the clinical relevance of the microscopic lesions detected. It is also unclear whether the development of sub-endocardial lesions in these cases were due to the initial injury or subsequent resuscitation attempts.

Post mortem studies such as the ones discussed here have considerable limitations. Firstly, they are typically retrospective and of small size. Post mortem histology can be difficult to interpret given tissue degradation and the clinical significance of any histological changes seen is of uncertain significance clinically. They often lack the details relating to severity of injury (such as ISS) and other clinical considerations and therefore it is more difficult to draw conclusions about the risks for the development of myocardial injury and how this manifests clinically. The administration of vasopressors, such as noradrenaline, may also contribute to ischaemic lesions due to coronary arterial constriction, but it is difficult to establish the role

of exogenous catecholamines in the development of these cardiac lesions. The limited number of studies however, report mutually supportive findings and do shed some light onto the timing and nature of the cellular damage experienced by the left ventricle as a result of trauma and haemorrhage.

1.5.2 Clinical manifestations of TISCI

One of the earliest studies relating to cardiac outcomes was performed retrospectively and took the form of a patient record and ECG review of patients admitted to critical care. It included 107 injured patients out of a population of 2,820 intensive care patients. Approximately 10% of the injured developed atrial tachyarrhythmias (Artucio *et al* 1990). A significant association between arrhythmia and mortality was not proven.

In 2011, Hadjizacharia *et al* reported the findings from a retrospective study spanning 7 years and designed to investigate incidence of and outcomes from atrial arrhythmias (including fibrillation, flutter and supraventricular tachycardia (SVT)) after trauma and in the critical care setting. 3499 patients were admitted over the study period and 6% developed an atrial arrhythmia (AA). The development of an AA was associated with older age, female gender injury severity and blunt trauma. AA was found to be an independent risk factor for mortality and if beta-blockers were administered to treat the AA, this was associated with a reduced mortality (Hadjizacharia *et al* 2011).

As well as arrhythmia, a link between injury and acute myocardial infarction (AMI) had previously been proposed. In a retrospective study of over 11,000 trauma patients, 19 developed an AMI and 5 were identified as experiencing an AMI truly related to injury. If AMI developed, it tended to be in older patients with previous comorbidities which predisposed to infarction (Moosikasuwan *et al* 2000).

The studies outlined above lend some support to the association between trauma and the development of adverse cardiac events (ACE) such as arrhythmia and MI but they do have significant limitations. Firstly, they are retrospective and involve a very small number of patients who actually developed the cardiac complication in question. Both studies focus on one specific ACE only and in the case of the 2000 study, patients who had experienced direct thoracic trauma were not excluded. Hadjizacharia's study included patients admitted to critical care for a variety of reasons and the trauma patients only represented a proportion of patients studied. The lack of detail including injury severity score and comorbidity for example also limits the utility of these studies in drawing conclusions about the association between non-cardiac trauma and incidence of ACE.

A larger study, although still retrospective, was published in 2005 and, involving more than 1 million American trauma patients, makes for more convincing reading (Ismailov *et al*, 2005). The risk of AMI after trauma was greatest in patients who had suffered direct, blunt cardiac injury (independent of pre-existing coronary artery disease) but injury to sites distant from the thorax such as the pelvis, was also associated with the development of ACE such as infarction. Coronary angiography findings confirmed that AMI was occurring via a non-atherosclerotic mechanism. The authors suggest that blunt trauma to the lower abdomen and pelvis may exert abnormal hydrostatic pressures leading to an increase in shear stresses which could potentially damage endothelium and in some cases, rupture coronary arteries. The study was the first to give evidence for trauma as a risk factor for AMI but did not comment on outcomes, incidence of other forms of ACE and, as a retrospective study, is subject to some of the limitations encountered in other clinical studies already discussed.

The following year, another study looking at atrial arrhythmia in critically ill trauma patients was published (Seguin *et al* 2006). The design of the study addressed some of the limitations of preceding work; namely that it was carried out prospectively, it looked specifically at

trauma patients rather than intensive care admissions as a whole, and more clinical information was available (such as injury severity and organ dysfunction scores). Some important findings were reported. These were that AA occurrence could be an indicator of the overall illness severity after injury and that the presence of systemic inflammatory response syndrome (SIRS), a recognised physiological occurrence after severe injury, was a risk factor for the development of atrial fibrillation (AF) in these patients. The severity of injury was also linked to the development of AF (over half of the patients who developed AF had 2 or more body regions injured).

Mortality rates have not been proven to be associated with the development of arrhythmia in the studies discussed. Despite this, the development of such a cardiac complication has significance clinically as it results in administration of drugs such as beta blockers, and could potentially lengthen a patient's stay in a critical care setting due to the need for continuous cardiac monitoring and treatment.

What is striking about the studies previously discussed here is that although functional cardiac outcomes were investigated following trauma, biochemical indicators of cardiomyocyte injury were not analysed as a method by which to detect and characterise cardiac injury and adverse events. Cardiac specific biomarkers have been an integral part of the work-up and monitoring of patients with acute cardiac events for decades. Point of care testing of troponins for example have provided a rapid, sensitive and specific means of stratifying risk of acute myocardial infarction for many years. An appreciation of the risk for cardiac injury in trauma has existed for a while and some critical care units routinely screen trauma patients for troponin rises in the 1st 24 hours after injury (Martin *et al* 2005).

In 2012 however, De'ath *et al*. conducted a retrospective study aiming to lend support to the existence of TISCI and investigate associated outcomes. 135 trauma patients requiring

intensive care admission were included. Data regarding injury severity, mechanism of injury, daily physiological data and survival were analysed.

The development of arrhythmia, AMI, angina, cardiogenic and cardiac shock were regarded as ACE. The study found that 13.3% of patients recruited developed ACE (most often SVT, with atrial fibrillation included within this classification) and these outcomes were more common in patients over 50 and those with pre-existing cardiac diagnoses. There was also an association between severity of injury and subsequent development of cardiac complications. Interestingly, 5 out of the 18 patients who developed ACE were under 50 years old and had no pre-existing cardiac history and were on no medication. This study also supported earlier studies with the observation that ACE could occur in the absence of direct thoracic injury. It was the first to perform biochemical analysis of well characterised cardiac biomarkers troponin I (TnI), brain natriuretic peptide (BNP) and heart fatty acid binding protein (H-FABP). Significantly higher admission biomarker levels were seen in those patients who developed ACE and in these patients, H-FABP and BNP remained elevated compared to patients without ACE at 24 and 72 hours. Patients who developed an ACE were more than twice as likely to die.

An association between the pro-inflammatory cytokine IL-6 and H-FABP was noted lending support for the previously postulated link between inflammation and secondary cardiac injury.

Following on from this study, De'Ath *et al* investigated the association between TISCI and inflammation in an attempt to elucidate some of the mechanisms underlying the cardiac injury previously reported to be associated with ACE and mortality. Cardiac biomarkers and cytokine levels were assessed at admission, 24 and 72 hours. They concluded that even at this acute stage after injury, there is an association between inflammation, cardiac injury and

ACE. Moreover, if cytokine levels AND cardiac biomarkers were both elevated on admission, there was a higher in-patient mortality rate. Therefore, this study lends support to the mechanistic theories regarding the contribution of post-traumatic inflammation to the development of TISCI which is associated with poorer clinical outcomes.

Naganathar *et al* in 2015 published the first prospective study investigating Trauma-Associated Cardiac Injury and Dysfunction, (TACID). This study confirmed the earlier findings from retrospective work regarding admission cardiac biomarker profiles and subsequent ACE development and increased mortality (Naganathar *et al* 2015). Again, 13% of trauma patients recruited developed an ACE with SVT (including atrial fibrillation) being the most frequent manifestation. Older patients tended to be at higher risk for experiencing an ACE but 50% of the ACE 'positive' patients were younger than 45 and with no history of cardiac disease. Nearly a third of those developing ACE were below 30 years of age. Patients with thoracic injury however were not excluded and indeed over half (59%) of the ACE group had experienced chest trauma.

Many of the studies discussed did not assess the clinical outcomes relating to ACE development. Naganathar *et al* however reported that there was an association between development of a cardiac complication and a longer critical care and overall hospital stay. The association between admission catecholamine levels and cardiac injury was also investigated and suggested that adrenaline (epinephrine) concentrations on admission were strongly associated with TISCI development.

Questions still remain regarding the underlying pathophysiology of TISCI and functional implications associated with the development of this complication; both in the acute and early stages after injury, longer term consequences and how cardiac injury and dysfunction actually contribute to the increased associated mortality and morbidity.

1.5.3 Cardiac biomarkers in TISCI

Cardiac specific biomarkers have been an integral part of the work-up and monitoring of patients with acute cardiac events for decades. Point of care testing of troponins for example have provided a rapid, sensitive and specific means of stratifying risk of acute myocardial infarction. An appreciation of the risk for cardiac injury in trauma has existed for a while and some critical care units routinely screen trauma patients for troponin rises in the 1st 24 hours after injury (Martin *et al* 2005).

It has been recognised for some time that cardiac biomarkers can be elevated in critically ill patients and this is not always a result of acute myocardial infarction resulting from coronary artery occlusion. (Lim *et al* 2006). A study involving 1081 trauma patients showed that Troponin I was elevated in 29% and a 'high' TnI (>5μg/L) was associated with a 44% mortality rate. Direct myocardial, or indeed thoracic injury however was not an independent predictor of raised TnI concentration. The authors concluded that elevated TnI post injury was related to overall burden of physiological disruption and not necessarily due to direct cardiac injury (Martin *et al* 2005). In another study, 35% of trauma patients admitted to critical care had elevated TnI in the absence of chest injury and elevated TnI was associated with hypotension and the development of MODS (Edouard *et al* 1998).

The development of cardiac complications after trauma-haemorrhage has been shown to be associated with blood levels of well recognised cardiac biomarkers such as H-FABP and troponin. De'ath *et al* in 2012 reported that serum biomarkers were elevated at point of admission to hospital and were associated with the subsequent development of ACE. For example, H-FABP was found to be 3 times higher in patients who later developed an ACE when compared to the levels of those who did not. H-FABP and BNP were detected early on in the admission of trauma patients with troponin rising later. 12 and 24 hour troponin levels were significantly higher in patients suffering an ACE compared to those who did not

experience cardiac complications. Not only was H-FABP found to be an early indicator of the risk of subsequent ACE, it was also found to correlate with the severity of injury with the highest levels being seen in those with the highest ISS. In 2015, Naganathar *et al* also reported the results of Trauma Associated Cardiac Injury and Dysfunction (TACID) study, a prospective study involving 300 trauma patients admitted to an urban major trauma centre. TACID echoed some of the findings from earlier work regarding biomarker levels and ACE incidence but also found that the development of ACE was associated with longer critical care and hospital admission and a 3-fold increase in hospital mortality rate (Naganathar *et al* 2015).

There are of course multiple factors to be considered when interpreting these results but these findings do lend weight to the argument regarding early development of cardiac injury and ACE after TH and also the utility of cardiac biomarkers in the both detection and risk stratification of patients for cardiac injury and dysfunction after TH.

1.5.4 H-FABP as a biomarker of TISCI

H-FABP is a small protein located in the cytosol of cardiomyocytes. It has a low plasma concentration under normal circumstances and it was first recognised as a marker of myocardial injury by Glatz *et al* in 1988. It is released rapidly during periods of cardiac ischaemia and has been demonstrated to identify AMI within 4 hours of symptom onset (McCann *et al* 2008). Immunohistochemistry studies demonstrated rapid decline in H-FABP from cardiomyocytes with reduced or absent immunoreactivity detected at 60 minutes of ischaemia (Watanabe *et al* 2003).

An autopsy study demonstrated that H-FABP was depleted from the myocardium of patients with sudden cardiac death (Kilcullen *et al* 2007), but the corresponding electron microscopy studies did not show features of myocyte necrosis. It can therefore be regarded as an early

indicator of cardiac ischaemia, even in the absence of established myocyte necrosis and in this way, can be regarded as superior to troponin in the detection of early myocardial ischaemia.

H-FABP is therefore particularly relevant as a biomarker in TISCI studies where regional ischaemia secondary to coronary occlusion is no necessarily present and therefore the associated myocyte damage may be more subtle. H-FABP has been described as an "excellent early marker" of myocardial injury in the context of heart failure and unstable angina in particular (Pelsers *et al* 2005).

H-FABP has utility not only in detecting acute episodes of ischaemia, but there is also evidence that it has a role in prognostication after cardiac ischaemia, being predictive of mortality post MI across a range of troponin concentrations (Kilcullen 2007). H-FABP has also been shown to identify patients at high risk of MI who were troponin negative (O'Donoghue *et al* 2006). H-FABP has also been demonstrated to significantly correlate with the development of adverse cardiac events and cardiac mortality (Nakata *et al* 2003).

Another potential advantage of H-FABP over troponin is the association of troponin with head injury. In a 2005 study, Martin *et al* assessed troponin levels in trauma patients admitted to critical care. They reported that patients with traumatic brain injury were more likely to have elevated TnI when compared to the remainder of the non-brain injured cohort (Martin *et al* 2005). This association with brain injury has not been demonstrated with H-FABP. A raised troponin level in a polytrauma patient with head injury may therefore be more complicated to interpret whereas an elevated H-FABP is less likely to be a result of brain injury.

Another potential benefit of H-FABP compared to troponins lies in the rapidity of its clearance after myocardial ischaemia. Levels typically normalise within 24 hours compared

to troponins which take longer to return to within reference range. H-FABP therefore would be useful in the early detection of subsequent episodes of myocardial ischaemia whereas these may be 'masked' by continuously high troponin levels (Pelsers & Glatz 2005).

There are some however some potential drawbacks to the use of H-FABP as a biomarker of TISCI. H-FABP has been shown to be expressed to a lesser extent by skeletal muscle (Pelsers *et al* 2005). Caution has to be taken therefore in interpreting the results when injuries include fractures and associated soft tissue damage are present as is indeed frequently the case in polytrauma patients.

Despite this potential limitation, H-FABP has shown real promise as an early biomarker of cardiomyocyte injury with resultant cardiac dysfunction after trauma-haemorrhage. This evidence however has remained largely in the clinical setting and there is a need for this to be validated in animal models of trauma-haemorrhage which could be used to further our knowledge regarding the functional implications of TISCI.

1.6 The Experimental Evidence for Trauma-Induced Secondary Cardiac Injury

There is a growing body of clinical evidence that supports the development of an indirect cardiac injury, occurring after a period of haemorrhagic shock and trauma, which culminates in impaired cardiac function and contributes to poorer outcomes and mortality after trauma.

Conditions of reduced tissue perfusion with subsequent reperfusion as well as the upregulation of inflammatory pathways are likely to coexist after severe injury and resuscitated blood loss. A combination of biological and physiological factors therefore, is likely to contribute to the development of this secondary cardiac injury. However, much remains unknown about the key mechanistic pathways in the development of this important

clinical outcome. The development of robust, translatable pre-clinical models of secondary cardiac injury will be critically important in the characterisation and treatment of the cardiac consequence of trauma and haemorrhagic shock.

1.6.1 Contemporary pre-clinical models of TISCI; methodology, mechanistic insights & limitations

A literature search was performed for studies relevant to cardiac dysfunction following trauma, haemorrhage or the combination of the two. A PubMed search was performed in 2013 using the search details ((“injuries” [subheading] OR “injuries” [All Fields] OR “trauma” [All Fields] OR “wounds and injuries” [MeSH Terms] OR (“wounds” [All Fields] AND “injuries” [All Fields] AND “haemorrhage” [MeSH Major Topic]) AND “heart” [MeSH Major Topic].

Applying these search criteria returned 205 abstracts. Abstracts published within the last 20 years were selected in order to capture a good range of studies and to gain an understanding of how models have changed and developed over time, particularly in a relatively rapidly evolving clinical setting. Studies investigating isolated brain or spinal injury were excluded, as were studies relating to burn injury. Each of the remaining abstracts were screened and only those deemed to be relevant were retrieved as full text articles. Publications not captured by the search criteria outlined above but having relevance to the scope of this literature review were also reviewed.

A search of the online database retrieved 205 abstracts, after screening, 31 of these were regarded to be relevant to this review. A further 21 abstracts were identified as having relevance to this topic and therefore have also been included. Certain authors have contributed several studies (for example I.R Chaudry’s team in the US) and there is therefore

a significant degree of overlap in terms of methodology and protocol design across the literature reviewed.

Species

The studies reviewed described the use of five species of animal, the largest animal used was a pig. The vast majority of the studies used a rat model. The availability of genetically modified mouse strains may have prompted the use of this species in studies investigating the mechanistic basis of cardiac injury, but mice were used in a minority of cases. Li *et al* in 2007 reported findings using a TNF-α "knock-out" strain to investigate the role of inflammation in the development of cardiac injury. 2 years earlier, Meng *et al* also used a genetically modified strain in order to demonstrate pro-inflammatory signalling pathways acting via toll-like receptor-4.

Rodent models dominate the literature. There is only one porcine model (Granfeldt *et al* 2012). The availability of genetically modified strains is one reason why these smaller animals are preferred and despite the genomic differences, smaller animals are invaluable in gaining insights into molecular and cellular mechanisms of trauma haemorrhage related cardiac pathophysiology. There are however, a number of limitations when using a mouse or rat when investigating cardiac dysfunction after trauma haemorrhage.

Technically, surgery and instrumentation are challenging in a small animal model requiring vessel cannulation. Blood volume is limited and this will influence the number of tests possible in a single animal. The small volumes available may also preclude repeat testing in the same animal over time making assessment of temporal trends difficult.

Baseline mean arterial blood pressure is similar across the species but resting heart rate is much higher in rats and mice and there are some structural differences in the cardiovascular systems of rodents compared to larger animals and humans who share similar anatomy and haemodynamic responses to blood loss. The small heart size and increased heart rate poses technical challenges for cardiac imaging such as echocardiography but the differences do not just impact on a technical front. Rodents are most active during the night and as such, their circadian rhythms are different to humans with higher nocturnal blood pressure during their active hours. There are differences in the action potential in rodents compared to humans with a lack of the plateau phase leading to a shorter action potential in rodents. There are differences in calcium flux with less reliance on the $Na^{+}/Ca2^{+}$ exchanger playing less of a role in rodents compared to humans and there are also differences in cardiac myosin isoforms with rodent hearts demonstrating a preferential shift towards the beta-myosin isoform under conditions of load change and / or hormonal influence (Hasenfuss. 1998). These physiological differences also need to be considered when selecting the most suitable species to answer the cardiovascular research question being posed but these differences, although important, have not been a barrier to using rodents in this type of research.

Animal models have been used to assess therapeutics with potential to prevent cardiac injury and / or dysfunction. In terms of therapeutic studies, due to the genomic differences between rodents and human, additional experiments in a larger animal may be required before conclusions about efficacy and safety can be made and this represents a drawback in the use of the smaller animals in therapeutic studies and as cardiac specific research moves forwards, larger animals may feature more often in the research.

Anaesthesia

There were a number of different anaesthesia strategies used in the papers reviewed here. 19 of the references implemented a protocol where a combination of inhalational and injectable agents were used for induction and maintenance of anaesthesia. In 16 of the references, intravenous or intraperitoneal drugs alone were used and 13 used inhaled gases. In one of the studies reviewed, it was unclear which anaesthesia strategy had been used. Isoflurane was the inhalational agent of choice in the majority of the studies. If an injectable agent was used alone, or in combination with, the inhaled agent, this tended to be pentobarbital.

A potential limitation of using an anaesthetic regimen which includes a short-acting barbiturate such as pentobarbitone relates to its relatively short half-life. As the drug is eliminated, 'top-up' doses may be required during the experiment. This has the draw-back of a variable and relatively uncontrolled depth of anaesthesia and, as a consequence, fluctuations in haemodynamic status and catecholamine release.

Isoflurane is a halogenated ether used for anaesthesia alone, or in combination with an injectable agent. As well as anaesthesia, it also has analgesic properties which make it an attractive choice for the injury models. The route of administration and short half-life mean that isoflurane can be easily titrated to produce a stable surgical plane of anaesthesia. Isoflurane can induce hypotension but is less cardio-depressive than other anaesthetic agents used in these models. This is obviously one potential benefit when conducting studies relating to cardiac performance.

Zou *et al* use ketamine as an anaesthetic agent in their rat trauma haemorrhage model. Ketamine has been deemed a favourable choice when anaesthetising trauma patients as it is less likely to cause hypotension. The addition of ketamine into the trauma models therefore may improve the translational potential. In the studies to date, ketamine is not used widely.

Another limitation of the animal models generally is the lack of medications routinely seen in clinical management of injured patients such as opiate analgesia. The inclusion of relevant drugs such as ketamine therefore, may answer such criticism.

Pre-Clinical Models of Trauma / Trauma-haemorrhage / Haemorrhage

Trauma is a complex, multi-modal disease. Pre-clinical models therefore have been designed in order to reproduce the complex physiology and biological conditions of haemorrhagic shock, injury or a combination of the two.

Trauma

In the majority of the experiments, injury took the form of soft tissue injury by means of a laparotomy. Guan *et al* in 2002 however, used a different approach and combined multiple fractures with blood loss in order to generate conditions of tissue ischaemia and inflammation. The fractures created varied in severity with the heaviest injury load comprising of bilateral femoral, tibial and humeral fracture in the same animal.

A modified Noble-Collip drum was used in a number of the studies (Wang *et al* 2013, Feng *et al* 2013, Yan *et al* 2010 and Tao *et al* 2005). This method was adopted when blunt, whole body trauma in the absence of haemorrhagic shock was being investigated. (Briefly, anaesthetised animals are placed in a rotating drum and the drum turned at a defined rate per minute in order inflict multiple, blunt force injuries on unrestrained animals as they repeatedly fall. Sham control animals are also placed in the drum but restrained to the inner wall so experience the revolution but without injury). The aim is to produce non-lethal mechanical trauma without concomitant circulatory compromise. Not all of the groups however, report on the appearance of the heart after the experiment and therefore it is not clear if direct cardiac trauma was sustained as a consequence of the technique used. None of the studies using this technique report any post mortem analysis being performed on the rest

of the cadaver and, apart from the information that the animals were alive at 24 hours, it therefore it is difficult to determine the exact injury severity and how reproducible this technique was.

As the purpose of the non-lethal trauma studies outlined above was to investigate secondary cardiac injury, thoracic trauma was avoided. Many investigators used laparotomy for the trauma component of the model. In a primate model designed to induce secondary lung injury however, a combination of tissue trauma, fracture and haemorrhagic shock were required (Pretorius *et al* 1987). Only 1 of the studies reviewed here included limb fractures in the protocol. A potential limitation of including fractures however, is the ability to recover the animal from anaesthesia and the addition of fracture to the study protocol can therefore limit the duration of the observation period. In the UK, animals are generally not recovered from anaesthesia if limb fracture without fixation has been performed. This type of model would require intensive animal monitoring and follow up and potentially additional home office approval. The addition of fractures to a protocol, although possible, will increase the severity of the injury and resultant inflammation, arguably increasing the translational potential for the model but, this will have its own challenges and limitations.

Haemorrhage

None of the studies adopt an uncontrolled bleeding protocol and there are two strategies used in order to induce haemorrhagic shock in the studies, namely, fixed pressure or fixed volume haemorrhage with controlled withdrawal of blood via an indwelling vascular catheter. Only 3 of the studies reviewed adopted a volume-controlled protocol (Meng *et al* 2005, Yang *et al* 2007 and Nachuraju *et al* 2011). In these experiments 30, 50 and 60% of estimated total blood volume was drawn respectively after performing a weight calculation in order to

estimate total blood volume. The remaining experiments, where blood loss was included in the study design, used a pressure-dependent protocol.

The most-severe form of haemorrhagic shock was described in the protocol of 4 studies reviewed (Granfeldt *et al* 2012, Sambol *et al* 2009 & 2011 and Yao *et al* 2006). In the 4 studies conducted by these 3 groups, blood was withdrawn until a target mean arterial pressure of 30 – 35mmHg was achieved. Kapoor *et al* in 1997 presented the results of experiments conducted in dogs with pressure-dependent haemorrhage to 50 ±5mmHg. The haemorrhagic shock induced in this study therefore represents the least severe (in terms of target MABP achieved) amongst the references presented in this review.

The majority of the studies adopted a fixed-pressure haemorrhage protocol with blood loss performed over a defined time-frame in order to achieve a pre-determined target blood pressure. The depth of hypotension is therefore well defined and haemodynamic responses can be characterised. A limitation of this approach, however, is that it is tightly controlled and therefore does not accurately reflect what happens clinically. The main advantage over the fixed-volume method is that the degree of hypotension can be tightly controlled and is reproducible. Small animal work using rodents tends to preclude the use of uncontrolled bleeding in the form of liver laceration etc due to technical considerations relating to the animals' size and total blood volume. Compensatory mechanisms may vary between controlled and uncontrolled bleeding and it has been demonstrated previously that indices of shock severity are lower in pressure-controlled bleeding compared to uncontrolled haemorrhage protocols. (Sondeen *et al* 2007). It may be argued that an uncontrolled haemorrhage protocol may be superior in terms of translational potential, but in mechanistic rodent studies, controlled haemorrhage is favoured in the literature.

Resuscitation

Only 4 of the combined trauma / haemorrhage studies reviewed did not include a resuscitation phase in the experimental design (Nachuraju *et al* 2011, Sato *et al* 2007, Gonzales *et al* 2006 and Meng *et al* 2005). In some cases, such as Granfeldt *et al* 2012, the impact of resuscitation strategy upon cardiac injury was one of the study aims. In the majority of studies however, the rationale for including a resuscitation phase is not implicitly stated and therefore one must deduce that it is performed in order to restore intravascular volume and blood pressure with the aim of monitoring cardiac function and the temporal patterns of the inflammatory response over the longer term. The introduction of a resuscitation phase also allows for the development of an ischaemia / reperfusion injury which has been postulated as a possible contributor to organ injury and dysfunction after trauma-haemorrhage.

Table 1.1 Protocols for Inducing Haemorrhagic Shock With, or Without Trauma in Pre-Clinical Models.

(Reference ranges for mice, rats, hamsters and dogs are taken from Wolfensohn & Lloyd (2013). Ranges for pigs are taken from Hannon *et al* 1989). SBP = systolic blood pressure.

Species	Normal SBP range (mmHg)	Fixed pressure shock	Fixed volume shock	References
Haemorrhage only				
Mouse	133 - 160		30%	Meng '05
Rat	84 - 134	35 ± 5mmHg 50mmHg 35 – 40mmHg 40mmHg		Yao '96, M Shahani '01 Yang '04, Sato '07 Soliman '12 Meldrum '97
Rat			60%	Gonzales '06, Letson '11
Hamster	150		50%	Nachuraju '11
Dog	100 - 178	50 ± 5mmHg 40mmHg		Kapoor '97 Sato '00, Coimbra '04
Pig	120 - 134	30 – 35mmHg		Granfeldt '12
Trauma - Haemorrhage				
Mouse		35 ± 5mmHg		Zhang '12, Nickel '09
Rat		40mmHg 35mmHg		Remmers '97&'98 Robinson '97, Angele' 98, Jarrar '00 (x2), Mizushima '00, Guan '02, Hsieh '06, Yu '07, Zou '07, Ba '08, Kan '08, Hsu '09, Tsai '12 Kuebler '03
Rat		35 – 40mmHg		Hsieh '05, Szalay '05, Hsieh '06, Yang '06, Hsu

				'07, Zou '09, Sambol '11, Jian '12
Rat		30 – 40mmHg	60%	Yang '07 Sambol '09

The choice of resuscitation fluid varies amongst the studies. 25 of the studies out of the 36 which include a resuscitation phase, report the use of crystalloid solutions intravenously. Ringer's lactate is the fluid of choice in these references and a fixed volume of 4 times the shed blood volume is used in each case. A protocol using twice the volume of shed blood in Ringer's was implemented in 3 studies (Coimbra *et al* 2004, Meldrum *et al* 1997 and Yao *et al* 1996).

Shed blood was reinfused after a period of haemorrhagic shock in 5 of the studies reviewed and in the remaining 3, shed blood and crystalloid were administered after the haemorrhage and shock phase (Granfeldt *et al* 2012, Guan *et al* 2002 and Shahani *et al* 2001).

Apart from the publications from Soliman *et al*, Granfeldt *et al* and Shahani *et al*, resuscitation fluids are administered in the absence of a pre-defined resuscitation end-point. For example, the majority of the studies relating to the investigation of the role of the sex hormones in the development of cardiac dysfunction, use a fixed volume resuscitation protocol where 4 times the volume of shed blood is returned to the animal in the form of lactated Ringer's solution. Guan *et al* in 2002 outline that shed blood is returned along with a fixed volume of 0.9% saline. In 2012, Soliman *et al* outline in their methodology that shed blood alone was returned with the aim of reaching a target mean arterial blood pressure of 50mmHg. Granfeldt *et al* and Shahani *et al* also targeted MAP and after blood was administered, crystalloid boluses were given to meet a pre-defined target blood pressure.

Fluid resuscitation was included in the protocol of most of the studies where animals had suffered blood loss. Lactated Ringer's solution was the commonest resuscitation fluid of choice and was used in isolation or in combination with blood.

The inclusion of a resuscitation phase in the models adds another level of technical difficulty, with insertion of a greater number of indwelling vascular catheters (if blood is to be returned via the intravenous route rather than through the arterial catheter), blood storage and the requirement for anti-coagulation to prevent coagulation of drawn blood and occlusion of multiple in-dwelling catheters.

In terms of translational potential, resuscitation of the animal will cause organ reperfusion, which in itself may contribute to pathophysiology in the form of ischaemia-reperfusion injury, and this is more likely to reflect the clinical scenario. The majority of the studies reviewed here employed a clear fluid resuscitation strategy with crystalloid solutions administered on the basis of the shed blood volume. For the majority of the studies, fluid is returned at a set volume. Shahani et el, Soliman *et al* and Granfeldt *et al* however target resuscitation in terms of mean arterial pressure. The latter 2 groups also administered a combination of shed blood and crystalloid. As clinical resuscitation practice moves away from the use of large volume crystalloid resuscitation in trauma, contemporary animal models of trauma haemorrhage would be expected to modify accordingly in order to optimise their translational potential. There appears to be a lack of emphasis upon targeted resuscitation, with a lack of pre-defined end-points of resuscitation. This approach could add another level of complexity to already challenging models, but it may be required to develop standardised, robust models in which to characterise the functional cardiac response.

Table 1.2 Protocols for Resuscitation in Pre-Clinical Models of H and THS.

SB = Shed blood, 4x refers to 4 times the volume of shed blood, 2x refers to twice the volume of shed blood.

Species	Shed blood	Crystalloid	Blood & Crystalloid
Mouse		4 x SB volume (Nickel '09)	
Rat	(Sambol '09 &'11, Soliman '12)	2 x SB volume (Yao '96, Meldrum) 4 x SB volume (Remmers '97, Robinson, Angele, Jarrar, Mizushima, Kuebler, Yang, Hsieh, Szalay, Yang, Hsu, Yu, Zou, Ba, Kan, Tsai)	(Guan '02)
Dog	(Kapoor '97, Sato '00)	2 x SB volume (Coimbra '04)	(Shahani '01)
Pig			(Granfeldt '12)

Diagnosis and Manifestations of Cardiac Injury in pre-clinical models

Rodent models have repeatedly demonstrated that cardiac function is depressed following trauma (Li *et al* 2007), (Li *et al* 2007), (Feng *et al* 2013), (Wang *et al* 2013), (Tao *et al* 2005), (Yan *et al* 2010), haemorrhagic shock (Shahani *et al* 2001), Meldrum *et al* 1997), Nachuraju *et al* 2011), (Yao *et al* 1996), (Soliman *et al* 2012), (Sato *et al* 2000) and trauma-haemorrhage (Zhang *et al* 2014), (Zhang *et al* 2012), (Kan *et al* 2008), (Liu *et al* 2012), (Sambol *et al* 2009), (Sambol *et al* 2011), (Yang *et al* 2004 & 2006), (Zou *et al* 2009), (Remmers *et al* 1998), (Hsu *et al* 2008), (Mizushima *et al* 2000), (Hsieh *et al* 2006), (Kuebler *et al* 2003), (Hsu *et al* 2009), (Yu *et al* 2007), (Yu *et al* 2008), (Yu *et al* 2005), (Tsai *et al* 2012) and (Szalay *et al* 2005).

Cardiac dysfunction has been demonstrated both in-vivo and ex-vivo using invasive and non-invasive haemodynamic monitoring. The most commonly adopted method involved the use of a heart performance analyser with or without a dilution technique to assess cardiac performance. Stroke volume was seen to decline, maximum rates of rise (+dP/dtmax) fell along with a decline on ventricular pressure (-dP/dtmax) and left ventricular developed pressures (LVDP). Ventricular peak systolic pressures, cardiac output, cardiac index and the delivery of oxygen (DO2) were all reported to decline after trauma with or without accompanying haemorrhagic shock.

Both systolic and diastolic dysfunction has been reported (Shahani *et al* 2001). Electrocardiogram (ECG) assessment has also allowed for the monitoring of rhythm disturbance and electrophysiological abnormalities have been reported (Sato *et al* 2007, Sambol *et al* 2009 and Letson & Dobson 2011).

Only 1 study elected to use well characterised cardiac specific biomarkers to diagnose cardiac injury for the purposes of their study. This group published experimental data regarding the

role of TNF- α and ROS in the development of cardiac injury and dysfunction in rats after mechanical trauma. They demonstrated that troponin I levels and Creatinine kinase (CK) activity were elevated in trauma rats when compared to sham controls at time-points up to 2 hours after injury (Feng *et al* 2013).

Human cardiovascular disease has been modelled in small animals for decades (Russell & Proctor 2006) and characterisation of left ventricular pressure-volume relationships, and the construction of pressure-volume loops, have been widely used in pre-clinical rodent models of cardiovascular disease. The miniaturisation of technology has allowed for the development of high-fidelity micro-manometers which can be used in small-animal studies and real-time generation of pressure-volume loops, have proven to be a valuable tool in characterisation of cardiovascular dynamics.

The use of sophisticated, invasive cardiac assessment has therefore been successfully employed in models of cardiac injury to demonstrate the functional implications of trauma-haemorrhage. These techniques are highly specialised and require a great deal of skill to perform in a robust fashion. There are also a number of limitations to be considered when using these techniques in rodent models of trauma-haemorrhage. Their placement is highly invasive and challenging requiring microsurgery and monitoring to ensure that the catheters remain in the correct place. There may also be issues in the placement of these micro-manometers into a cardiovascular system which has been subject to severe haemorrhage and is therefore 'shut-down'. Appropriate calibration of these systems may also be difficult as viscosity changes with haemorrhage and then subsequent resuscitation. The use of these systems in small animals may be limited by the need for frequent recalibration. Greatly reduced circulating volumes may preclude frequent blood taking for recalibration, particularly in models with severely shocked animals who are already very unstable.

Ex vivo heart systems such as the isolated retrograde-perfused Langendorff heart were also used by a number of the researchers (Sambol *et al* 2009, Sambol *et al* 2011, Du *et al* 2015, Wang *et al* 2013 and Tao *et al* 200). They have proved useful in studies of ischaemia / reperfusion and cardiac disease such as heart failure and have been a useful piece of the cardiovascular researcher's toolkit when assessing the impact of therapeutics upon cardiac physiology. The addition of a pressure transducing system allows for the measurement of indices of ventricular performance.

The use of these apparatus however poses significant technical challenges to the researcher particularly when using small, delicate murine hearts. Crystalloid perfusion of the heart may cause tissue oedema and due to its low oncotic pressure, coronary artery flow rates are much higher than in vivo. Removing the heart from the body will also remove important neuro-hormonal influences and therefore reduce translational potential and retrograde perfusion of the heart with clear fluid is obviously far removed from the in vivo situation.

The timing of onset of cardiac dysfunction has been, in the majority of studies, reported as occurring between 2 and 5 hours after injury (Yang *et al* 2006, Yang *et al* 2004, Mizushima *et al* 2000, Meng *et al* 2005 and Szalay *et al* 2005). Feng *et al* in 2013 reported a 'hyper-acute' onset of dysfunction of within 60-minutes of trauma but this is not typical. Longer studies have been performed with animals being recovered from anaesthesia and then undergoing cardiac functional assessment at a later stage. Cardiac dysfunction onset has been demonstrated to occur up to 48-hours post injury (Remmers *et al* 1998, Kuebler *et al* 2003, Tao *et al* 2005, Liu *et al* 2012 and Wang *et al* 2013).

While the identification of molecules and biomarkers relevant to the development of secondary cardiac injury can be easily taken into the clinical setting, investigating cardiac dysfunction with the use of functional assessment in trauma patients is more challenging.

There is therefore a need to take the research presented here forward. To develop models which encompass the ischaemia, inflammation, reperfusion which contribute to organ dysfunction and to develop ways of characterising cardiac function in this setting which are relevant clinically.

Pathophysiology

Identification of the key effector molecules and pathways involved in the development of this specific type of cardiac injury was the aim in the majority of the research studies presented here. As such, small animal models feature most frequently.

Endocrine function

Gender differences in the inflammatory and immune responses in general have been well documented. 16 of the studies reviewed, many of which have been conducted by I.H. Chaudry's research team in the US, characterise the contribution of gender-specific endocrine signalling to the inflammatory response after trauma-haemorrhage specifically. Because many of these studies were performed by the same group, there is significant overlap in terms of species chosen and other aspects of the methodology such as resuscitation strategy and burden of injury. In the majority of these studies therefore, rats are the species used, haemorrhage takes the form of controlled, pressure - dependent bleeding to a MAP of 35-40mmHg and soft tissue injury in the form of a laparotomy. Broadly speaking, the aims of these studies include the elucidation of key endocrine pathways that confer immunological benefit and cardioprotection to females compared to males after trauma haemorrhage and to identify therapeutic agents that have the ability to provide males with these benefits or antagonise the potential detrimental inflammatory signalling that predisposes them to worse outcomes.

Shahani *et al* in 2001 published the only study investigating the role of adrenergic signalling in the development of cardiac inflammation and dysfunction after haemorrhagic shock. They concluded that the α1-adrenergic pathway was initiated in response to haemorrhage and this was associated with depressed myocardial function. This effect was partially blocked with the use of a TNF-α blocking antibody and, in another experimental group, with the administration of prazosin hydrochloride before the onset of haemorrhage (Shahani *et al* 2001).

Apoptosis & Autophagy

The role of apoptotic and autophagy pathways specifically have been investigated in a smaller number of these references with 8 studies focussing upon relevant pathways and molecules. Of these, Guan *et al* in 2002 and Tsai *et al* in 2012 investigated the development of cardiomyocyte apoptosis in response to the combination of trauma and haemorrhagic shock while the remainder (Wang *et al* 2013, Feng *et al* 2013, Yan *et al* 2010, Li *et al* 2007 and Tao *et al* 2005) subjected animals to blunt trauma (most often in the form of the modified Noble-Collip drum whole body trauma, which has been described earlier) in the absence of haemorrhage. Broadly speaking, the hypothesis being that injury at sites other than the heart set into motion inflammatory cascades culminating in cardiomyocyte cell death which was demonstrated in these studies in injured animals when compared to sham controls.

Pro-Inflammatory Pathways & Cytokines

Pro-inflammatory mediators such as TNF- α and the interleukins feature frequently in the body of literature reviewed here. There is also significant overlap with studies elucidating the role of the sex hormones as these inflammatory cytokines are downstream effectors of the pathways involved. 6 of the studies investigate the role of signalling via NF-KB and the production of TNF – α on the development of cardiac injury and dysfunction.

Investigators have measured levels of these cytokines most frequently in plasma of animals after haemorrhage and / or trauma but there are a number of studies where levels of cytokine expression within the myocardial tissue itself has been performed.

The toll-like receptor (TLR) pathways have drawn much interest in trauma research due to their role in damage-associated molecular pattern (DAMP) signalling. TLRs such as TLR-4 are regulators of pro-inflammatory signalling cascades and as such, investigators have been keen to identify their role in organ dysfunction after trauma-haemorrhage and identify pivotal molecules which could provide therapeutic targets. In order to investigate the relationship between TLR-4 and TNF- α as a downstream effector in haemorrhagic shock and cardiac dysfunction, Meng *et al* haemorrhaged mice with a defective TLR-4 receptor and compared the TNF- α response and cardiac function with wild-type. The group also used genetic strains to investigate TNF receptor signalling. They reported that TLR-4 signalling was involved in generating TNF - α in haemorrhagic shock and that depression of myocardial function was modulated via the p55 TNF – α receptor specifically.

Li *et al* in 2007 also took advantage of the availability of genetically modified strains to investigate inflammatory signalling pathways in the development of cardiomyocyte injury when they developed their mouse trauma model. Serum from TNF – α knock-out mice was used to demonstrate the role of this cytokine in the development of cardiomyocyte apoptosis after non-lethal mechanical injury. Healthy cardiomyocytes cultured with plasma taken from the TNF- α deficient strain did not develop the higher incidence of apoptosis that was demonstrated in cadiomyocytes cultured in plasma removed from injured wild-type mice.

In 2013, Feng *et al* observed that mechanical trauma led to an increase in myocardial and serum TNF- α concentrations which was associated with cardiac dysfunction. The group went on to demonstrate that serum from trauma animals, when incubated with cardiomyocytes

from a control group, decreased contractile amplitude. This outcome, however, was less pronounced if a TNF – α antagonist was also applied supporting their hypothesis that TNF- α has a role in the development of cardiac dysfunction after trauma.

Reactive Oxygen Species

The earliest study investigating the role of oxidative stress and free radical production in the development of cardiac dysfunction after haemorrhage was published in 1997 by Kapoor *et al*. Another 3 studies have focussed upon contribution of the oxidative pathways (Feng *et al* 2013, Li *et al* 2007 and Tao *et al* 2005), but always in the context of isolated trauma.

Lymphatics

The gut represents an organ which is vulnerable to ischaemia in haemorrhagic shock as remaining blood is diverted in order to maintain circulation to other organs. The potential for ischaemia, coupled with the huge surface area that the intestinal organ possesses has raised the possibility that the gut may be involved in the development of organ dysfunction after haemorrhage. Specifically in relation to this review, Sambol *et al* published the only study investigating the role of mesenteric lymph in the development of cardiac dysfunction secondary to trauma and haemorrhagic shock. The group hypothesise that lymph, in conditions of trauma and shock, contains factors which have a negatively inotropic effect on the heart. One group of animals was treated with LDL with the intention of preventing intestinal lymph from entering the bloodstream. Cardiac function was analysed using an ex vivo isolated heart system. The group report that this was protective and infer that myocardial dysfunction after trauma and shock is likely related to factors present in lymphatic fluid.

Conclusions

Trauma and haemorrhage represent a spectrum of pathophysiological states in humans. Mild injury without accompanying blood loss will not be expected to instigate the same degree of inflammatory response and physiological derangement as the polytrauma patient with soft tissue and bony injury, severe blood loss, haemorrhagic shock, organ ischaemia and subsequent resuscitation and reperfusion.

This review demonstrates the variability amongst animal models which have been developed to investigate cardiac sequelae of trauma and haemorrhage. Protocols which have separated bleeding from injury have provided mechanistic insights into cardiac injury and dysfunction but it may be said that the ideal model would be one in which both conditions are present. Injury in isolation may be enough to precipitate cardiac injury, however, the absence of shock in these models may offer too simplistic a view. To optimise the translational potential of these models to the severely injured patient in the resuscitation room, hypoperfusion and all the cellular implications that it brings should ideally be acting in parallel with injury and inflammation.

This review of contemporary animal models in published work, has identified common methodological approaches used in the detection and characterisation of trauma-induced secondary cardiac injury. In vivo studies have already yielded important insights into the cellular and molecular mechanisms driving cardiac injury and dysfunction, but the understanding of the pathophysiology underpinning TISCI remains relatively poorly understood. There is a relative paucity of knowledge regarding clinically-relevant biomarkers and how these relate to the development of cardiac injury and dysfunction in the pre-clinical models. As we better understand the temporal patterns of biomarker release in humans, this

needs to be validated in the animal models to facilitate development of relevant models of injury and haemorrhage.

A significant limitation of the small animal studies is the lack of uncontrolled haemorrhage protocols. It can be argued that controlled bleeding does not accurately reflect the clinical situation and this therefore limits the translational potential. That being said, the reproducibility and standardisation gained with the use of a controlled haemorrhage model may represent a significant advantage, particularly perhaps when designing a model in which to assess therapeutic agents. This issue has to be considered however when drawing conclusions from studies designed in this way. It is vitally important, however, that the model is designed carefully in order to appropriately address the question being posed and allow for successful translation into human studies.

This review has highlighted some of the limitations associated with widely used models. Although there has to be an acceptance that no one model will perfectly recreate the complex pathophysiology associated with injury and shock, there is no doubt that animal models still have much to offer us in our quest to understand how cardiac injury and dysfunction occurs and ultimately, how this can be limited or prevented. There is a need for in vivo models capable of comprehensively characterising cardiac functional outcomes in the context of TISCI. Such models will be particularly critical for the development of therapeutics.

1.7 Proposed Therapeutic Strategies to Treat TISCI

No clinical studies have been undertaken to identify drugs which could specifically prevent or limit the cardiac injury which develops as a consequence of trauma-haemorrhage (TH). A great number of studies have focussed upon aspects of resuscitation such as prevention of

coagulopathy, however there is a relative paucity of research investigating prevention of TISCI specifically with ‘cardio-protective’ resuscitation strategies.

It can of course be said that any improvement in overall haemodynamic status will have the presumed beneficial effect of improving myocardial blood flow, but, as previously discussed, restoration of blood flow to the myocardium after an antecedent period of ischaemia, as with other organs, can potentiate changes in cellular and tissue homeostasis (ischaemia-reperfusion injury) which can instigate organ failure and contributes to high morbidity and mortality at a later stage. The heart could be regarded to be especially at risk due to its inherent inability to reduce its workload during acute hypotension; in fact, the metabolic demands placed on the organ during this time of physiological stress is even greater as the work rate must increase in order to maintain cardiac output. Whereas the cerebrovascular and renovascular circulations for example may benefit as the heart increases its work rate to raise cardiac output, the coronary circulation is compromised further with a reduction in diastolic filling time as heart rate increases. The myocardium therefore rapidly accrues a metabolic deficit under these circumstances with associated cellular derangement and this is also accompanied by the inflammatory consequences of shock and injury during ischaemia and upon reperfusion (Kalogeris *et al* 2012).

As knowledge regarding the pathophysiological processes behind the development of secondary cardiac injury is gained the focus shifts onto potential ways to modulate these key pathways in order to ameliorate the cardiac injury resulting from trauma-haemorrhage. A small number of pre-clinical studies have investigated therapeutic agents with the potential to provide cardio-protection after haemorrhage or combined trauma-haemorrhage.

Interventions investigated have included the use of strategies as diverse as whole body hypothermia to protect 'key' organs including the heart (Alam *et al* 2010) to targeting specific molecules with monoclonal antibodies (Yang *et al* 2006).

1.7.1 Vascular endothelium and microcirculation

The importance of protecting the endothelium from the effects of ischaemia and reperfusion injury, such as the situation of coronary artery occlusion followed by revascularisation, has long been appreciated.

Endothelial injury has been observed as an indirect consequence of blood loss and injury. It is thought that this may contribute to impaired perfusion and cellular homeostasis and this was the rationale behind the use of the amino acid L-arginine in these studies. L-arginine is a substrate for the production of cNOS, levels of which had been observed to fall after trauma-haemorrhage. In 1998, Angele *et al* employed a rat model of TH and administered L-arginine as an adjuvant to fluid resuscitation. They reported improved cardiac output and organ blood flow (measured using thermodilution technique) compared to a control group (Angele *et al* 1998).

Pentoxyfilline was administered at resuscitation to haemorrhaged dogs with subsequent improvements in cardiac function, cardiac index and a reduction in inflammation. This study was limited by the small number of animals and the relatively short period of observation, however, this demonstrated how targeting the microcirculation by altering red blood cell morphology could be another aspect of the vascular system to investigate (Coimbra *et al* 2004).

1.7.2 Hormonal therapies

Irshad Chaudry's team in the United States have undertaken a number of studies investigating the role of sex hormones in the development of cardiac dysfunction after TH and have

offered evidence that progesterone (Kuebler *et al* 2003) and oestrogen therapy may reduce the severity of cardiovascular injury when used in their rat model of TH. They have demonstrated a dose-dependent improvement in left ventricular performance when oestrogen and oestrogen receptor agonists, including diarylpropinonitrile (DPN), were administered 2 hours after the onset of TH and the beneficial cardiac effects persisted for more than 6 hours. There have been a number of studies reporting the beneficial effects of oestrogens after T-H, but this study provided insights into both the specific receptor sub-types involved in mediating this cardio-protection as well as demonstrating a dose-response (Ba *et al* 2008).

It is not only oestrogens and testosterone which have been investigated in the context of trauma and injury mediated organ injury. The administration of metoclopramide was shown to improve cardiac and hepatocellular outcomes and reduce inflammation (as measured by IL-6 production) due to its effect of increasing prolactin secretion from the anterior pituitary of rats subjected to TH (Jarrar *et al* 2000).

1.7.3 Regulation of metabolic function

An end-point of the cellular consequences of ischaemia involves the mitochondrial generation of adenosine triphosphate (ATP). Enzymes including pyruvate dehydrogenase (PDH) are essential for ATP generation and during periods of hypoxia, activity of this enzyme is reduced (Andersen *et al* 2015).

This observation was the basis of a 2015 study which used a rat model of 55% blood loss to investigate the potential beneficial effects of a drug which restored the balance of enzyme acetylation and therefore improved the availability of the functional enzyme needed for ATP generation during ischaemia. Administration of a deacetylase inhibitor was shown to enhance cardiac PDH and reduce mortality from 75% - 37.5%. There was also an observed reduction in the expression of pro-apoptotic caspase-3 as well as pro-inflammatory mediators. PDH

activity was the only measured component of the electron transport chain and the mechanism by which the effects were mediated however remain unclear (Chang *et al* 2015). Similar improvements in haemodynamics and survival had been previously reported in 2006 with the use of the histone deacetylase inhibitor valproic acid, but this had been given as a pre-treatment hours before the onset of haemorrhagic shock (Gonzales *et al* 2006).

1.7.4 Targeting inflammation and oxygen species generation

Strategies employed to modulate the generation of reactive oxygen species (ROS) and their impact upon cardiac function have included the administration of exogenous nitric oxide (Nachuraju *et al* 2011) and modulation of immune-mediated ROS generation. Insulin has recognised anti-inflammatory and anti-oxidant properties and as such this has been investigated as a therapeutic agent in TISCI. Animals that received insulin 10 minutes after injury was sustained were observed to generate less ROS, have lower serum troponin levels and demonstrated better cardiac function when compared to controls. Insulin therapy was therefore identified as having anti-inflammatory and anti-oxidant properties which would be beneficial in limiting cardiac dysfunction (Feng *et al* 2013).

The administration of hydrogen sulphide (H_2S) was demonstrated to be of benefit in terms of survival, haemodynamic outcomes and organ injury after haemorrhage. The improvements in haemodynamic function reported were attributed to the anti-inflammatory properties of H2S (although it has also been reported as having pro-inflammatory activity under certain conditions (Szabo 2007) and this therefore may be a dose-related response). A single bolus of H2S improved blood pressure and was associated with reduced generation of reactive oxygen species and a down-regulation of pro-inflammatory mediators (Ganster *et al* 2010).

The use of the anti-oxidant resveratrol was investigated as a potential adjunct to fluid resuscitation. 10-minutes after resuscitation after T-H, a single bolus of resveratrol was

administered to rats and left ventricular contractility was assessed using an intra-ventricular pressure transducer. Rats treated with the agent showed improved LV performance and reduced heart rates compared to controls at 2 hours. There was also an associated reduction in inflammation (plasma TNF-α assay) and when cardiac tissue ATP levels were compared, T-H controls were found to have significantly reduced levels. Reduced levels of ATP were restored however with treatment (this improvement was not seen in animals pre-treated with the Sirt-1 inhibitor suggesting that resveratrol mediated its cardio-protective effects via a Sirt-1-dependent pathway (Jian *et al* 2012). This study therefore identifies a potential cardioprotective agent and also describes its mechanism of action in bringing about its cardioprotective effects.

Anti-inflammatory agents have also been identified for the prevention of TISCI in trauma without haemorrhage. Quercetin is an anti-oxidant found in fruit and vegetables and this was administered as a pre-treatment before injury was induced. In-vivo cardiac assessment at 12 hours post trauma revealed improved left ventricular performance when treatment animals were compared to controls. Heart tissue analysis determined that there was a reduction in both cardiomyocyte apoptosis and circulating TNF-α in treated animals (Jing *et al*., 2016). This was the first time that this dietary flavonoid was tested in this context and the results are encouraging. However, there are limitations in terms of the methodology; a Noble-Collip drum was used to induce mechanical, non-lethal injury and, as previously discussed, there are issues regarding standardisation of injury and therefore inflammation with the use of this technique. Quercetin was also given before trauma and this therefore has implications for translational potential.

Another naturally occurring anti-oxidant found in plants, resveratrol, has also been identified as an anti-inflammatory, anti-oxidant agent with potential applications in trauma-

haemorrhage as a cardio-protective agent with additional benefits for other organs including the gut as well (Liu *et al* 2015).

The platelet inhibitor dipyridamole is also known to have anti-inflammatory effects and this commonly used cardiovascular drug was identified as a potential therapeutic agent by Soliman *et al* in 2012. When administered to rats after haemorrhage, there was a reduction in the size of the inflammatory infiltrate into the myocardium. Cardiac performance measured in isolated hearts was improved and plasma TNF – α levels were noted to be lower in the treatment group compared to controls (Soliman *et al* 2012). A possible limitation of this use of this drug however lies with its anti-platelet effect. It is unclear how the use of this therapeutic would contribute to bleeding and the development or potentiation of coagulopathy when used in an uncontrolled haemorrhage setting.

Research using transgenic mouse strains have provided insights into key molecules and effectors involved in the development of TISCI. The pro-inflammatory signalling pathways in particular have been investigated (Zhang *et al* 2012 and Meng *et al* 2005). The development of monoclonal antibodies has allowed for the selective blockade of molecules and therefore pathways thought to be detrimental to cardiac function. Anti-IL-6 antibodies administered after T-H has been shown to improve cardiac function and also to reduce cardiac inflammation but the development of cardiomyocyte inflammation and injury is multifactorial and therefore although benefit has been seen with these antibodies, the precise mechanism of their action remains unknown (Yang *et al* 2007).

1.7.5 General strengths and limitations of the therapeutic studies

The therapeutic studies briefly discussed here have covered a wide range of pathophysiological factors contributing to the development of TISCI. General limitations of the therapeutic studies include the relative small number which combine both trauma and

haemorrhage. This would therefore reduce the overall physiological 'burden' of injury and make these studies less translatable to the most severely injured patients who experience the greatest organ dysfunction, mortality and morbidity. There have been a variety of methods used to assess cardiac performance, ranging from thermodilution techniques to assess cardiac output and organ blood flow (Coimbra *et al* 2004 and Angele *et al* 1998) to the insertion of left ventricular pressure transducers (Ba *et al* 2008). Study length and therefore the observation period was variable between studies, but generally studies were conducted to include at least a 2-hour observation period after trauma-haemorrhage, extending beyond 24 hours in some cases (Gonzales *et al* 2006). There have been some encouraging advances made in the identification of novel therapeutics which could be used as adjuncts to resuscitation to improve cardiac outcomes. The emphasis needs to be on moving these studies out of the pre-clinical arena with the implementation of clinical trials of those with most promise. Carefully designed, robust pre-clinical models which reflect the human condition of TISCI will be vital in order to achieve this aim.

1.8 The Quest for Novel Cardioplegic Agents and their Potential Application in Trauma Research

Cardiac injury and dysfunction is well-recognised in the context of low out-put states other than that relating to haemorrhagic shock. In some cases (including some of the published work previously discussed) observations made in studies related to shock in the context of sepsis have led to the trial of the same agents for organ protection after trauma-haemorrhage. Innovations in trauma research therefore can come from an appreciation of advances being made in different fields where similar challenges relating to organ dysfunction and protection are being faced.

The early 21st century saw a drive to develop novel, more 'myocardial friendly' cardioplegic agents that could be used in complex cardiac bypass surgery, particularly with the challenges of an ageing patient group with multiple comorbidities. Observations made regarding the cell biology and electrophysiology of natural hibernators such as small rodents (and described by Geoffrey Dobson and Hayley Letson), gave rise to the idea of developing a 'polarizing' cardioplegic agent which would arrest the cell membrane at a more physiologically acceptable level for the duration of bypass. To this end, a solution containing a combination of drugs was produced which was associated with improved myocardial outcomes in terms of inflammation and function when compared to the older, more traditional high-potassium solutions.

Adenosine, lidocaine and magnesium (ALM), three drugs used widely in clinical practice, were combined to produce a 'polarizing' cardioplegic agent. The rationale for this particular combination arises from the physiology of hibernating animals and a desire to mimic this by arresting the heart at its resting membrane potential of -80mV (Dobson & Letson 2016). No single agent would have the ability to achieve this, but it is possible using a combination of drugs each with different electrophysiological properties.

Lidocaine blocks the movement of Na+ into cells, thereby inhibiting the sodium influx required to generate the upstroke of the action potential. Adenosine is a class V anti-arrhythmic agent and acts on the atrio-ventricular (AV) node to induce a transient heart block and terminate AV-node dependent supraventricular tachycardia (SVT). Adenosine exerts this effect by opening K+ATP channels and decreasing the length of the action potential and it was for this property that it was selected. Calcium entry into cells is observed during ischaemia-reperfusion. During ischaemia, intracellular ATP levels and pH drop and ion transport becomes disordered. Intracellular and mitochondrial calcium levels increase which can lead to cell swelling and eventual rupture (Kalogeris *et al* 2012). Magnesium was added

to adenosine and lidocaine in order to reduce calcium entry and limit cell injury mediated in this way and to prevent arrhythmia development (Dobson & Letson 2016).

ALM was studied for this role in an isolated perfused rat heart model (Dobson & Jones 2004) and later in a canine model (Corvera *et al* 2005). These studies confirmed the drug to be safe to use and with improved cardiac outcomes when compared to alternative solutions. Three prospective, randomised controlled trials using the combination of adenosine and lignocaine in cardiac surgery (one of which was a paediatric study) have shown favourable outcomes, including improved haemodynamics, decreased cardiac biomarker levels and shorter in-patient stay when compared to patients treated with high-potassium cardioplegia (Jin *et al* 2008).

Adenosine in combination with lidocaine +/- magnesium has also been reported as having cardiac benefits in a broad range of settings including sepsis (Griffin *et al* 2014 and Granfeldt *et al* 2014), preventing arrhythmia post cardiac arrest (Granfeldt 2013) and also in the context of haemorrhagic shock (Letson *et al* 2011). Table 1.3 gives the half-lives and cardioprotective mechanisms of adenosine, lidocaine and magnesium.

1.8.1 Adenosine, Lidocaine and Magnesium in haemorrhage models

Interest in the combination of ALM as a therapeutic agent in HS is relatively recent. A group of researchers who have since demonstrated its haemodynamic benefits in both rat (Griffin *et al* 2014) and porcine models of sepsis (Granfeldt *et al* 2014) and in reversing coagulopathy rat model of severe haemorrhage (Letson *et al* 2011) also observed that the use of ALM in a rat model of HS significantly improved the animals' haemodynamic status, reduced the volume of fluid resuscitation required and also led to 100% survival compared to controls (Letson *et al* 2011).

Table 1.3 Half-Lives and Proposed Mechanisms of Cardioprotection of Adenosine, Lidocaine and Magnesium.

ICAM = intracellular adhesion molecule, VCAM = vascular cell adhesion molecule, eNOS = endothelial nitric oxide synthase, NFκB = nuclear factor kappa-light-chain enhancer of activated B cells, I-R = ischaemia-reperfusion, ROS = reactive oxygen species.

Agent	Half-life	Electrophysiological	Inflammation & immune	Other
Adenosine	<10 seconds	Negative inotropy, chronotropy and dromotropy (Pelleg '97, Belardinelli '98, Canyon '04)	Reduced signaling *via* NFκB, reduced endothelial expression of ICAM, VCAM (Bouma '10) Reduced TNF-α & neutrophil adhesion molecule expression (Dobson 2016)	Coronary vessel dilatation (Ely '92) Induces eNOS (Bouma '10) Anticholinergic (Belardinelli '95) Reduces cardiac ischaemia-reperfusion (I-R) injury (Vinten-Johansen '99) Stabilises glycocalyx in I-R (Platts '03)
Lidocaine	2 hours	Down regulation of voltage-dependent Na+ channels, reduces Ca2+ accumulation and negative inotrope (Wilson '93)	Reduced signaling *via* NFκB (Bouma '10) ROS scavenging and impaired neutrophil activation (Shi '12)	Coronary vessel dilatation (Ely '92) Slows ATP utilization (Canyon '04)
Magnesium	5 hours	Ca2+ antagonist (Dobson '16)		Essential for cell metabolism and ionic regulation, antiadrenergic (Dobson '16)

Table 1.4 below gives examples of studies where adenosine, in combination with lidocaine and magnesium, has been shown to confer cardioprotection and survival benefit in animal models of TH specifically.

Table 1.4 Adenosine, Lidocaine and Magnesium in Pre-Clinical THS Research.

ATP = adenosine triphosphate, $MgCL_2$ = magnesium chloride, NaCl = sodium chloride, MAP = mean arterial pressure, ALM = combined adenosine, lidocaine and magnesium, Mg = magnesium, A = adenosine, L = lidocaine, TH = trauma-haemorrhage.

Author	Year	Model	Agent/s	Main findings
Robinson	1997	Rat Trauma-haemorrhage Crystalloid resuscitation	ATP-MgCL2	Improved cardiac contractile function
Letson	2011	Rat Haemorrhage Crystalloid resuscitation	NaCl/ALM & NaCl/Mg	Improved MAP, reduced incidence of arrhythmia and 100% survival in ALM treated
Letson	2011	Rat Haemorrhage Crystalloid resuscitation	NaCl/ALM, NaCl/A/Mg, NaCl/Mg & NaCl/L/M	Higher MAP in ALM treated
Letson	2011	Rat Haemorrhage Crystalloid resuscitation	NaCl/ALM	Reversal of TH coagulopathy
Granfeldt	2012	Pig Haemorrhage Blood resuscitation	NaCl/AL & NaCl/ALM	Improved haemodynamic stability and cardiac and renal function
Granfeldt	2014	Pig Haemorrhage Blood resuscitation	NaCl/ALM	Improved cardiovascular, metabolic, acid-base outcomes with improved renal function in ALM treated animals

More recently, a rat-to-pig translational study was also performed whereby pigs were subjected to a 75% haemorrhage were treated with a bolus of ALM in normal saline (Granfeldt *et al* 2014). This study corroborated the beneficial haemodynamic effects of ALM

when used as part of the resuscitation fluid regimen in haemorrhagic shock. Mean arterial blood pressure, cardiac output and stroke volume were reported to increase significantly, along with a reduced heart rate, lower lactate and base excess when compared to a control group who had been resuscitated with blood and saline alone. The use of ALM in these haemorrhage models also favoured lower volume resuscitation; important for the avoidance of complications resulting from high volume clear fluid resuscitation including pulmonary complications and coagulopathy.

Despite encouraging results being reported in haemorrhage models using rats and pigs, ALM has not, to the best of my knowledge, been investigated in the context of haemorrhagic shock accompanied by injury.

1.9 Summary

Severe trauma is associated with significant mortality and morbidity. As advances are made in the acute management of bleeding patients, research needs to keep pace with the new challenges related to the severe physiological derangement patients have suffered yet survived. There is growing clinical evidence for the development of a secondary cardiac injury after trauma-haemorrhage which is associated with myocardial dysfunction and worse clinical outcomes.

Pre-clinical research, predominantly in small animal models, supports the clinical data and is providing insights into mediators and mechanisms driving the development of cardiac injury. An acute onset cardiac injury characterised by an increase in systemic and cardiac inflammatory molecules, ROS generation and associated with impaired left ventricular performance has been described. The use of knock-out mouse strains and hormone agonists

and antagonists has provided insights into key molecular processes in both driving and preventing cardiac injury after trauma-haemorrhage. There is however a relative lack of studies assessing clinically-relevant cardiac biomarkers and their association with cardiac dysfunction.

Robust, translatable animal models continue to be vital for the investigation of cardiac injury and dysfunction. There are a number of well-designed pre-clinical models in the contemporary literature, but the focus has been largely on inflammation and less work has been undertaken which links the biomarker evidence with functional outcomes. There also needs to be a drive towards developing therapeutics which can be used acutely to limit the degree of cardiovascular dysfunction resulting from resuscitated trauma-haemorrhage.

1.10 Project Aims and Objectives

The overall aim was to investigate the functional consequences of trauma-induced secondary cardiac injury on the myocardium. The objectives were therefore as follows:

1. Validate the use of H-FABP, a clinical cardiac biomarker of TISCI, in our existing in vivo model of trauma-haemorrhage and establish the nature of the relationship between H-FABP and severity of injury in the animal model.

2. Characterise the acute functional myocardial response to trauma-haemorrhage in the animal model of H-FABP defined TISCI.

3. Develop a resuscitated model of TISCI in which to assess the functional left ventricular response to resuscitation after antecedent trauma-haemorrhage and to quantify the biomarker response with restoration of baseline pre-load conditions.

4. Test the efficacy of ALM as an adjunct to fluid resuscitation in improving haemodynamic outcomes and survival after combined trauma-haemorrhage in the murine model of TISCI.

Chapter Two

Materials and Methods

Chapter Two

2 Materials and Methods

The recurring materials and methods for all the experimental chapters to follow are outlined in this chapter.

2.1 The Murine Model of Trauma-Haemorrhage

All animal procedures described in this thesis are regulated under a specific animal project licence (Procedure Project Licence (PPL) – PC5F29685) approved by the Animal welfare and Ethical Review Body at Queen Mary University of London, and the UK Home Office, in accordance with the EU directive 2010/63/EU on the protection of animals used for scientific purposes. The PPL includes all the experimental procedures required for the pre-clinical studies, including the induction of haemorrhage and traumatic injury, imaging procedures, dosing substances and blood / tissue samplings. This PPL was renewed on December 2016 and has been granted for 5 years (to December 2021). Previously to 2016, we used the PPL 70/7348 which expired on the 30/11/2016.

2.1.1 Mice

Male C57BL/6 wild-type mice weighing 25 – 30 grams were supplied by Charles River Laboratories (Margate, UK). This strain was used in all experiments. Animals were housed in accordance with the UK Home Office Guidance in the Operation of Animals (Scientific Procedures) Act 1986 and received a standard diet and water ad libitum prior to undergoing anaesthesia. Mice were randomly allocated to experimental group.

2.1.2 Anaesthesia

Isoflurane (Abbott Labs ltd, Berkshire, UK) in combination with 100% medical oxygen was used for both induction and maintenance of general anaesthesia throughout the duration of the experiments which were all 'non-recovery'. 100% oxygen was used due to the centre's previous experience with injury and haemorrhage models and it has been deemed appropriate for a model of this severity with associated mortality.

An animal was taken from their cage and transferred into a sealed anaesthetic induction chamber. Oxygen and isoflurane was delivered into the chamber and when the animal was anaesthetised, they were rapidly transferred to the scanner platform (or surgical table) where isoflurane and oxygen were delivered continuously via nose cone to maintain a surgical plane of anaesthesia. No surgery was commenced until a surgical plane of anaesthesia, assessed with reaction to paw pinch, was achieved and checks were performed intermittently throughout the experiment. "Depth" of anaesthesia was assessed with a combination of reaction to external stimulus such as paw pinch, and, after surgical placement of catheters, haemodynamic monitoring (if there was an unexpected increase in MAP during the experiment for example, reaction to stimulus was checked in order to assess adequacy of anaesthesia). Anaesthetic delivery was controlled with the titration of isoflurane as appropriate. All experiments were 'terminal' meaning that no animals were recovered from anaesthesia after surgery, and all were culled at the end of the experiment whilst still anaesthetised.

2.1.3 Surgery

After induction of anaesthesia and transfer to either the scanner platform or surgical table, a lubricated rectal probe was inserted and temperature maintained at 36.0 +/- 1^{o}C with the use of heating pads under the platform (or surgical table) and heat lamps. 3 of the paws were

taped to maintain stability during surgery, with the 4th left free in order to monitor adequacy of anaesthesia. Prior to making the first incision, reaction to pain was assessed with a paw pinch on the unrestrained limb. No incision was made if the animal was not sufficiently anaesthetised (as discussed above).

A neck dissection was performed and the left jugular vein was identified. A venotomy was performed and a length of polyethylene tubing (internal diameter 0.28mm, Portex. Smith's Medical Int. Ltd. Kent, UK) which had been pre-flushed with heparinised saline (25 IU/mL) was inserted and secured with nylon suture. The right carotid artery was then cannulated in the same fashion. After carotid cannulation, a pressure transducer (Capto SP 844, AD Instruments. Chalgrove, UK) was connected to the line by way of a three-way connector. The neck wound was covered in a moistened gauze to prevent drying out of tissues and the wound was checked intermittently for evidence of line displacement and bleeding. If either of these situations developed, the animal was euthanised and removed from the experimental data.

2.1.4 Haemorrhage

The animals were allowed to stabilise for 5 minutes after cannulation and injury. A 'baseline' mean arterial blood pressure (MABP) was recorded at this point. Mice then underwent a pressure-controlled haemorrhage of arterial blood via the carotid catheter over 10 minutes to achieve a target MABP of 60 – 70mmHg or 30 – 40mmHg (depending upon the experimental group allocation). The target blood pressure was then maintained over a 60-minute period with removal of blood as required via the carotid cannula. Shed blood was kept warm in a 1mL syringe containing 0.05mL of heparinised saline (25 IU/mL) and occasionally agitated to prevent thrombus formation. This volume of heparinised saline was taken into account when recording volumes of shed blood.

2.1.5 Trauma

In models including an injury component, trauma took the form of bilateral hind limb fracture, laparotomy and rectus muscle crushing. Fractures were performed using a closed, manual 3-point bending technique. A 2cm midline laparotomy was performed, with internal inspection of the abdominal viscera in order to exclude inadvertent iatrogenic injury and / or bleeding. The rectus muscle was crushed using forceps in a systematic fashion in each animal. The laparotomy was then closed using 5.0 monofilament suture material (Ethicon, UK). Moist gauze was then applied to cover the neck and laparotomy wound sites.

2.1.6 Observation periods

After trauma-haemorrhage and resuscitation episodes, animals underwent a 60-minute observation period. During this time, core temperature was continuously monitored and maintained with the use of a heat mat and lamps. Temperature, MABP and % of inhaled anaesthetic required to maintain a surgical plane of anaesthesia were recorded at 10-minute intervals. In-dwelling vascular catheters were intermittently flushed with small volumes of heparinised saline and wound coverings were monitored and kept hydrated.

(Refer to the experimental groups outlined for each chapter in tables 3.1, 4.1, 5.1 and 6.1 and experimental outlines for each experimental chapter given in figures 3.1, 4.1, 5.1and 6.1).

2.2 Echocardiographic Assessment of the Left Ventricle

Echocardiography was performed by a second investigator who had received relevant training in the techniques. This second investigator was the same person for each experiment and they were blinded to the experimental group the animal was assigned to. However, due to the nature of these experiments, the most severely shocked animals were easily identified on echocardiographic assessment (with obvious volume depletion of the left ventricle after

severe haemorrhage) and in this way, complete blinding of the second investigator was not possible. This was also true for the ALM experiments due to the immediate bradycardia induced by the drug which was visible during real-time imaging as the drug was delivered. It was therefore obvious to the second investigator if ALM had been administered, but they were not able to distinguish between higher or lower dosage as the extent of the bradycardia was similar regardless of dose and were therefore blinded in this respect in the *chapter six* experiments.

Imaging of the left ventricle was performed at baseline, and at defined time-points after trauma-haemorrhage and resuscitation, using the Vevo 770 high-resolution in vivo micro-imaging system (Visualsonics Inc. Toronto, Canada). Animals were anaesthetised before any scanning was conducted as previously described.

A lubricated rectal temperature probe was inserted and their paws were taped to electrocardiogram (ECG) pads which had been coated with a layer of conductance cream. The anterior thoracic wall was prepared with depilatory cream and warmed ultrasound transmission gel (Aquagel, Parker laboratories Inc., NJ, USA) was applied to the chest before applying the ultrasound probe. Modified parasternal long and short axis views were obtained. The short axis view was used to obtain an image of the left ventricle at the level of the papillary muscles. A 2-dimensional cine-loop was recorded. Scanning was converted to M-mode at this point and an image of the left ventricle was acquired. Continuous core temperature, EGC and heart rate monitoring was performed throughout the duration of the scanning procedure. Temperature was maintained at 36.0 +/- 1^{o}C for the duration of the procedure.

Qualitative and quantitative measurements were taken using analytical software (Visualsonics. Toronto, Canada). M-mode measurements were made using the programme

callipers. Measurements were taken in systole and diastole as follows; Intraventricular septum (IVSs, IVSd), left ventricle internal diameter (LVIDs and LVIDd) and left ventricle posterior wall (LVPWs and LVPWd). Using these measurements, the software automatically calculated stroke volume (SV), cardiac output (CO), ejection fraction (EF) and fractional shortening (FS). The measurements were carried out in triplicate and the average of these values calculated.

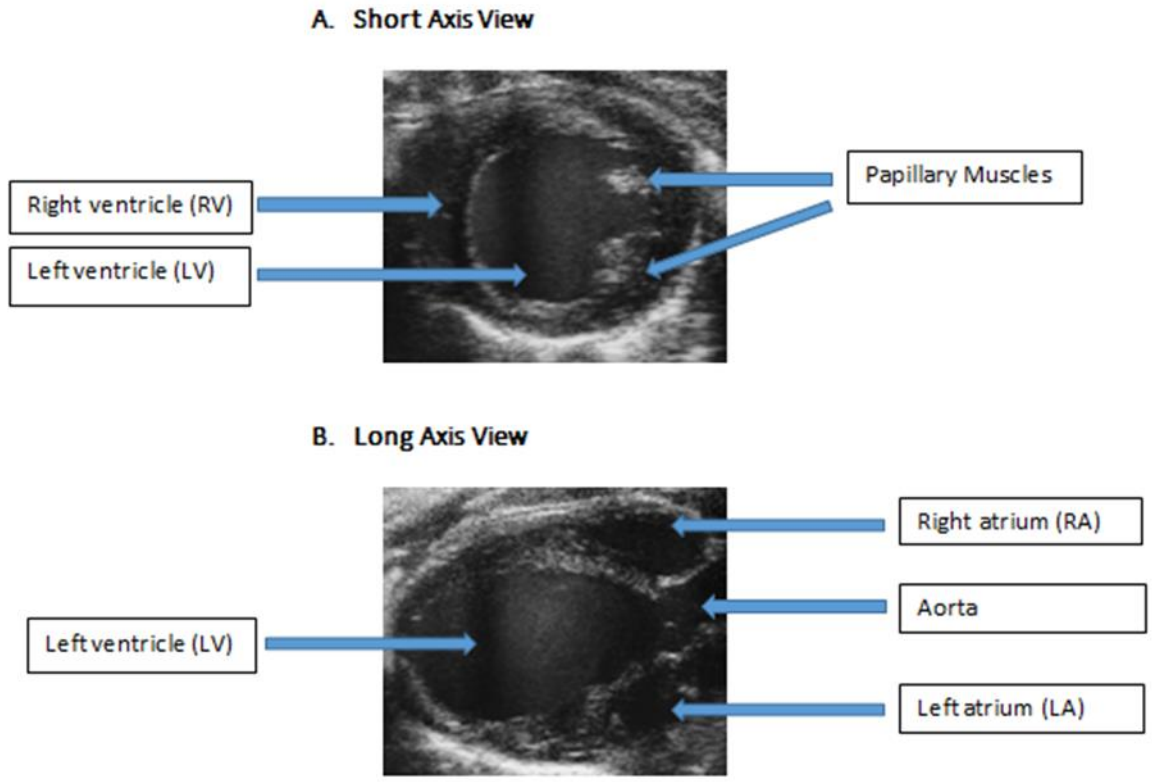

Figure 2.1 Echocardiographic assessment of the left ventricle in B mode imaging.

A shows the short axis view of the left ventricle (LV) and right ventricle (RV) at the level of the papillary muscles. B shows the long axis view (still in B mode imaging) of the LV, right atrium (RA), left atrium (LA) and aorta.

2.3 Resuscitation

2.3.1 Fluid resuscitation

In experiments where fluid resuscitation was included in the protocol, fluid was administered via the jugular catheter over a 5 – 10 minute period. In the clinical setting of major trauma, blood products and other fluid is preferentially delivered through central veins (often the jugular or subclavian) during resuscitation and therefore administration *via* a larger vein in this model more closely resembles the clinical setting.

The first resuscitation phase in all experiments saw the transfusion of whole blood which had been previously withdrawn during the haemorrhage phase. Shed blood was stored in a 1mL syringe and kept warm *ex vivo* before being administered as a bolus over 5 minutes *via* the jugular catheter. Haemodynamic parameters of heart rate and blood pressure were monitored closely to ensure a response to the blood resuscitation. If no response was seen, the neck structures and catheters were checked to ensure correct placement and to exclude extravasation of blood into the tissues. If the catheters were found to be dislodged, blocked or that blood was being lost into the tissues, the experiment was terminated and the animal replaced.

If the return of shed blood was not adequate to resuscitate the animal to baseline parameters, small boluses of warmed crystalloid (Vetivex11. Dechra Veterinary Products, Shrewsbury, UK) were then administered. (Vetivex is a sterile, non-pyrogenic Hartmann's solution and each 100mL contains sodium chloride 600mg, sodium lactate 317mg, potassium chloride 40mg and calcium chloride dehydrate 27mg). Volumes of intravenous crystalloid administered were recorded in all experiments. In experiments where SV was used as an end-point for resuscitation, real-time echocardiography was performed throughout the resuscitation phase in order to monitor the response to fluid boluses. In addition to the shed

blood, boluses of crystalloid solution were then administered in order to restore the SV to the baseline level which was taken to represent the completion of the resuscitation phase. Volumes of crystalloid administered to achieve the echocardiographically defined end-point in these experiments were recorded.

In *chapter six* experiments, a second resuscitation phase was performed at the end of this 60-minute monitoring period. During this time, which lasted 5 – 10 minutes, crystalloid alone was administered through the jugular line with the aim of restoring baseline SV once again. In some cases, the SV stopped incrementing in response to intravenous fluid. In this instance, if a previous increase in response to fluid had been witnessed and if satisfied that the jugular line remained appropriately sited, a failure to respond to a further fluid bolus was taken to represent the completion of this resuscitation phase and another period of monitoring was commenced with no further administration of fluid.

2.3.2 End-points of fluid resuscitation

The end-point of resuscitation was based either on MABP or on left ventricle volaemic status which was determined by means of echocardiography. When MABP was used as an end-point, pressures within +/- 5mmHg of the baseline recording were accepted as indicating completion of the resuscitation phase. If the MABP was lower than the baseline and further fluid boluses were not leading to a positive increment in blood pressure, resuscitation was regarded as being completed.

In all subsequent experiments, the end-point of resuscitation was based upon stroke volume recordings in response to fluid boluses. In these experiments, the aim was to restore left ventricle stroke volume (SV) to baseline and resuscitation was deemed to be 'completed' when the SV was within +/- 5µL of baseline measurement. However, if SV plateaued at

lower than the baseline measurement and further fluid boluses were not increasing the SV, this point was accepted to represent the completion of the resuscitation phase.

2.4 Adenosine, Lidocaine and Magnesium

Prior to commencing the experiment, mice were assigned to either ALM or non-ALM resuscitation groups. Any animals that did not survive to complete the 60-minute TH phase were excluded and replaced to achieve the target N value.

Animals in the non-ALM treated control group were resuscitated as outlined above with a combination of shed blood and crystalloid administered in order to achieve a target SV. ALM-treated mice received a combination of adenosine, lidocaine and magnesium in either a low or high dose "one-shot" regimen which was administered after the return of shed blood during the first resuscitation phase.

The lower-dose administered comprised of adenosine 0.54mcg/g, lidocaine hydrochloride 1.63mcg/g and magnesium sulphate 0.6mcg/g while the high dose group received a doubling of all the ALM components (adenosine 1.12mcg/g, lidocaine hydrochloride 3.26mcg/g and magnesium sulphate1.2mcg/g). The lower dose regimen mirrors that administered in the experiments of Granfeldt *et al* where this dose was used in a porcine haemorrhage model.

Adenosine, lidocaine and magnesium doses were prepared according to weight and suspended in 0.5mL of warmed Hartmann's solution (Vetivex). This solution was then administered after transfusion of shed blood in the first resuscitation phase and the 0.5mL volume included in the total volume of crystalloid administered during the first resuscitation phase. During intravenous infusion, blood pressure, heart rate and ECG were continuously monitored and echocardiographic assessment performed throughout. The Hartmann's

solution used to deliver the drugs was included in the volume of crystalloid administered to reach target SV during the first resuscitation phase. No ALM was administered during the second of the resuscitation phases in any of the experiments.

2.4.1 Terminal blood sampling and storage of whole organs and samples

At the end of the experiment, terminal exsanguination was performed via the carotid catheter. If the catheter has become dislodged, or had become blocked, blood was not taken from any other route. Cardiac puncture was avoided due to the requirement of these experiments to avoid direct cardiac injury which could impact upon biomarker assessment. In these circumstances, functional echocardiographic and haemodynamic data was included in the results but no serum biomarker analysis could be performed.

Blood was dispensed into a 1.1ml Z-Gel microtube (Starstedt. Westphalia, Germany) before being centrifuged at room temperature for serum separation. The resulting serum was harvested and transferred into an Eppendorf tube (Eppendorf UK Ltd. Stevenage, UK) before being frozen at -80^{o}C. Serum samples were stored at this temperature before being thawed in a water bath at 37^{o}C for immediate assay.

The hearts were removed immediately after the end of the experiment. The analysis of this cardiac tissue was performed in studies by Centre for Trauma Sciences PhD student, Sriveena Naganathar, and described in her thesis “Trauma Induced Secondary Cardiac Injury: clinical manifestations and underlying mechanisms”.

2.5 Assessment of Tissue Perfusion

In addition to cardiovascular assessment in terms of MABP, heart rate and left ventricle functional parameters, the peripheral perfusion status of experimental animals was also performed with assessment of metabolic acidosis and cardiac index.

2.5.1 Blood lactate measurement

Blood lactate was measured and used as an index of shock and tissue perfusion. Lactate concentrations were assessed using the Accutrend Lactate monitor (Roche, Mannheim, Germany). In all of the experiments when a terminal blood sample could be taken, this blood was collected into a 1mL syringe and the first 0.05mL were discarded. A drop of blood was then immediately applied to the monitor according to the manufacturer's instructions. Blood lactate was then recorded in mmol/L.

In chapters 3 and 4, blood lactate measurements were recorded before the completion of the experiment. In these cases, in order to minimise the blood lost for sampling and therefore limit the haemodynamic instability resulting from further blood loss, a small volume of arterial blood was drawn back using the blood pressure transducer apparatus and a drop of blood was collected from the carotid catheter before the remaining blood in the line was flushed back into the animal. This blood was then analysed using the technique outlined above.

2.5.2 Cardiac index

In these experiments, cardiac index (CI) was calculated as an additional means to quantify tissue perfusion on an individual basis. CI was calculated retrospectively after completion of the experimental work and was derived using echocardiographic readings (cardiac output)

and the weight of the individual animals which was determined prior to the induction of anaesthesia. Cardiac index was calculated using the following formula;

$$CI\ (mL/min/Kg) = (CO\ (mL/min) / gram) \times 1000$$

2.6 Cardiac Biomarker Assessment

2.6.1 Enzyme-linked immunosorbent assays

Serum heart fatty acid-binding protein (H-FABP) and troponin I (TnI) were analysed using commercially available mouse specific enzyme-linked immunosorbent assay (ELISA) kits (supplied by Life Diagnostics Inc., West Chester, PA, USA.). ELISAs were performed in accordance with the manufacturer's instructions.

2.6.2 ELISA standard curves

Standard curves for all ELISAs were plotted using Graphpad Prism software (version 6.0h) and unknown concentrations were interpolated from the standard curve. If readings fell outside of the standard curve, samples were diluted as appropriate and retested. Corrected biomarker concentrations were calculated by multiplying concentrations by the appropriate dilution factor.

2.7 Statistical Analysis

Haemodynamic and echocardiographic readouts and blood analysis results were stored on a database created using Microsoft Excel software for Macintosh. Data are expressed as mean +/- standard deviation of the mean (SD). Statistical analysis was performed using Graphpad Prism software for Macintosh (version 6.0h).

Parametric data was analysed using Student t test or one-way ANOVA. Non-parametric data was analysed with Kruskall-Wallis and Mann-Whitney U test analysis. 2-tailed $P<0.05$ was taken to represent significance in all statistical analysis. The strength of linear relationships (correlation assessment) was performed using Pearson's correlation coefficient (for normally distributed data). Spearman's rank correlation coefficient was used to assess the relationship between non-parametric variables. Derived r or rs values were interpreted as follows; 0 -0.19 – 'very weak', 0.20 - 0.39 – 'weak', 0.40 - 0.59 – 'moderate', 0.60 - 0.79 – 'strong' and 0.80 – 1.0 – 'very strong'.

CHAPTER THREE

Validation of Heart-Type fatty Acid Binding Protein (H-FABP) as a Biomarker of TISCI in the 1 Hour Murine Model of Trauma-Haemorrhage

Chapter Three

3 Validation of Heart-Type fatty Acid Binding Protein (H-FABP) as a Biomarker of TISCI in the 1 Hour Murine Model of Trauma-Haemorrhage

3.1 Introduction

The development of secondary cardiac injury is associated with worse outcomes in injured patients. A growing body of literature is suggestive of distinct biomarker profiles associated with trauma-induced secondary injury (TISCI) and the development of adverse cardiac events (ACEs) including tachyarrhythmia and cardiogenic shock. The development of TISCI has been found to be independent of pre-existing heart disease and can occur in the absence of direct thoracic trauma. De'Ath *et al* reported the findings of a retrospective study of 135 patients admitted to a level one major trauma centre. 13.3% of patients recruited developed an ACE and these patients had significantly higher levels of cardiac specific biomarkers on admission when compared to patients who did not develop ACE over 28 days. Admission Heart fatty acid binding protein (H-FABP) concentrations were 3 times higher in those patients who went on to develop an ACE when compared to those who did not. Systolic blood pressure was found to be associated with the development of ACE with the likelihood increased if systolic blood pressure was below 100 mmHg on admission (De'Ath *et al* 2012).

A prospective study conducted at the same centre and published in 2015 demonstrated that admission levels of H-FABP correlated with mortality. As well as an association with mortality after trauma, an elevated H-FABP on admission also appeared to have predictive value with respect to the development of ACE. Elevated serum levels of H-FABP were

therefore taken to represent the development of TISCI and when coupled with cardiac dysfunction, this resulted in increased mortality and morbidity (Naganathar *et al* 2015).

The pathophysiology underlying the development of TISCI and associated cardiac dysfunction remain unclear. Mechanical trauma may instigate an inflammatory response, which is detrimental on cardiac function. Pro-inflammatory cytokines such as TNF-α and IL-6 may be associated with admission cardiac biomarkers (De'Ath *et al* 2013) and associated with secondary cardiac injury and dysfunction (Yang *et al* 2004) Apoptotic pathways in cardiomyocytes were triggered when they were cultured with serum from injured animals but this was reduced with TNF-alpha blockade (Li *et al* 2007) These studies may point towards an inflammatory driver of TISCI as a result of injury and as part of a systemic inflammatory response, however, cardiac dysfunction has also been evident in haemorrhagic shock without concomitant mechanical injury (Horton *et al* 1989).

Previous studies therefore support the existence of an indirect myocardial specific injury post trauma-haemorrhage. The release of proteins such as H-FABP into the blood from myocardial tissue may be an indication of occult cardiac damage that occurs soon after injury. What is less clear is the precise pathophysiological processes involved and the impact of this cellular injury on myocardial function. There is a need for clinically relevant in vivo models of TISCI in which to assess the translational relevance of H-FABP related to the pathophysiology of TISCI. The ideal animal model would demonstrate appropriate injury burden and severity of haemorrhagic shock and display biomarker profiles similar to those seen clinically in our patients.

The relationship between acute trauma, with or without concomitant haemorrhagic shock and serum H-FABP levels has not previously been described in a pre-clinical TH model. In order to investigate the development of cardiac dysfunction as a consequence of TH, it will first be

important to demonstrate that H-FABP is an appropriate cardiac biomarker in this setting and does rise, reflecting cardiomyocyte injury, as quickly as 60 minutes after TH in the pre-clinical model.

3.2 Study Aims

The overall aim of this study was to develop a mouse model of TISCI and characterise the acute cardiac response to trauma and/or haemorrhage. The principle objective was to confirm that TISCI, demonstrable by a rise in serum H-FABP concentration, occurred as soon as 60 minutes after the onset of TH and that the magnitude of this rise was comparable to the clinical setting.

3.3 Materials and Methods

The broad materials and methods are discussed in *Chapter 2*.

3.3.1 Experimental groups

Before the start of the experiment, animals were randomly assigned to 1 of 5 experimental groups; Sham control (S), Trauma (T), Haemorrhage (H), Trauma-Haemorrhage at 60 – 70mmHg (TH 60 – 70) or Trauma-Haemorrhage at 30 – 40mmHg (TH 30 – 40).

Table 3.1 Experimental Groups, interventions and final N number for the *Chapter three* studies.

S = Sham, T = Trauma, H = Haemorrhage, TH = Trauma-Haemorrhage.

Intervention	Experimental Group				
	S	T	H	TH 60-70	TH 30-40
Cannulation	Yes	Yes	Yes	Yes	Yes
Pressure-dependent haemorrhage	No	No	Yes. 30-40mmHg	Yes. 60-70mmHg	Yes. 30-40mmHg
Laparotomy & Fracture	No	Yes	No	Yes	Yes
Final N number	6	6	6	6	6

3.3.2 Experimental outline for *Chapter three* studies.

Figure 3.1 below shows the experimental process for the T, H and TH groups for the 1 hour studies.

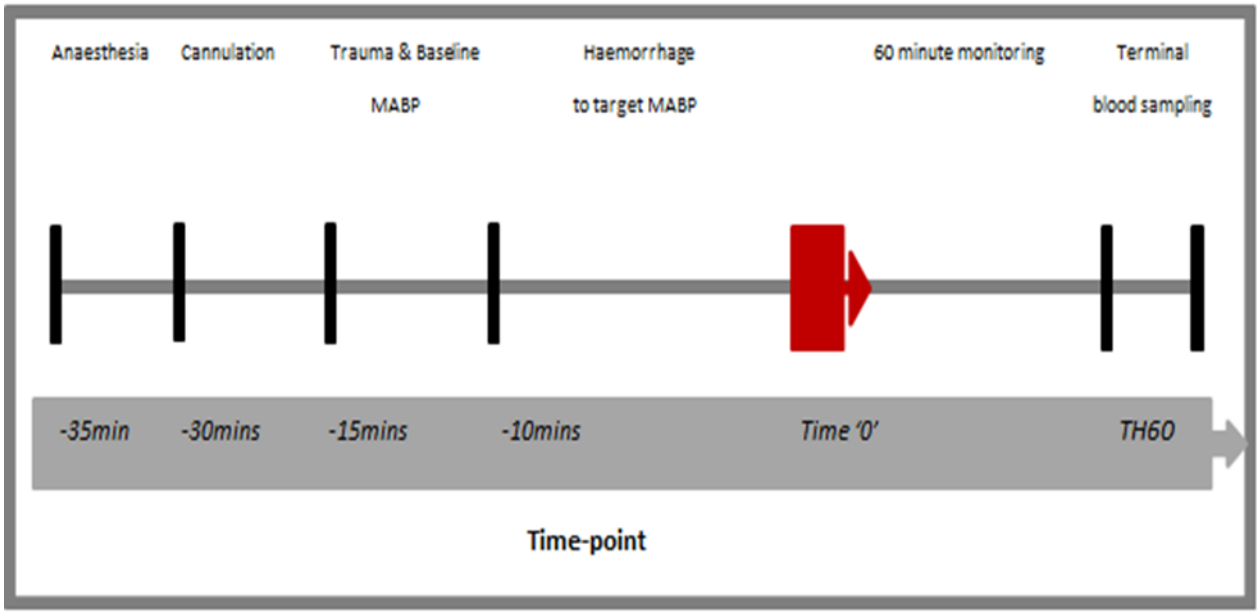

Figure 3.1 Experimental outline for chapter three studies.
Interventions, observations and the time-frame in which they occur are shown. TH60 refers to the 60th minute at target blood pressure in haemorrhage groups.

The experimental period began after the traumatic injuries were inflicted in the T and TH groups. In the H and S groups, this was commenced 5 minutes after the carotid cannulation was completed thus allowing for the mean time taken to complete the traumatisation. Mice in the H and TH groups underwent a pressure-dependent haemorrhage phase after cannulation and injury. Blood withdrawal via the carotid cannula was then performed over a 10-minute period to bring the MAP into target range of 30 – 40mmHg. Further blood withdrawals were performed throughout the remaining experimental period in order to keep the MAP within the target range. In order to investigate the effect of a less severe degree of haemorrhagic shock and trauma, one group of TH animals were haemorrhaged to a target MAP of 60 -70mmHg.

The experimental period was completed 60 minutes after the target MAP was reached in the H and TH groups. In the T and S groups, the experiment was completed after 70 minutes, allowing for the 10 minutes of bleeding phase in the H and TH groups.

All animals were euthanized at the end of the experimental period with exsanguination via the carotid catheter.

Blood was separated and stored as previously described. Cardiac tissue was harvested post mortem and supplied to Dr Naganathar for analysis as previously discussed (refer to section 2.4.1).

3.4 Results

3.4.1 Characterisation of the fixed-pressure TH model of TISCI

A total of 41 mice were used in this study. There were no deaths in the S, T or TH 60-70 groups. Due to vascular catheter issues such as blockage or dislodgement, 8 mice were excluded from the study. There were 2 deaths before the end of the experimental period in the TH 30-40 mmHg group and these were replaced in order to achieve N=6 mice in each of the 5 experimental groups for final analysis.

Mean arterial pressures after carotid catheter insertion were similar across the 5 groups (fig. 3.1). (S = 86.83 ± 2.79 mmHg, T = 84.33 ± 8.91 mmHg, H = 81.33 ± 4.03 mmHg, TH 60-70 = 86.33 ± 6.31 mmHg, TH 30-40 = 79.83 ± 7.57 mmHg; $P>0.05$ between all groups). The mean arterial blood pressure of the H and TH 30-40 groups did not differ significantly at the end of the experimental period (H = 31 ± 3.58 mmHg and TH 30-40 = 32.33 ± 3.72 mmHg).

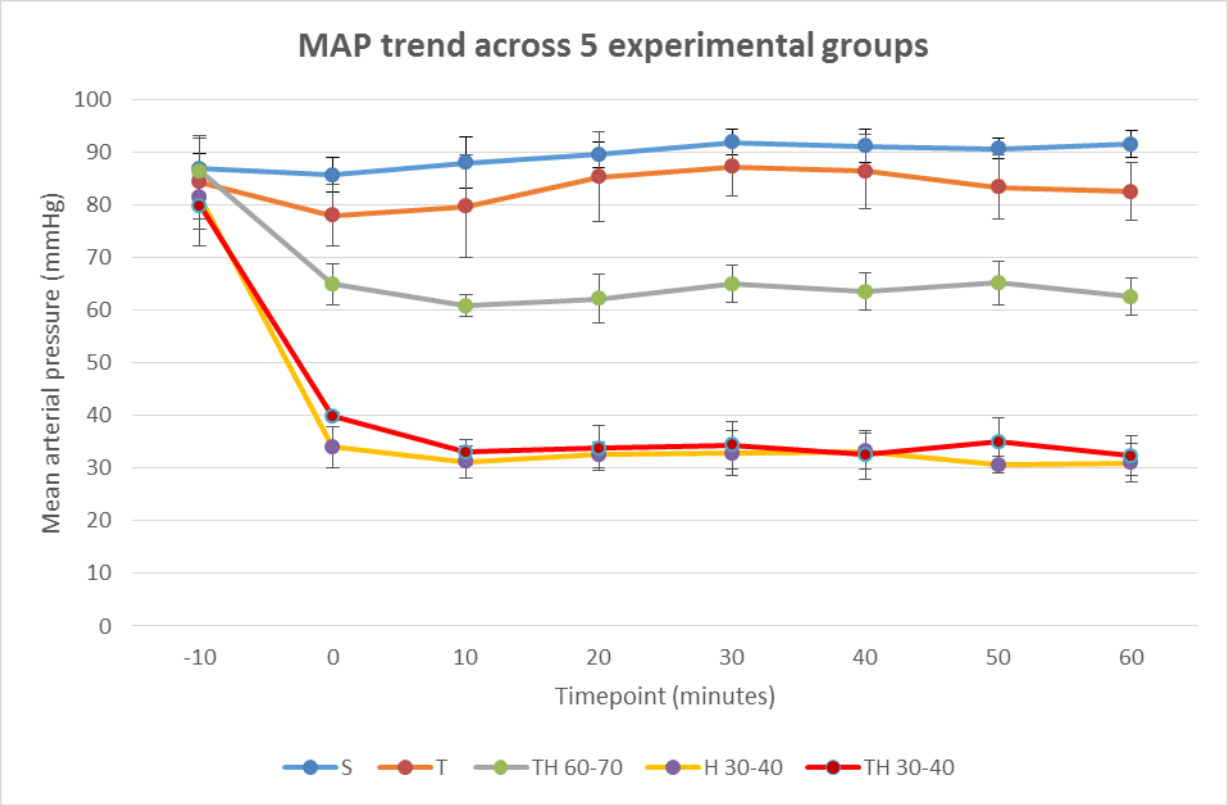

Figure 3.2 Mean arterial blood pressure (MABP) trends over 60 minutes.
Data is shown for the 5 groups throughout the experimental period. Dots represent the mean, vertical bars represent the standard deviations of the means.

Venesection of 14.88 ± 2.79%, 32.75 ± 3.91% and 33.38 ± 3.03% of the estimated total blood volume (calculated based upon 0.07mL per gram of body weight) was required to achieve and maintain target mean arterial pressure in the TH 60-70, TH 30-40 and H groups respectively. There was no statistically significant difference in % blood loss between the TH 30-40 and H groups ($P>0.05$) but the difference was significant when these 2 groups were compared to the TH 60-70 animals ($P<0.05$ and $P<0.01$ respectively). Refer to figure 3.3 below.

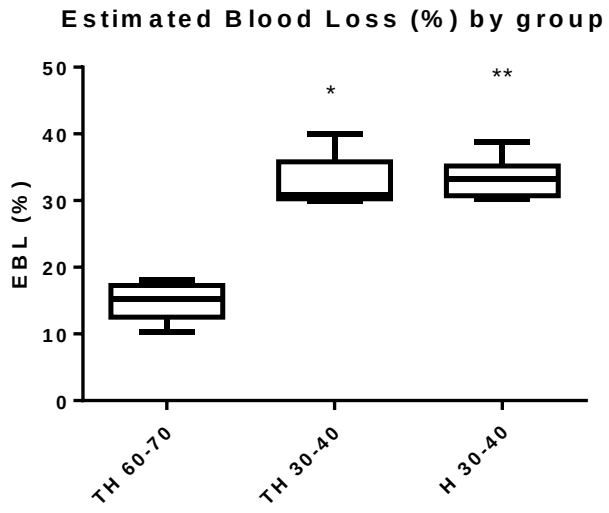

Figure 3.3 Blood loss in the fixed-pressure haemorrhage model.
Box and whisker plots showing estimated blood loss as a percentage of total blood volume in the haemorrhaged groups**.** Median values (horizontal line within box), interquartile range (box) and range (error bars) are shown. *P<0.05, **P<0.01 *versus* TH 60-70.

3.4.2 Blood lactate after 1 hour of trauma-haemorrhage

Blood lactate measurements were made at the end of the 60 minute experimental period (S = 0.93 ± 0.17 mmol/L, T = 0.92 ± 0.4 mmol/L, TH 60-70 = 1.9 ± 0.26 mmol/L, H = 4.47 ± 1.53 and TH 30-40 = 4.45 ± 0.86 mmol/L). There was no statistically significant difference (P>0.05) between the lactate concentrations of S, T and TH-60-70 groups but H 30-40 and TH 30-40 groups had significantly higher arterial lactate levels when compared to the S, T and TH 60-70 groups (P<0.01) (fig. 3.4). Trauma in the absence of haemorrhage did not produce a significantly higher lactate level when compared to sham controls but when trauma was coupled with the most severe haemorrhage (exceeding 30% of blood volume), significantly higher lactate levels were recorded (P<0.05). The most severe degree of fixed-pressure haemorrhage alone resulted in similar levels of lactate to those seen when trauma and haemorrhage were combined. For the same target MAP over 60 minutes, the addition of trauma did not lead to significantly higher arterial lactate concentrations (P<0.05) (fig.3.4).

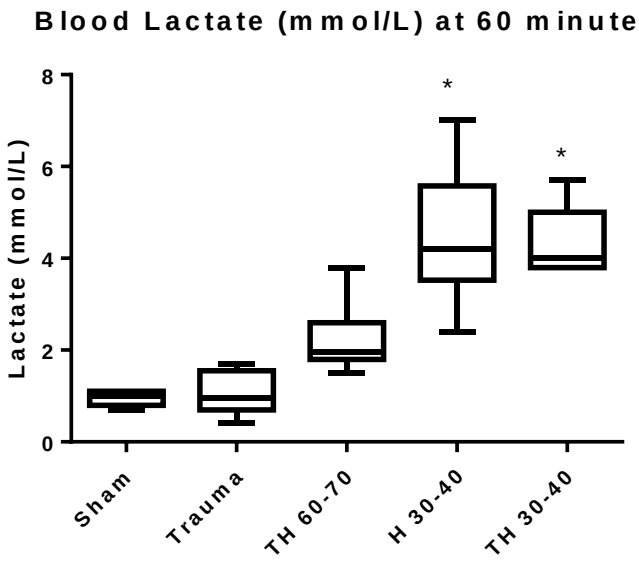

Figure 3.4 Arterial blood lactate concentrations in the fixed-pressure haemorrhage model.
Box and whisker plots showing arterial blood lactate levels at 60 minutes. Median values (horizontal line within box), interquartile range (box) and range (error bars) are shown. *P<0.01 *versus* S, T and TH 60-70.

3.4.3 The relationship between serum H-FABP and trauma-haemorrhage

At the end of the 60 minute experimental period, blood was taken via the carotid catheter, serum isolated and this underwent batch analysis for H-FABP concentration (mean values and SDs were as follows; S = 3.54 ± 3.06ng/mL, T = 28.04 ± 21.10ng/mL, H = 64.50 ± 38.17ng/mL, TH 60-70 = 80.04 ± 49.1ng/mL and TH 30-40 = 166.69 ± 38.64ng/mL). Serum H-FABP concentrations were significantly raised in T, H, TH 60-70 and TH 30-40 groups when compared to sham operated controls (P<0.05) (fig. 3.5).

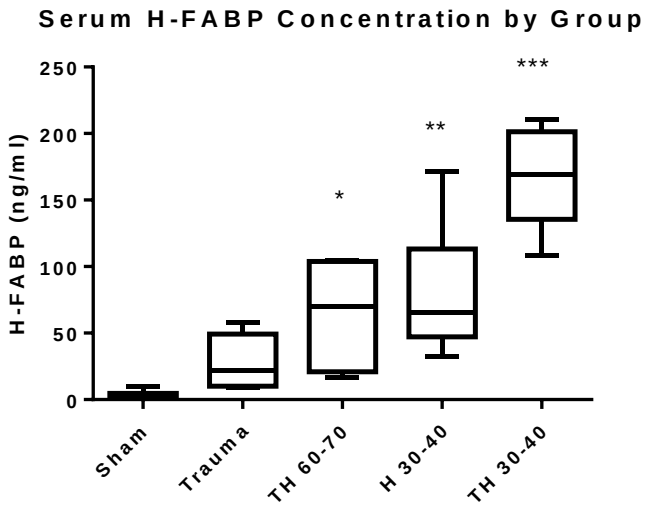

Figure 3.5 Serum H-FABP concentrations in the 5 experimental groups.
Box and whisker plots showing median values (horizontal lines within box), interquartile range (box) and range (error bars) are shown. *P<0.05, **P<0.01 and ***P<0.001 *versus* S.

Post-hoc analysis revealed that H-FABP concentration in TH 30-40 group was significantly higher versus T (P<0.001), H (P<0.001) and TH 60-70 (P<0.01). Serum H-FABP is therefore elevated after 60 minutes of trauma and or haemorrhage with the highest serum concentrations being seen in the model combining injury with the more severe degree of blood loss.

H-FABP level correlated positively with the estimated blood loss (r= 0.4812, 95 per cent confidence interval (C.I.) 0.00346 – 0.7801, p <0.05). Serum H-FABP negatively correlated with mean MAP over 60 minutes (r=-0.8092, C.I. -0.9075 to -0.6270, p<0.05).

3.5 Discussion

These experiments have shown that serum H-FABP concentrations are raised in response to trauma-haemorrhage as quickly as 60 minutes after the onset of TH. The highest concentrations were seen in the most severe form of trauma combined with haemorrhagic shock although levels were also significantly elevated when injured mice with a lesser degree

of shock were compared to non-injured or haemorrhaged controls. H-FABP concentration negatively correlated with MAP over the 60-minute TH phase and positively correlated with blood loss.

The aim of these studies was to validate H-FABP as a biomarker of trauma-induced secondary cardiac injury and to develop a translatable model in which to investigate myocardial function acutely after TH.

The mouse model developed in these studies successfully reproduces the biomarker profiles reported in clinical studies of TISCI. H-FABP levels have been shown to rise as quickly as 60 minutes after TH in the absence of thoracic injury and the magnitude of this rise is similar to that seen in previous clinical studies of cardiac injury after trauma. The mean level of H-FABP in TH30-40mmHg at 60 minutes was 166.69 ± 38.44 while De'Ath *et al* reported values of up to 200ng/ml on admission, in patients who later developed an ACE (De'Ath *et al* 2012).

H-FABP concentrations were significantly higher when TH animals were compared to sham controls. The H-FABP concentrations in the trauma group were numerically higher when compared to sham but his was not statistically significant. This is an interesting observation however as it may support previous work which has suggested a link between acute inflammation (systemic inflammatory response syndrome) and the development of myocardial dysfunction (De'Ath *et al* 2013) also IL-6 and cardio-depression after TH (Yang *et al* 2004). Mechanical trauma alone has been previously associated with cardiomyocyte apoptosis (Tao *et al* 2005). The injury inflicted in the absence of trauma may have precipitated inflammatory pathways, which drive TISCI and lead to the elevated H-FABP levels reported here. This finding needs to be interpreted with caution however, as the MABP in the T group by the end of the experiment was lower than that reported in the sham group

therefore there may have been a degree of hypotension in this group when compared to baseline blood pressure. Care was taken to ensure there were no sources of bleeding in the T group. Inspection of the abdomen post laparotomy was performed to exclude iatrogenic injury leading to internal haemorrhage and post-mortem analysis of the femoral fractures did not reveal gross haemorrhage into the hind limb. Mean lactate concentrations were similar between S and T groups and significantly lower than haemorrhage groups suggesting that although the T group had a lower MAP at the end of the experiment, there was no significant lactatemia, making the incidence of tissue ischaemia less likely.

The highest H-FABP concentrations were seen in serum of animals experiencing a combination of trauma and haemorrhage. Haemorrhage alone to MAP 30-40 for 60 minutes however produced H-FABP levels which were significantly higher than controls, and higher (but not significantly) than TH 60-70. Blood loss as a percentage of total body weight was found to be in the region of 32 – 34% in the H and TH 30-40 group and the difference between the two groups was not significant. Lactate levels in these 2 groups were not significantly different with the H group actually having slightly higher mean values. This again supports the hypothesis that hypotension during a 60 minute period was perhaps not the only driver of TISCI and the higher H-FABP concentration.

Reperfusion injury and associated myocardial inflammation has been reported as contributing to the cardiac injury seen after a period of ischaemia (Ashraf *et al* 1995 and Lefer *et al* 1993) I have presented here findings for an un-resuscitated model of TH and therefore the pathophysiological drivers of the TISCI in these animals are not dependent upon the reperfusion of the myocardium. Within 60 minutes of TH in patients, many may not have received intravenous fluid resuscitation, particularly if they are not hypotensive in the pre-hospital setting. It may be that the higher H-FABP concentrations seen at 24 hours (De'Ath *et*

al 2012) may be due to ongoing cardiac 'leak' of H-FABP, or there may now be other processes involved now with fluid resuscitation and other interventions occurring.

A pilot study was also conducted during the course of these studies to investigate troponin I (TnI) as a potential biomarker in this setting. TnI levels were not elevated (<0.05ng/ml) at 60 minutes post TH when these animals were compared to sham (data not shown). This also supports the findings of De'Ath *et al* who reported admission levels of <0.05ng/ml in patients who went on to develop ACEs after trauma (De'Ath *et al* 2012). This pattern of biomarker release is likely to reflect the kinetics of H-FABP and TnI. H-FABP is released more quickly into the circulation in response to ischaemia when compared to troponin by virtue of its location within the cytoplasm. The low levels of TnI detected at 60 minutes of TH in the small study also supports the translational potential of this model to investigate TISCI as this reflects findings in the literature.

These studies have therefore validated the use of H-FABP as a biomarker of TISCI in a murine model of TH. The results presented here also, for the first time, demonstrate the dose-dependent nature of the H-FABP rise in response to TH at 60 minutes. No overt cardiac dysfunction was observed in these 60-minute experiments however and it is therefore unclear how the raised H-FABP levels correspond to the development of myocardial dysfunction at this acute stage and in the longer term.

3.6 Limitations

Trauma, in the clinical setting, represents a multi-factorial condition which poses many challenges when constructing a robust, translatable pre-clinical model. It is not possible to reproduce the uncontrolled haemorrhage seen clinically in small animal models of TH. Fixed-

pressure haemorrhage as demonstrated here, therefore will not reflect the conditions seen in patients with uncontrolled blood loss. For example, the rate and depth of shock is controlled and may therefore limit the extent of tissue ischaemia and associated phenomena such as trauma-induced coagulopathy which can result (Frankel *et al* 2007). The benefit of the fixed-pressure method used here however, has allowed for an assessment of the correlation between severity of blood loss and the resultant rise of H-FABP. Anaesthesia is induced before the onset of traumatization and bleeding and this obviously does not reflect the clinical setting. However, in order to limit the impact of anaesthesia choice in this experiment, isoflurane was specifically chosen as it is less cardio-depressant when compared to other anaesthetic agents available for *in vivo* studies using mice. Sham controls were anaesthetised in the same way as the other 3 groups and demonstrated the lowest lactate levels which suggests that anaesthesia was not negatively impacting upon cardiovascular reflexes or homeostatic mechanisms in these animals. This supports the use of isoflurane as the anaesthetic agent in these experiments. The sham animals also had the lowest H-FABP concentrations at the end of the experiment which indicates that the anaesthetic regimen was not causing direct myocardial injury under these conditions.

Despite the limitations when performing this type of translational work, mice have proven valuable in cardiovascular research fields and the availability of micro-imaging systems has meant that these techniques have been successfully applied to this model of TH in order to discover more about myocardial function in the acute phase after injury and haemorrhage. Small animal models of TH are technically difficult to successfully master and reproduce. Another potential drawback is the small circulating volume and therefore limited blood available for biochemical analysis.

This study has focussed on the first 60 minutes after trauma-haemorrhage. This is a very short window and the aim was to determine whether H-FABP would be significantly in the murine

model this quickly. The drawback of this however is that the very short study duration has not allowed for the development of overt adverse cardiac events. No comment can therefore me made between the association between myocardial cellular injury (diagnosed on the basis of H-FABP rise) and subsequent ACE development or the nature of the ACE that may arise on this background.

3.7 Conclusions

It has been possible to develop a murine model of trauma-induced secondary cardiac injury, which demonstrates biomarker profiles similar to those seen in clinical studies. Movement from 'bedside-to-bench' in this case has been invaluable in developing a translatable model in which to further investigate the functional implications of this cardiac injury after trauma haemorrhage. A dose-dependent increase in circulating H-FABP in response to TH has been shown here for the first time.

The development of this pre-clinical model of TISCI, which mirrors the cardiac biomarker profiles of trauma patients at 60 minutes, provides an opportunity to investigate the functional cardiac outcomes associated with the development of the biomarker-proven insult. Characterisation of cardiac function in this small animal model of TISCI will prove invaluable in furthering our understanding of functional consequences of the acute damage inflicted upon a vitally important terminally differentiated cell population within the heart. There is a need therefore to characterise myocardial function in the context of elevated H-FABP levels in this animal model.

CHAPTER FOUR

In-Vivo Assessment of the Myocardial Response to Trauma-Haemorrhage using Non-Invasive Micro-Echocardiography

Chapter Four

4 In-Vivo Assessment of the Myocardial Response to Trauma-Haemorrhage using Non-Invasive Micro-Echocardiography

4.1 Introduction

The previous chapter of this thesis has demonstrated the presence of biomarker proven TISCI at 60 minutes post onset of trauma and haemorrhagic shock in the pre-clinical model. Circulating H-FABP levels have been shown to rise in-line with increasing severity of insult. This cardiac-specific biomarker can therefore be considered an indirect indicator of cardiomyocyte damage as a result of blood loss and remote injury.

The application of *in vivo* functional assessment to the model described previously offers the potential to identify therapeutic targets which limit the extent of cardiac injury and dysfunction. This small animal model of TISCI has successfully demonstrated the presence of cardiomyocyte injury after just 60-minutes of trauma with haemorrhage; the impact of injury and severe blood loss with resultant H-FABP rise on myocardial function *in vivo*, remains to be elucidated.

4.2 Important Considerations for the use of Echocardiography in Rodent Models of Cardiovascular Disease

Echocardiography is a widely used non-invasive imaging tool with which to visualise cardiac structure and assess function in in vivo rodent models of cardiovascular disease. The development of high frequency (greater than 10MHz) probes for use specifically in small

animals has allowed for good quality imaging of the rapidly beating left ventricle in rats and mice. Genetically manipulated mouse strains have proven invaluable in the development of murine models of cardiovascular pathology and echocardiography has been a useful tool in the assessment of cardiac structure and function in these disease states (such as mouse models of cardiomyopathy and regional ischaemia), and in the development of therapeutics (Rottman *et al* 2007).

Table 4.1 Baseline LV functional parameters and heart rate in conscious C57BL/6 mice.

Mean values are given with standard deviations. (Data taken from Rottman *et al* 2007).

LVID = left ventricular internal diameter, LVPW = left ventricular posterior wall, IVSd = interventricular septal, s = end systole, d = end diastole. HR = heart rate (beats per minute).

	Systolic Measurements (cm)		Diastolic Measurements (cm)			
Measurement	LVIDs	LVPWs	IVSd	LVIDd	LVPWd	HR
Values (Mean and SD)	0.124 ± 0.019	0.138 ± 0.025	0.092 ± 0.014	0.302 ± 0.032	0.093 ± 0.023	683 ± 63

An important consideration when using *in-vivo* imaging in rodent models is the impact of anaesthesia. Performing imaging in conscious animals would obviously be the most desirable scenario in order to limit the effect of sedatives and anaesthetic agents upon normal physiology, however, there are obvious logistical and humane barriers to doing this. Physical restraint of conscious animals to facilitate scanning can be done but this in itself leads to physiological changes such as increased sympathetic tone, which will invariably have a bearing on readouts relating to cardiac performance such as heart rate (Yang *et al* 1999).

In order to minimise discomfort and distress, and allow reproducibility of good quality images, animals can be anaesthetised for the duration of the imaging procedure. The choice of anaesthetic agent in this case is of importance however as commonly used anaesthetics will vary in the associated degree of cardio-depression they induce (Rottman *et al* 2003). An agent with the least negative chronotropic and inotropic effects will therefore be favourable, particularly in the context of haemorrhage models where normal homeostatic mechanisms are vital in allowing physiological compensation and survival.

Inhaled isoflurane is a sensible choice of agent for studying left ventricular function in mice due to its ease of titration and rapid reversibility. Isoflurane has also been reported to have fewer cardio-depressant effects when compared to other agents commonly used to anaesthetise rodents (Kohn. 1997).

Body temperature will affect the transmission of ultrasound waves through tissues and therefore, to ensure consistent readouts, the animals' temperature should be monitored and controlled throughout the duration of the scanning procedure (Lahoutte. 2007).

Other cardiovascular imaging modalities are available for use in small animals. Computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI) and micro-single photon emission computed tomography (microSPECT) have all been used to assess cardiac function in rodents but the need for sophisticated equipment and radioactive tracers mean that the successful application of these technologies is more complex and comes with additional costs (Golestani *et al* 2010). The pharmacokinetics of commonly used tracers, for example, dictates the time between scanning, as tracers need to be cleared before another scan can take place and this could therefore limit studies requiring serial evaluation.

The advancement of ultrasound technology for use in animals coupled with the translational potential of this imaging modality means that echocardiography remains highly

popular in pre-clinical cardiovascular research to identify and monitor the progression of cardiac pathophysiology. The application of non-invasive cardiac ultrasonography in the model of TISCI will allow for rapid, serial assessment of the left ventricle before and after a period of trauma-haemorrhage. This will enable us to answer questions regarding cardiac function and the response of the left ventricle to the development of TISCI.

4.3 Study Aims

Determine the feasibility of using micro-imaging in the existing murine trauma-haemorrhage model. Assess the impact of 60 minutes of trauma and haemorrhage on systolic function of the left ventricle and correlate cardiac performance with severity of biomarker proven TISCI.

4.4 Materials and Methods

The broad materials and methods have been previously discussed in *Chapter Two*.

4.4.1 Experimental groups for *Chapter Four* studies

Table 4.2 Experimental groups, interventions and final N number for the Chapter Four studies.

S = Sham, T = Trauma, TH = Trauma-Haemorrhage, Echo = Echocardiography

Intervention	Experimental Groups			
	S	T	TH 60-70	TH 30-40
Cannulation	Yes	Yes	Yes	Yes
Pressure-dependent haemorrhage	No	No	Yes. 60-70mmHg	Yes. 30-40mmHg
Laparotomy & Fracture	No	Yes	Yes	Yes
Baseline Echo	Yes	Yes	Yes	Yes
60-minute Echo	Yes	Yes	Yes	Yes
Final N number	6	6	6	6

4.4.2 Experimental outline for *Chapter Four* studies.

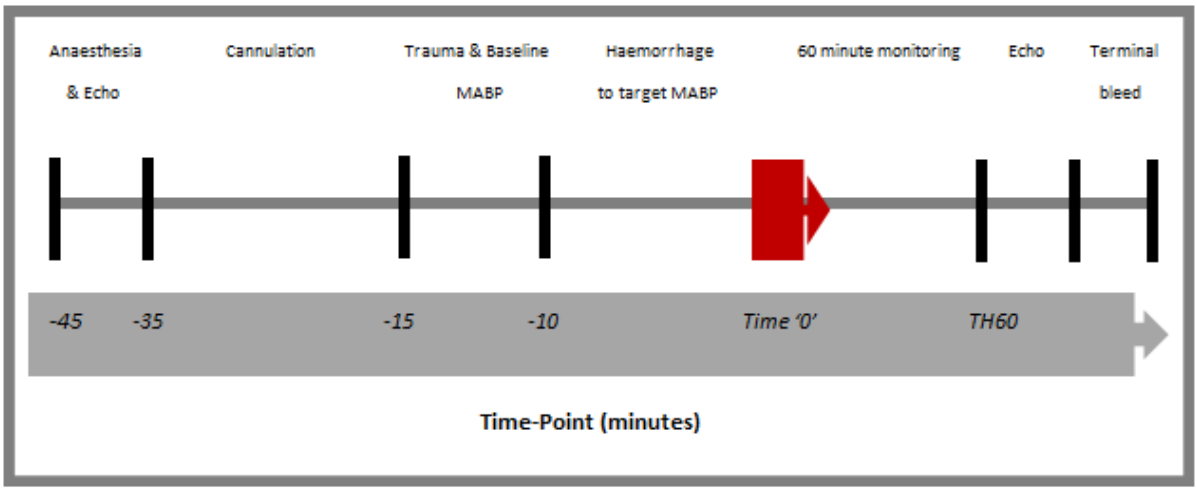

Figure 4.1 Experimental outline for the 1 hour echocardiography studies.
'TH60' refers to the 60th minute at target blood pressure in the trauma and haemorrhage groups.

4.5 Results

A total of 28 mice were used in this study. Technical difficulties with carotid catheter (loss of patency and lack of reliable signal transduction) led to the exclusion of 2 animals. There were 2 deaths in the TH 30-40 group before the end of the experimental period and these animals were replaced to give N=6 for all groups. There were no deaths in the S, T or TH 60-70 groups before the end of the experiment.

4.5.1 Haemodynamic characterisation of the trauma-haemorrhage model

There was no significant difference between the MAPs after insertion of the carotid catheter (S = 89.33 ± 5.99 mmHg, T = 85.5 ± 4.72 mmHg, TH 60-70 = 86.5 ±4.42 mmHg and TH 30-40 = 87.2 ± 7.69 mmHg). At the end of the experimental period, there was no statistically significant difference between the S and T group ($P<0.05$) whereas, the haemorrhaged groups were significantly more hypotensive when compared to S and T ($P<0.001$). Mice in the TH

30-40 group were significantly more hypotensive than TH 60-70 mice at 60 minutes (P<0.01). (S= 89.67 ± 10.61 mmHg, T= 90 ± 6.16 mmHg, TH 60-70 = 58.17 ± 13.3 mmHg and TH 30-40 = 32.4 ± 4.51 mmHg).

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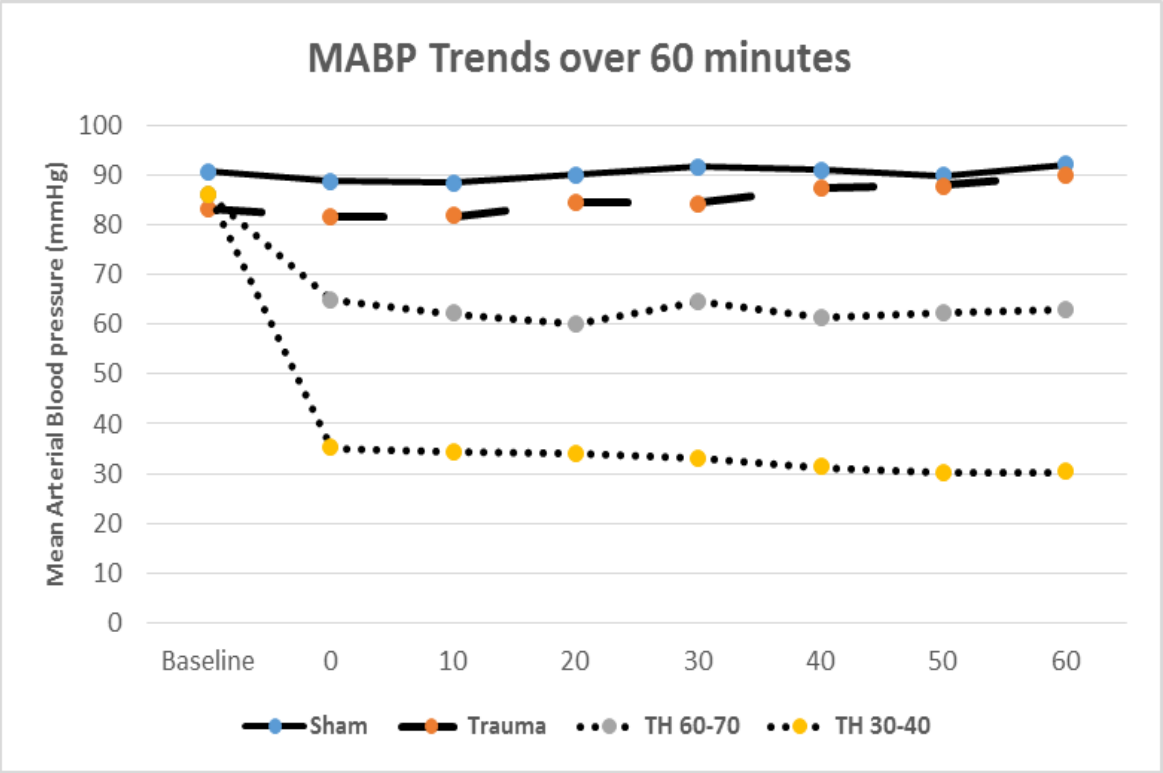


Figure 4.2 MABP trends during the 60 minute TH phase in 4 experimental groups. Line graph shows the trends in MABP across the experimental period of 60 minutes. P<0.05 TH60-70 and TH30-40 compared to Sham and Trauma, P<0.01 Th30-40 compared to TH60-70.

Comparison of estimated blood loss (calculated as a percentage of total blood volume) in the 2 haemorrhage groups revealed a significantly higher % blood loss in the TH 30-40 group (P<0.05) compared to those animals in the TH 60-70 group (Fig. 4.3).

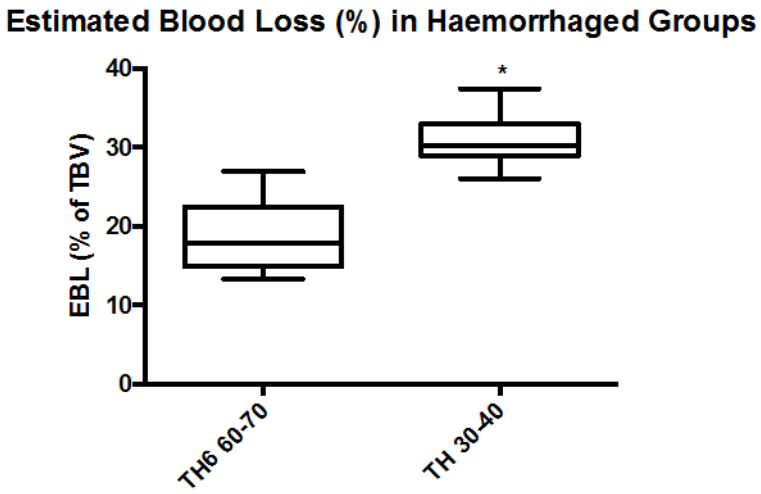


Figure 4.3 Blood loss in the haemorrhaged groups.
Estimated blood loss is shown as a percentage of the total blood volume (calculated based upon individual animal's weight). Box and whisker plots showing median values (horizontal lines within box), interquartile range (box) and range (whiskers) are shown. *P<0.05 TH60-70 *versus* TH 30-40.

4.5.2 The functional myocardial response to non-resuscitated TH

Echocardiography was performed at baseline and again after 60 minutes in each animal.

Table 4.3 presents the mean and standard deviations (SD) for LV functional parameters (stroke volume (SV), cardiac output (CO), ejection fraction (EF) and fractional shortening (FS) and heart rate (HR) at baseline. There was no significant difference in SV, CO, EF, FS or HR at baseline when the 4 groups were compared (P>0.05).

Table 4.3 Baseline LV outcomes.

Mean values are given for stroke volume (SV), cardiac output (CO), ejection fraction (EF), fractional shortening (FS) and heart rate (HR) with standard deviation also given. P<0.05 as significant.

Experimental group	Stroke Volume (SV) µL	Cardiac Output (CO) mL/min	Ejection Fraction (EF) %	Fractional Shortening (FS) %	Heart Rate (HR) BPM
Sham (*n*=6)	36.26 ± 6.09	14.55 ± 3.37	60.16 ± 6.70	31.78 ± 4.48	399.33 ± 57.43
Trauma (*n*=6)	41.31 ± 9.03	18.62 ± 4.32	51.39 ± 14.85	32.24 ± 5.72	449.5 ± 23.65
TH 60-70 (*n*=6)	40.95 ± 8.28	18.60 ± 3.59	58.20 ± 4.94	30.61 ± 3.30	455.17± 32.16
TH 30-40 (*n*=5)	36.04 ± 2.35	16.50 ± 0.82	57.49 ± 6.40	29.93 ± 5.68	457.2 ± 37.00
P value	0.4389	0.1499	0.4092	0.8494	0.0667

Functional analysis at 60 minutes revealed a significantly lower SV and CO when the haemorrhage groups were compared to S and T groups (fig. 4.4 A-B). The greatest significance was seen when TH 30-40 were compared to S or T (P<0.001).

Table 4.4 Left ventricular outcomes at 60 minutes.

Mean values are given for Stroke volume (SV), cardiac output (CO), Ejection fraction (EF), Fractional shortening (FS) and Heart rate (HR) with standard deviations also shown. P<0.05 taken as significant.

Experimental group	Stroke Volume (SV) µL	Cardiac Output (CO) mL/min	Ejection Fraction (EF) %	Fractional Shortening (FS) %	Heart Rate (HR) BPM
Sham (*n*=6)	27.46 ± 7.14	12.57 ± 1.81	66.35 ± 10.43	36.04 ± 8.08	446.5 ± 46.77
Trauma (*n*=6)	27.66 ± 7.77	14.25 ± 3.498	54.51 ± 14.86	36.20 ± 14.50	504.33 ± 43.12
TH 60-70 (*n*=6)	13.80 ± 5.95	7.82 ± 3.38	56.30 ± 14.47	28.72 ± 9.22	563.5 ± 66.09
TH 30-40 (*n*=5)	3.46 ± 1.95	1.92 ± 0.95	71.16 ± 10.13	39.67 ± 8.16	573.6 ± 76.56
P value	<0.0001	<0.0001	0.1232	0.3718	0.0157

The reduction seen in CO was also significantly lower in the TH 30-40 group when compared to TH 60-70 (P<0.01). CO in the T group appeared to be higher than S (14.25 ± 3.50 mL/min and 12.57 ± 1.81 respectively) but this difference was not significantly significant (P>0.05) and the CO in this group was higher at baseline so this result is not unexpected.

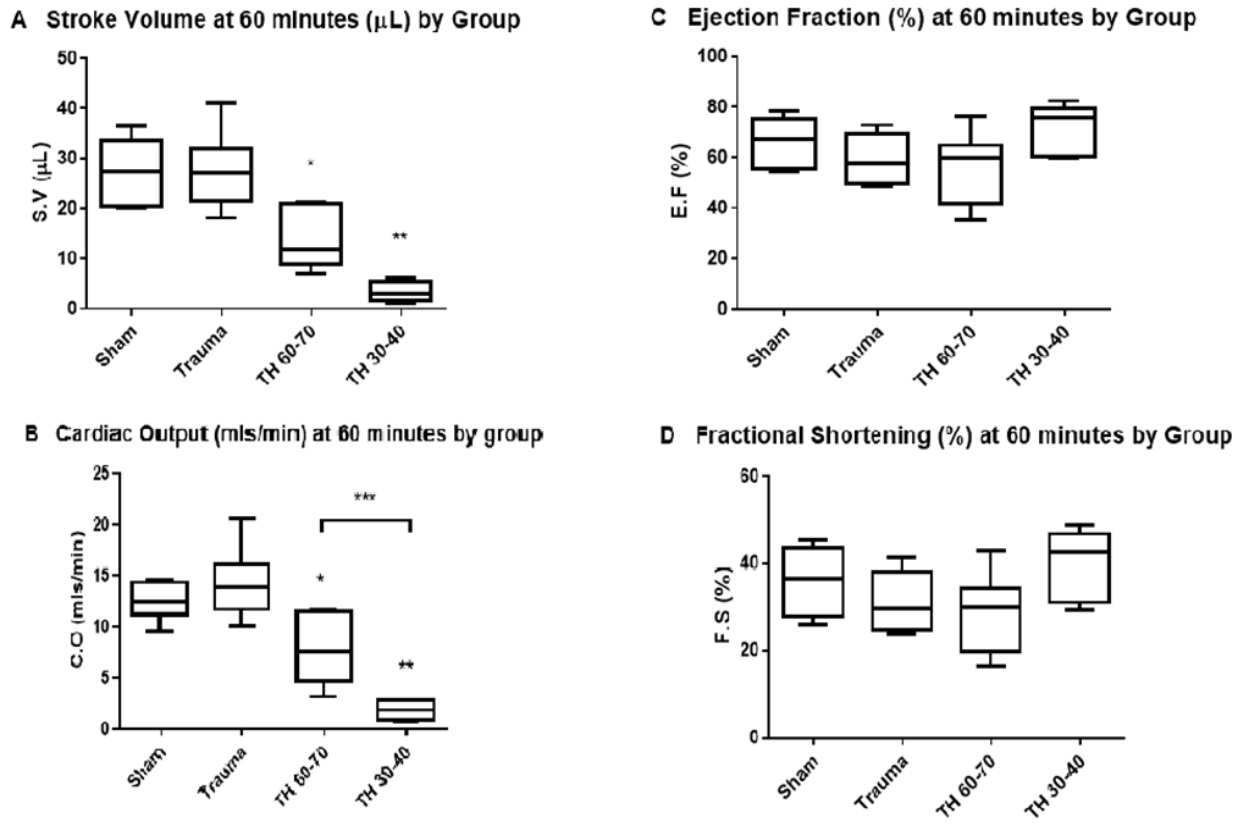


Figure 4.4 Left ventricular functional outcomes at 60 minutes.
Box and whisker plot showing **A** Stroke Volume (SV), **B** Cardiac output (C.O), **C** Ejection fraction (E.F) and **D** Fractional shortening (F.S) at 60 minutes. Median values (horizontal lines within box), interquartile range (box) and range (whiskers) are shown *P<0.05, **P<0.001 *versus* sham or trauma. ***P<0.01 TH 60-70 versus TH 30-40.

Ejection fraction and fractional shortening did not have any statistically significant difference across the 4 groups at 6o minutes (P>0.05).

The heart rate tended to be numerically higher in the T *versus* S group but this was not statistically significant. HR was significantly higher in the haemorrhaged groups (P<0.05 and P<0.01) when compared to S and T (fig 4.5).

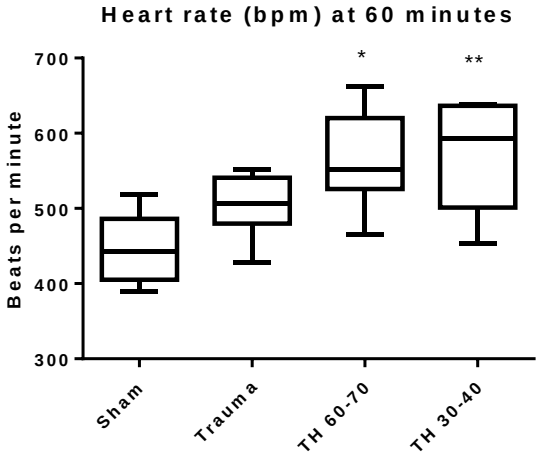


Figure 4.5 Heart rate at 60 minutes.
Box and whisker plot showing heart rate (HR) at 60 minutes. Median values (horizontal lines within box), interquartile range (box) and range (whiskers) are shown. *P<0.05 *versus* sham, **P<0.01 *versus* sham.

4.5.3 Perfusion status after trauma-haemorrhage

Cardiac index (calculated as litres per gram of body weight per minute) was derived as an indicator of perfusion using individual animal's weight and cardiac output at specified time-points. There was no statistically significant difference between the groups in terms of cardiac index at baseline (refer to table 4.5 below). Cardiac index was significantly lower in the TH 60-70 and TH 30-40 groups when compared to Sham and trauma groups. The TH 30-40 group had significantly lower cardiac index when compared to the less severe TH 60-70 group (P=0.0049).

Table 4.5 Cardiac index changes after 60 minutes of trauma-haemorrhage.

Data is shown for the four experimental groups at baseline and then at the end of the experimental period. (Mean is given with standard deviation in parenthesis). P values are also given and calculated using unpaired t-test (comparison between baseline and 60 minutes) and one-way ANOVA (comparison between the 4 groups at baseline and 60 minutes). $P<0.05$ is considered significant.

	Cardiac Index (L/g/min)		
	Baseline	60 Minutes	P Value
Sham	58.23 (± 11.30)	50.372 (± 4.74)	0.1472
Trauma	67.76 (± 13.70)	51.974 (± 11.57)	0.0538
TH 60-70	67.77 (± 11.078)	28.028 (± 12.79)	0.0002
TH 30-40	66.97 (± 7.86)	8.386 (± 3.40)	<0.0001
P Value	0.3897	<0.0001	

The relationship between cardiac index at baseline and after 60-minutes of TH without resuscitation is illustrated in fig.4.6. Cardiac index will, by virtue of how it is derived, reflect the drop in cardiac output seen after TH but can be considered an indicator of how cardiac output is related to the size and therefore the perfusion status of the animal as a whole. For example, 2 animals may have similar cardiac output after an hour of hypotension, but a larger animal (with, by inference, a larger surface area) may be more compromised in terms of their peripheral perfusion status. In order to support this, blood lactate concentrations at the end of the experiments were recorded and a correlation analysis was performed to assess the relationship with cardiac index (fig.4.7).

A B

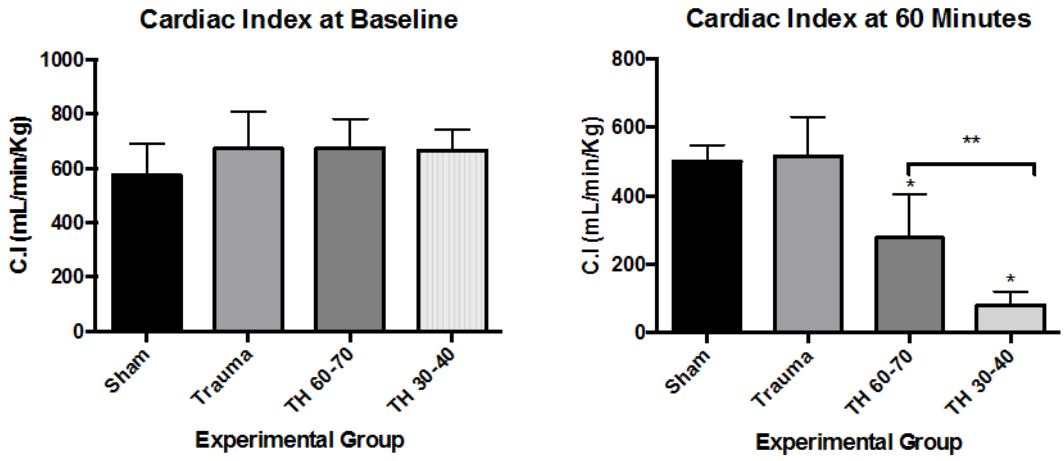


Figure 4.6 Cardiac index at baseline and at 60 minutes.
Graphs show cardiac index at A) Baseline and B) 60 minutes. *P<0.01 when group is compared to Sham or Trauma groups. **P<0.01 when TH 60-70 *versus* TH 30-40.

Blood lactate concentration was significantly higher at 60 minutes in the TH 30-40 animals (P<0.05) when compared to the other 3 experimental groups (fig.4.7).

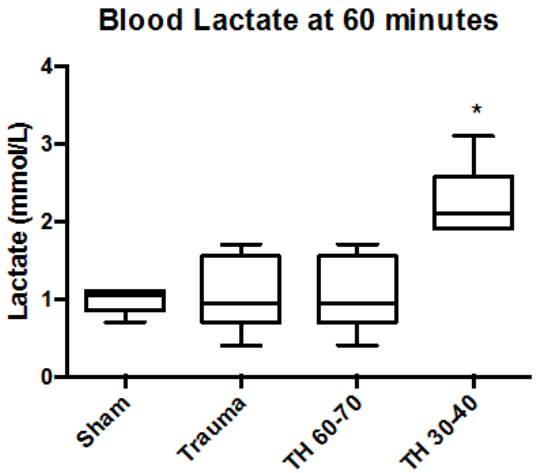


Figure 4.7 Blood lactate concentrations at 60 minutes.
Median values (horizontal lines within box), interquartile range (box) and range (whiskers) are shown. *P < 0.05 *versus* Sham, Trauma and TH 60-70.

4.5.4 The relationship between lactate and cardiac index

There was a negative correlation between cardiac index and blood lactate concentration when all of the animals were assessed with the higher lactate concentrations being recorded in animals with lower cardiac index (fig. 4.8).

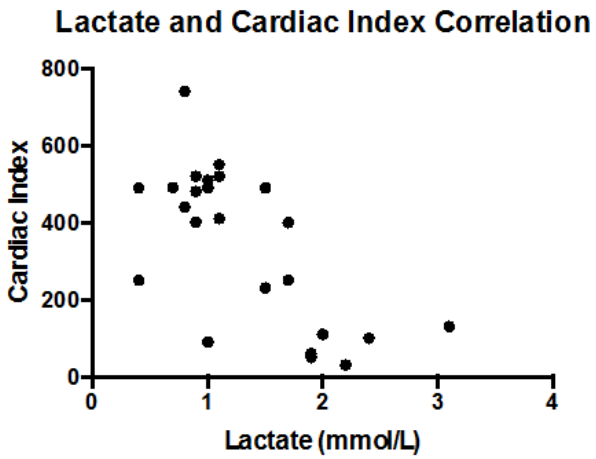


Figure 4.8 Correlation between lactate and cardiac index at 60 minutes.
Scatter graph demonstrates the correlation between blood lactate concentration and cardiac index after 60 minutes of trauma-haemorrhage. Spearman r = -0.6206 (95% confidence interval -0.8231 – 0.2781. P value (2-tailed) = 0.0012).

4.5.5 Serum H-FABP

Serum H-FABP was highest in the TH 30-40 mice (fig. 4.9). This increase was significant when compared to sham controls. Serum concentrations of H-FABP were higher in the serum of T and TH 60-70 mice, but this was not statistically significant (P>0.05). When assessed for a linear relationship between CO and Serum H-FABP, a negative correlation was found. As CO fell, H-FABP increased with Spearman r = -0.6959 (C.I. -0.8811 to -0.3259. Two-tailed P value = 0.0013).

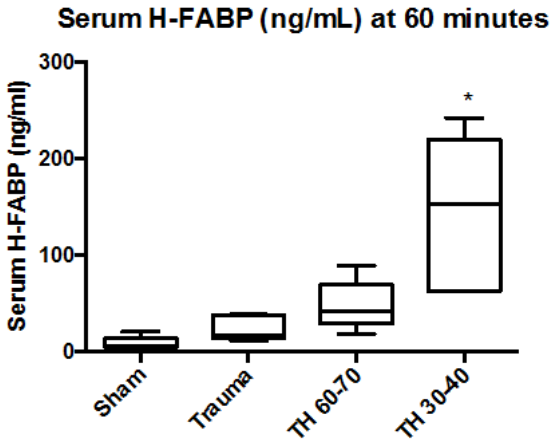


Figure 4.9 Serum H-FABP concentration at 60 minutes in the 4 experimental groups. Box and whisker plots showing serum H-FABP at 60 minutes. Median values (horizontal lines within box), interquartile range (box) and range (whiskers) are shown. *P<0.01 v*ersus* Sham, Trauma and TH 60-70.

4.5.6 The correlation between perfusion and serum H-FABP

Serum H-FABP analysis revealed that there was a tendency for higher H-FABP concentrations in animals subjected to increasing severity of insult. This biomarker rise was significant when TH 30-40 mice were compared to Sham, Trauma and TH 60-70 groups (fig. 4.9). Correlation analysis performed to assess the relationship between H-FABP and cardiac index revealed that there was a statistically significant, strong negative relationship between the two with the highest H-FABP concentrations being seen in the animals with the lowest cardiac indices (P<0.05) (fig.4.10).

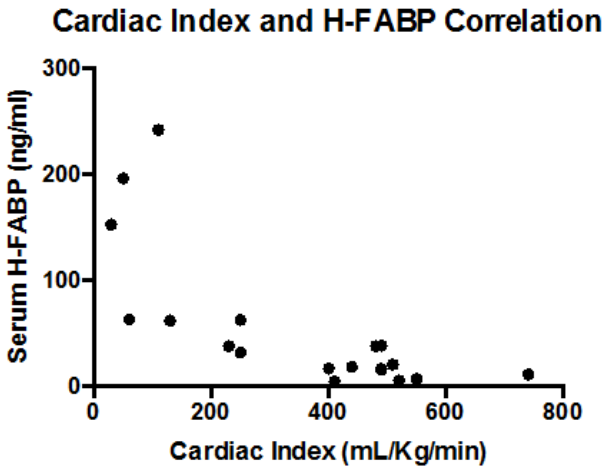


Figure 4.10 Correlation between cardiac index and serum H-FABP concentration. Scatter plot shows the correlation between cardiac index and serum H-FABP at the end of the experiment. Spearman r = -0.7985, 95% confidence interval -0.9192 - -0.5406, P value (2 tailed) <0.0001.

4.6 Discussion

Non-invasive echocardiography has been demonstrated to be a feasible cardiac imaging modality in this murine model of TH and has been used successfully in these experiments to assess left ventricular systolic function before and after trauma-haemorrhage with repeated imaging at specified time-points in the same animal. To the best of my knowledge, this is the first example of this imaging modality being used in a murine model of TISCI.

Serum H-FABP levels at 1 hour reported here corroborate the data presented in the previous chapter with a dose-dependent increase in relation to severity of trauma and haemorrhagic shock at 60 minutes. This study confirms that serum H-FABP levels at 60 minutes correlate with cardiac output with the highest biomarker values reported for the animals experiencing the lowest cardiac output states as a direct result of blood loss. This finding is interesting and one can speculate that it provides an insight into the degree of cardiac output compromise

experienced by patients who present to the emergency department with hypotension and serum H-FABP level rises of a similar magnitude.

There was no significant difference in baseline MAP between any of the groups. S and T groups did not differ significantly at the end of the 60 minute experimental period whereas the TH 30-40 group was significantly more hypotensive than S, T and TH 60-70 as expected. Blood loss as a percentage of body weight was significantly lower in the TH 60-70 group (mean 18.63 ± 3.50% compared to 35.83 ± 5.05% in TH 30-40). Baseline analysis of the left ventricle revealed that there were no significant differences between the groups in terms of LV parameters SV, CO, EF and FS. We can therefore be more reassured that any differences seen at the end of the experimental period were not due to intrinsic differences in left ventricular function between the groups. Heart rate at baseline tended to be higher in the trauma, TH 60-70 and TH 30-40 groups but this was not significant.

At the end of the 60 minute period, SV and CO were significantly lower in the TH groups when compared to S and T with the greatest reduction being seen in the TH 30-40 mice. SV was comparable between the S and T mice. CO tended to be higher in T compared to S but this was found to be non-significant. The higher CO with similar SV seen in these non-haemorrhage groups can be explained by the increased HR (446.5 ±46.77 bpm *vs* 504.33 ± 43.12 bpm) in the injured mice. This increased heart rate may explain the difference in CO but was not statistically significant and likely reflects the expected physiological response to injury. Although there was a trend toward a higher heart rate in the more severe haemorrhage group, this was not significant when compared to TH 60-70. This may be regarded as somewhat surprising as a greater degree of tachycardia may be expected in those animals experiencing a greater percentage blood loss. A technical consideration here relates to acquisition of a signal from the peripherally placed ECG (and therefore HR) pads. In severe shock, it is possible that the resulting vasoconstriction resulted in poorer signal acquisition

and therefore an artefactual relatively reduced heart rate. Another important consideration however, relates to the phenomenon of bradycardia arising in rodents after haemorrhage. Severe haemorrhage in rats has been shown to be associated with a bradycardia response thought to be modulated *via* vagal efferent pathways. Furthermore, tissue injury has also been shown to modulate these reflex pathways (Little *et al* 1989). In this model of severe haemorrhage and tissue injury therefore, it is possible that these vagally-mediated pathways are upregulated and if not significantly lowering the HR, may be limiting the resultant tachycardia. Given that the only mortality that occurred during these experiments was in the most severe haemorrhage group, it may also be the case that the animals were so severely shocked that they were no longer able to compensate or mount a further tachycardia response and this physiological observation may hint at imminent decompensation. The cardiac output and stroke volume data in these animals would support this as it illustrates the severity of the fall in left ventricular outflow however at this stage in the experiment.

The drop in CO and SV after trauma-haemorrhage is dramatic in the data presented here. Blood loss equating to approximately 19% of total blood volume in the TH 60-70 group resulted in a reduction of SV by 66.3% and CO by 58% while a 36% haemorrhage resulted in a percentage reduction of stroke volume of 90.39% and CO was reduced by 88.36% in the TH 30-40 mice. In this, and the preceding study, mortality during the TH phase was only seen in the TH 30-40 group and this may reflect the precarious cardiovascular situation at this degree of blood loss. (Post-mortem analysis of the laparotomy wound and the femoral fracture sites precluded the possibility of bleeding at these sites as a source of ongoing haemorrhage).

Given that myocardial perfusion is dependent upon filling of the coronary arteries during diastole, this combination of reduced SV and shorter diastole as a result of the increased heart rate, may go some way to explaining the possible cardiac ischaemia and cardiac biomarker

rise seen in this model. Serum analysis revealed significantly higher H-FABP concentrations in the TH 30-40 mice when compared to all other groups with a numerical tendency for concentration to rise with severity of insult. Sympathetic drive in response to injury and blood loss may generate increased myocardial mechanical and metabolic activity that the coronary flow is not able to reconcile under these conditions. The resultant myocardial ischaemia, demonstrated by a rise in circulating H-FABP, may set in motion pathways which later lead to the development of overt cardiac dysfunction and, as referred to earlier, there is a need to perform longer term studies to evaluate myocardial function longer-term.

Cardiac index has been used in previous animal models of trauma-haemorrhage as a haemodynamic outcome measure (Coimbra *et al* 2004) but tends to be measured using thermodilution techniques rather than in the way described here with echocardiographically derived cardiac output measurement. Changes in cardiac index, used as a measure of cardiac performance and relates cardiac output to the size of the individual (in these experiments based upon weight) also serve to highlight the extent of the haemodynamic compromise experienced by the animals in the most severe haemorrhage group. Baseline measurements were similar throughout the 4 groups and although a small decline was seen in the sham and trauma groups at 60-minutes, this was not significant. Both haemorrhage groups had significantly lower cardiac index when compared to sham and trauma at 60-minutes and the TH 30-40 group animals had a significantly worse cardiac index at 60-minutes when compared to the less hypotensive group members. A limitation regarding the analysis of cardiac index however relates to the weight of animals used as experimental animals were only used if they were within the defined weight range.

Fractional shortening and ejection fraction were also recorded before and after TH. There was no significant difference seen in these parameters when 60-minute data was compared to baseline. Fractional shortening assesses the degree of shortening of the diameter of the LV

between end-diastole and end-systole and therefore acts as an approximation of myocardial contractility. This 60-minute model of TH did not therefore demonstrate any significant acute change in myocardial contractility when using this systolic functional outcome. Haemorrhagic shock has previously been demonstrated to negatively impact upon myocardial contractility with or without accompanying mechanical injury. Mechanical cardiac dysfunction has previously been reported as soon as 30 minutes after trauma (Feng *et al* 2013) but the dysfunction was not seen in vivo but rather within isolated heart systems and in preparations of isolated cardiac myocytes. In the majority of the studies, overt cardiac dysfunction, if seen, typically occurs beyond 1 hour (Remmers *et al* 1998 and Hsu *et al* 2009), and in some cases up to 24 hours after initial TH. (Sambol *et al* 2009) Therefore, the duration of the studies reported here has demonstrated a significant rise in H-FABP over a 60-minute period, but may not be long enough to allow for the development of overt myocardial dysfunction, as assessed with echocardiography. The omission of resuscitation in the model may also account for the apparent lack of overt cardiac dysfunction. The vast majority of small animal models in the contemporary literature regarding cardiac dysfunction after trauma-haemorrhage include resuscitation in their protocols. Reperfusion of previously ischaemic myocardial tissue may set in motion a number of pathophysiological pathways, both locally and distant, culminating in ventricular dysfunction. Inflammatory mediators generated in response to distant injury and hypoxia for example, may only impact upon the heart upon restoration of circulating volume and therefore in states of hypovolaemia, as in the un-resuscitated model presented here, the full impact of these factors may not be appreciable.

Many studies have identified the presence of TISCI and there have been a number of different methods employed in order to assess cardiac function after trauma haemorrhage in animal models. The contemporary literature in this field (discussed previously) has however, generally focussed upon indirect techniques to explore changes in left ventricular function in

relation to TISCI such as the thermodilution methods. Methods such as the isolated perfused heart system have also been beneficial in providing a controlled circulatory system in which to assess therapeutics particularly. However, such ex vivo techniques have limitations, including removal of the heart from the ongoing exposure to circulating mediators constituting part of the milieu of haemorrhagic shock and inflammation.

Imaging the *in vivo* model means that the cardiac function that we see here is within the context of the myriad of neuro-hormonal changes which are occurring in response to trauma and haemorrhagic shock and in this way, could be considered to be more translatable to the clinical scenario. These experiments have demonstrated that, despite technical constraints such as anaesthesia, the animals' haemodynamic responses and compensatory mechanisms are still responsive and trends reflect those which we see in humans after significant haemorrhage i.e. tachycardia to maintain cardiac output in attempts to maintain adequate cardiac output and perfusion.

The application of non-invasive imaging to the previously described small animal model of TISCI has allowed for the characterization of LV function in the acute stages after TH in the presence of biomarker proven cardiac injury. This un-resuscitated model has demonstrated the development of biomarker-proven TISCI in a 60 minute model of hypotension and trauma. Imaging the left ventricle in these studies has demonstrated the impact of haemorrhagic shock and injury on left ventricle functional parameters and has also allowed for the assessment of a 'personalised' marker of haemodynamic status in relation to habitus, reported as cardiac index.

Clinical studies have highlighted the need for functional assessment of the myocardium in the presence of TISCI. However, many questions still remain about the underlying pathophysiology and functional consequences of TISCI. The development of this *in vivo*

model of TISCI will prove useful in the further characterization of the functional myocardial outcomes associated with this clinically important pathophysiology. No overt myocardial dysfunction was demonstrated during these experiments at 60-minutes, even in the context of raised serum H-FABP concentrations. A longer-term model is therefore warranted in which to observe for the development of dysfunction beyond 60 minutes after initial severe TH.

4.7 Limitations

Some of the commonly encountered limitations when performing these types of studies in small animal models have already been discussed. Small animal work however is challenging and the small size and blood volumes means that there are restrictions on blood sampling frequency and monitoring. Despite these issues, I believe that these studies have demonstrated the feasibility of performing robust, reproducible *in vivo* cardiac imaging in a complex trauma-haemorrhage model.

4.8 Conclusions

Echocardiography is a non-invasive imaging modality, which provides the clinician with rapid, reproducible structural and functional information. Transthoracic echocardiography (TTE) has, in more recent years, been proposed as an adjunct to more traditional methods of assessing volume status and monitoring response to fluid therapy in adult trauma patients. (Porter *et al* 2015) Although there are no specific recommendations regarding its role in this aspect of trauma care at present, this serves to demonstrate the potential wide-ranging applications of cardiac ultrasound and how pre-clinical models will need to keep pace with developments in the clinical arena.

The application of this highly relevant and translatable imaging modality to this *in vivo* model of TISCI therefore represents a more sophisticated platform in which to further our knowledge regarding myocardial function in TISCI. The studies presented in this thesis thus far have focused upon the impact of trauma with hypoperfusion resulting from haemorrhage. Future work is needed to assess the impact of reperfusion on the myocardium. There is a need therefore to take the TH model described here forwards and develop a longer-term, resuscitated model of TISCI. The addition of echocardiography to the previously described TH model is feasible and has provided important insights into acute myocardial function and offers an opportunity to assess how the ventricle responds to changes in haemodynamic status. For these reasons, the next studies will include echocardiography as a means of assessing response of the left ventricle, and to monitor for the development of overt myocardial dysfunction, at later stages after TH and resuscitation. The clinical studies relating to TISCI (De'Ath *et al* 2012 and Naganathar *et al* 2015) observed biomarker rise and the development of adverse cardiac events in trauma-haemorrhage patients. For this reason, subsequent studies will focus on the TH pre-clinical model with combined injury and haemorrhagic shock as a precipitant of TISCI.

CHAPTER FIVE

Developing a Murine Model of Resuscitated Trauma-Haemorrhage to Investigate the Longer Term Functional Consequences of TISCI

Chapter Five

5 Developing a Murine Model of Resuscitated Trauma-Haemorrhage to Investigate the Longer Term Functional Consequences of TISCI

5.1 Introduction

The data previously presented in this thesis demonstrates the presence of biomarker-proven secondary cardiac injury after a 60-minute period of trauma and haemorrhagic shock.

What remains less clear is the impact of this cellular injury upon myocardial function in the longer term and how the cardiac biomarker profile changes over time and with resuscitation. In order to investigate this, it is necessary to develop the existing murine model of TISCI and, using the non-invasive imaging techniques discussed previously, restore circulating volume guided by baseline pre-load conditions. *Chapter four* demonstrated the predictable nature of the decline in blood pressure, cardiac output, stroke volume and left ventricular end-diastolic pressure after 60-minutes of trauma haemorrhage and how these relate to cardiomyocyte injury as assessed by circulating H-FABP levels. This raises questions regarding how the left ventricle will function in the longer-term with this established secondary cardiac injury. The duration of the experiment could simply be prolonged until the inevitable demise of the animal, but the restoration of pre-load and sequential in vivo imaging would be more informative with regards to observing how the injured myocardium responds to resuscitation over time and how this relates to biomarker levels.

The addition of a resuscitation phase to the established *in vivo* model, will allow for functional assessment of the heart, *in vivo*, in real-time and at time-points further down the line from the initial TH. Replacement of intravascular volume, and therefore pre-load to the left ventricle, affords us the opportunity of characterising the myocardial response to resuscitation after an initial 60-minutes of reduced perfusion and subsequent cellular injury with the release of H-FABP. The addition of resuscitation to the existing model, introduces reperfusion to the myocardium thereby mirroring the clinical situation when intra-venous fluid is administered to a casualty after a preceding period of blood loss and reduced perfusion pressures. Resuscitating the existing murine model of TISCI in this way therefore more closely resembles the clinical setting. An hour of TH followed by fluid resuscitation with an increase in organ perfusion pressures, exposes the myocardium to both ischaemia and then subsequent reperfusion and renders it more translatable to the clinical scenario.

Many models of resuscitated TH exist in the literature and these have been discussed previously in this thesis. Contemporary models of cardiac injury and dysfunction after TH however, demonstrate a variety of resuscitation strategies. The choice of resuscitation fluid may range from purely clear fluid solutions to blood only or in some instances, a combination of the two. Goals, or end-points, of resuscitation may be fixed for example, a pre-defined volume of fluid being delivered which signifies the completion of resuscitation or may be more dynamic**,** for example, titrating fluid in order to reach a pre-defined haemodynamic end-point such as arterial blood pressure. The route of fluid delivery may also vary although the vast majority of protocols involve intravenous fluid administration.

At low ventricular end-diastolic volumes, as demonstrated in the previous chapter in response to controlled haemorrhage, stroke volume can be quickly increased with the administration of an intravenous fluid bolus. Cardiac output will also rise in line with the stroke volume in the context of resuscitation with fluid. Perfusion to the tissues increases, along with the delivery of oxygen. Administration of resuscitative fluid will continue to elevate the stroke volume and cardiac output until a plateau point. Beyond this point, further fluid will not serve to increase these systolic parameters and further increase of preload may in fact prove to be detrimental, leading to complications including accumulation of pulmonary fluid (Weidemann *et al* 2006).

It is therefore important when developing a model in which to investigate TISCI that the interventions performed are not contributing to the entity being investigated. Cardiac failure as a result of over exuberant fluid resuscitation for example, would not therefore truly represent dysfunction resulting from trauma and haemorrhage and the purposes of these experiments are not to investigate resuscitation strategies and resultant complications. The acquisition of a baseline scan in these experiments is therefore incredibly useful as it provides a target for tailored resuscitation in these studies which would, one hopes, avoid over-filling the heart and the complications that this may cause. The nature of echocardiography means that scanning can be performed to rapidly, reliably and reproducibly assess the filling status of the left ventricle and inform the decision regarding the requirement of further fluid boluses. Having the baseline data means that there can be greater confidence that the left ventricle is not being overfilled (although it is of course possible that a heart subject to TISCI may still be overfilled if subjected to restoration of baseline preload conditions). Other clinical parameters, such as blood lactate concentration can be used to determine the response to fluid administration. Urine output, arterial oxygenation, pH and conscious level are also

parameters which have value in determining adequacy of resuscitation, but the mouse model is not amenable to having these monitored due to practical considerations.

In spite of the widespread use of echocardiography in pre-clinical cardiovascular research, to the best of my knowledge, none of the contemporary trauma-haemorrhage models use non-invasive imaging of the heart in order to establish the extent and adequacy of resuscitation. One of the benefits of this novel approach is the ability to view, in real-time, the volume status of the left ventricle and therefore the pre-load conditions. The ability to scan animals before surgery and TH will establish individual baseline conditions of the left ventricle, which can then serve as an appropriate end-point in a goal-directed resuscitation strategy implemented in a model designed to investigate cardiac function in association with TISCI.

5.2 Study Aims

The overall aim of these studies is to introduce a resuscitation phase into the established model of TISCI and monitor left ventricle and biomarker response.

This will be achieved by fulfilling the following objectives:

1. Determine whether echocardiography can be used to assess fluid response.
2. Compare MABP and stroke volume as end-points for completion of resuscitation.
3. Conduct a 60-minute monitoring period after completion of resuscitation in which to observe the haemodynamic response to fluid and establish whether longer studies are feasible.
4. Measure H-FABP levels 2 hours after TH to determine the response to resuscitation and how this is affected by the end-point of resuscitation.

5.3 Materials and Methods

The broad materials and methods have been previously described in *Chapter Two*.

Animals were randomly allocated into either MABP or SV-directed resuscitation groups. Only animals that survived the 60-minute post trauma haemorrhage monitoring phase were included. Animals dying before the 'TH60' time-point were replaced.

After completion of the 60-minute monitoring phase after trauma-haemorrhage, animals were resuscitated with blood and crystalloid according to their group allocation.

5.3.1 Experimental groups for the *Chapter Five* studies

There are two experimental groups for the following studies. The groups and the interventions undertaken in the groups are outlined in table 5.1 below.

Table 5.1 Experimental groups, interventions and final N number for the Chapter Five studies.

MABP = Mean arterial blood pressure, Echo = Echocardiography, CSL = Crystalloid, SV = Stroke volume.

Intervention	Experimental Groups	
	MABP Directed Resuscitation Group	Stroke Volume Directed Resuscitation Group
Cannulation	Carotid & Jugular	Carotid & Jugular
Pressure-dependent haemorrhage	Yes. 30-40mmHg	Yes. 30-40mmHg
Laparotomy & Fracture	Yes	Yes
Baseline Echo	Yes	Yes
Resuscitation Fluid	Shed blood & CSL via jugular catheter	Shed blood & CSL via jugular catheter
Resuscitation Endpoint	Baseline MABP+/-5mmHg	Baseline SV+/-5µL
Final N number	10	10

5.3.2 Blood pressure directed resuscitation

Animals allocated to the blood-pressure end-point group received intravenous fluid boluses with the intention of restoring baseline MABP +/- 5mmHg. Fluid administration was conducted between 5 – 10 minutes and completion of the resuscitation period in this group was confirmed with reaching a MABP within the target range (or the failure of the MABP to increment appropriately despite earlier response and increase of MABP in absence of on-going haemorrhage and delivery of appropriate percentage of isoflurane). After reaching the target MABP, a monitoring period was then commenced whereby temperature and MABP were continuously monitored, echocardiographic assessment was made at specific time-

points but no further fluid boluses were administered. Total volumes of shed blood and crystalloid administered were recorded.

5.3.3 Stroke volume directed resuscitation

Baseline stroke volume (SV) of each mouse was calculated, as above, after anaesthesia but before surgery. The average stroke volume of 3 repeated measurements was recorded and used as the target for subsequent resuscitation. The resuscitation phase commenced as for the MABP group, with the intravenous infusion of shed blood, followed by a variable volume of crystalloid. Throughout the fluid administration procedure, imaging was performed continuously in order to monitor the response to fluid delivery and to guide the administration of further fluid boluses. Attainment of baseline SV indicated the completion of the resuscitation phase. In animals where the SV increased in response to fluid but the response reached a plateau and no further increment occurred in response to a bolus, this point was taken to represent the completion of the resuscitation phase. Volumes of shed blood and crystalloid administered were recorded. Animals then began a period of monitoring with intermittent echocardiographic assessment and continuous temperature and MABP monitoring. No further fluid boluses were delivered during the monitoring phase.

5.3.4 Experimental outline for *Chapter Five* studies.

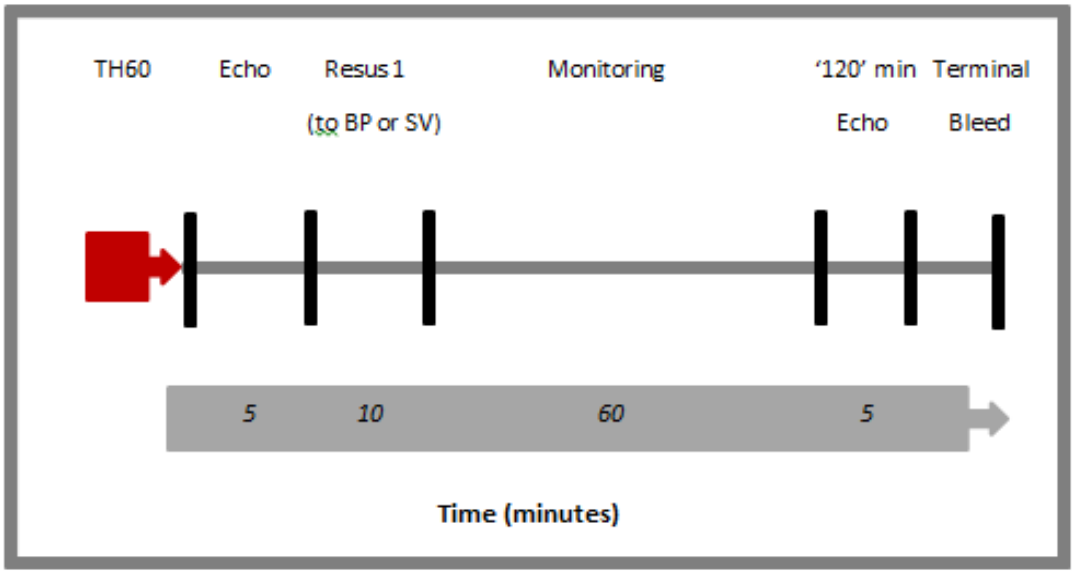


Figure 5.1 Experimental outline.
Schematic representation of the experimental outline for the 2 hour resuscitation studies. The red arrow indicates the end of the 60 minute trauma-haemorrhage phase. The average time taken for echo, resuscitation and monitoring are shown. Echo = echocardiography, Resus = resuscitation, min = minute.

5.4 Results

A total of 26 mice were used in these experiments. All underwent anaesthesia and baseline scanning. 2 in the MABP-directed group died during the TH phase and another 2 were excluded due to technical issues with the jugular line (dislodged / blocked and therefore unable to use for resuscitation). In the SV-directed group, 2 died during the TH phase before receiving any resuscitation fluid. It was not possible to draw blood from the arterial catheter at the end of the experiment for one animal in the SV-directed resuscitation group making the N number for H-FABP and lactate analysis was therefore N=9 for the SV-directed resuscitation group but functional haemodynamic and echocardiographic data was available for all 10 animals in both groups.

5.4.1 Baseline haemodynamics and left ventricular function

There was no statistically significant difference between the 2 groups in terms of MABP and heart rate at baseline (P>0.05) (table 5.2).

Table 5.2 Haemodynamic outcomes in the 2 experimental groups at baseline.

Averages are presented with standard deviations in parenthesis. MABP = mean arterial blood pressure, SV = stroke volume, BPM = beats per minute.

Measurement	MABP-guided (n=10)	SV-guided (n=10)	P value (2-tailed)
MABP (mmHg)	80 (± 4.14)	78.1 (± 4.13)	0.3306
HR (BPM)	450.38 (± 65.13)	448.08 (± 39.67)	0.9934

There was no statistically significant difference in LV functional parameters at baseline when the 2 experimental groups were compared (P>0.05) (see table 5.3). Cardiac index was calculated for each animal as previously described in *Chapter Two*.

Table 5.3 Baseline left ventricular systolic function.

Descriptive numerical values for LV systolic function obtained in 2 experimental groups at baseline by means of high-frequency transthoracic echocardiography. Mean values are presented with standard deviations in parenthesis. MABP = mean arterial blood pressure, SV = stroke volume, CO = cardiac output, EF = ejection fraction, FS = fractional shortening, LVEDV = left ventricular end diastolic volume. 2-tailed P values are also given.

Measurement	MABP-guided (n=10)	SV-guided (n=10)	P value (2-tailed)
SV (μL)	44.19 (± 5.12)	45.68 (± 3.03)	0.4370
CO (mL/min)	19.87 (± 3.73)	23.81 (± 7.71)	0.2837
EF (%)	62.41 (± 7.16)	58.92 (± 7.32)	0.2954
FS (%)	33.43 (± 5.19)	32.75 (± 5.51)	0.7769
LVEDV* (μL)	72.81 (± 13.13)	79.51 (± 10.25)	0.2286

*****LVEDV calculated retrospectively using the formula (SV/EF) x 100.

5.4.2 Haemodynamic Characterisation of the TH Model

Figure 5.2 summarises the volume of blood drawn *via* the arterial catheter in order to achieve the target MABP of 30 – 40 mmHg. The volumes required to induce hypotension were slightly higher in the SV-directed group (mean 40.17%, SD 4.87% compared to mean 36.80%, SD 4.42%), but were not significantly different between the 2 groups (P>0.05).

The target MABP was reached over a 10-minute period in all animals and maintained throughout a 60-minute TH period. Figure 5.3 illustrates the MABP trends over this time in the 2 groups. There was no statistically significant difference seen between the 2 groups at any of the time-points (P>0.05).

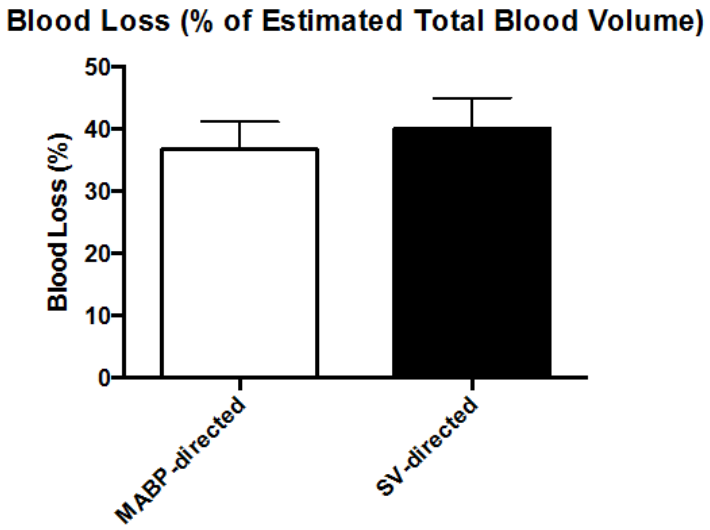


Figure 5.2 Blood loss required to achieve target MABP in the 2 experimental groups. Blood loss is expressed as a percentage of total blood volume (calculated as body weight x 0.07mL). P=0.1431.

At the completion of the 60-minute TH phase, heart rate was recorded and there was no significant difference between the groups at this point (P>0.05). The MABP-directed group had a mean heart rate of 607 ± 108.71bpm and SV-directed group 575 ± 39bpm (P=0.4033).

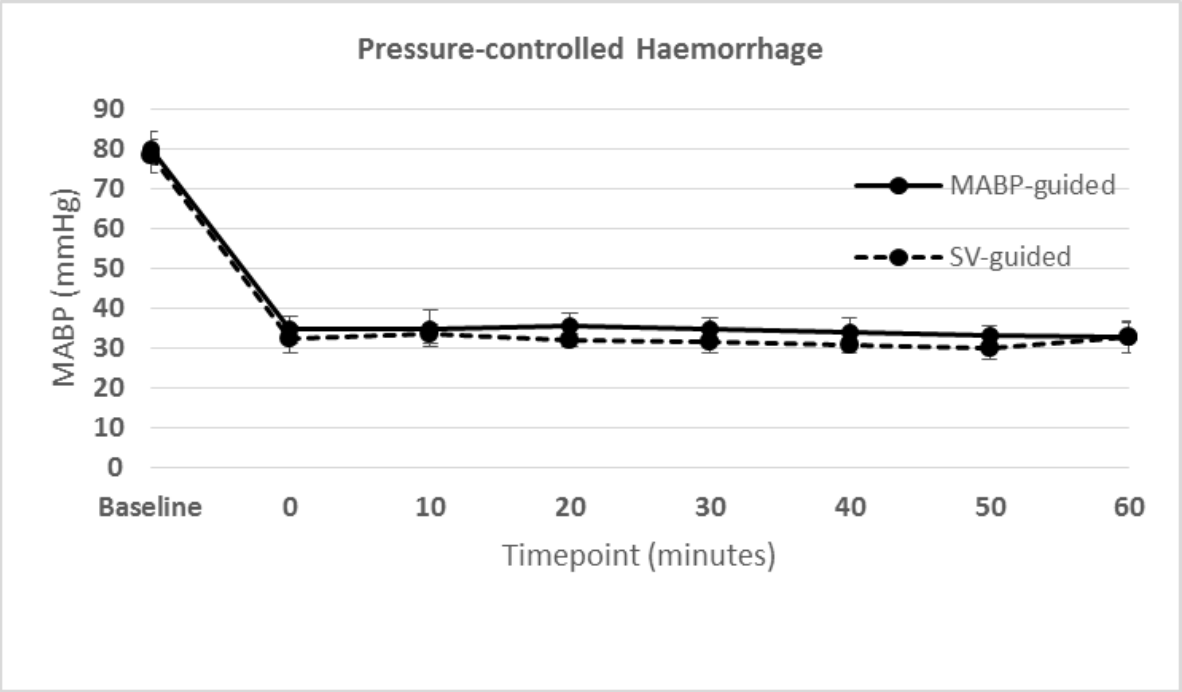


Figure 5.3 MABP trends across the 60 minute TH phase.
Line graph shows the MABP at baseline and the trend in response to fixed-pressure haemorrhage. Dots represent the mean MABP for the group at a given time-point. Vertical bars represent the standard deviation of the mean.

Functional LV parameters after 60 minutes of TH were assessed using echocardiography and Table 5.4 summarises the results obtained for the 2 groups.

SV, CO, LVEDV and CI were significantly lower than baseline in each animal. Each group experienced a comparable reduction however, with no statistically significant differences were seen when the groups were compared to each other at TH60 time-point.

Table 5.4 LV systolic functional outcomes and cardiac index at TH60.

Mean values are given with SDs in parenthesis for stroke volume (SV), cardiac output (CO), ejection fraction (EF), fractional shortening (FS) and left ventricular end diastolic volume (LVEDV). 2-tailed P values are also given.

Measurement	MABP-directed (n=10)	SV-directed (n=10)	P value (2-tailed)
SV (μL)	5.82 ± (2.56)	6.75 ± (3.63)	0.7246
CO (ml/min)	3.39 ± (1.15)	3.98 ± (1.82)	0.3936
EF (%)	65.74 ± (11.83)	61.07 ± (17.21)	0.4885
FS (%)	33.95 ± (7.51)	31.63 ± (11.10)	0.5906
LVEDV (μL)	13.16 ± (9.76)	12.49 ± (11.68)	0.8940

Animals were resuscitated as previously described immediately after completion of the TH60 ultrasound scan. Warmed, shed blood was returned *via* the venous catheter and this was followed by crystalloid infusion.

5.4.3 Fluid volumes required for resuscitation

A B

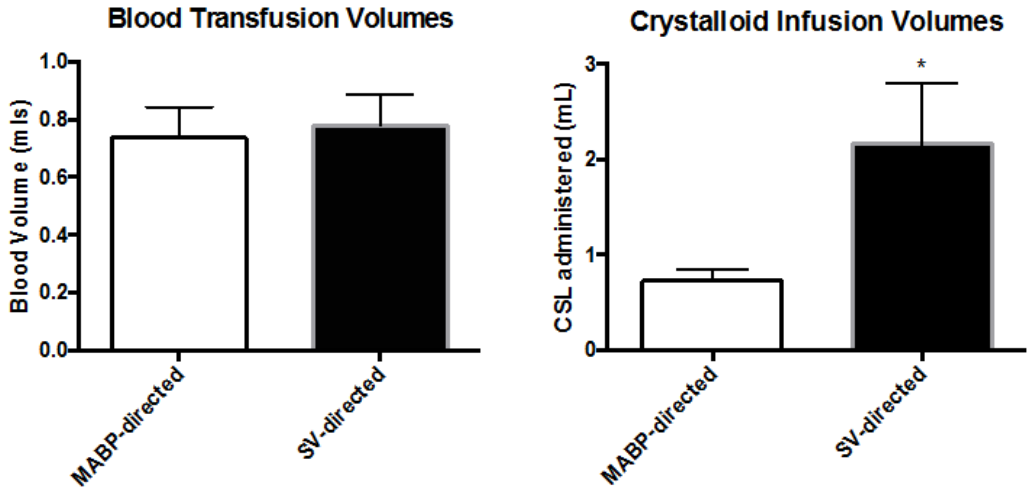


Figure 5.4 Fluid resuscitation volumes.
Bar charts illustrate; **A**. Volumes of shed blood transfused and **B**. Crystalloid administered to achieve end-point of resuscitation. *P<0.001 *versus* MABP-guided group.

Animals in the MABP-directed group received a mean blood volume of 0.74 ± 0.11mL whilst mice in the SV group received a mean of 0.78 ± 0.11mL. There was no significant difference between the groups in terms of the volume of blood infused at the commencement of the resuscitation phase (P>0.05). However, the SV-guided resuscitation group received significantly higher volumes of crystalloid (mean 2.16, SD 0.64mL compared to mean 0.726, SD 0.115, P<0.05). Data is illustrated in figure 5.4 above.

5.4.4 End-Points of Resuscitation

A B

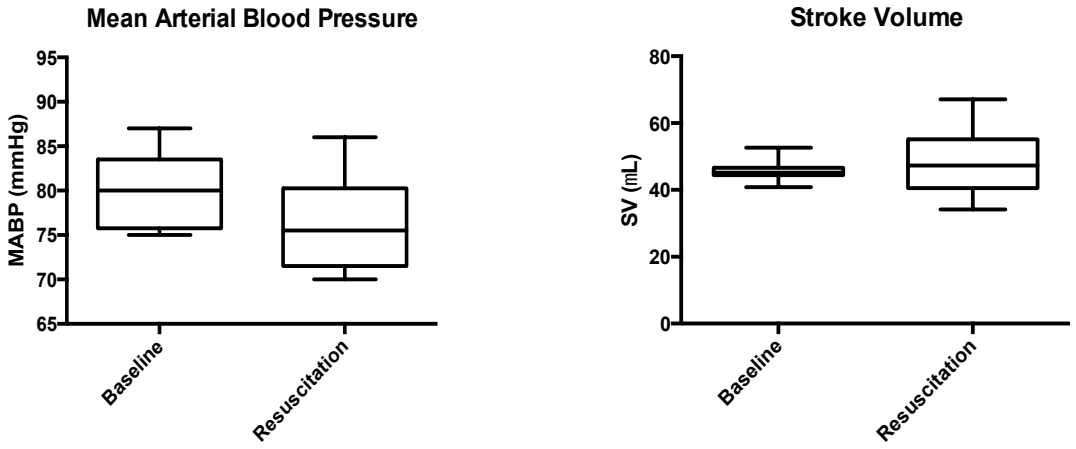


Figure 5.5 MABP and stroke volume at baseline and post resuscitation.
Box and whisker plots show **A**. MABP at baseline and after resuscitation in the MABP-directed group and **B**. SV at baseline and at completion of resuscitation in the SV-directed group. Whiskers represent range, box represents interquartile ranges and horizontal line the median value.

There was no statistically significant difference in MABP at baseline and post-resuscitation in these animals (P>0.05) (fig.5A).There was no significant difference between baseline and post-resuscitation SV in this experimental group (P>0.05) (Fig. 5B).

LVEDV was calculated at the end of the experiment using stroke volume and ejection fraction, and assessment of the strength of correlation between SV and LVEDV at baseline and after completion of resuscitation was calculated and a strong positive correlation between the two was shown (fig.5.6A & B).

A B

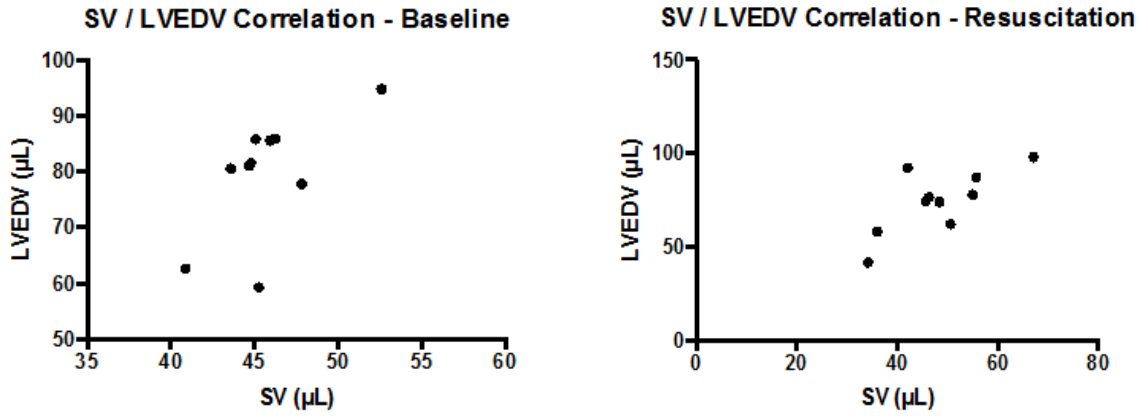


Figure 5.6 Correlation between SV and LVEDV in the SV-resuscitation group. Scatter plots show the correlation between SV and LVEDV at A. Baseline and B. Post resuscitation. Pearson r was calculated for both sets of data. Pearson r=0.6354 (95% C.I 0.009699 – 0.9036, 2-tailed P value = 0.0483) at baseline and r=0.7319 (95% C.I 0.1897 – 0.9320, 2-tailed P value = 0.0161) after resuscitation.

5.4.5 Haemodynamic trends

All animals reached their target MABP or SV with fluid resuscitation. Blood pressure increased in both groups after resuscitation and was then observed to decline in both groups throughout the following 60-minute monitoring period. The MABP tended to be higher in the SV-directed group during this phase of monitoring but this difference was not significantly different at any of the time-points (P>0.05) (fig.5.7).

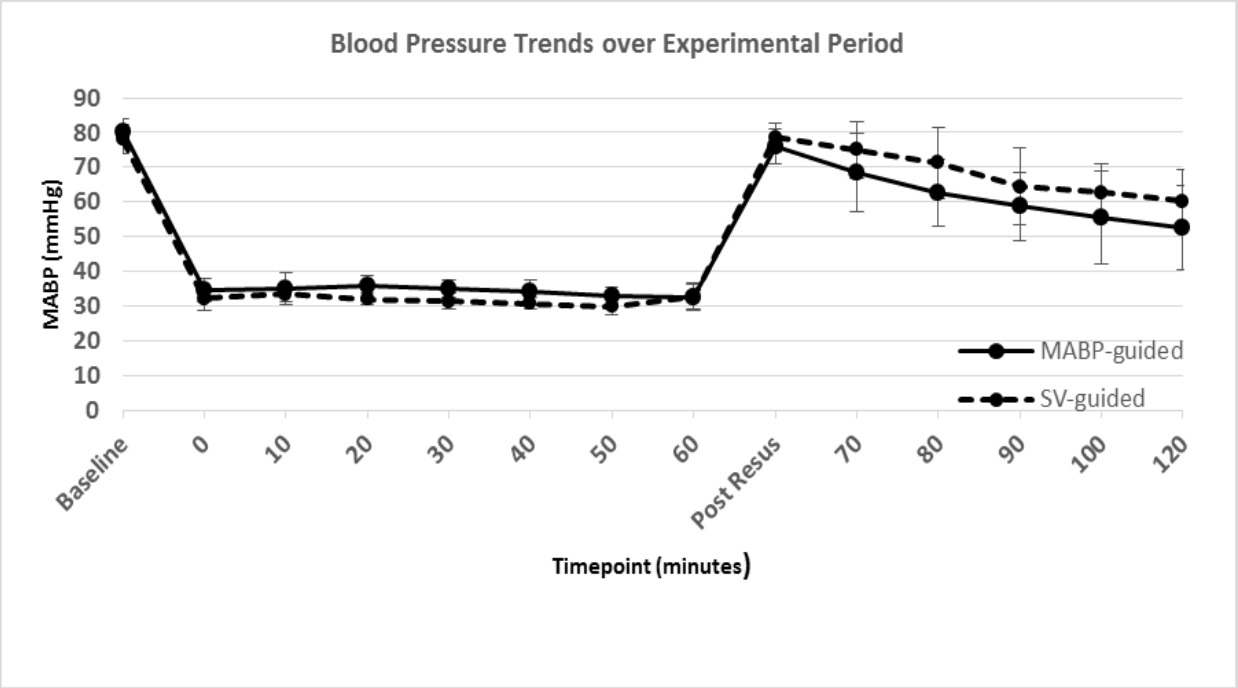


Figure 5.7 Trends in MABP over 2 hours.
Line graph showing trends in MABP in response to TH and resuscitation in the MAPB and SV-directed groups. Dots represent the mean for the group at a given time-point. Vertical bars represent the standard deviations from the mean.

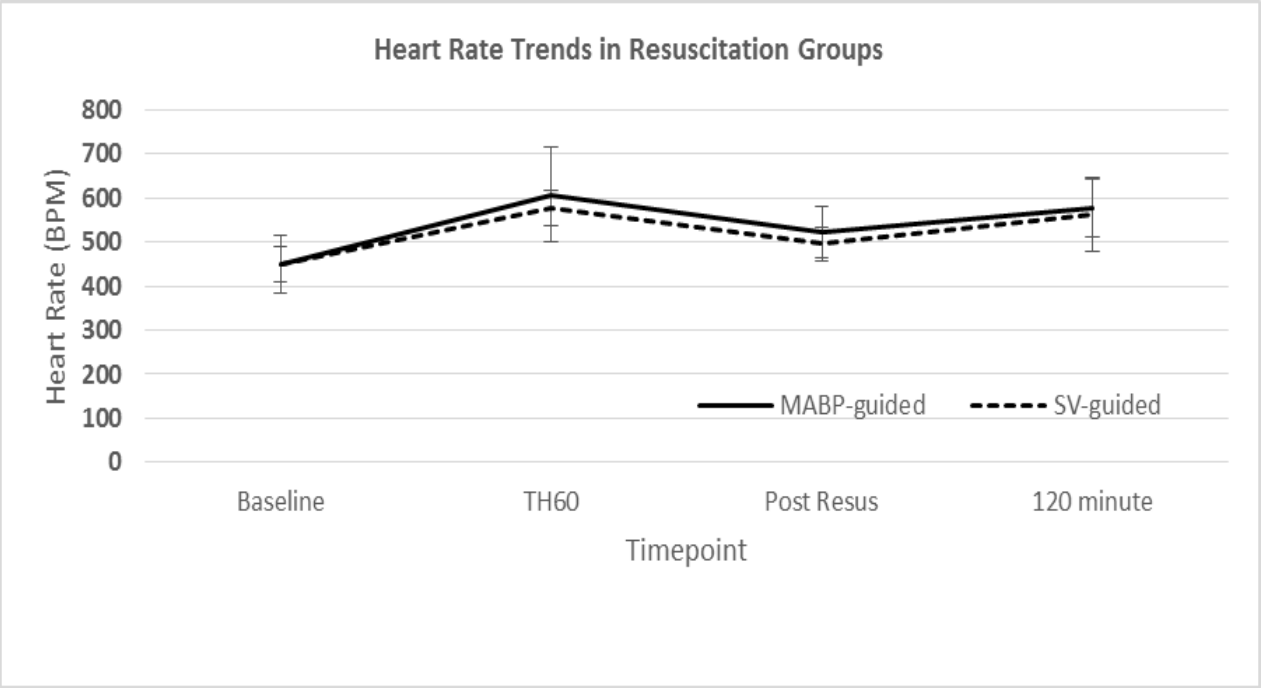


Figure 5.8 Heart rate trends over 2 hours.
Line graph shows trends in heart rate in response to TH and resuscitation in the MABP and SV-directed groups. Dots represent mean for the group at a given time-point. Vertical bars represent the standard deviations from the mean.

In both of the groups, heart rate was higher after a 60-minute period of TH and fell after resuscitation. Heart rate tended to be lower throughout the experimental period in the SV-directed group but this did not reach significance at any time-point (P>0.05) (Fig.5.8).

Core body temperature was monitored throughout the experiment in both groups. Figure 5.9 illustrates the changes in temperature over the course of the experiment and there was no statistically significant difference in temperature between the 2 groups at any of the time-points (P>0.05). The SV-directed group however tend to show less variability in core temperature after resuscitation although this was not statistically significant (P>0.05).

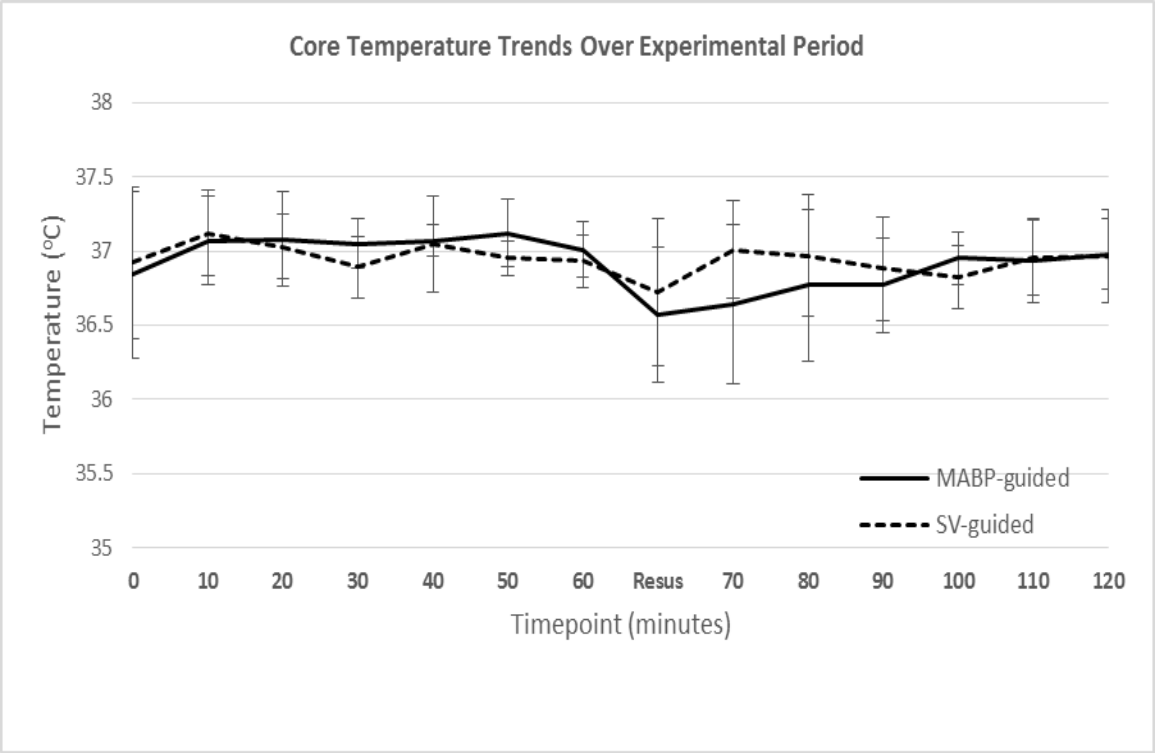


Figure 5.9 Core temperature trends over 2 hours.
Line graph showing the trends in temperature in both experimental groups over 2 hours. Dots represent the mean for the group at a given time-point. Vertical bars represent the standard deviations from the mean.

5.4.6 Functional assessment of the left ventricle after resuscitated trauma-haemorrhage

Left ventricular systolic functional outcomes were assessed at pre-defined time-points in the 2 groups. Tables 5.5 and 5.6 below show the data for LV systolic function and LVEDV after resuscitation and at 120 minutes respectively.

Table 5.5 LV systolic functional outcomes and LVEDV post resuscitation.

Mean values are given with SDs in parenthesis. *Indicates statistical significance when values of the 2 groups are compared.

Measurement	MABP-directed	SV-directed	P-value (2-tailed)
SV (µL)	25.58 ± (8.57)	48.1 ± (9.77)	<0.0001*
CO (mL/min)	13.04 ± (3.85)	25.17 ± (5.62)	<0.001*
EF (%)	59.61 ± (11.69)	67.71 ± (8.13)	0.0887
FS (%)	31.41 ± (7.84)	36.54 ± (8.60)	0.1798
LVEDV (µL)	45.26 ± (13.36)	74.27 ± (15.96)	0.0005*

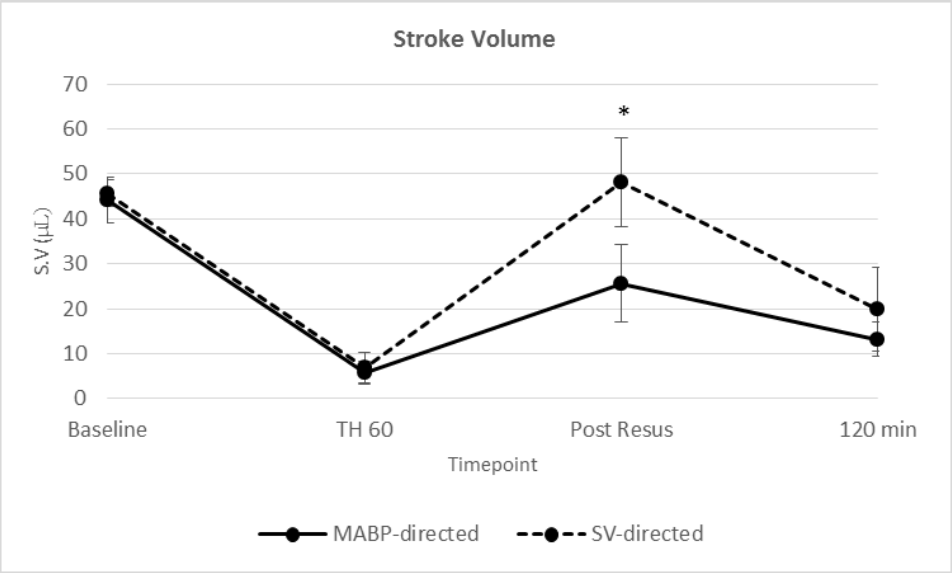
Table 5.6 LV systolic functional outcomes and LVEDV at the end of the study.

Means are given with SDs in parenthesis. *Indicates statistical significance when values form the 2 groups are compared.

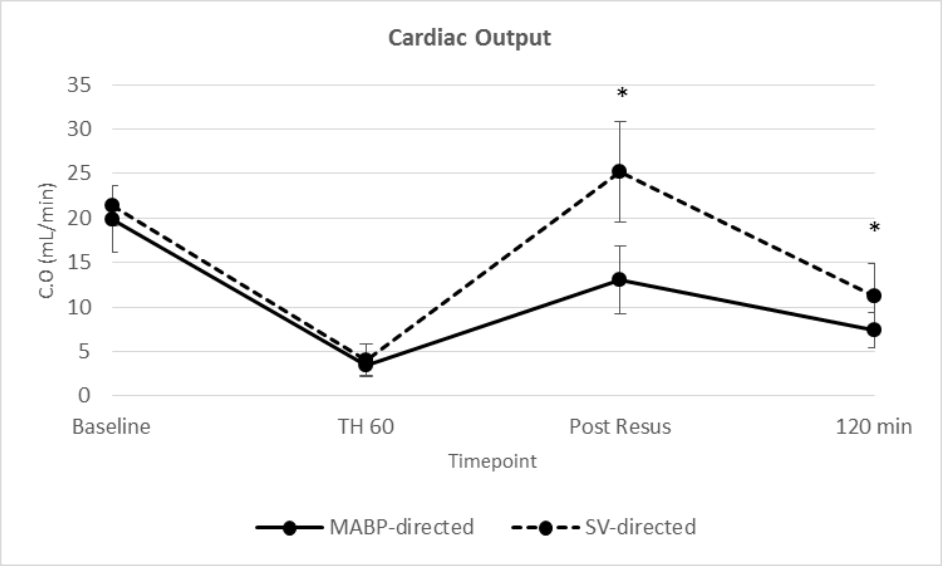
Measurement	MABP-directed	SV-directed	P-value (2-tailed)
SV (µL)	13.17 ± (3.81)	19.89 ± (9.31)	0.0617
CO (mL/min)	7.41 ± (2.03)	11.27 ± (3.68)	0.0152*
EF (%)	51.99 ± (13.32)	65.64 ± (17.63)	0.0820
FS (%)	27.12 ± (8.41)	37.38 ± (14.65)	0.0868
LVEDV (µL)	25.22 ± (7.33)	32.47 ± (16.54)	0.2641

Figure 5.10 illustrates the trends in stroke volume, cardiac output, ejection fraction and fractional shortening over the 120-minute experimental period. There was no statistically significant difference between the groups at baseline and at TH60 (P>0.05).

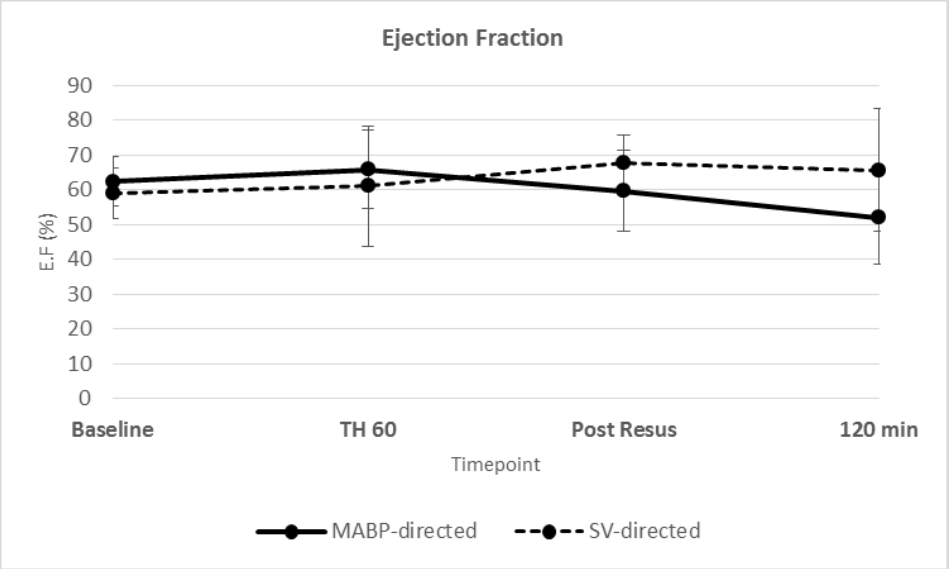
A.



B.



C.



D

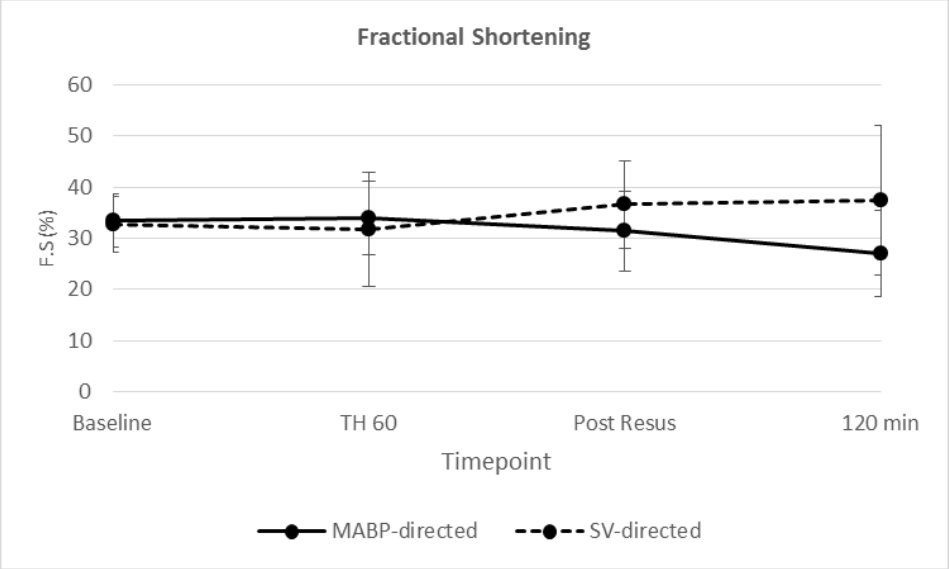


Figure 5.10 LV systolic functional trends over 2 hours.
Line graphs represent changes in **A**. SV, **B**. CO, **C**. EF and **D**. FS over time. Dots represent the mean for the group at a given time-point. Vertical bars represent the standard deviations from the mean.

After resuscitation, the SV-directed animals had a numerically higher ejection fraction and fractional shortening when compared to the MABP-directed group but this did not reach

significance (see table 5.4). At the end of the experiment, the animals in the SV-directed group had significantly higher cardiac output when compared to MABP-directed group animals (refer to table 5.5).

Left ventricular end-diastolic volume was calculated using stroke volume and ejection fraction for each animal and the data is given in figure 5.11. LVEDV was higher in the SV-directed group animals at all time-points but this only reached significance at the post-resuscitation time-point (P<0.05).

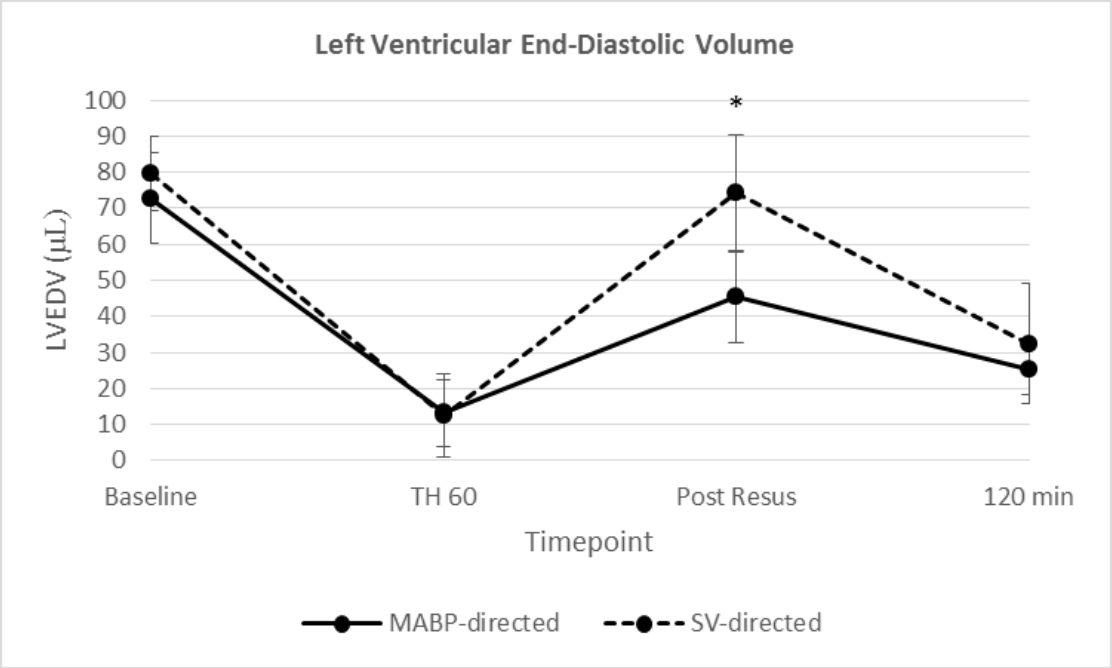


Figure 5.11 LVEDV trends over 2 hours.
Line graph shows changes in calculated LVEDV over the course of the experimental period. Dots represent the mean for the group at a given time-point. Vertical bars represent the standard deviations from the mean. *P<0.05.

5.4.7 Perfusion Indices after TH with Resuscitation

Blood lactate concentrations were recorded at time-points throughout the experimental period as an indicator of tissue perfusion at TH60, after resuscitation and at the end of the experiment.

Arterial blood lactate concentrations were seen to rise and fall in both groups in response to TH and subsequent fluid resuscitation and this data is shown in figure 5.12. The SV-directed group animals had significantly higher blood lactate levels after 60-minutes of TH. Although the lactate levels fell with subsequent resuscitation, they remained higher than in the MABP-directed group at this point and at the end of the experiment, this group again had more severe lactatemia (although this was not significant at this time-point).

Cardiac index was calculated (as previously described) in order to provide another measurement of an individual's tissue perfusion status in response to TH with resuscitation. Trends in C.I are given for the entire experimental period in fig 5.13. As anticipated, C.I drops to a similar extent in both of the groups after TH. It then rises after fluid resuscitation. At 120 minutes, the cardiac index is significantly higher in the SV-directed group ($P<0.05$ with absolute values for the 2 groups are given in table 5.7 below).

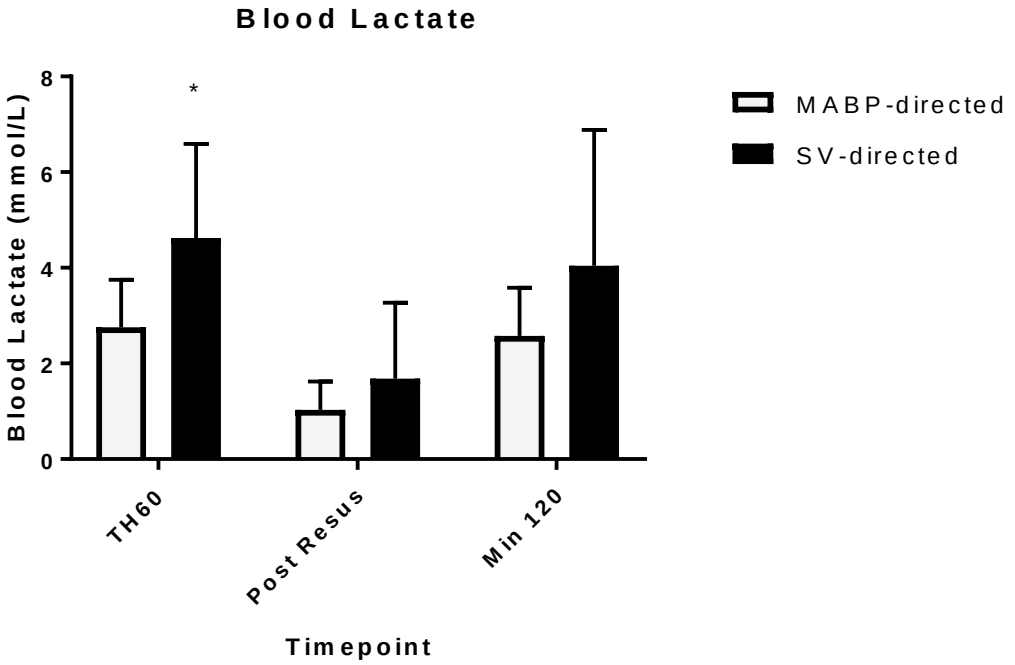


Figure 5.12 Blood lactate concentrations.
Column graphs show lactate concentrations in the 2 groups at 3 experimental time-points. *P<0.05 when the 2 groups are compared at TH60. Lactate concentrations after resuscitation were significantly lower in both groups, when compared to TH60 and 120 minutes.

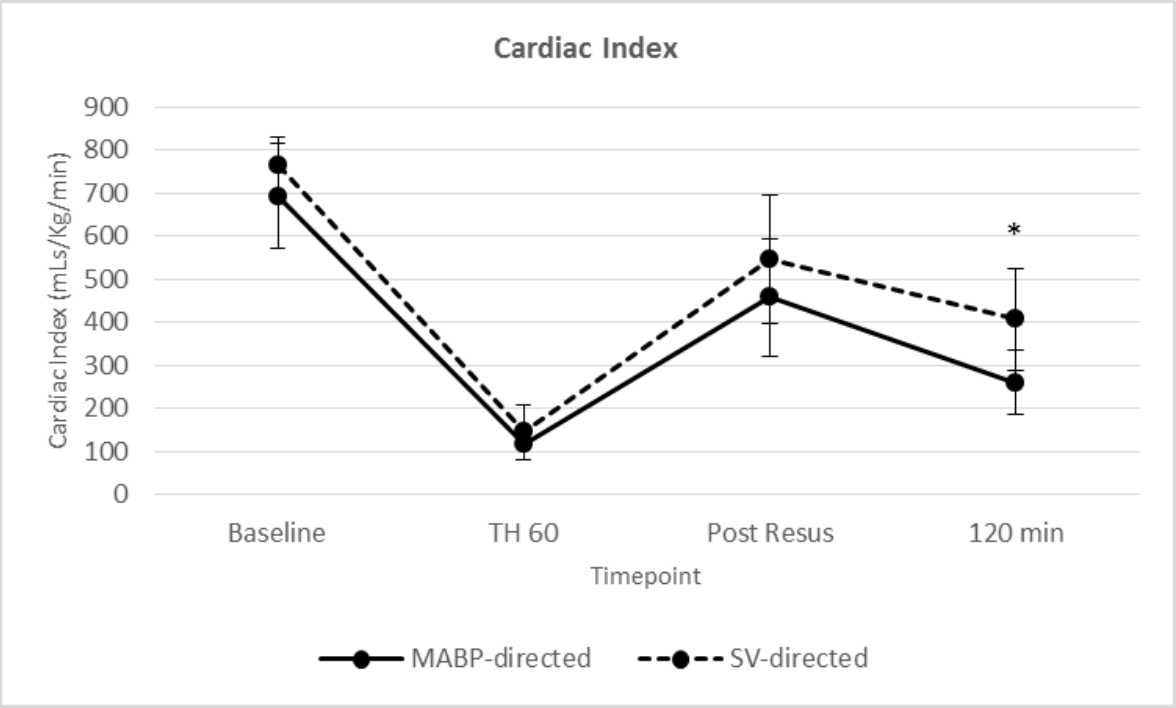


Figure 5.13 Trends in cardiac index.
Line graph showing the trends in calculated cardiac index across the 2 hours in the 2 experimental groups. Dots represent the mean for the group at a given time-point. Vertical bars represent the standard deviations from the mean. *P<0.05.

Table 5.7 Cardiac index results after resuscitation and at 120 minutes.

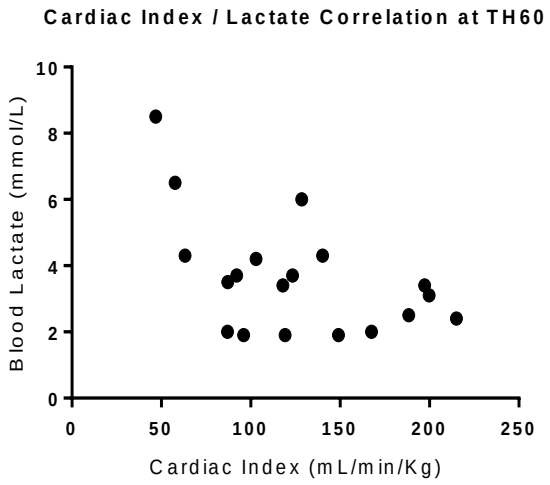
Mean values are given with standard deviations in parenthesis. P values are also shown and * represents statistical significance (P<0.05) when the 2 groups are compared.

Time-point	MABP-directed	SV-directed	P value
Baseline	692.8 (±121.6)	763.96 (±64.1)	0.1102
TH60	118.40 (±37.81)	145.7 (± 63.06)	0.3150
Post resuscitation	457.80 (±137.2)	545.52 (±148.1)	0.2799
120 minutes	260.61 (±75.41)	408 (±118.8)	0.0078*

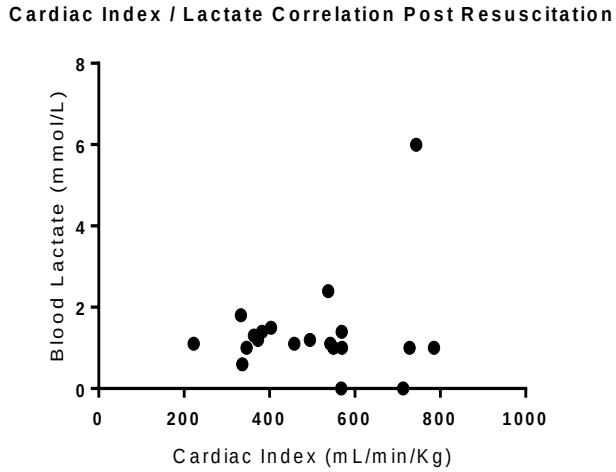
5.4.8 The relationship between cardiac index and blood lactate

The nature and the degree of correlation between cardiac index and lactate, both used as indicators of perfusion, was assessed. There was a negative correlation between the 2 parameters with the highest blood lactate being seen at the lowest cardiac index (Fig.5.14). There was therefore a relationship between C.I and lactate but the strength of this association was variable depending upon the experimental time-point with the strongest association seen at TH60.

A



B



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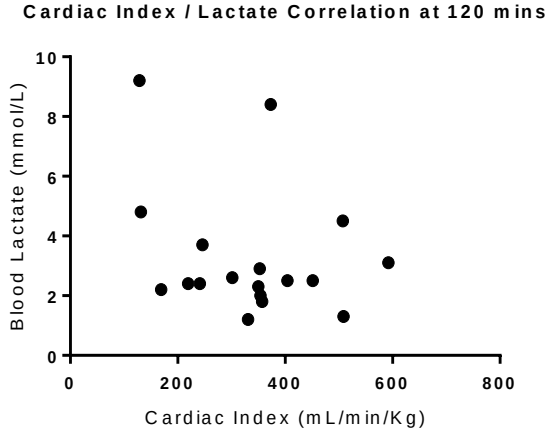


Figure 5.14 Correlation between lactate and cardiac index.
Scatter charts show the correlation between cardiac index and blood lactate concentration at **A**. TH60. Pearson r = -0.5211 (P<0.05), **B**. Post resuscitation. r = -0.2065 (P=0.3824) and **C**: At end of experiment r = -0.2817 (P=0.2574).

5.4.9 Cardiac biomarkers

Cardiac biomarkers were also analysed. H-FABP analysis revealed that both groups had elevated blood concentrations at 120 minutes. There was no significant difference between the 2 groups in terms of H-FABP concentration at the end of the experiment (P>0.05)

(Fig.5.15). There is a greater spread of H-FABP serum concentrations in this compared to the studies in preceding chapters *three* and *four*. The lowest concentrations were seen in the SV-directed group with serum analysed from one animal yielding an H-FABP concentration of 57.97ng/mL (all other animals recorded levels in excess of 150ng/mL). This individual had one of the highest % blood losses, but serial lactate measurements were the lowest within the group. This suggests that, despite comparable severity of haemorrhagic shock, haemodynamic compensation was better in this individual and this may go some way to explain the elevated, but relatively lower H-FABP in this case. Troponin T levels were also measured in the 2 groups but were not detectable in the vast majority at 120 minutes (data not shown).

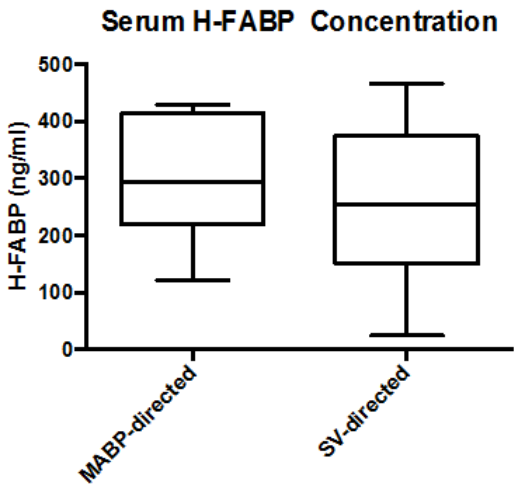


Figure 5.15 Serum H-FABP concentrations.
Box and whisker plots show the serum H-FABP concentrations at the end of the study for the 2 experimental groups. Whiskers represent the entire range, the box represents interquartile ranges and horizontal line the median value.

5.4.10 Survival

There were 4 deaths (2 in each experimental group) during the 60-minute TH phase before the commencement of resuscitation. This was comparable to the mortality seen during the same phase in chapters *three* and *four*, and demonstrates that the animals in these experiments are subject to a potentially lethal severity of trauma-haemorrhage.

There was 1 death in the SV-directed group after resuscitation but before the end of the experiment. There were no deaths in the MABP-directed group but one animal was seen to have a period of haemodynamic instability which was associated with the onset of a cardiac arrhythmia during the final 10 minutes of the experiment (data not shown).

5.5 Discussion

The application of transthoracic echo in our mouse model of TH presented here, has been beneficial in two ways. Not only has it been demonstrated here as a useful tool in the development of a resuscitated model of TISCI, but it has also allowed for *in vivo* assessment of myocardial function after TH with resuscitation. The addition of a resuscitation phase has allowed for the studies in *chapter three* and *four* to be lengthened to 2 hours with good overall survival. Resuscitation to SV requires higher volumes of fluid when compared to the MABP-directed group but this was not associated with significantly higher blood pressure recordings but cardiac output and cardiac index at 2 hours was significantly higher in the SV-directed resuscitation group suggesting that in terms of LVS function, this approach was favourable. H-FABP levels were high in all animals, irrespective of resuscitation strategy and there was no significant difference between the 2 groups.

These studies have demonstrated that H-FABP concentrations continue to rise beyond the 1 hour mark, and this supports clinical data also published by our group, where H-FABP continued to rise up until 24 hours after admission in severely injured patients who developed

adverse cardiac events (De'Ath *et al* 2012). In terms of cardiac events in these studies, 1 animal in the MABP-directed group had a recorded period of arrhythmia towards the end of the experimental period.

Baseline echocardiographic assessment, heart rate and MABP recordings showed that there was no significant difference between the animals in terms of haemodynamic parameters at baseline and after 60 minutes of trauma-haemorrhage. Both groups required a similar percentage blood loss to achieve the target MABP of 30-40mmHg and there was no significant difference in the MABP blood pressure between the groups for the maintained duration of the 60-minute TH phase.

Given the comparable blood loss and blood pressure, it is interesting to note that the SV-directed group did however have higher mean and median lactate concentrations at TH60. This suggests that this group, despite being haemorrhaged to the same target MABP range, were in a more severe shock state in terms of reduced peripheral perfusion and associated accumulation of lactic acid. This group had higher recorded lactate concentrations at every subsequent time-point compared to the MABP-directed group and the only post-resuscitation mortality was in this second group. This perhaps reflects that, despite the same degree of hypotension induced by controlled haemorrhage, some of these animals were more severely shocked and indeed, the animal with the highest recorded lactate at TH60 was the same animal that died before the end of the study.

The end-point of resuscitation had a significant impact upon the volume of fluid required to adequately resuscitate the animal. Both groups received similar volumes of whole blood back at the commencement of the resuscitation period. Resuscitation targeted to restoration of baseline blood pressure was associated with lower volumes of crystalloid administration. In order to achieve baseline stroke volume however, animals required at least double the volume

of crystalloid be given after the return of shed blood (Fig.3B). This demonstrates that a regimen of fluid resuscitation guided by arterial blood pressure alone was not enough to restore intravascular volume measured by left ventricular volume. This is an important finding as this could have particular relevance when designing models in which to assess cardiac function after trauma-haemorrhage. Occult 'under-filling' (I.e. normotension but with significantly reduced LVEDV as reported here) could lead to ongoing physiological derangement, such as tachycardia, as the heart attempts to compensate and maintain cardiac output. A continuous tachycardia in response to fluid depletion, coupled with higher myocardial metabolic demand will lead to shortened diastolic filling times of the coronary arteries and may lead to myocardial ischaemia with resultant biomarker rise and dysfunction.

The MABP in both groups was comparable after resuscitation but one could postulate that in the MABP-directed animals, a larger contribution was being made by neuro-hormonal mechanisms relating to blood pressure homeostasis, particularly after an antecedent period of shock. It could be suggested that these animals remain reliant upon peripheral vasoconstriction mediated by endogenous catecholamines to offset the continuing relative hypovolaemia. Although not significant, these animals also demonstrated higher heart rates throughout the experiment after resuscitation, a finding which supports a greater degree of compensation occurring in this group. Given the lack of other available physiological parameters however, this is difficult to confirm with certainty and the lactate data (with higher lactates recorded in the SV-directed group at the end of the experiment) would not be supportive of this.

The fall in arterial lactate concentrations after resuscitation may reflect the relative hypovolaemia experienced by the MABP-directed group. Although the SV-group lactates were higher at each time-point, the fall in blood lactate between TH60 and 20-minutes after resuscitation was greater in the SV-directed group. Despite these animals having a higher

mean lactate concentration at the end of the TH phase, the extent of the drop in concentration suggests that these animals were able to correct the significantly higher lactate levels accumulated during the first 60 minutes.

Despite the important differences seen in the physiology of the 2 groups, there are some similarities seen in haemodynamic trends across the experimental period. Irrespective of the method and relative 'adequacy' of the resuscitation, all animals demonstrated a decline in LV systolic parameters and blood pressure over the final 60 minutes of the experiment. Close monitoring of the surgical sites and post-mortem assessment was undertaken to ensure that there was no further uncontrolled blood loss which could account for this. Blood was drawn during the final 60 minutes for lactate measurement, but this was a very small volume and was followed by a small, compensatory bolus of crystalloid. Isoflurane, chosen in part due to its ease of titration, was adjusted throughout each experiment in order to minimise and standardise its impact upon haemodynamic outcomes. Isoflurane concentration were maintained between 0.5-1% in all animals during the final hour of the experiment (data not shown). The decline in blood pressure, stroke volume and cardiac output is therefore likely to reflect the redistribution of fluid occurring during this hour. Intravascular fluid depletion in the TH60 phase would have resulted in the shift of fluid from the interstitial into the vascular space in order to compensate and maintain perfusion as haemorrhage was occurring (evident during the haemorrhage phase with the need to draw small volumes of blood intermittently in order to maintain the blood pressure in the target range). Upon resuscitation, this fluid 'debt' generated during haemorrhage would have been repaid with the movement of fluid from the intravascular back to the interstitium. Although this could provide an explanation for the reported drop in MABP ad CO, this doesn't explain why, after resuscitation, these parameters continue to decline beyond the baseline level. For example, in the SV-directed group, the mean CO at baseline was 21.39mL/min compared to 11.27mL/min at the end of the

experiment (refer to Fig.9B). The rebound in lactate with higher levels recorded at the end of the experiment compared to after resuscitation, also suggest that there is again a state of acidosis and worsening shock (Fig.12). Despite the administration of at least 2 times the original haemorrhage volume in both groups, haemodynamic and metabolic outcomes deteriorate in the final 60 minutes, in the absence of ongoing haemorrhage.

The decline in haemodynamic performance after controlled haemorrhage and resuscitation seen here is not novel. A rodent haemorrhage study performed by Wang *et al* in 1991 demonstrated that cardiac output (measured using an *in vivo* dilution method) normalised after resuscitation with up to 4 times the haemorrhage volume in Ringer's lactate, but the CO was not sustained. (Wang & Chaudry 1991) Ongoing haemodynamic compromise despite resuscitation is likely to result from a complex interplay between neuro-hormonal homeostatic mechanisms. Haemorrhage and injury may also instigate an inflammatory response which leads to changes in the vasculature leading to third space fluid losses which has been well documented in sepsis and systemic inflammatory response syndrome. Resuscitation with crystalloids has been previously demonstrated to impair endothelial integrity and microvascular responsiveness (Torres *et al* 2013). The use of a combined shed blood and crystalloid regimen in these experiments therefore may have potentially allowed for the development of endothelial changes with resultant fluid extravasation and decline in intravascular filling status and elevated blood lactate concentration.

Serum H-FABP concentrations were elevated in all of the animals in both of the experimental groups and support the findings of the earlier experiments. The serum H-FABP tended to be higher in the MABP-directed group but this was not statistically significant. The H-FABP concentration is not reduced using the SV-directed resuscitation protocol and the serum concentrations are higher than those seen in the earlier 1 hour experiments (*chapter three* and *chapter four*). This suggests that the cardiomyocyte injury leading to the release of this

cardiac-specific biomarker is ongoing and not reduced with either resuscitation protocol. Higher levels of H-FABP in these 2 hour experiments may indicate that, despite restoration of pre-load, there is continuing cardiomyocyte damage. The results may have been different with a more 'clinical' resuscitation protocol with further fluid boluses administered in response to declining haemodynamic parameters. For the purposes of these mechanistic studies, a protocol of restoring baseline conditions (MABP or SV) was chosen in order to monitor the LV response to initial hypovolaemia and then subsequent resuscitation with standardised resuscitation at defined intervals rather than administering intermittent boluses of fluid for the duration of the study. This approach clearly deviates from the clinical practice of hypotensive resuscitation and therefore limits the translational potential of these experiments in that regard. Bolus resuscitation as employed here may allow for TISCI to worsen in the intervening periods as MABP declines. However, if hypotensive resuscitation had been used with intermittent fluid boluses administered to keep MABP above a defined level throughout, TISCI may not be prevented or reduced as the LV remains relatively, persistently under-filled. Both approaches therefore have their pitfalls when considering mechanistic studies.

Echocardiography has proven a useful guide to resuscitation in these studies. It did not however, identify any acute structural abnormalities or regional wall motion abnormalities resulting from a period of TH. A single episode of arrhythmia was captured on the ECG trace and then confirmed with imaging. In terms of left ventricular systolic function, these studies did not identify any significant loss of contractile function (regarded in terms of fractional shortening). The SV-directed group tended to have a slightly higher fractional shortening at the end of the experiment, while the MABP-directed group showed a steady decline over the 120 minutes but this was not statistically significant (Fig.9D). According to Starling's law, the force of contraction is related to the volume of blood in the LV at the end of diastole;

given that there was no significant difference in LVEDV between the 2 groups at 120 minutes, perhaps this is to be expected. There is a difference between the 2 groups in terms of the temporal trend of FS over 120 minute (Fig. 9C) with a persistent decline in FS seen in the MABP-directed group over time. The reason for this is unclear.

Apart from the isolated case of arrhythmia, the heart appears to respond as expected to resuscitation in these studies and the decline in SV, CO and CI appears to be a result of fluid redistribution rather than an intrinsic contractile failure. Despite this, there is biomarker evidence of increasing cardiomyocyte injury. It may be that the 2 hour timeframe of these experiments was not adequate to allow for the development of overt LV dysfunction and longer studies would be needed to test this hypothesis.

5.6 Limitations

The aims of these studies were met but the development of this resuscitated model of trauma-haemorrhage was complex and required the accomplishment of a number of new skills. Vessel cannulation in itself is difficult and needs to be performed in a consistent way. Having previous experience with arterial cannulation meant that I was able to become proficient at jugular cannulation relatively quickly and without having to sacrifice animals in order to practice but there were cases where loss of lines led to removal of animals from the data and necessitated the use of another animal to replace them. Adding a second line did however lengthen the anaesthetic and surgical time and the initial experiments were inevitably a little longer than the last because of this.

As well as the technical challenges of siting and manipulating an animal with additional lines, there are considerations related to the overall small size of the animal. Relatively small total

blood volume has obvious limitations on the number and frequency of blood tests that can be performed without further compromising survival, particularly in TH models where animals have lost significant circulating volume prior to resuscitation. For example, it would have been useful to perform biomarker analysis at a number of time-points but the serum volumes required for assay made this impracticable. The emphasis was on establishing cardiac biomarker levels at the end of the experiment and there was therefore no blood available for performing other analysis. Assessment of acid-base, haemoglobin levels for example could have been a useful means by which to ensure standardisation of the haemorrhage phase and modes of resuscitation but this was not possible.

Questions remain regarding fluid redistribution after resuscitation and assessment of inflammation and vascular permeability may have provided insights into this, but were beyond the remit of these studies.

5.7 Conclusions

Echocardiography is a reproducible, non-invasive, quick imaging technique which has already shown promise as a tool for fluid management in critically ill patients (Boyd *et al* 2016). These studies have demonstrated that micro-echocardiography, a highly translatable imaging modality, can be robustly applied to our existing murine model of TH. Not only can it be used as a method of assessing cardiac structure and function, but it has also provided an insight into the volumetric status of animals after TH and subsequent resuscitation. A major benefit of this approach is that we can accurately guide restoration of baseline pre-load conditions to the LV. This also gives confidence that we are appropriately restoring

intravascular volume the before going on to assess cardiac function and dysfunction as a consequence of TH. Resuscitation is carried out with the intention of restoring normal physiology. It could be viewed as particularly relevant in the case of TH models of cardiac injury as the organ of interest is particularly susceptible to the impact of inadequate resuscitation as it struggles to compensate to maintain cardiac output and jeopardises its own perfusion further as it mounts and maintains tachycardia and increases contractility.

The studies presented here have demonstrated cardiomyocyte injury in the resuscitated model of TH regardless of resuscitation protocol. H-FABP concentrations were elevated despite fluid resuscitation and were generally higher than those recorded in the 60-minute studies in *chapter three* and *four* so one can speculate that the cardiomyocyte damage is worsening with time and despite fluid administration (this cannot be said with absolute certainty due to the lack of serial H-FABP measurements in these experiments). An adverse cardiac events (in the form of arrhythmia) was recorded, but only in a single animal. This suggests that whereas ACEs were not detected in the 60-minute studies, a longer time-frame has been required to assess for myocardial dysfunction. There is therefore a need to implement longer experimental periods to assess for the development of overt cardiac dysfunction in more animals. The addition of resuscitation to the original 1 hour model discussed in *chapter three* allows for longer studies with acceptable survival rates in which to carry out such studies and it is this model that will be taken forward into the next studies.

CHAPTER SIX

Investigating Adenosine, lignocaine and magnesium as a Cardiovascular 'Rescue' Agent in the Murine Model of TISCI

Chapter Six

6 Investigating Adenosine, Lignocaine and Magnesium as a Cardiovascular 'Rescue' Agent in the Murine Model of TISCI

6.1 Introduction

The previous chapters of this thesis have demonstrated a predictable decline in MABP and LV systolic functional outcomes despite resuscitation. This observation is by no means novel. Well-characterised physiological responses to haemorrhage have outlined the nature of fluid redistribution acutely during haemorrhage, and in the acute, intermediate and longer term after resuscitation. Starling forces acting across capillary beds during haemorrhage, for example, result in fluid movement from the interstitium into the vascular compartment in an attempt to compensate for blood loss. The interstitium therefore can be viewed as representing a reservoir of further fluid to sacrifice to the intravascular space if the losses continue (this phenomenon of the 'internal transfusion' (Levick 2000) is evidenced in animal models of haemorrhage with the need to intermittently draw blood after initial haemorrhage in order to maintain a target MABP). Upon fluid resuscitation, pre- and post-capillary pressures are subject to change again, this time to favour fluid movement from the intravascular space to the interstitum and intracellular compartment thereby repaying the fluid 'debt' accrued during the haemorrhagic shock phase.

It is well known therefore that THS and HS models require a higher volume fluid resuscitation compared to the shed blood volume. Simply returning the same volume of shed blood will initially restore MABP for example, but as fluid redistributes, intravascular volume will begin to decrease once again. A 1991 study using a rat model of trauma-

haemorrhage reported that crystalloid resuscitation with 4x the volume of the initial blood loss, was enough to initially restore CO and total peripheral resistance, but CO was not maintained (Wang *et al* 1991). This observations made by this group is likely to have led to the adoption of the 4x volume of blood loss fluid replacement strategy in multiple studies published by the same authors.

Ongoing haemodynamic impairment post TH with resuscitation is not new to the intensivist or anaesthetist either. A point is usually reached after severe shock where vasoactive substances are required in an attempt to improve haemodynamic status. Choice of agent and timing of implementation is controversial (Mongarden *et al* 2009) and the use of more commonly used vasoactive substances such as noradrenaline in humans usually requires invasive monitoring, titration and central vein cannulation which usually limits their use to the theatre and critical care setting.

There is a need to identify novel therapeutic agents which can be used in the acute phase after TH which can a) prevent or ameliorate this cardiovascular decline and eventual cardiovascular collapse ('buying time' during initial resuscitation and transfer to a place of definitive care) and b) limit the extent of the cardiac injury and dysfunction and facilitate cardiac recovery. A cardioplegic agent comprising of adenosine, lidocaine and magnesium ("ALM") has been previously investigated and has shown promise as an adjunct to small-volume, hypotensive resuscitation in rats (Letson & Dobson 2011) and pigs (Granfeldt *et al* 2012) subjected to haemorrhagic shock. The use of this combination of drugs has been demonstrated to dramatically improve survival and haemodynamic outcomes in treatment animals compared to controls in these studies. What remains unclear however, is whether this drug combination has efficacy in a model where severe haemorrhage is combined with trauma, and what, if any, impact it has upon H-FABP levels and LV systolic function in a combined TH model.

6.2 Study Aim

The principle aim of this study is to investigate the use of ALM, when given as an adjunct to blood and crystalloid resuscitation, as a potential cardiovascular 'rescue' agent with the ability to improve survival and limit / prevent the haemodynamic deterioration previously characterised in the murine model of TISCI. The impact of the addition of ALM to resuscitation protocol on H-FABP levels and LV systolic function will also be investigated.

6.3 Materials and Methods

The broad materials and methods have been previously discussed in *Chapter Two*.

6.3.1 Experimental design

Prior to commencing the experiment, mice were randomly assigned to either ALM or non-ALM resuscitation groups (refer to table 6.1 for experimental groups). Animals that did not survive the 60-minute TH phase at 30-40mmHg were excluded and replaced. Animals in the non-ALM group were resuscitated as outlined above. ALM-treated mice received a combination of adenosine, lidocaine and magnesium in either a low or high dose "one-shot" regimen. The lower-dose administered comprised adenosine 0.54mcg/g, lidocaine hydrochloride 1.63mcg/g and magnesium sulphate 0.6mcg/g (based on the previous work in the porcine model of HS as described by Granfeldt *et al* in 2012) while the high dose group received a doubling of all the ALM components (adenosine 1.12mcg/g, lidocaine hydrochloride 3.26mcg/g and magnesium sulphate1.2mcg/g).

Adenosine, lidocaine and magnesium doses were prepared according to weight and suspended in 0.5mL of crystalloid. This solution was then administered after transfusion of shed blood in the first resuscitation phase. During intravenous infusion, blood pressure, heart rate and ECG were continuously monitored and echocardiographic assessment performed throughout. The Hartmann's solution (Vetivex) used to deliver the drugs was included in the volume of crystalloid administered to reach target SV during the first resuscitation phase. No ALM was administered during the second of the resuscitation phases and crystalloid alone (entire shed blood volume having been returned during the first resuscitation phase) was administered to resuscitate the animals to achieve baseline stroke volume.

Table 6.1 Experimental groups, interventions and final N number for the Chapter Six studies.

MABP = Mean arterial blood pressure, Echo = Echocardiography, CSL= Crystalloid, SV = stroke volume, ALM = adenosine/lidocaine/magnesium.

Intervention	Experimental Groups		
	Control	ALM	ALM Double-Dose
Cannulation	Carotid & Jugular	Carotid & Jugular	Carotid & Jugular
Pressure-dependent haemorrhage?	Yes. 30-40mmHg	Yes. 30-40mmHg	Yes. 30-40mmHg
Laparotomy & Fractures	Yes	Yes	Yes
Baseline Echo?	Yes	Yes	Yes
Resus Fluid (Resus 1)	Shed blood & CSL via jugular catheter	Shed blood & CSL & ALM via jugular catheter	Shed blood & CSL & ALM via jugular catheter
ALM dose	N/A	Adenosine 0.54mcg/g, lidocaine hydrochloride 1.63mcg/g & magnesium sulphate 0.6mcg/g	Adenosine 1.12mcg/g, lidocaine hydrochloride 3.26mcg/g & magnesium sulphate1.2mcg/g

Resuscitation Endpoint (Resus 1 + 2)	Baseline SV+/-5µL (or non-responsive SV)	Baseline SV+/-5µL (or non-responsive SV)	Baseline SV+/-5µL (or non-responsive SV)
Resus fluid (Resus 2)	CSL only	CSL only	CSL only
Final N number	10	10	10

6.3.2 Experimental outline for *chapter six* studies.

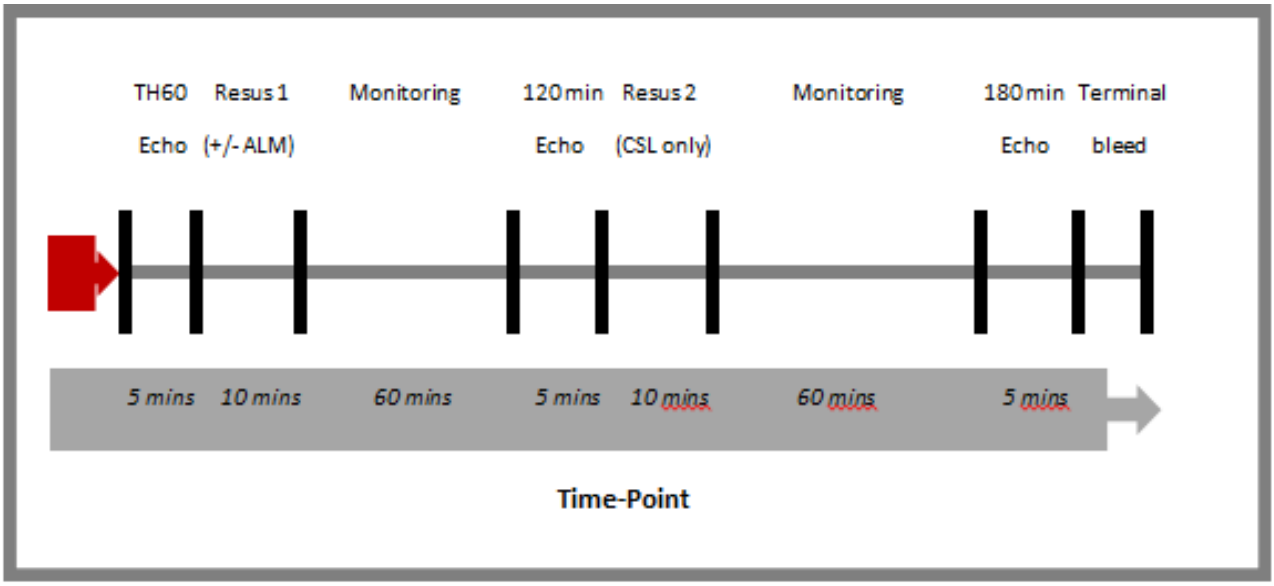


Figure 6.1 Experimental outline for the ALM treatment studies.
Schematic showing the experimental outline for the 3 hour ALM studies. The red arrow represents the end of the 60 minute TH phase. Interventions and observations and their average timings are given.

6.4 Results

A total of 37 animals were used in these experiments. Any animal that survived to receive the 1st resuscitation fluid +/- ALM was included. Any animal that died at any time before this point was replaced in order to achieve n=10 in all groups. 2 animals had an abnormal

appearance of the left ventricle at baseline assessment and were therefore removed from the study.

Echocardiographic assessment of the left ventricle was performed at baseline and SV, CO, EF and FS were calculated with the average of three readings being recorded.

6.4.1 Baseline left ventricular systolic function

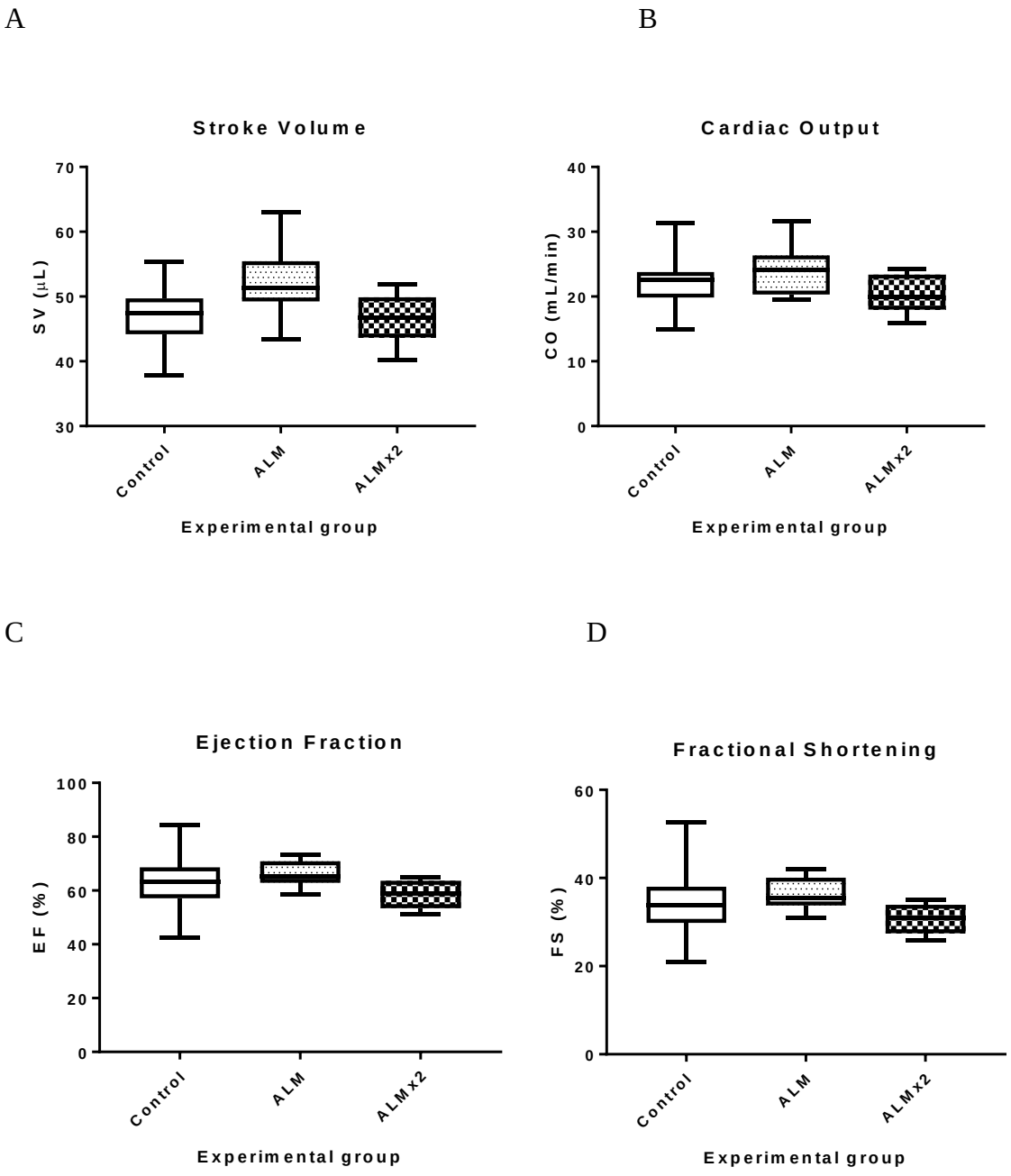


Figure 6.2 Left ventricular systolic functional outcomes at baseline.
Box and whisker plots show the LVS functional outcomes in the three experimental groups. Data is shown for **A.** Cardiac output (CO), B. Stroke volume (SV), C. Ejection fraction (EF) and D. Fractional shortening (FS). Box and whisker plots show the median (horizontal line), interquartile ranges (box) and range (whiskers).

Although there was greater variability between control group animals, there were no significant differences between the 3 groups at baseline in terms of LVS function assessed with echocardiography (fig.6.2). 2 animals assigned to the control group did demonstrate a structurally abnormal LV appearance on baseline echo and these were excluded and replaced, however, no animals were excluded from the final analysis based on non-significant baseline LVS functional variability. LVEDV was calculated for each animal at baseline (using SV and EF) and no statistically significant differences were observed (fig.6.3) between the animals in the 3 groups.

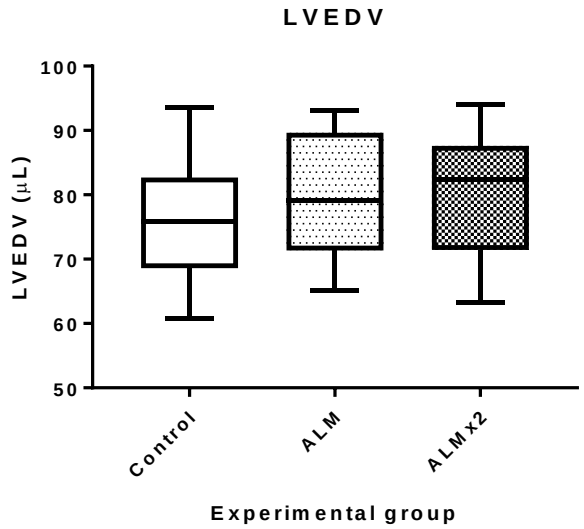


Figure 6.3 LVEDV at baseline.
Box and whisker plots represent the calculated LVEDV at baseline in the 3 groups. Box and whisker plots show median (horizontal line), interquartile ranges (box) and range (whiskers).

6.4.2 Haemodynamic characterisation of the TH model

Animals in the three experimental groups underwent a similar percentage blood loss in order to achieve the target MABP of 30-40mmHg (fig.6.4). Estimated total blood volume was calculated as 0.07 x grams of total body weight. Mean percentage blood loss was 39.71

(±3.88) %, 37.44 (±3.27) % and 40.41 (±2.40) % in the control, ALM and ALMx2 groups respectively.

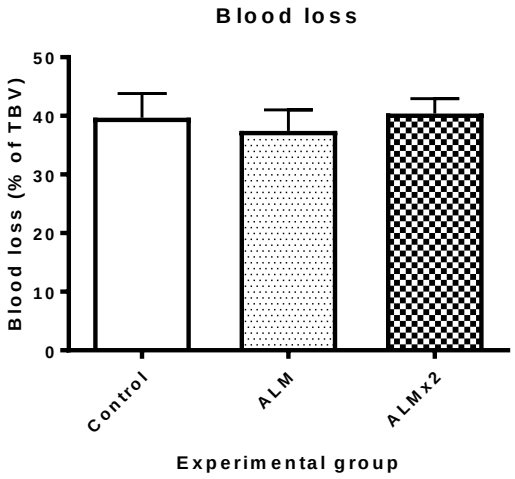


Figure 6.4 Blood loss required to achieve target MABP in the 3 groups.
Colum graphs show the blood loss as a percentage of total blood volume (derived from body weight) for control and ALM treated groups.

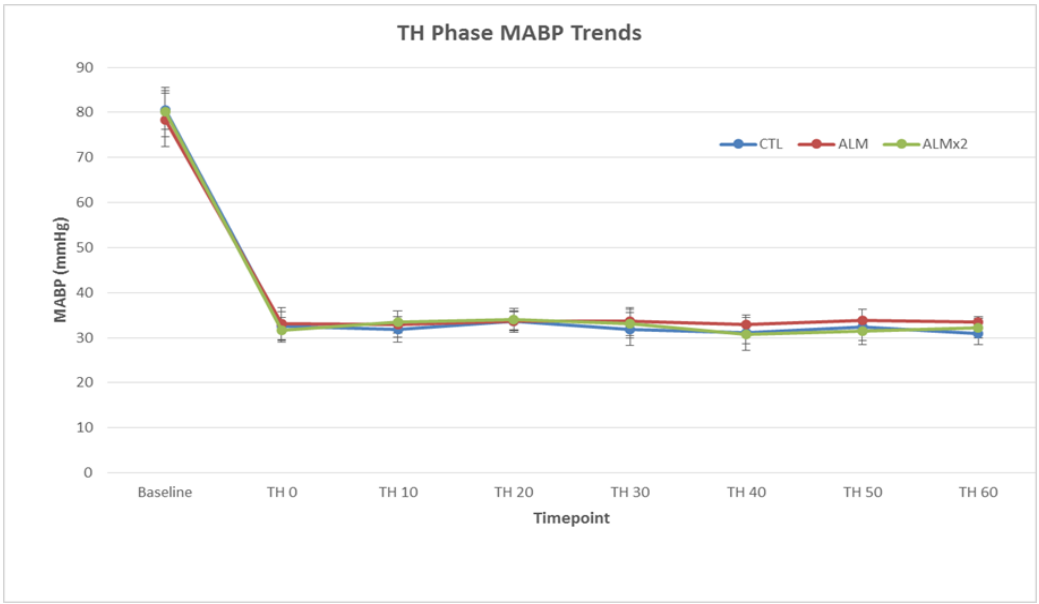


Figure 6.5 MABP trends across the 60 minute TH phase.
Line graph showing the MABP trends in the 3 groups at baseline and then at 10-minute intervals until reaching the TH60 time-point. Dots represent the mean value, vertical lines represent standard deviations.

Figure 6.5 above shows the trend in MABP at 10-minute intervals over the 1 hour TH phase. There was no significant difference between the groups in terms of MABP at baseline and at every subsequent time-point over the first hour of the experiment. All animals were kept within the target MABP range of 30-40mmHg for the duration of the haemorrhage phase.

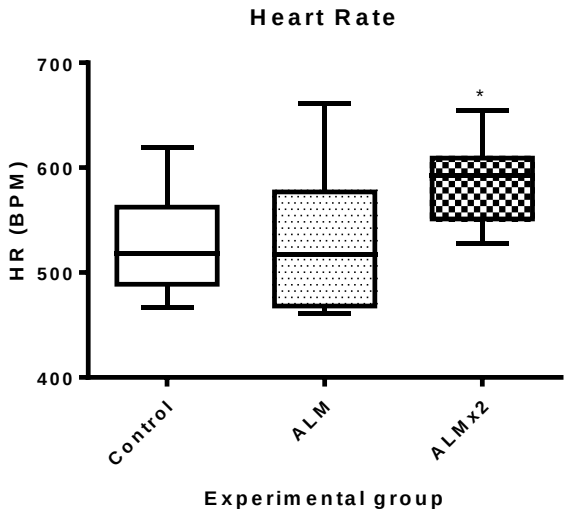


Figure 6.6 Heart rate at completion of TH60.
Box and whisker plots show heart rates in the 3 groups at the end of the TH60 phase. Plots represent the median (horizontal line), interquartile ranges (box) and range (whiskers). *P<0.05 when compared to control and lower dose ALM group.

All animals mounted a relative tachycardia in response to haemorrhage (data not shown). At the point of completion of the 60-minute TH phase, despite a similar percentage blood loss and blood pressure in the 3 groups, the higher dose ALM group demonstrated significantly higher heart rates (P<0.05) (figure 6.6).

6.4.3 Left ventricular Systolic Function after Trauma-Haemorrhage

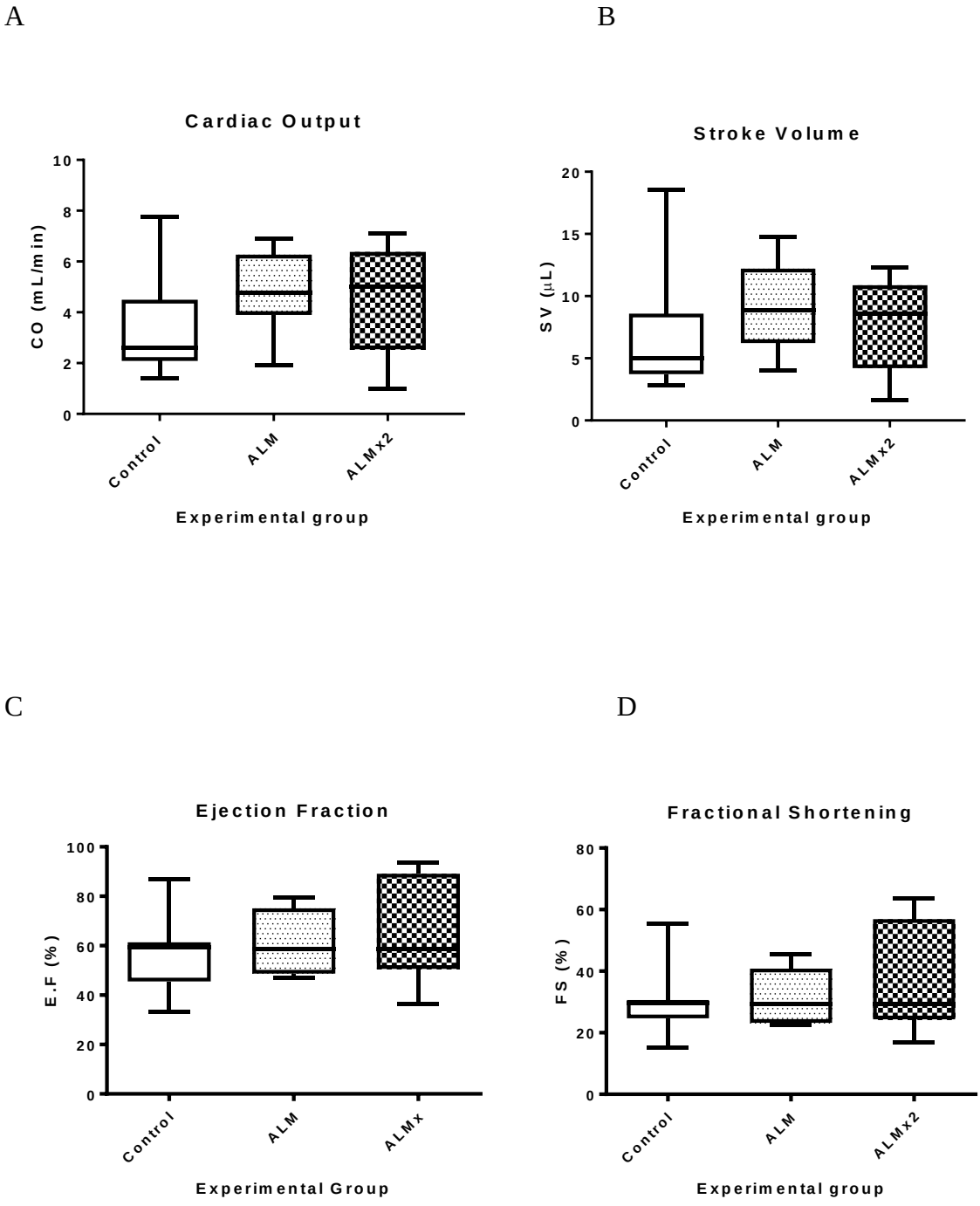


Figure 6.7 Left ventricular systolic function after TH60.
Box and whisker plots show the **A.** Cardiac output (CO), **B.** Stroke volume (SV), **C**. Ejection fraction (EF) and **D**. Fractional shortening (FS) at the completion of TH60 for the 3 groups. Box and whisker plots show median (horizontal line), interquartile ranges (box) and range (whiskers).

Echocardiographic assessment was performed at the end of the 60-minute TH period. All animals showed a reduction in CO and SV compared to baseline. There was no significant difference in CO, SV, EF or FS at TH60 when the 3 groups were compared. LVEDV was calculated after 60-minutes of TH and there was no significant difference seen when the 3 groups were compared (P>0.05) (fig.6.8).

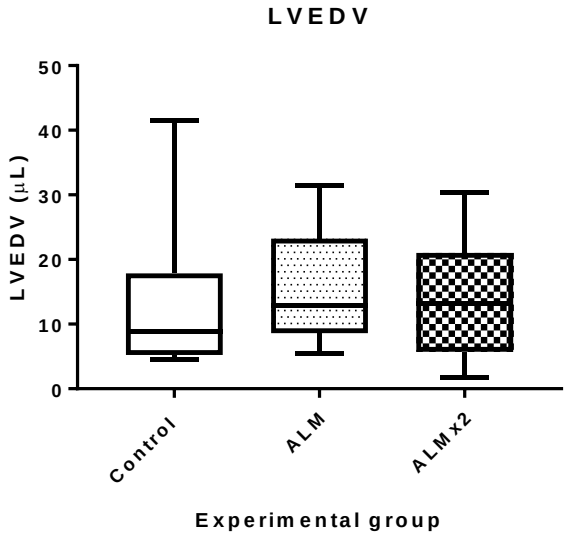


Figure 6.8 LVEDV at completion of TH60.
Box and whisker plots show the calculated LVEDV after 60 minutes of TH for the 3 groups. Plots show the median (horizontal line), interquartile ranges (box) and range (whiskers).

6.4.4 Resuscitation 1 Fluid Requirement

Animals that survived the initial 60-minute TH period underwent echocardiographic assessment and were then resuscitated. Echocardiographic assessment of SV was performed during resuscitation and resuscitation was deemed 'complete' when baseline SV+/- 5µL was achieved (or the SV stopped incrementing in response to further fluid boluses). All animals positively incremented their SV in response to fluid and target SV was achieved.

Animals treated with ALM required significantly less intravenous crystalloid for resuscitation ($P<0.05$) when compared to control animals. Animals treated with higher dose ALM tended to require less fluid than the lower dose group but this difference was not statistically significant (Fig.6.9).

A B

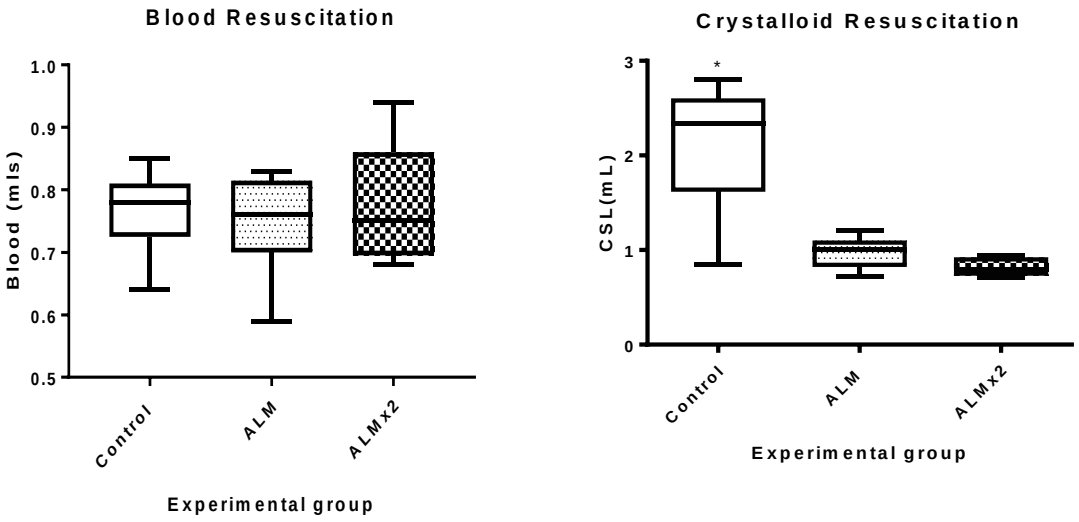
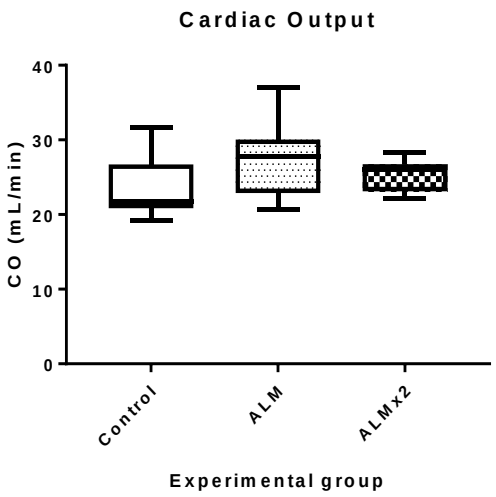


Figure 6.9 Volumes of fluid administered during resuscitation 1.
Box and whisker plots show the volumes of fluid resuscitation required to achieve SV. **A.** Shed blood and **B.** crystalloid administered during the first resuscitation to achieve target SV are shown. Box and whisker plots show median (horizontal line), interquartile ranges (box) and range (whiskers). *$P<0.05$ when controls compared to ALM and high dose ALM.

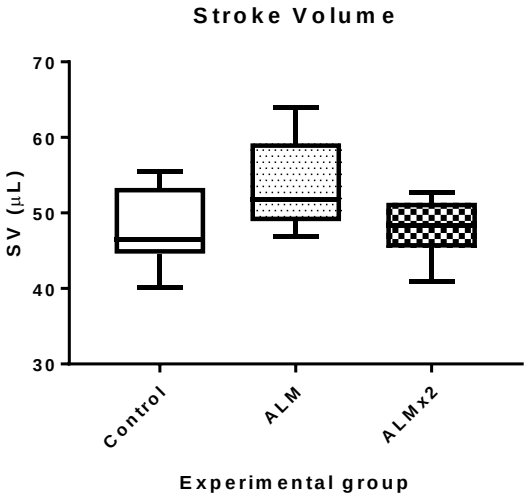
6.4.5 The left ventricular functional response to ALM resuscitation

After completion of resuscitation, echocardiography was used to assess LVS function. All animals, irrespective of experimental group, showed improvement in CO, SV and LVEDV when compared to TH60 outcomes. The left ventricle had filled in response to fluid resuscitation and SV, CO and LVEDV had risen accordingly.

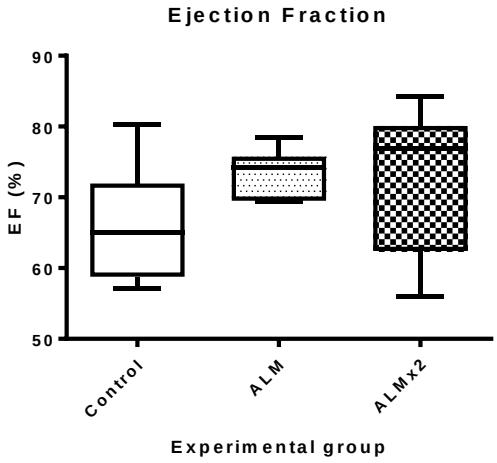
A



B



C



D

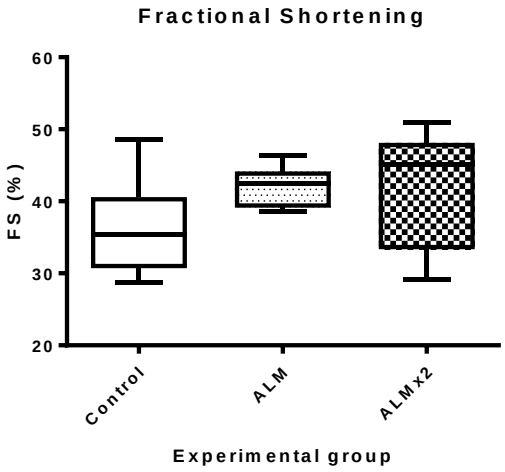


Figure 6.10 Left ventricular systolic function in response to ALM resuscitation.
Box and whisker plots show the response of the LV to ALM resuscitation in addition to blood and CSL. **A**. CO, **B.** SV, **C.** EF and **D**. FS after completion of resuscitation 1 are shown. Box and whisker plots show median (horizontal line), interquartile ranges (box) and range (whiskers).

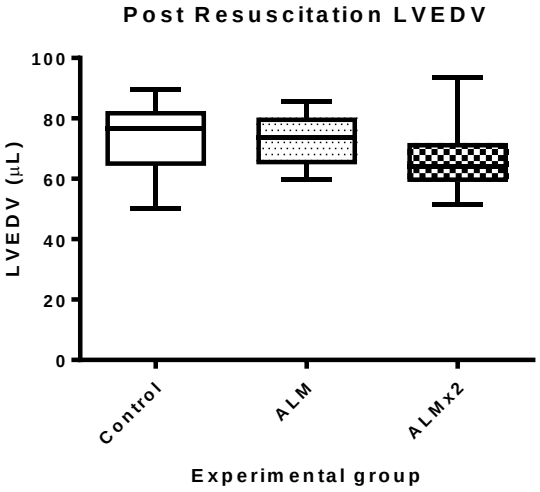


Figure 6.11 LVEDV after resuscitation with, and without, ALM.
Box and whisker plots showing the calculated LVEDV in the 3 groups. Plots show median (horizontal line), interquartile ranges (box) and range (whiskers).

There was no significant difference in the response to fluid in terms of LVS function and LVEDV among the 3 groups (figs. 6.10 and 6.11) after completion of resuscitation with blood and crystalloid ($P>0.05$).

Resuscitation was guided by stroke volume. Table 6.2 below gives the mean and standard deviations for SV at baseline and after resuscitation 1 and 2.

Table 6.2 Stroke volume changes from baseline in response to resuscitation.

The table gives the figures for stroke volume and how these change in response to fluid resuscitation with, and without ALM. Mean values are given along with standard deviations in parenthesis. ALMx2= 'double-dose' ALM.

Time-point	Stroke Volume (µL)		
	Baseline	Resuscitation 1	Resuscitation 2
Control (N=10)	47.07 ± (5.28)	47.87 ± (5.00)	47.87 ± (8.53)
ALM (N=10)	52.44 ± (5.59)	53.65 ± (5.90)	51.16 ± (5.88)

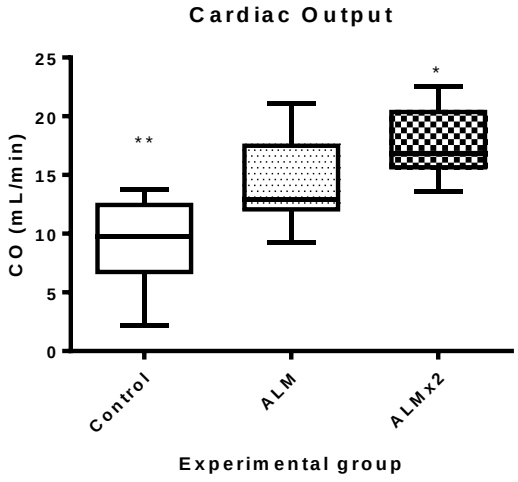
ALM x 2 (N=10)	46.75 ± (3.63)	48.00 ± (3.90)	43.39 ± (4.75)

Resuscitation titrated to baseline SV (as a surrogate for LVEDV and therefore pre-load) was therefore achieved in all animals at resuscitation 1. The volume of fluid required however to achieve this was highly variable with control animals requiring significantly higher volumes compared to the treatment groups (P>0.05) (fig.6.9).

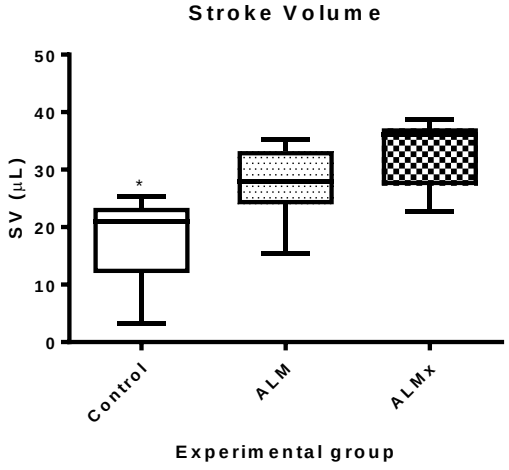
After completion of SV-directed resuscitation, animals were monitored for 60 minutes with no further fluid administration performed. Echo was then performed at the end of this monitoring phase (the '120-minute' point) to assess LVS function and assess LVEDV and Cardiac index.

6.4.6 Left ventricular function at 120 minutes

A



B



C D

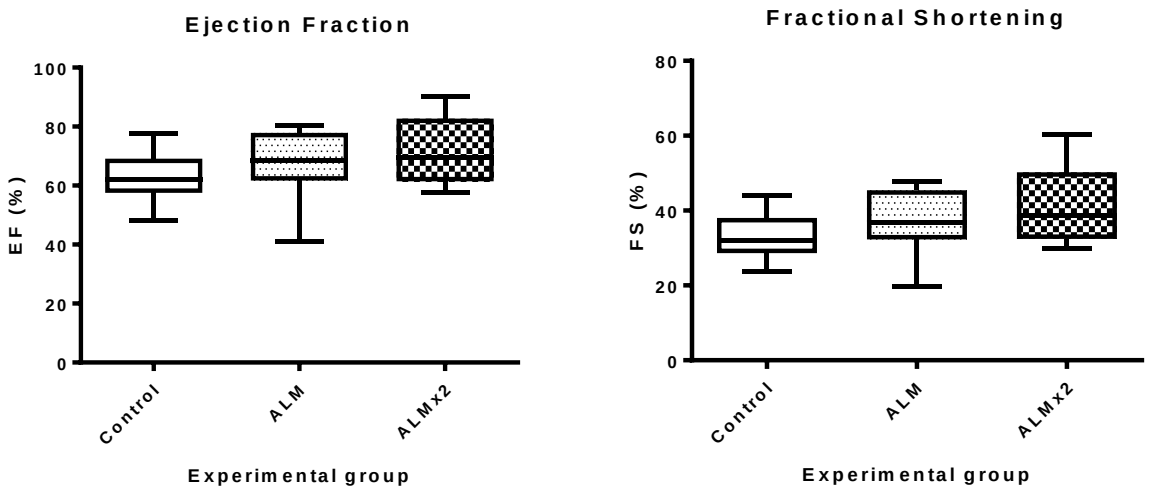


Figure 6.12 Left ventricular systolic functional outcomes at 120 minutes.
Box and whisker plots show **A**. CO, **B**. SV, **C**. EF and **D**. FS at 120-minutes. Plots show median (horizontal line), interquartile ranges (box) and range (whiskers). CO *P<0.05 when ALMx2 compared to control and lower dose ALM, **P<0.01 when controls compared to ALM and ALMx2. SV *P<0.01 when controls compared to both ALM and ALMx2 groups.

At 120 minutes, CO was significantly higher in treatment animals, at either dose when compared to controls (P<0.05). The double-dose (ALMx2) animals also had significantly higher CO when compared to the lower dose ALM group (P<0.05) (fig.6.12).

Stroke volume and LVEDV was significantly lower in the control animals when compared to the treatment groups (P<0.05) but there was no difference between the 2 ALM-treated groups (fig.6.12 & 6.13).

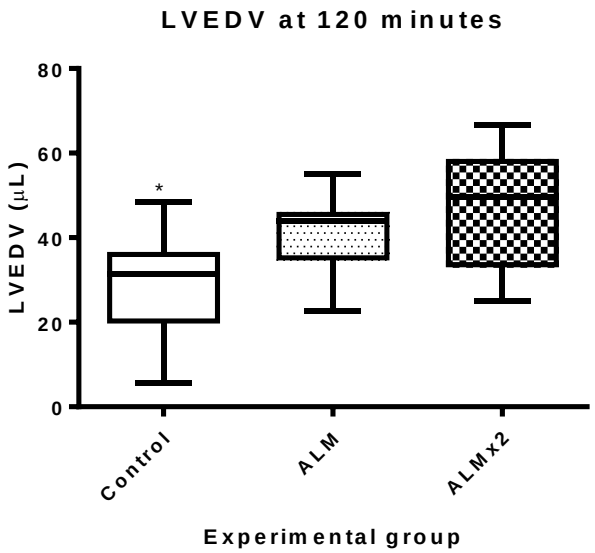


Figure 6.13 LVEDV at 120 minutes.
Box and whisker plots show calculated LVEDV at 120 minutes in the 3 groups. Plots show the median (horizontal line), interquartile ranges (box) and range (whiskers). *P<0.05 when controls were compared to ALM and ALMx2 groups.

6.4.7 Resuscitation 2 fluid requirement

All animals in each of the groups survived to receive a second resuscitation. Animals in the control group required significantly higher volumes of CSL during this resuscitation to reach baseline SV when compared to ALM and ALMx2 treated animals (P<0.01) (fig.6.14).

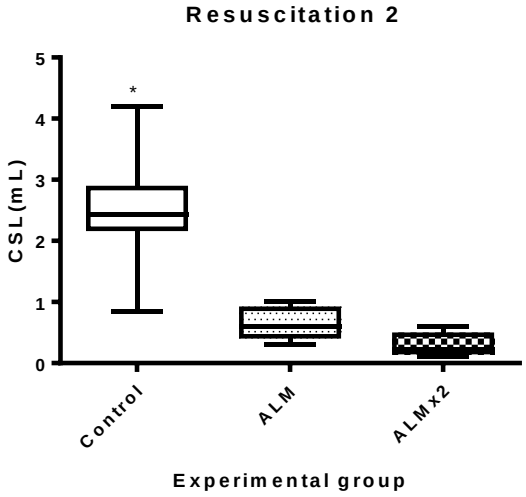


Figure 6.14 Volumes of crystalloid administered during resuscitation 2.
Box and whisker plots show the CSL volumes required to achieve target SV in the 3 groups. Plots show the median (horizontal line), interquartile ranges (box) and range (whiskers). *P<0.01 when controls were compared to both ALM and ALMx2 groups.

After completion of resuscitation (i.e. with the restoration of baseline SV), echo assessment was performed in order to assess function. Figure 6.15 gives SV, CO, EF and FS outcomes for the groups after resuscitation. There was no significant differences seen when the groups were compared in terms of LVS function (P>0.05).

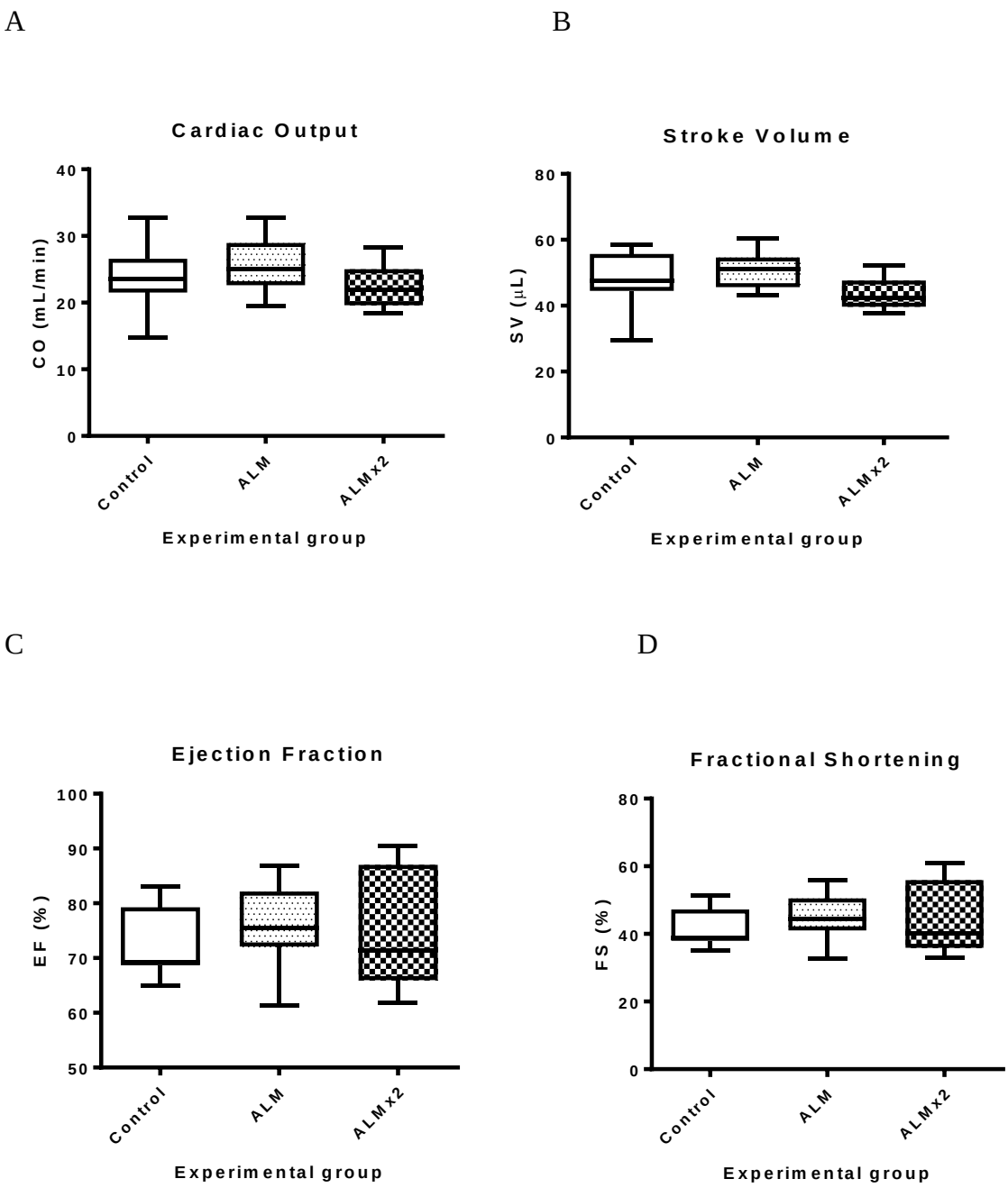


Figure 6.15 Left ventricular systolic functional outcomes after resuscitation 2.
Box and whisker plots show left ventricular systolic function **A.** CO, **B.** SV, **C.** EF and **D**. FS after completion of the 2nd resuscitation. Plots show median (horizontal line), interquartile ranges (box) and range (whiskers).

LVEDV was calculated after completion of fluid resuscitation. There was no difference between the groups in terms of LVEDV after resuscitation 2 (fig.6.16).

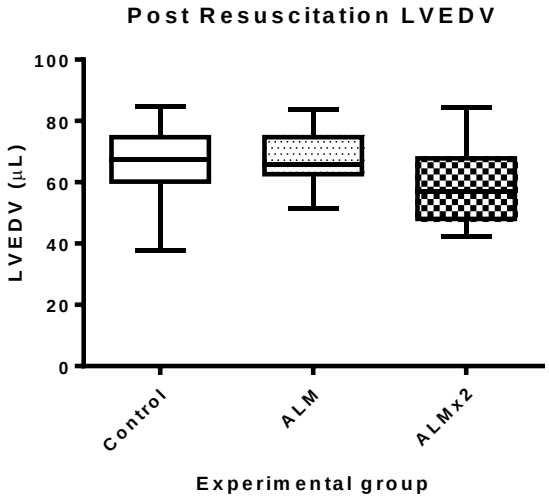


Figure 6.16 LVEDV after completion of resuscitation 2.
Box and whisker plots show the calculated LVEDV at the end of resuscitation phase 2 in the 3 groups. Plots show the median (horizontal line), interquartile ranges (box) and range (whiskers).

6.4.8 The relationship between LVEDV and resuscitation

Due to the need for real-time imaging and technical limitations, SV was employed as a surrogate indicator of LVEDV and therefore pre-load.

Table 6.3 LVEDV changes from baseline in response to resuscitation.

The table gives the values for calculated LVEDV at different time-points throughout the 3 hour studies. Mean values are given along with standard deviations in parenthesis. * denotes P<0.05 when compared to baseline.

Time-point	Left ventricle end-diastolic volume (µL)		
	Baseline	Resuscitation 1	Resuscitation 2
Control (N=10)	76.17 ± (10.15)	73.74 ± (11.85)	66.18 ± (13.62)
ALM (N=10)	79.89 ±(9.76)	73.31 (±8.37)	67.86 ± (10.07)*
ALM x 2 (N=10)	80.35 ±(10.10)	67.23 (±12.56)*	59.08 ± (12.97*

All animals positively incremented SV at both resuscitation points, however the LVEDV, calculated retrospectively, did indeed decrease from baseline levels at both resuscitation points (table 6.3).

There was no significant difference in LVEDV between the groups after resuscitation. There was however a trend, across all groups, for the LVEDV to fall and it was lower at resuscitation 2 compared to baseline in all groups. The LVEDV at resuscitation 1 was significantly lower in the ALMx2 group and was also significantly lower when compared to baseline LVEDV in the ALM and ALMx2 groups at resuscitation 2 ($P<0.05$).

After completion of a second resuscitation, animals were monitored for a further 60-minute period without administration of any further fluid boluses. During this second period of post-resuscitation monitoring, 4 of the 10 control animals died and therefore did not undergo echocardiographic assessment of LVS function at the 180-minute time-point. 1 animal in the control group decompensated during the 180-minute scan and died after image acquisition. This animal was therefore included in the data analysis for 180-minute LVS function and blood was available for end-experiment H-FABP and lactate analysis. It was however classed as a non-survivor taking the total number of non-survivors to 5.

6.4.9 Left ventricular function at 180 Minutes

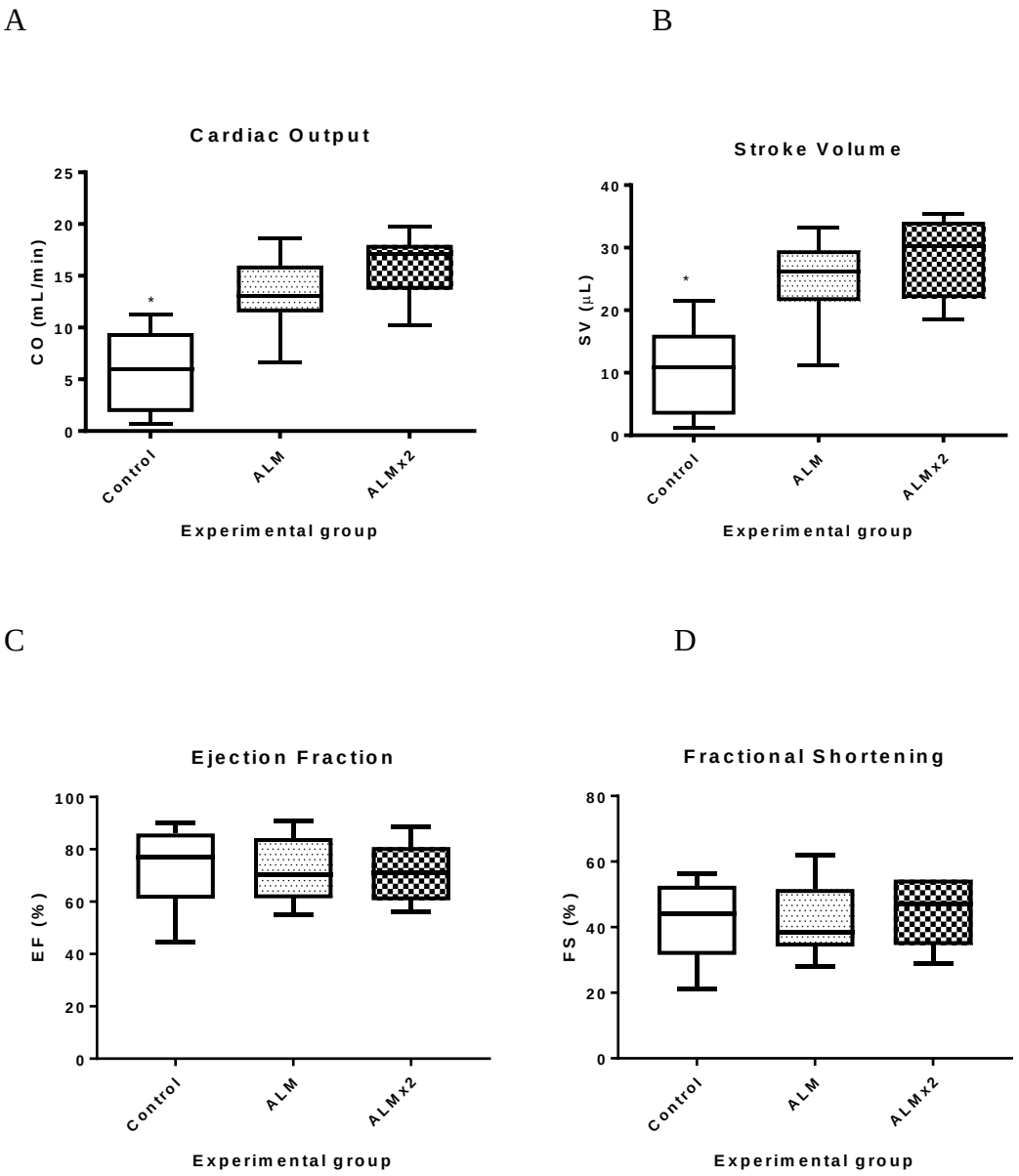


Figure 6.17 Left ventricular systolic function at 180 minutes.
Box and whisker plots show LVS outcomes at 180 minutes in the 3 groups. **A.** CO, **B.** SV, **C.** EF and **D.** FS at 180 minutes are all shown. Box and whisker plots show median (horizontal line), interquartile ranges (box) and range (whiskers). *P<0.0001 when controls compared to ALM treated groups. SV *P<0.0001 when controls were compared to ALM treated groups. N=6 for control group at 180 min time-point.

Echo assessment at 180 minutes revealed a significantly lower CO and SV in controls compared to the treated animals. EF and FS were similar for the 3 groups (fig.6.17). LVEDV was significantly lower in the control animals at 180 minutes (P<0.0001) (fig.6.18).

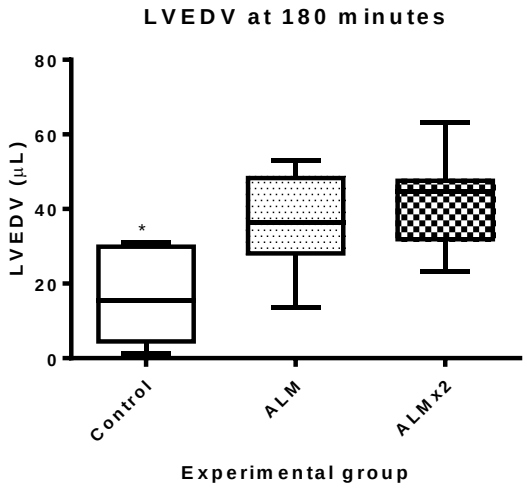


Figure 6.18 LVEDV at 180 minutes.
Box and whisker plots show calculated LVEDV at 180 minutes in the 3 groups. Plots show the median (horizontal line), interquartile ranges (box) and range (whiskers). *P<0.0001 when controls compared to treated groups at any dose. N=6 for control group at 180 min time-point.

6.4.10 Haemodynamic & temperature trends over the entire study period

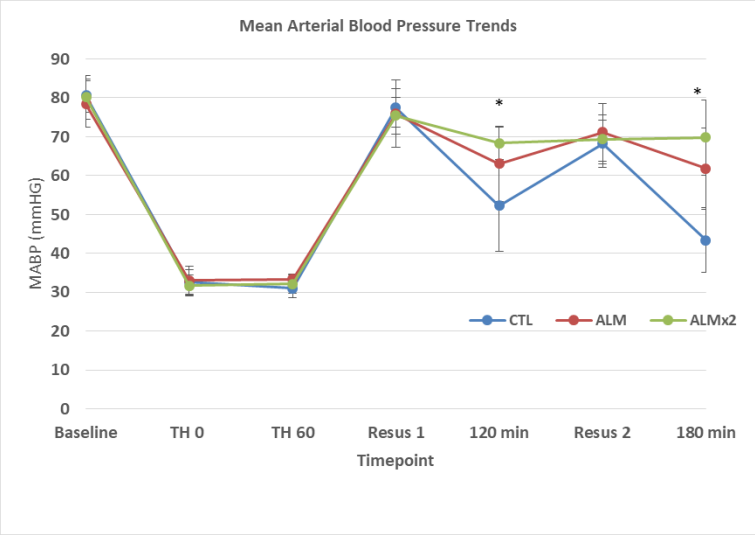


Figure 6.19 MABP trends across 180 minutes.
Line graph shows the MABP trends across the study period of 180 minutes in the 3 groups. Dots represent the mean for the group, vertical lines represent standard deviations from the mean. *P<0.01 controls *versus* ALM and ALMx2 groups. N=6 for control group at 180min time-point.

Figure 6.19 above illustrates the trend in MABP from baseline to the end of the experiment at 180 minutes. There was no significant difference between the groups at baseline, TH60 and after completion of the resuscitation 1. However, control animals tended to have lower MABPs at this point forwards at the 120-minute and 180-minute time-points, the blood pressure in these animals was significantly lower when compared to ALM and ALMx2 groups (P<0.01). The higher dose ALM group animals tended to do the best in terms of blood pressure maintenance and stability, but this improvement did not reach significance when compared to the lower dose group.

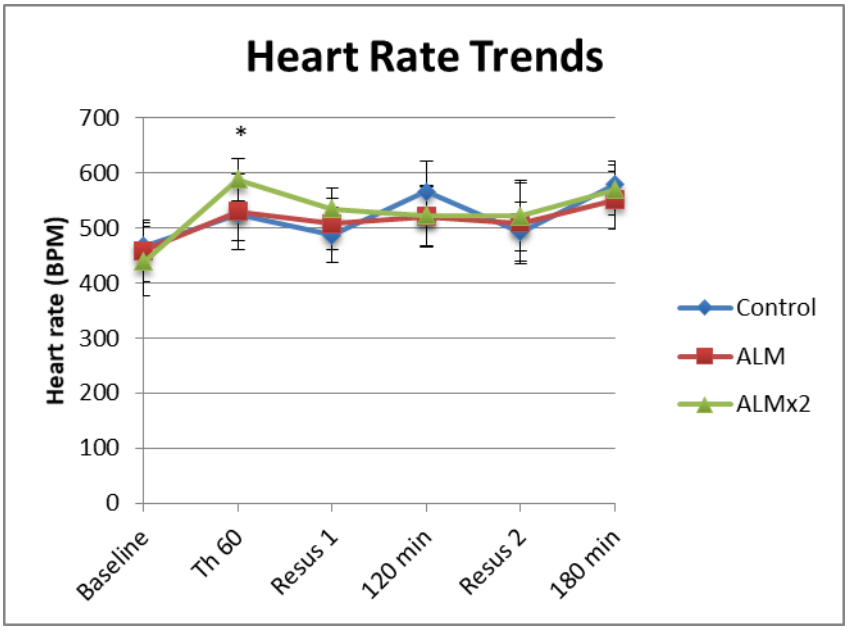


Figure 6.20 Heart rate trends across 180 minutes.
Line graph shows the trends in heart rates across the study period of 180 minutes in the 3 groups. Markers represent mean cardiac index value at specific time-points. Vertical lines represent standard deviations. *P<0.05 ALMx2 *versus* ALM and controls. N=6 for control group at 180 min time-point.

Significantly higher heart rates were recorded for the ALMx2 group at TH 60 (P<0.05) but there was no statistically significant difference seen when the groups were compared at any time-point over the remainder of the experiment (fig.6.20). As would be expected, heart rates were seen to increase relative to baseline in response to haemorrhage.

Importantly, ALM treated animals, although experiencing a transient, self-limiting bradycardia lasting a couple of seconds, in response to the bolus of ALM (data not shown), these treated animals did not develop any sustained relative bradycardia when compared to controls.

The temperature of each animal was monitored for the duration of the experiment and regulated with the use of heat lamps. Warmed blood and crystalloid was administered at each resuscitation point in order to limit the drop in temperature but despite these measures, a drop

in temperature was observed at these points. Generally, ALM and ALMx2 treated animals were easier to regulate and the control group became relatively hypothermic in the final 60-minute monitoring phase, and technically required more intensive temperature monitoring and adjustment of the heat source when compared to animals in the other 2 groups (fig.6.21).

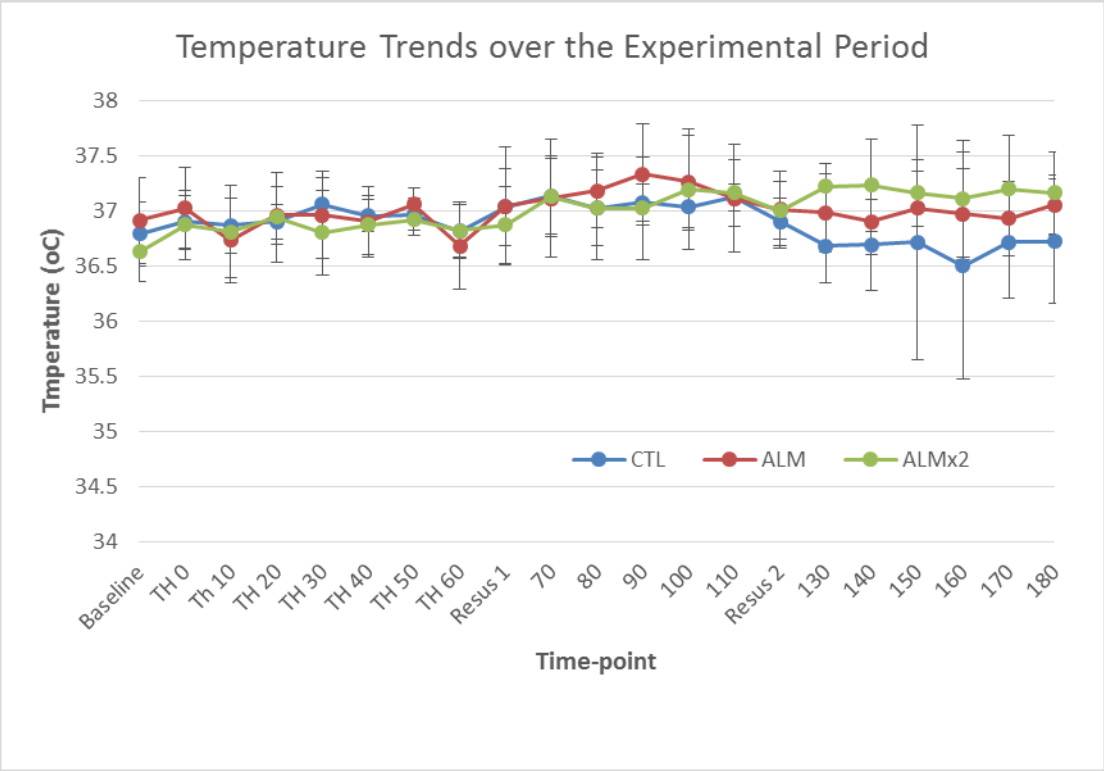


Figure 6.21 Core temperature trends across 180 minutes.
Line graph shows temperature trends in the 3 groups over the 180 minute study period. Dots represent mean, vertical lines represent standard deviations. N=6 for control group at 180 min time-point.

6.4.11 Trends in left ventricle function

LVS functional parameters were collated and line graphs represent the temporal changes in CO, SV, EF and FS at baseline and over the course of the experiment.

CO was significantly different when ALMx2 animals were compared to both controls and the lower dose ALM group at 120-minutes ($P<0.05$) (fig.6.22). CO was significantly lower in the control group compared to ALM (at any dose) at 180 minutes ($P<0.01$). At 180 minutes, there was no significant difference between the two treatment groups ($P>0.05$).

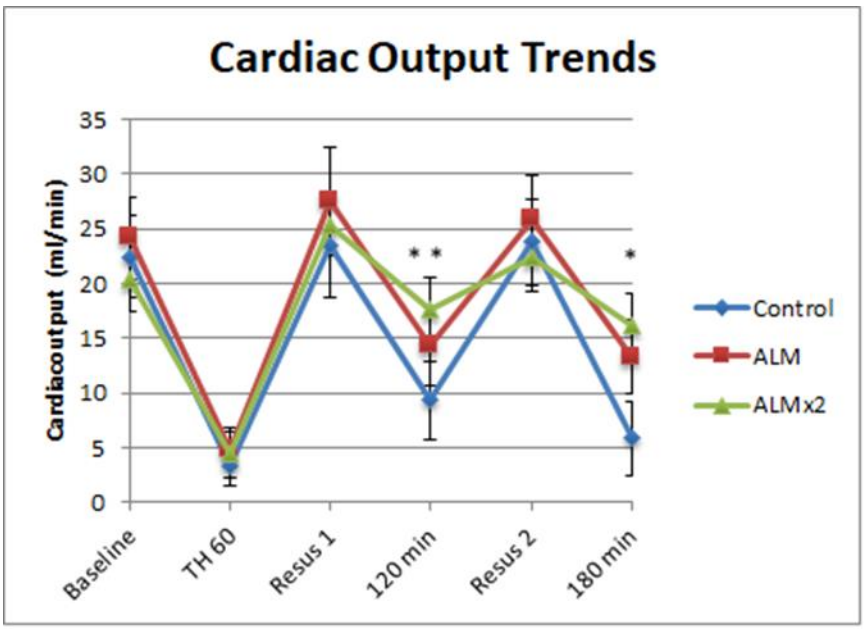


Figure 6.22 Trends in cardiac output across 180 minutes.
Line graph shows the trends in CO in response to TH with subsequent resuscitation in the 3 groups. Dots represent the mean, vertical bars represent standard deviations. *P<0.01 controls *versus* ALM treated (any dose), **P<0.05 ALM *versus* ALMx2. N=6 for control group at 180 min time-point.

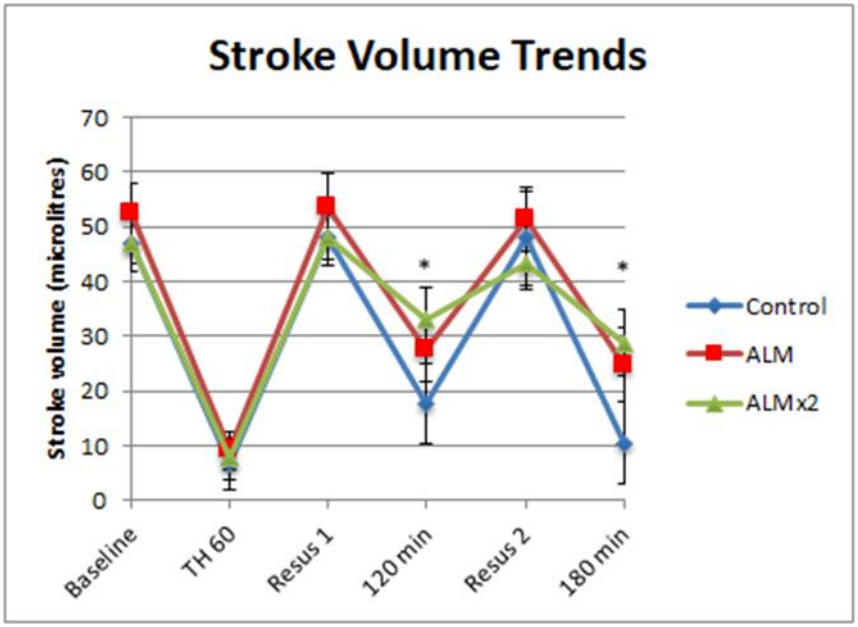


Figure 6.23 Trends in stroke volume across 180 minutes.
Line graph shows the trends in SV in response to TH with subsequent resuscitation in the 3 groups. Dots represent the mean, vertical bars represent standard deviations. *P<0.01 controls *versus* ALM (at either dose). N=6 for control group at 180 min time-point.

SV was significantly lower in the control group when compared to both ALM groups at 120 and 180-minute time-points (P<0.01). There was no statistically significant difference between the 2 ALM groups at any point over the 180 minutes (fig.6.22).

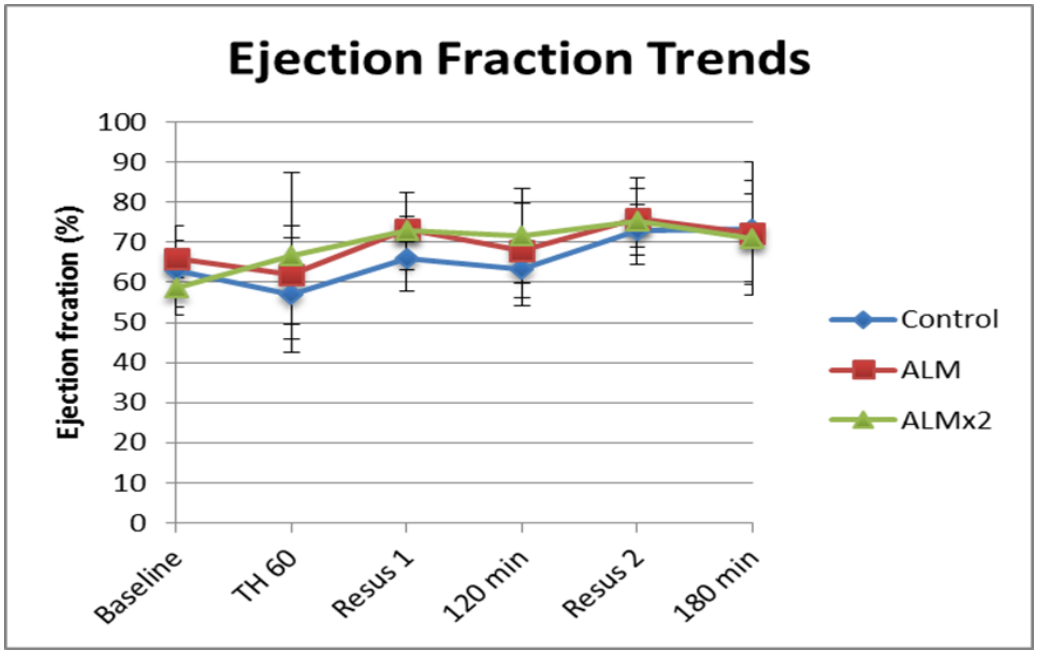


Figure 6.24 Trends in ejection fraction across 180 minutes.
Line graph shows the trends in EF in response to TH with subsequent resuscitation in the 3 groups. Dots represent the mean, vertical bars represent standard deviations. N=6 for control group at 180 min time-point.

Ejection fraction varied across the course of the 180-minutes but there were no significant differences between the groups at any of the time-points analysed (fig.6.24).

There was no statistically significant difference at any time-point between the 3 groups when fractional shortening was analysed over the 180-minute period.

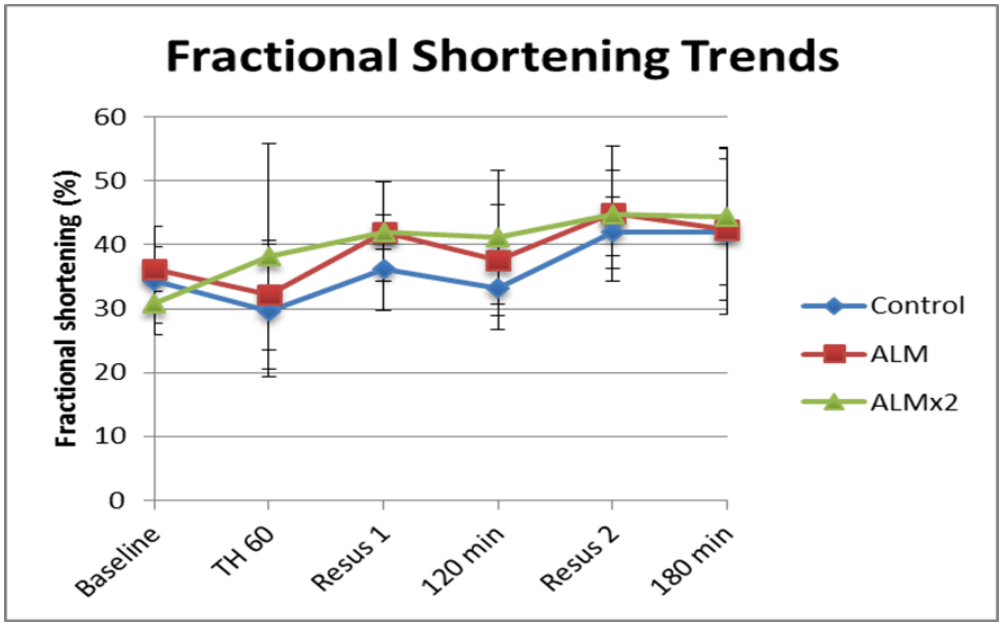


Figure 6.25 Trends in fractional shortening across 180 minutes.
Line graph shows the trends in FS in response to TH with subsequent resuscitation in the 3 groups. Dots represent the mean, vertical bars represent standard deviations. N=6 for control group at 180 min time-point.

Control animals tended to have lower percentage FS from TH60 onwards, but this did not reach significance at any of the time-points analysed (fig.6.25).

LVEDV was calculated using SV and EF. LVEDV was taken as the pre-load to the left ventricle and, as expected, fell dramatically in all of the animals as a consequence of haemorrhage and rose in response to fluid resuscitation (titrated to SV). LVEDV fell in the animals during the 2 post-resuscitation monitoring periods, regardless of group allocation however, the LVEDV in the control animals was significantly lower than the ALM and ALMx2 groups at 120 and 180 minutes ($P<0.05$ and $P<0.01$ respectively). At the end of the experiment, the LVEDVs of control animals was approaching that of the TH60 value, despite 2 intervening periods of resuscitation (fig.6.26).

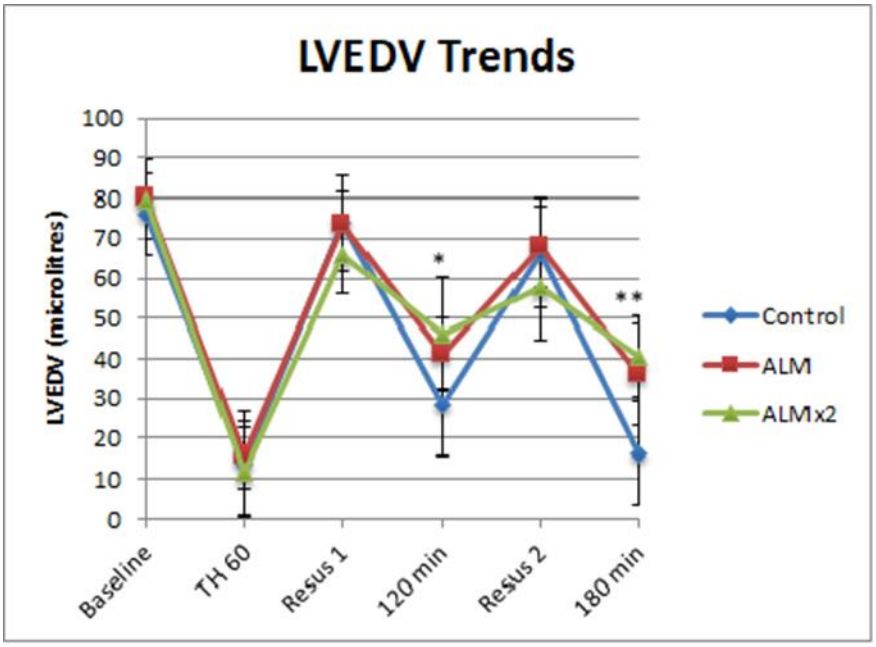


Figure 6.26 Trends in LVEDV across 180 minutes.
Line graph shows the trends in calculated LVEDV in response to TH with subsequent resuscitation in the 3 groups. Dots represent the mean, vertical bars represent standard deviations. *P<0.05 controls *versus* ALM (either dose) at 120 minutes, *P<0.01 controls *versus* ALM at 180 minutes. N=6 for control group at 180 min time-point.

6.4.12 Perfusion indices

Blood lactate was measured at the end of the experiment with blood drawn for terminal exsanguination. In animals who were dying before the 180 minute time-point, if blood was available, this was withdrawn and analysed prior to death and recorded as ‘end experiment lactate’.

Figure 6.27 shows the end experiment lactate concentrations with the highest being recorded in the control group. These levels were significantly higher in the controls when compared to the ALM group (P<0.05). Blood was taken and lactate recorded for animals who died before the 180 minute time-point in the control group meaning that pre-morbid lactates were included in the analysis. These levels were the higher than for survivors at 180 minutes and

therefore increased the mean level for the control group. ALMx2 group tended to have lower lactate concentrations at 180-minutes, but this was not significant when compared to controls.

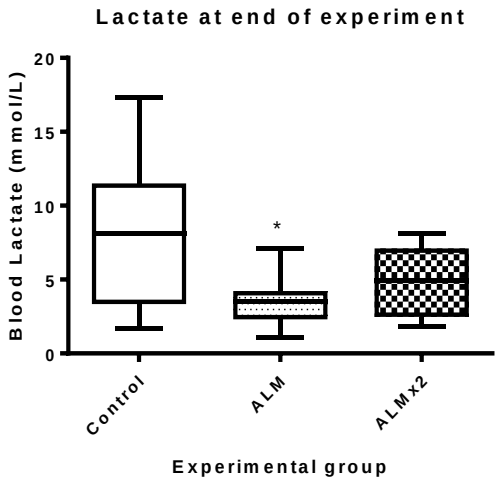


Figure 6.27 Blood lactate concentrations.
Box and whisker plots show the blood lactate concentrations at the end of the experiment in the 3 groups. Plots show median (horizontal line), interquartile ranges (box) and range (whiskers). *P<0.05 when compared to control group. N=8 for ALMx2 group at the end of the experiment due to inability to draw blood *via* the catheter. N=10 for the ALM and Control groups.

Cardiac index was calculated using CO and individual body weight as a way to quantify individual animal's perfusion status relative to body surface area. CI, and by extrapolation, peripheral perfusion, was similar at baseline, TH60 and at both resuscitation phases. Only 6 of the control animals survived to complete the experiment and undergo LVS functional assessment and CI analysis. Means and standard deviations are shown in table 6.4 below, temporal trends in cardiac index are given in figure 6.27.

Table 6.4 Cardiac index changes over time.

The table gives the calculated cardiac index values for the 3 groups at different time-points. N=6 for control group at 180 min time-point. Mean values are given with standard deviations in parenthesis. P values are shown.
Resus = resuscitation.

Group	Experimental Time-Point					
	Baseline	TH60	Resus 1	120 minutes	Resus 2	180 minutes
Control	804.67 (±136.10)	116.28 (±62.53)	843.58 (± 114.48)	333.68 (± 130.10)	857.19 (± 168.98)	203.08 (± 127.71)
ALM	855.93 (± 142.94)	170.18 (±63.59)	975.08 (± 178.10)	507.15 (± 134.32)	917.42 (± 147.41)	474.68 (± 134.58)
ALMx2	750.6 (± 105.86)	163.67 (±78.17)	934.04 (± 81.08)	649.49 (± 109.74)	823.99 (± 90.15)	595.93 (± 107.45)
P value (2-tailed)	0.2091	0.1772	0.0896	<0.0001	0.3310	<0.0001

Cardiac index was significantly lower in the control group at 120 and 180-minutes when compared to the ALM treated animals (at any dosage, $P<0.0001$). Similarly to the trend seen in LVEDV, CI was approaching TH60 levels in the control group at the end of the experiment. The CI in ALMx2 animals tended to be highest at these time-points, but this was not significantly higher than the lower-dose group animals ($P>0.05$).

The results suggest that animals receiving a bolus of ALM, of either dose, during the first resuscitation, maintain higher cardiac index levels. In the control group, by the end of the experiment, cardiac index had fallen to a level similar to that reached at the end of the TH phase.

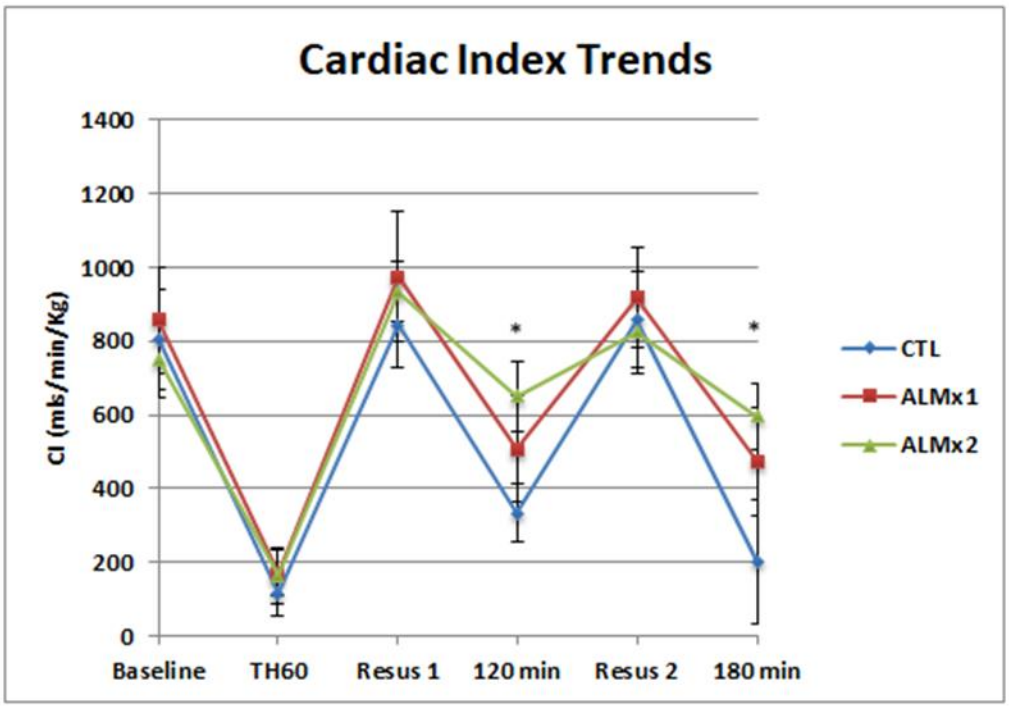


Figure 6.28 Cardiac index trends across 180 minutes.
Line graph shows the trends in calculated cardiac index over 180 minutes and in response to TH and subsequent resuscitation. Dots represent mean cardiac index value at specific time-points. Vertical lines represent standard deviations. * P<0.0001 when controls are compared to ALM and ALMx2. N=6 for control group at 180 min time-point.

In contrast, the ALM treated animals at 180 minutes had at least double the cardiac index seen in control mice. ALM treated animals therefore had significantly improved perfusion at 120 and 180 minutes when compared to controls. This is somewhat supported by the lactate data with control animals having significantly higher blood concentrations of lactate at 180 minutes when compared to treatment groups. However. ALM treated animals still had lactate concentrations above the normal range and data for the control group includes lactates recorded in non-survivors and these pre-terminal blood lactate levels in non-survivors were the highest recorded resulting in an elevated mean value for this group. There was no statistically significant difference in the lactate concentrations when lower dose ALM treatment was compared to the higher dose treatment group (fig.6.28).

6.4.13 Observed cardiac dysfunction

ECG monitoring was performed while the animals were on the echo scanning platform. ECG analysis was performed therefore for the duration of any imaging, and also was prompted when there was any unexpected change or unexplained variation in MABP. 5 of the 10 control animals did not survive the 180-minutes and ECG traces recorded showed evidence of atrial fibrillation which was coupled with labile blood pressure before the onset of profound hypotension which occurred as a pre-terminal event.

There were no observed cases of arrhythmia in the lower dose ALM group, however, 4 animals who received the higher dose preparation had witnessed episodes of arrhythmia with accompanying haemodynamic instability. In all cases, this was recorded in the second 60-minute monitoring phase after resuscitation 2. In 3 out of the 4 animals, this persisted until the end of the experiment but all animals were alive at 180-minutes in this group.

6.4.14 Cardiac Biomarkers

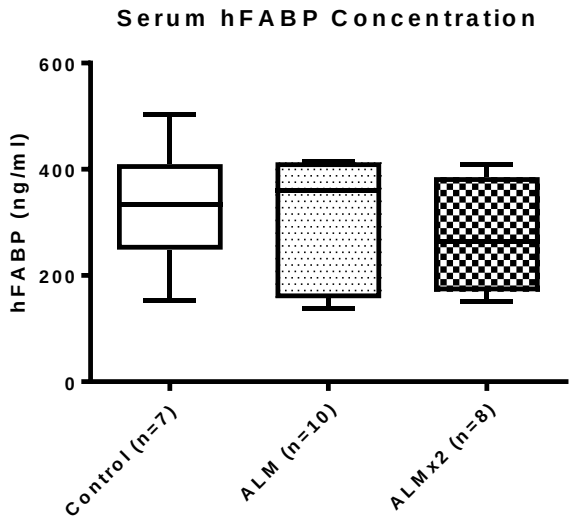


Figure 6.29 Serum H-FABP at 180 minutes.
Box and whisker plots show the serum H-FABP concentrations at the end of the study in the 3 groups. Plots show median (horizontal line), interquartile ranges (box) and range (whiskers). N=8 for the ALMx2 group at the end of the experiment.

Serum H-FABP concentrations were assessed for each animal where blood was available at the end of the experiment. In animals that died before the end of the experiment, a sample was taken in the agonal phase. This however was not possible in a number (due to low MABP and therefore technically unable to draw blood from the carotid catheter). In 2 of the ALMx2 animals, the line was not patent at the very end of the experiment and therefore no blood could be drawn.

Mean H-FABP concentrations were 339.81 (±106.72) ng/mL, 313.09 (±109.71) ng/mL and 273.74 (±97.04) ng/mL in the controls, ALM and ALMx2 groups respectively. All groups demonstrated abnormally high levels of the cardiac specific biomarker. There was a trend for the ALM groups to have lower H-FABP concentrations when compared to controls, but this did not reach statistical significance ($P>0.05$) (fig.6.29).

6.4.15 Survival

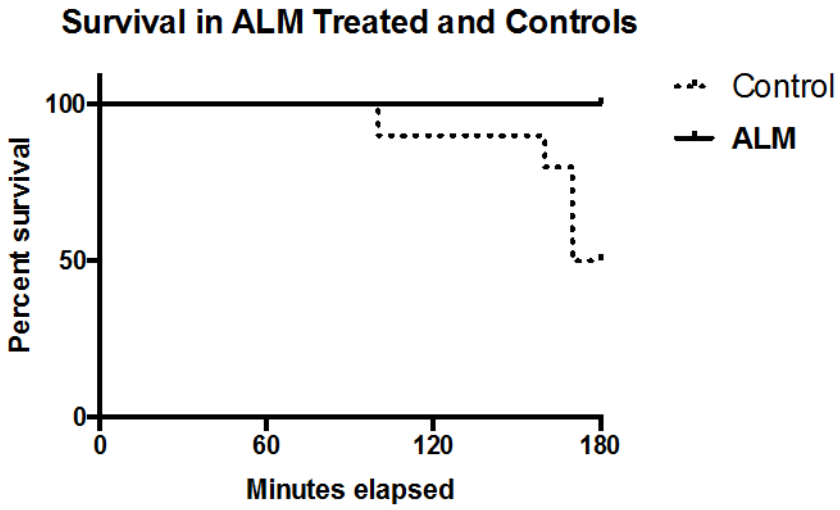


Figure 6.30 Kaplan-Meier survival analysis for the ALM studies.
Kaplan–Meier survival analysis shows the proportion of animals alive at 60, 120 and 180 minutes in control and ALM (at any dose) treated groups. P=0.01 (Log-rank (Mantel-Cox test)).

100% of the treated animals (single and double dose) survived the 3 hours (fig.6.30). 50% of the control animals did not survive the 180 minute experimental period and were observed to have arrhythmia as a pre-terminal event. 4 of the ALMx2 group developed arrhythmia in the final 60 minutes of the experiment, but all survived to undergo final echo assessment and terminal blood sampling.

6.4.16 Six-hour studies

Given the encouraging results of the 3 hour studies with lower dose ALM in terms of improved haemodynamics, smaller fluid required for resuscitation and 100% survival, a

smaller pilot study was conducted in order to investigate whether the beneficial responses persist beyond the 3 hour experimental period. The lower dose regimen was chosen due to the better survival outcome compared to that of the double-dose. 5 animals were treated with ALM at the lower dose (as previously described), 5 control animals were resuscitated with shed blood and crystalloid alone (as outlined above). The methodology was the same as for the shorter experiments, apart from the final monitoring period after the second resuscitation, which lasted for 4 hours rather than 1. No further fluid boluses were administered during this phase as the aim was to determine the trends in MABP, HR and LVS function. H-FABP, lactate were also assessed at the end of the experiment as previously described.

Baseline MABP and LVS functional parameters were similar for treatment and controls at baseline and after 60-minutes of TH (data not shown). Mean blood loss was 39.82 ±2.49% and 40.38 ±2.61% in controls and ALM-treated respectively and there was no significant difference.

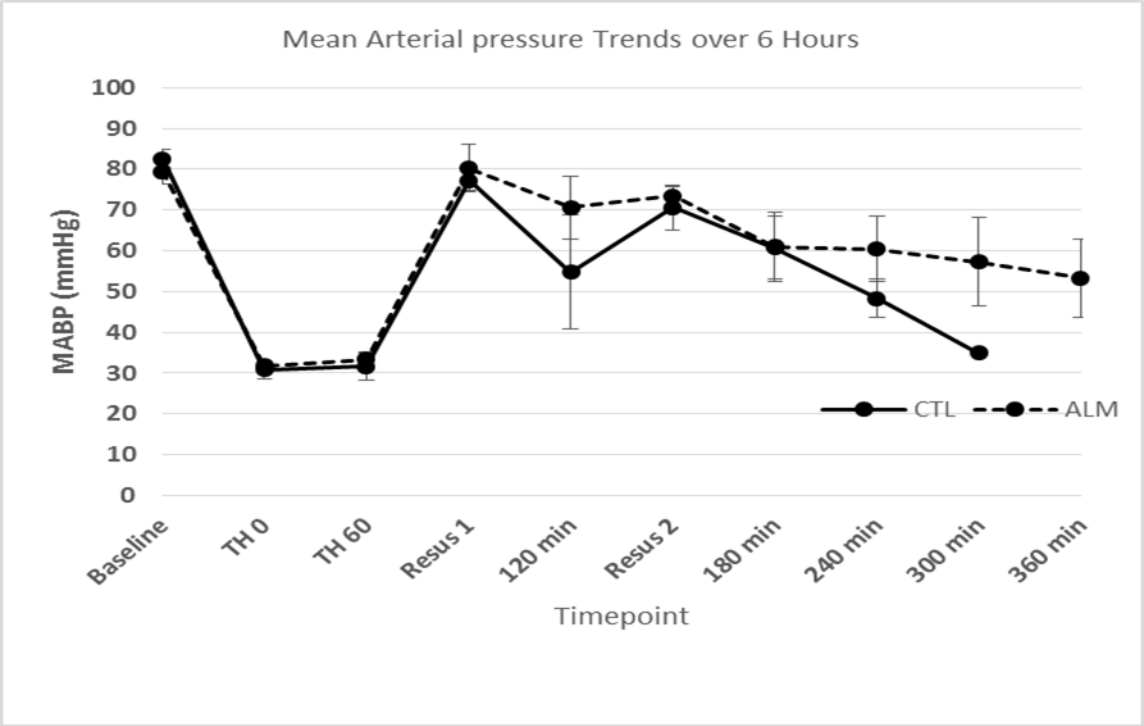


Figure 6.31 MABP trends across 6 hours.
Line graph shows MABP trends over the 6-hour experimental period and in response to TH with subsequent resuscitation in ALM-treated and control animals. Dots represent the means, vertical lines the standard deviation.

Fluid volumes administered, MABP, HR and LVS trends up until 180 minutes mirrored those seen in the initial 3-hour studies with significantly less fluid and significantly improved LVS function at 180 minutes compared to controls. At the end of the experiment (this referred to blood taken immediately prior to death if the animal did not survive), the controls tended to have higher lactate concentrations, but this did not reach significance (mean concentrations were 6.88 ±1.39mmol/L and 4.52 ±1.31mmol/L in controls and ALM animals respectively).

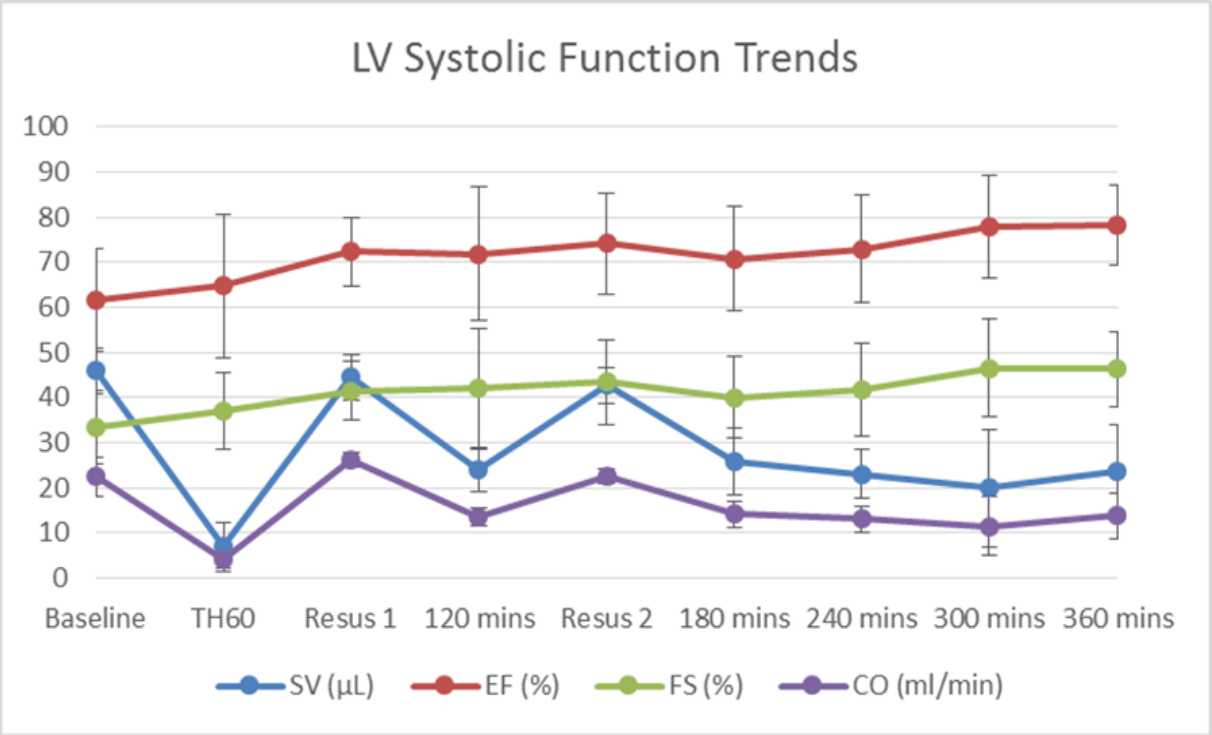


Figure 6.32 Trends in left ventricular systolic function across 6 hours.
Line graph shows the trends in LV systolic function over 6 hours in response to TH with subsequent resuscitation in ALM treated animals. Dots represent the mean, vertical bars standard deviation.

The trends in MABP and LV systolic function for the ALM treated animals are given in figures 6.31 and 6.32. The trends seen in the 3 hour studies are repeated here up until 180 minutes. Beyond 3 hours, CO and SV decline before showing a slight increase between 300 and 360 minutes.

At the 240 minute time-point, 2 of the control animals had died. Only 1 survived until 300 minutes, and subsequently died at 330 minutes, 30 minutes before the 6-hour point. No control animals therefore survived the entire experiment and all developed arrhythmia as a pre-terminal event. The 100% survival rate previously seen in the shorter studies was not seen at 6 hours with 1 of the ALM group dying at 280 minutes (fig.6.33).

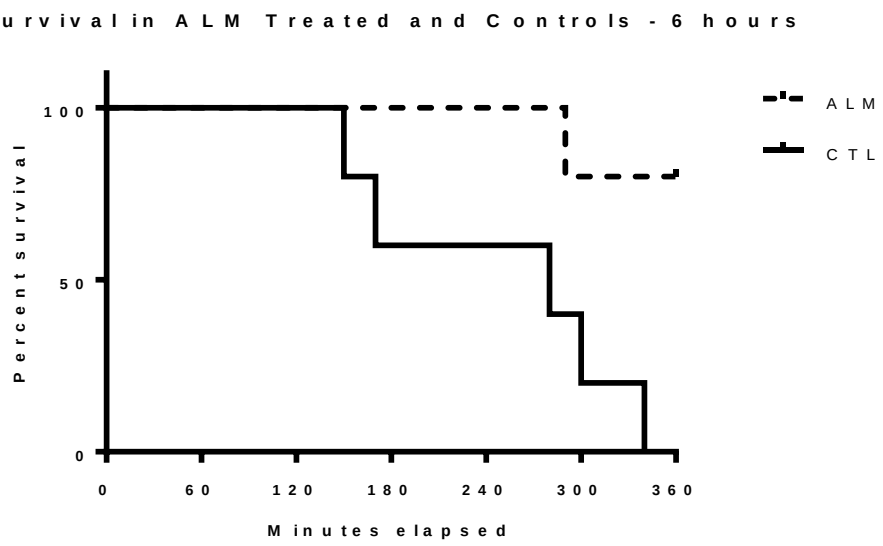


Figure 6.33 Kaplan-Meier survival analysis for the 6 hour studies.
Kaplan-Meier survival analysis shows the proportion of animals alive at 60,120,180,240,300 and 360 minutes in the control and ALM treated group. P=0.01 (Log-rank (Mantel-Cox test)). N=5 for ALM and control groups.

Blood sampling was performed at the end of the experiment. Serum was analysed, as previously described, for H-FABP and concentrations shown in figure 6.33. In the single ALM-treated animal that did not survive, no blood could be drawn during the agonal phase and therefore n=4 for this group.

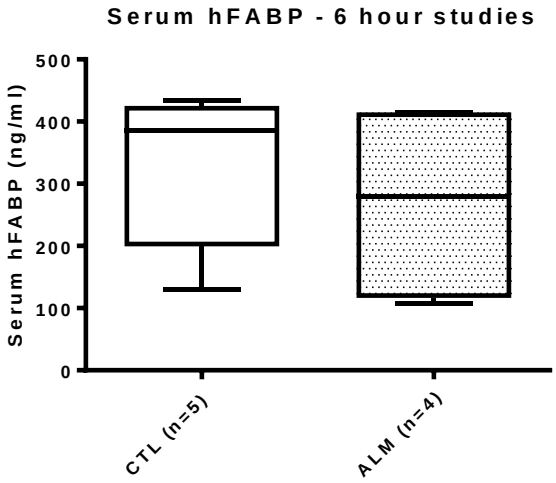


Figure 6.34 Serum H-FABP concentrations at 6 hours.
Box and whisker plots show serum H-FABP concentrations at the end of the 6 hour studies (or at point of death if the animal did not survive). Plots show the median (horizontal line), interquartile ranges (box) and range (whiskers).

All animals had elevated H-FABP concentrations at the end of the experiment (fig.6.33), but there was no statistically significant difference in the H-FABP levels between the two groups (means were 327.06 ± 114.06ng/mL and 270.15 ±144.94ng/mL in controls and ALM animals respectively). It is important to note when interpreting these results that the longest a control animal survived for was 330 minutes, with the other deaths at 280 minutes (2 mice), 170 and 140 minutes. Where available, blood was analysed for H-FABP in non-survivors with blood taken just prior to death and individual H-FABP concentrations were higher in the control animals than the treatment group but this is likely to reflect the severity of the physiological derangement pre-mortem. Numerically lower H-FABP levels in the ALM group may not therefore reflect a direct protective effect of ALM on cardiomyocytes but rather an indirect effect due to improved haemodynamics and / or longer term survival allowing for clearance of previously released H-FABP from the circulation.

6.5 Discussion

This study supports the findings of previous studies by other groups using haemorrhage-only models, with a 100% survival rate seen in the animals treated with a single intravenous dose of ALM during resuscitation after shock (Letson *et al* 2011). 50% of the controls died, the earliest occurring before the 120 minute scan. Those control animals who survived the 120-minutes did so with LV systolic parameters and MABP approaching values seen after the initial TH phase, such was the extent of their haemodynamic decline. ALM treated animals however survived the 3-hour studies and did so with improved haemodynamic parameters when compared to controls. This study has shown that ALM, when used as an adjunct to blood and crystalloid resuscitation fluid has efficacy in a rodent model combining trauma with severe haemorrhage.

Baseline MABP, HR and LV systolic functional parameters were similar in controls and treatment groups. All animals experienced a similar percentage blood loss during the TH phase of the experiment in order to achieve the target MABP of 30-40mmHg. There was no significant difference between the groups in terms of MABP or echo outcomes at completion of the TH phase of the experiment. One could reasonably expect therefore, that animals had experienced a similar degree of physiological derangement up until this point before commencement of resuscitation. However, the ALMx2 animals had a significantly higher HR prior to resuscitation. Given the similar degree of blood loss and plane of anaesthesia, the reason for this is not immediately clear. This difference was seen before commencement of resuscitation and was therefore not related to ALM treatment.

Resuscitation with crystalloid alone has been previously shown not to fully restore or maintain microvascular blood flow or cardiac output (Robinson 1997). Previous chapters of this thesis also echo some of these observations. The inclusion of blood in the resuscitation

regimen in this study discussed here has also been shown to be inadequate in terms of restoring baseline pre-load and haemodynamics and crystalloid was required in all animals in order to restore baseline SV and CO.

There was no significant difference between the groups in terms of the volume of blood delivered but there was a striking difference in the volume of crystalloid needed to restore SV. ALM-treated animals required significantly less fluid than controls. Control animals required up to 3-times more crystalloid. This is an interesting observation as this suggests that ALM has an immediate effect upon the haemodynamic status of treatment animals. Delivering the ALM as a rapid bolus dose therefore has enabled an appreciation of the rapidity of onset of its action.

The observations here regarding fluid resuscitation volumes support some previous work (Letson *et al* 2011) but some important differences are noted. Letson *et al* report an upward trend in MAP seen in the 60 minutes following ALM treatment in their rat model. They were however, investigating NaCl/ALM as an 'ultra-small' bolus for early resuscitation after haemorrhage alone and as such, they used 7.5% (hypertonic) saline in their studies whereas Hartmann's was the crystalloid used here. The addition of injury in our model also sets into motion the cascade of systemic responses to trauma which will contribute to cardiovascular dysfunction.

After completion of the resuscitation phases, a 60-minute period of monitoring was undertaken and no further fluid boluses were delivered. This strategy has allowed us to appreciate a predictable decline in MABP and LV systolic functional outcomes despite previous resuscitation which had restored pre-load. In these studies, ALM-treatment did not arrest this decline completely, but the animals treated with ALM, at either dose, did not deteriorate as far or as rapidly as controls. MABP, cardiac output and stroke volume were

significantly higher in treatment animals at 120 minutes and this was despite significantly lower volumes of resuscitation fluid being given to these animals. Ejection fraction and fractional shortening were not significantly different when the three groups were compared. FS tended to be higher in the ALMx2 group but this did not reach significance. Given that FS is an indicator of myocardial contractility, this finding suggests that ALM was not having an impact upon the force of contraction and the heart was not 'working harder' as a consequence of the drug. This is an important observation as, given what we already know regarding cardiomyocyte injury in TH, any therapeutic that caused further 'stress' on the myocardium may not be favourable in this context. This observation is only speculation however as, due to technical limitations, shock index could not be calculated and used as an indirect indicator for LV stroke work in this model. Published work using ALM in haemorrhagic shock does not include data relating to this aspect of cardiac function so this question remains unanswered.

All animals survived the 60-minute post resuscitation monitoring phase but controls had experienced significant cardiovascular deterioration by the 120-minute time-point and were in worse physiological condition than the ALM-treated animals upon entering the second resuscitation phase. Control animals required significantly higher volumes of crystalloid in order to restore SV at this point. Although not significant, ALM-treated animals required even less fluid than they did in the first resuscitation and ALM high dose animals required the smallest volumes. This reflects the improved SV of the ALM treated animals at the time of reaching 120 minutes; less fluid was required to push this up to baseline levels again.

This second resuscitation was again targeted to the baseline SV. The rationale for this decision was that SV provided a rapidly assessable surrogate for LVEDV and the aim was to restore pre-load. At both resuscitation points, SV was successfully restored to within target

range to all animals. However, when LVEDV was later calculated, it was found to fall at each resuscitation point, in each group, despite restoration of the SV to within the target range.

The decline in LVEDV was not significant when baseline was compared to resuscitation 2 in the control group but there was a significant drop from baseline in the ALM and ALMx2 groups and it is also interesting to note that in the higher dosage group, the fall in LVEDV between baseline and resuscitation 1 was significant whereas the fall in LVEDV in the single-dose group did not reach significance until the baseline and resuscitation 2 values were compared.

It is possible that ALM has an impact upon the pre-load conditions of the heart, independent of the volaemic status of the animal, which has been shown to be improved with ALM treatment at either dose. Cardiac output and index was improved in the treatment animals and it may be the case therefore, that peripheral perfusion was improved and a lower LVEDV reflects improved micro-circulation in the periphery rather than a truly reduced intravascular volume and LVEDV in the case of the ALM treated animals. This may also be supported by the temperature trend data with treated animals appearing to maintain thermoregulatory capability and maintain stable temperature whereas controls were less able to maintain temperature in the latter stages of the experiment and were more dependent upon external heat sources. Metabolic acidosis (reflected by higher lactates in the control animals) is known to instigate a peripheral vasodilatation in decompensated shock (Levick). Control animals therefore may have entered the decompensation phase with peripheral vasodilatation feeding into a fatal spiral of falling central pressures and this was the reason for the drop in LVEDV in this group.

Isoflurane was titrated against response to MABP and stimuli throughout the experiments (data not shown). Control animals required lower levels of isoflurane and in the final 60-

minutes, many were receiving only 0.5%. In comparison, ALM animals often required the dose to be increased (above 1.5% in some cases) in the final 60 minutes as they showed signs of regaining consciousness. This observation may reflect improved cerebral perfusion in the treatment-groups at this late stage but this is of course difficult to quantify with accuracy. It can also be suggested that ALM has a direct action on the CNS. Adenosine and lignocaine have been previously shown to cross the blood brain barrier (Partridge 1994, Dobson 2016). Although an interesting observation, any further comment on this however is beyond the limitations of this study.

Arrhythmia was seen in control animals as a pre-terminal event. Arrhythmia was however also seen in the ALM animals, but only in those receiving higher dose treatment. 4 of the 10 ALMx2 animals developed an arrhythmia in the final 60 minutes of the experiment and this was not in the context of a sudden decline in MABP (although the MABP was noted to become labile in these animals with arrhythmia onset). The development of arrhythmia in these animals differed from the controls as the onset did not appear to be related to physiological *extremis,* in fact the animals had been stable with satisfactory blood pressure prior to onset.

The higher lactate levels seen in the ALMx2 animals, although not significant, may possibly reflect the haemodynamic instability associated with the onset of arrhythmia which was intermittent but persisted for the remainder of the experiment in these animals.

A possible explanation lies with the doubling of the lignocaine dose in this group as the drug is known to induce arrhythmia at toxic levels (Brown 1980). Magnesium is known to stabilise the myocyte membranes. Adenosine has been used to prevent the development of arrhythmia, in a variety of settings across a range of doses in the literature but its role in the development of the arrhythmias seen in these studies is unclear.

As demonstrated in the previous chapters of this thesis, HFABP is elevated after 60-minutes of trauma-haemorrhage and the biomarker is a well characterised marker of myocyte ischaemia and necrosis. Other studies have demonstrated that adenosine has the ability reduce the extent of necrosis in regional ischaemia (Canyon *et al* 2004) and troponin levels post cardiac surgery in clinical trials when adenosine-lignocaine cardioplegia (Jin *et al* 2008). In our model, myocardial injury has been demonstrated with HFABP rise as a consequence of TH. In *chapter 5*, resuscitated 2 hour models demonstrated HFABP levels of a similar magnitude seen here at 3 hours. It is encouraging that the biomarker levels do not rise in response to ALM treatment and, although not significant, levels tended to be lower in ALM treated mice (the limited numbers having HFABP analysed for the reasons given earlier, means that there may be a power issue when interpreting this data). This may be seen as evidence for a reduction in the ongoing myocyte injury suggested in previous chapters, but it could also be related to improved renal perfusion pressures as a consequence of enhanced CO in the treatment groups. HFABP is renally excreted and therefore the lower levels may reflect improved renal perfusion and excretion of the molecule after its initial release during the TH phase.

6-hour experiments

The principle aim for conducting these longer experiments was to assess the feasibility of performing longer studies and to investigate whether the beneficial haemodynamic effect and survival outcomes previously seen with ALM treatment persisted.

Statistical analysis is limited due to the small numbers used in this study. This was a study designed to gain a better understanding of the longer-term consequences of using a single bolus of ALM. Initially an N of 10 for each group was planned but logistical reasons meant

that greater numbers were not achievable within the time available. However, the limited results are useful and provide some informative data regarding survival and longer-term functional outcomes and this data has therefore been included here.

The animals did not show any significant differences in terms of baseline MABP, HR or LVS function. They were haemorrhaged to a similar extent. Trends in fluid requirement, MABP, temperature and LVS function were similar to those in the 3-hour studies at the 3 hour time-point. In the 3 hours beyond the 3 hour time-point, the gradual decline in MABP continued in controls and ALM group. CO and SV declined past the 180 minute point but in the final 60 minutes of the experiment appeared to plateau and then a slight increase in these parameters was seen. EF and FS also show little change in the final 60 minutes.

At the 360 minute time-point, the MABP for the 4 survivors was 53.25 ± 9.52mmHg. No control animals survived the 6 hours and 1 ALM treated animal died at 280 minutes. The small number of controls surviving beyond 180 minutes makes comparison difficult and would require a larger study. The practical considerations of running these 6 hour experiments make them incredibly time-consuming and challenging. Previous rodent H models with ALM treatment have not continued as long as 6 hours and therefore, although limited by small group size, this study has provided some additional insights into the longer term haemodynamic effects of ALM when administered as an adjunct to fluid resuscitation after TH.

6.6 Limitations

This is a complex, technically challenging model involving multiple phases which subjects animals to extreme physiological derangement. There are some notable limitations regarding

the design and practical aspects of this study. Limitations relating to species and method of haemorrhage have been discussed in previous chapters.

ALM has been identified as novel therapeutic agent for the acute management of the shocked patient. In these studies, bleeding had been arrested by the time of ALM administration. It is not possible therefore to comment on its efficacy in a situation of on-going bleeding.

Mice have a limited circulating volume and this limits the investigations that can be performed, particularly in a model with a 40% blood loss. Further blood taking can lead to death due to the precarious haemodynamic state of the animal. Temporal patterns of cardiac biomarkers such as H-FABP may have addressed some of the questions raised in this study, but blood volume is a limiting factor. Blood analysis of the drug doses may have helped to investigate the cause for arrhythmias in the double dose group but again, blood volume is prohibitive.

A major limitation of the 6 hour studies is that these are underpowered, reflecting logistical issues which arose. The aim was to gauge the longer-term response to ALM and therefore the data from these studies is of interest but must be interpreted cautiously.

6.7 Conclusions

ALM has been shown to be beneficial when used as an adjunct to blood and crystalloid resuscitation after 60 minutes of TH. There is also some limited evidence for a dose-dependent response. This study used a bolus rather than infusion but despite the short half-life of adenosine in particular, we have seen lasting effects in terms of improved haemodynamics and LV systolic outcomes.

Single agent studies would be of value in determining additive / synergistic contributions of the three drugs. A single agent study may also help identify the cause of the arrhythmia seen in the high dose group; this being a major limitation to the use of the combination drug at this dose.

The mechanisms of action of ALM are still unclear but they are likely to be multiple and to include cardio-specific and systemic pathways. An advantage of having this model established in mice is that it would allow for the use of genetically-modified strains with which to further our understanding of the actions of adenosine in particular. For example, the availability of adenosine receptor knock-out strains may yield insights into the pathways involved in protective effects of ALM in TISCI.

This is the first time this combination drug has been used in the context of both trauma and haemorrhage. Studies in rats and pigs have been in the context of haemorrhagic shock without concomitant injury and therefore, lack the sequelae of combined TH.

These studies are also, to the best of my knowledge, the first example of echocardiography being used to assess in vivo left ventricular systolic function in response to TH with ALM resuscitation.

CHAPTER SEVEN

Conclusions

Chapter Seven

7 Conclusions

7.1 Summary of Project Findings

The aims of this project, as laid out in *chapter one*, were all met. The movement from "bedside-to –bench" to validate H-FABP as a marker of TISCI in our previous model of TH was successful. H-FABP was detected at significant concentrations as early as 60-minutes after trauma haemorrhage, in a time frame and magnitude that supports the clinical data. This research has also, for the first time, demonstrated the dose-dependent nature of the cardiac-specific biomarker rise in response to different severities of trauma + / or haemorrhage. The validation of the use of H-FABP as a biomarker of TISCI in the murine model was an important first step in this project as it confirmed established cardiomyocyte injury after 60-minutes, before going on to investigate the functional implications of this injury.

Chapter four demonstrated the successful application of non-invasive micro-imaging to characterise the functional systolic response of the left ventricle to TH. The novel use of this modality in a small animal model of trauma-haemorrhage has yielded insights into the LV performance in the acute phase after injury and blood loss and highlighted the dramatic reduction in cardiac output and stroke volume seen in response to severe blood loss. This emphasises the precarious state of the haemodynamic system at this point and helps to clarify why H-FABP levels are significantly raised at 60-minutes. Severely reduced cardiac output coupled with elevated heart rate will compromise filling of the coronary circulation during a shortened diastole. The left ventricular myocardium however is subject to increased

physiological demands however and the elevated H-FABP levels are likely to represent the resultant cardiomyocyte ischaemia. The H-FABP levels seen after 60-minutes of MABP of 30-40mmHg in chapters three and four are similar and therefore support that this is a reproducible model which demonstrates a H-FABP rise comparable to that seen in the clinical studies.

The model of TH presented here includes a severe form of haemorrhage which was fatal in some cases and associated with a mortality of approximately 25% during the initial 60-minute TH period. The mortality rate increased with time and the 3-hour studies in chapter six were associated with 50% mortality in non-ALM treated animals that had survived to receive resuscitation fluid.

A resuscitation model was developed so that the effects of the initial 60-minutes of severe TH could be assessed in the longer-term and the functional response of the myocardium, previously shown to have developed TISCI, could be assessed. In order to limit cardiomyocyte injury resulting from ‘under-resuscitation’, a decision was made to utilise the in-vivo imaging system to ensure ‘adequate’ re-filling of the left ventricle. Using this approach ensured that cardiac injury, and associated elevations in H-FABP concentration, was predominantly a consequence of the initial 60-minute insult and not due to either under or overfilling of the ventricle. A comparison of SV-guided and MABP-guided resuscitation was therefore conducted.

Additional intravascular catheters, the need to transfer the animals and the repeated use of scanning made for a complex, technically challenging model. Echocardiographic assessment of the filling status of the LV was used as a targeted end-point of resuscitation and to facilitate restoration of adequate, pre-load. Comparison of two resuscitation strategies

usefully demonstrated the relative inadequacy (in terms of LV volumetric status) of resuscitation targeted to MABP. Many of the contemporary models address the need for more fluid resuscitation relative to blood loss, but this project highlights, in more precise physiological terms, the potential pitfalls of using a less direct marker of intravascular volumetric filling status. The techniques used in these studies were robust and reproducible and trends in haemodynamic decline were predictable in animals resuscitated by either means.

The data presented here supports that of other researchers who have identified the development of an acute cardiac injury associated with cardiovascular dysfunction after THS (Hsieh *et al* 2006, Kuebler *et al* 2003, Kapoor *et al*, 1997). Having identified this consequence of TH, there has been an acknowledgement that therapeutic agents need to be identified which could treat TISCI.

The results of the ALM studies presented in chapter six are very exciting in that regard. The hypothesis was that the use of this drug, as an adjunct to fluid resuscitation, would improve haemodynamic stability after TH as suggested by previous authors (Dobson *et al* 2016, Granfeldt *et al* 2014). These studies were the first to use this combination drug in a mouse model of haemorrhage and trauma, and the assessment of the LVS response by means of echo added a functional assessment to the haemodynamic, lactate and biomarker data. ALM, used at 2 different doses as a single bolus, was shown to confer haemodynamic stability as well as a survival benefit (also demonstrated in 2011 by Letson and Dobson with the rat haemorrhage model) at 3 and 6 hours. Together, these studies have shown that ALM has great potential as

a treatment for haemodynamic instability which was shown to be associated with biomarker proven TISCI in this mouse model.

7.2 Limitations and Strengths of the Project

7.2.1 Limitations of the project

Some limitations have discussed in the previous chapters and here I will summarise the general limitations that need to be considered and also discuss study specific limitations relating to specific methodologies used.

Technical limitations

There are significant challenges associated with the use of a small animal such as a mouse for studies of this nature. This may also go some way to explaining the predominance of rat models within the contemporary literature.

Vessel cannulation is difficult and time consuming, especially in the early stages of mastering the techniques. Despite steps taken to prevent lumen occlusion, blockage of lines in some cases resulted in a lack of blood available for lactate and biomarker analysis at the end of the experiment. Despite this, cardiac and haemodynamic assessment is still valid in these animals and the decision was made to include this data despite a lack in accompanying blood results in a small number of cases.

A fixed-pressure haemorrhage protocol was used in all of the studies presented here. This is currently the only practical way to induce haemorrhagic shock in mice due to their size. Steps were taken to attempt to keep the rate of onset and the depth of shock the same for all animals

but, as has been demonstrated by these studies, markers of impaired perfusion status, such as lactate, were variable amongst animals in the same experimental group. This may indicate that the severity of shock was not identical in each animal in a particular group. Some may have been bled more quickly in the first few minutes for example leading to a more rapid onset of shock.

Small animals only possess small blood volumes and this has obvious implications for the number of samples that can be taken and often precludes repeated sampling in the same animal due to resultant haemodynamic instability. Analysis of H-FABP, for example, would have been useful at different points in the same experiment but the volumes of blood required to generate enough serum for the assay made this impossible.

Young, male mice were used for all of the studies. This therefore limits the translational potential when considering older trauma patients and those with pre-existing cardiovascular disease – an important and clinically very challenging patient group.

Limitations of the echocardiography studies.

With the appropriate training, echocardiography is rapid and reproducible. Imaging the left ventricle in a haemorrhaged animal however is more difficult due to the profound loss of volume from the LV and the tachycardia that results from haemorrhage. This is therefore not a straightforward undertaking in this model and acquisition of images and the derived LV outcomes are difficult and subject to a potential loss of accuracy when placing callipers on the M mode images. A second investigator with considerable experience in micro-imaging conducted all of the scans for these studies in an attempt to limit inaccuracy and to avoid inter-observer bias. Three sets of measurements were taken at each point and the mean of these calculated to improve accuracy.

Performing the imaging required transfer of the animal and in a couple of instances, lines became dislodged resulting in exsangination if the carotid catheter was lost, or inability to resuscitate according to protocol if the jugular line became misplaced. Due to loss of lines in this way in the first experiments, additional anchoring sutures were placed to prevent this and a small transfer 'scoop' was made which facilitated transfer of the animal to the scanner after surgery and line insertion. These measures led to a reduction in the number of lines dislodged in subsequent experiments.

Limitations of the resuscitation studies.

Stroke volume was used as a surrogate for LVEDV due to practical considerations. Real-time calculation of the LVEDV was not possible with the scanner used and therefore SV served as a rapid way to determine filling status during resuscitation. This has limitations however as SV doesn't necessarily directly reflect the volumetric status of the LV alone and subject to other influences.

Shed, and therefore whole, blood was used to resuscitate animals during the first resuscitation phase. If a second resuscitation was included, this was by means of administration of Hartmann's, a crystalloid solution. Such a resuscitation strategy has considerable differences when compared to clinical approaches. There is no possibility of transfusing individual components such as packed red cells in studies of this kind, reinfusion of the shed blood meant that there was no further blood available at the next resuscitation point. This is a potential drawback in the use of mouse models in THS research generally.

Limitations of the ALM studies.

The addition of ALM to resuscitation fluid has been shown in this project to have benefits related to haemodynamic outcomes, fluid requirements and survival. This project has not elucidated the mechanisms responsible for these findings and one can only speculate.

This model is not one of on-going bleeding and no further blood was drawn after the initial 60 minute TH phase. This means that although the benefits of ALM are convincing in this model, it is in the absence of on-going haemorrhage.

In the course of this project, I have developed a robust, reproducible small animal model of biomarker-proven TISCI. The contemporary pre-clinical models of trauma-haemorrhage were reviewed and drawn upon in order to develop a model capable of addressing questions regarding TISCI and cardiac dysfunction.

The model is incredibly complex involves both trauma and severe haemorrhage. The combination of injury with haemorrhage was important in terms of improving the translational potential of the model. TISCI has been demonstrated clinically to be associated with haemorrhage and trauma of high severity. The addition of injury to haemorrhage will instigate inflammation and therefore is more likely to represent the clinical scenario however, the *chapter three* studies demonstrated that H-FABP was elevated above normal in the mice subjected to haemorrhage alone. The use of a haemorrhage only murine model therefore may still represent the clinical condition and would likely prove less technically challenging and would also allow for a recovery model to be more easily developed. Echocardiography could then potentially be performed in the days and even weeks following haemorrhagic shock in order to monitor for the development of overt cardiac dysfunction after resuscitated haemorrhagic shock. More work on this haemorrhage only model of TISCI is therefore warranted.

Rodent models of trauma and haemorrhage are challenging. This project has demonstrated that the challenges are not insurmountable however and there is still a role for these models in investigating the physiology associated with trauma.

7.2.2 Strengths of the Project

Trauma is a complex, multifactorial disease and therefore the development of robust, translatable animal models is a significant challenge.

The centre for trauma sciences have previously used a mouse model of trauma haemorrhage to investigate conditions such as acute traumatic coagulopathy (Frith *et al* 2010). This project has therefore benefitted from the availability of a previously validated murine model of injury and haemorrhage in which to conduct these studies and I was trained by co-workers with significant expertise in the field of pre-clinical research and trauma research in particular.

Considering that haemodynamic function was to be assessed in this project, close attention was paid to the design of the model, for example, inhaled isoflurane was selected as the anaesthetic agent due to ease of titration, rapid elimination and the fact that it is one of the least cardio-depressive agents amongst the variety available for pre-clinical work. A degree of hypotension was inevitable with anaesthesia, but isoflurane was used in an attempt to limit this and therefore minimise the impact of anaesthesia on cardiovascular function.

This project has, for the first time, demonstrated a dose dependent rise in H-FABP with increasing severity of trauma-haemorrhage. The magnitude of the biomarker rise is similar to that seen in clinical studies at 1 hour making H-FABP a valid biomarker of TISCI in this animal model. The studies in *chapter three* were important as establishing the presence and magnitude of this biomarker rise was vital in determining that cardiac injury was present within the model at the degree of haemorrhagic shock induced, before proceeding to assess haemodynamic outcomes in the hours after injury onset.

This project took the existing mouse TH model and developed it to include venous cannulation and a resuscitation phase and in vivo myocardial imaging. A sophisticated model has therefore been developed which is complex yet benefits from good reproducibility. The

ability to image the left ventricle in real-time has allowed for multiple images to be acquired at different experimental time-points with resuscitation tailored to the individual animal rather than a 'one-size fits all' approach to fluid resuscitation. Mice do not require ventilation and as such, effects of positive pressure ventilation on cardiac blood flow and function were not an issue in this model.

A second investigator with considerable in-vivo imaging expertise carried out the echocardiographic assessment of the LV. There was therefore no potential for inter-observer variability. His expertise also allowed for rapid, reproducible assessment of the left ventricle meaning that animals weren't subjected to lengthy scan times and frequent re-positioning. This investigator was not involved in the surgery and haemorrhage aspect of the studies and was therefore not generally aware of the blood pressure prior to performing the imaging. In some cases, if the animal was particularly unstable prior to transferring to the scan bed for example, it was necessary to discuss this but this was the case in only a minority of the severe haemorrhage animals.

Echocardiography is of huge clinical relevance and the ability to quantitatively assess the left ventricle under TH conditions makes this project highly translatable and relevant. As previously discussed, ultrasound imaging is cheap, reproducible and already used in the resuscitation bay as an adjunct to resuscitation. It is portable and images of the heart can be acquired quickly without the need to transfer unstable patients for more lengthy cardiac imaging studies. These features make echocardiography, as applied in this project, a realistic and attractive option for future clinical studies of TISCI. Echocardiography is a frequently used clinical imaging modality. It is already used in the resuscitation bay and there is some suggestion that its use should be broadened and it could have utility in volumetric assessment of trauma patients and used to assist in fluid resuscitation. This project has demonstrated the disparity between MABP and LV volumetric status as markers of the 'adequacy' of fluid

administration and therefore, could also support the argument for a role for echocardiography in guiding resuscitation of shocked trauma patients.

Mouse models are ideal for mechanistic studies and are widely used in cardiovascular research. The creation of this TH model serves as a great platform for future studies. Using genetically modified mouse strains in this setting could address outstanding questions regarding the inflammatory aspects of TISCI development and may be useful in determining the mechanisms underlying the cardio-protective effects of ALM therapy that have been reported here. The techniques and protocols used for this project could also easily be adapted for a rat model of TH in which to assess myocardial function. This may have additional benefits including higher blood volume available for more frequent testing and the opportunity to catheterise the urinary bladder for urine output assessment.

This project is the first to demonstrate the haemodynamic and survival benefits of ALM in a mouse model of trauma with haemorrhagic shock. The use of echocardiography to assess LV function before, during and after ALM therapy is another strength of this project. There is a real need to identify therapeutics for use in trauma patients in both the civilian and military settings. The findings of this project lend support to the use of this drug combination to maintain haemodynamic function, reduce the need for high-volume fluid resuscitation and favour survival in a setting where there has been blood loss resulting in hypotension with severe decline of LV function and a dramatic fall in cardiac index. The decision was made to deliver the ALM bolus after reinfusion of shed blood and this was taken in order to optimise the translational potential of these studies as any therapeutic delivered clinically is likely to be administered after initial blood product based resuscitation. The findings of this project regarding the haemodynamic benefits of ALM are incredibly exciting as this project has identified a drug which is potentially life-saving and warrants further attention in clinical trauma haemorrhage studies.

7.3 Future Work

The development of this model offers exciting opportunities for further research into TISCI. The sophisticated model, combining bony and soft tissue injury, pressure-controlled haemorrhagic shock, blood and crystalloid resuscitation and in vivo cardiac imaging, represents a complex, but achievable, pre-clinical platform in which to advance our knowledge of the pathophysiology of TISCI.

This project has addressed some of the functional questions surrounding TISCI but there are opportunities to further our knowledge. For example, this project has not included an assessment of the contribution of inflammation to the development of TISCI and this is an incredible important aspect in the development of cardiac complications after TH and as such, has been the focus of work by many research teams. The methodology described in these studies however, could be applied to knock-out mice to investigate the roles of specific immune signalling pathways in the development of, or protection from TISCI. The use of knock-out mice in such studies however would not be without its own limitations. One of the most important, but frequently overlooked, considerations relates to the persistence of regions of genetic material neighbouring the 'knock-out' gene. These "flanking regions" can be transferred onto the selected genetic background and have implications for observed phenotype. Knock-out mice may also have differences that have not been specifically selected for such as altered motor function and behavioural changes (Eisener-Dorman *et al* 2009).

As more sophisticated imaging techniques and miniaturisation become available, these could add a fresh perspective to pre-clinical TISCI research. More advanced ultrasound imaging could provide a more accurate quantitative determination of myocardial contractility in response to TH and resuscitation. For example, speckle-tracking (or 'myocardial deformation

imaging') echocardiography is a more recently developed technique allowing for direct quantification of myocardial contractile function rather than relying on the LVEF which provides an indirect estimate. This technique 'tracks' changes in myocardial segment length and the rate at which this is occurring. It can therefore detect more subtle changes in contractile function compared to older techniques (Bansal & Kasliwal, 2013).

Nuclear imaging modalities such as cardiac single-photon emission computed tomography (SPECT) could also be employed to characterise perfusion and metabolic demands of the left ventricle in THS. Left ventricular pressure-transducer catheters (used in some rat models of THS) are available for murine work and this could also be explored as a method of assessing LV systolic function in mouse studies.

Although ALM has shown promise in these studies to slow the rate of haemodynamic decline and prevent early mortality, the precise mechanisms of action remain unclear. Again, genetically-modified mice with adenosine receptor mutations may provide a way of investigating its action further, as would the use of this model with receptor agonists / antagonists.

TISCI in this project has been modelled in young, male mice, presumed to be free from cardiovascular disease. As more research is carried out relating to older trauma patients, and those with pre-existing cardiac disease, the strain of mouse selected could be tailored in order to address questions about the pathophysiology of TISCI in older people with pre-existing vascular disease, and the efficacy of novel therapeutics in this case.

Clinical studies of TISCI have identified biomarker rises in TH patients which are associated with the development of ACE (De'Ath *et al* 2013, Naganathar *et al* 2015). There is still much to learn regarding the long term cardiac outcomes in TH survivors and clinical studies are needed to investigate the longer term impact of TISCI. For example, do survivors with high

admission H-FABP levels have a higher risk for ACE in the longer term? Should we be monitoring survivors in the months / years after discharge for the onset of a ‘trauma-related cardiomyopathy’? Would follow-up echocardiography identify structural and / or functional changes beyond discharge? These are the type of questions that could usefully be addressed with clinical studies in the future.

There is a need for therapeutic agents which can be used clinically to prevent death and limit the organ dysfunction resulting from major trauma. The work presented in this thesis has identified a therapeutic agent which shows efficacy in trauma with haemorrhagic shock in the pre-clinical model. The use of ALM as an adjunct to fluid resuscitation has shown dramatic effects on survival, fluid requirement and haemodynamics. A clinical study is therefore currently being designed in which to test this therapeutic clinically.

7.4 Conclusions

This thesis has successfully completed all of the study aims. It has demonstrated, for the first time, a dose-dependent cardiac-specific biomarker rise with increasing severity of injury in the murine model of THS, on a magnitude similar to that reported in clinical studies of TISCI. The use of echocardiography has provided important insights into *in vivo* myocardial function in the minutes and hours after trauma-haemorrhage with cardiac injury.

The development of a robust resuscitated model of TISCI has enabled us to perform longer studies in which to observe changes in cardiac function up to 6 hours after haemorrhage and also to investigate the functional implications of 60-minutes of TH followed by restoration of volume to the left ventricle. Trends in haemodynamic decline and myocardial functional outcomes after TH with, and without resuscitation, have been identified. The project also highlights the impact of resuscitation guided by MABP which resulted in persistent relative hypovolaemia of the left ventricle when compared to the individual's baseline conditions and the SV-guided resuscitation approach.

Improved haemodynamic outcomes and survival were seen with the addition of ALM to resuscitation fluid. ALM has therefore been shown here to have great promise as a cardiovascular rescue agent after acute injury and blood loss and consideration should be given to investigating its role in the clinical setting.

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