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THE EFFECTS OF TOURNIQUET ASSOCIATED ISCHEMIA REPERFUSION
INJURY ON THE DEVELOPMENT OF SECONDARY INJURY IN A BLAST-
RELATED MODEL OF LOWER LIMB EXTREMITY TRAUMA

by

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Thesis submitted to the Faculty of the
Molecular and Cell Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2019



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

SCHOOL OF MEDICINE GRADUATE PROGRAMS

Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814



APPROVAL OF THE DOCTORAL DISSERTATION IN THE MOLECULAR AND CELL BIOLOGY GRADUATE PROGRAM

Title of Dissertation: "The effects of tourniquet associated ischemia reperfusion injury on the development of secondary injury in a blast related model of lower limb extremity trauma"

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Doctor of Philosophy Degree
August 5, 2019

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ACKNOWLEDGMENTS

I would like to thank Dr Thomas Davis for his direction and support in determining the focus of this study over the past 3 years. His advice on transitioning from clinical to basic science research has allowed me to understand both ends of the spectrum of translational research and will provide a bedrock for my future efforts. I would also like to thank my Committee members for their time and input in shaping the direction and scope of this work.

My sincere gratitude goes to CAPT Eric Elster USN, Professor and Chair of the Department of Surgery at the Uniformed Services University, and Surgeon Captain Rory Rickard Royal Navy, Defence Professor of Surgery, for the opportunity to undertake my studies at USUHS. I am honored to have been afforded the chance not only to achieve my PhD, but also to play a part in strengthening the US-UK Defense research relationship; a collaboration that has delivered so many of the recent innovations in combat casualty care and military medicine.

Acknowledgement and my sincere thanks go to MAJ Erica Barkei, veterinary pathologist at Naval Medical Research Center (NMRC), for all her assistance with histological specimens and the grading and interpretation of injured tissue samples. I am indebted for all the support and assistance provided by the staff of the Regenerative Medicine Department at NMRC, whose efforts have enabled me to complete my research.

DEDICATION

To my wife Sarah and daughter Merryn,
to my parents, Eleanor and Ian, and sister Alison,
for your enduring support and love.

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November 5, 2019

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ABSTRACT

“The Effects of Tourniquet Associated Ischemia Reperfusion Injury on The Development of Secondary Injury in A Blast-Related Model of Lower Limb Extremity Trauma”

Philip James Spreadborough, Molecular and Cell Biology PhD Program, 2019

Thesis directed by: Dr Thomas Davis, Professor, Department of Surgery

Blast accounted for 70% of all mechanisms of injury during recent conflicts in Iraq and Afghanistan. Improved pre-hospital survival, partly due to tourniquet use in controlling haemorrhage, means that secondary injury as the result of a dysregulated immune response to polytrauma is now a significant challenge. Multiple organ dysfunction syndrome (MODS) increases mortality 6-fold and quadruples the requirement for assistance in daily living at 2 years. Heterotopic ossification (HO) occurred in 65% of blast-related traumatic amputees, many of whom had tourniquets applied during care at point of wounding. This research sought to establish how blast exposure may potentiate the onset and effects of tourniquet-induced ischemia reperfusion injury (IRI) with the subsequent development of either local or systemic secondary injury.

A pre-clinical study was performed in a rodent model to assess the effect of a 120 kPa systemic blast overpressure on the development of IRI following either 90 or 150 minutes of hind-limb tourniquet application. A further study was performed to assess the effect of tourniquet application in an established blast-associated lower extremity

traumatic amputation model. Analysis of blood biomarkers and tissue gene expression was performed to assess changes in inflammatory mediators and signaling pathways, with microCT imaging to assess HO formation.

Our results demonstrate that blast exposure following 150 minutes of tourniquet application increases markers of IRI and end organ injury through increased proinflammatory signaling. Tourniquets also increase proinflammatory signaling, oxidative stress and damage molecule signaling in a complex injured limb above that associated with traumatic amputation alone. This increases early remote organ dysfunction as well as increasing total bone formation at 12 weeks post-injury.

Hemorrhage remains the leading cause of preventable death in combat. Tourniquets play an important role in the control of catastrophic hemorrhage. This research highlights that mechanism of injury can reduce the duration of safe tourniquet application, after which it may amplify the systemic inflammatory response and predispose to secondary injury. It is recommended that the risk of secondary injury be reduced by maintaining short clinical timelines, promoting regular review of ongoing need for tourniquet and considering removal once risk of catastrophic hemorrhage has ceased.

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LIST OF ABBREVIATIONS

ALK2	activin-like kinase 2
AKI	acute kidney injury
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
ALT	alanine aminotransferase
ALB	albumin
ALP	alkaline phosphatase
ANOVA	analysis of variance
AST	aspartate transaminase
BOP	blast overpressure
BUN	blood urea nitrogen
BMP	bone morphogenetic protein
BALF	bronchoalveolar lavage fluid
CXCL1	C-X-C motif chemokine ligand 1
CXCL2	C-X-C motif chemokine ligand 2
CIRP	cold-inducible RNA-binding protein
Cr	creatinine
CK	creatine kinase
DAMP	danger-associated molecular patterns
DVT	deep vein thrombus
N ₂ S	dinitrogen-sulphide
FOP	Fibrodysplasia Ossificans Progressiva
mtDNA	free mitochondrial DNA
GSSG	glutathione
GSW	gunshot wounds
HSP	heat shock proteins
HO	heterotopic ossification
HMGB1	high-mobility group box 1
HIF-1 α	hypoxia-inducible factor 1-alpha
IED	improvised explosive device
iNOS	inducible nitric oxide synthase
ISS	injury severity score
IL	interleukin
IQR	interquartile range
KIM-1	kidney injury molecule-1
kPa	kilopascals
LDH	lactate dehydrogenase
MIP-2 α	macrophage inflammatory protein 2-alpha
MHC	major histocompatibility class
MnSOD	manganese superoxide dismutase
MTF	medical treatment facilities
MODS	multiple organ dysfunction syndrome

MPO	myeloperoxidase
NK	natural killer
NET	neutrophil extracellular traps
NGAL	neutrophil gelatinase-associated lipocalin
NISS	New Injury Severity Score
NLRP3	NOD-like receptor protein-3
OPGL	osteoprotegerin ligand
PAMP	pathogen-associated molecular patterns
PRR	pattern recognition receptors
PBLI	primary blast lung injury
PFC	prolonged field care
PE	pulmonary embolism
PSI	pounds per square inch
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
sRAGE	soluble receptor for advanced glycation end-products
SOD	superoxide dismutase
SIRS	Systemic Inflammatory Response Syndrome
NLR	NOD-like receptor
TLR4	Toll-like receptor 4
TEM	transmitting electron microscopy
TBI	traumatic brain injury
TREM-1	triggering receptor expressed on myeloid cells-1
UK	United Kingdom
US	United States
ZOI	zone of injury

CHAPTER 1: Introduction

THE CHANGING INCIDENCE AND ETIOLOGY OF BLAST RELATED INJURIES

During recent conflicts in Iraq and Afghanistan, there has been a changing pattern in the mechanism of injury sustained by our service personnel, with 70% of all injuries (109; 241) now resulting from blast explosions. Extremity wounds comprised 34-39% of injuries (109; 241), with just under half of these affecting the lower limbs (242). Combat casualties in Afghanistan sustained more extensive lower limb trauma due to blast, frequently resulting in proximal traumatic amputations. Improvised explosive devices (IEDs) led to an increased number of deaths between 2001 and 2010, with the percentage due to IEDs rising year on year during that period (117). Blast injuries are not confined to the military environment. Emerging terrorist threats (130; 137) and civilian accidents (146) makes this mechanism of injury equally applicable to civilian patient care (2).

The etiology of injuries sustained following blast are often related to the mechanisms by which it exerts its effect, and as such are frequently categorized that way. These mechanisms are typically referred to as primary, secondary, tertiary and quaternary injuries. Primary blast injuries occur directly from initial over, and then under, pressurization of the air relative to atmospheric pressure generated by the rapid expansion of compressed gas from the explosion, the peak of which is proportional to the energy of the blast (15). Air-filled organs, such as the lung, ear, gastrointestinal tract and brain, can sustain a barotrauma injury which may manifest immediately, such as a ruptured tympanic membrane, or develop over time, as with blast lung which results from the progression and maturation of pulmonary contusions and hemorrhage. Secondary blast

injuries are sustained as a result of debris from the blast causing blunt or penetrating injuries to the victim. This is the most common type of injury and accounts for the majority of mortality and morbidity associated with blast injuries. Tertiary injuries occur as a result of the individual being thrown by the primary blast wave, resulting in secondary injuries upon impact when landing, while quaternary injuries cover those not included in the former categories. These could include smoke inhalation injuries or burns, particularly with enclosed blasts.

The Department of Veteran Affairs has shown the incidence of blast related injuries between 2005 and 2009 to range from 1.7 to 4.5 per 1,000 deployed, with a spike in incidence to 83 per 1,000 during the 2007 Iraq troop surge amongst US Army Brigade Combat Teams. Musculoskeletal extremity injuries had the highest incidence, with 82% of them attributable to explosions (98). These numbers are likely to underestimate the burden in combat arms of the military, where vehicle or dismounted blast injuries are more likely to occur when compared to non-combatant service personnel deployed to rear echelon support roles. Analysis of probability of survival using UK data from Operation BANNER in Northern Ireland in 1969 to 1997 through to Operations TELIC and HERRICK in Iraq and Afghanistan has shown that the mean New Injury Severity Score (NISS) associated with 50% probability of survival has increased from 29 to 43 over the past four decades (184). In clinical terms this means an injury severity associated with a 50% chance of dying during Op BANNER carried an approximate 70% chance of survival during Iraq or over 90% in Afghanistan. Conversely, injuries during current conflicts in Afghanistan associated with 50% risk of mortality would have been 95% fatal during earlier operations in Northern Ireland (184). Despite a rise in the severity of

injuries sustained following blast-related trauma, survival has improved. Reasons for this include increased medical training and ‘buddy-buddy’ care at the point of wounding with early hemorrhage control, improved body armor, forward deployment of prehospital physicians and surgical teams delivering early damage control resuscitation and surgery, and the rapid retrieval and evacuation of casualties from the battle space (170; 215).

A study of 100 personnel wounded-in-action in Afghanistan between 2007-2010 who survived to medical treatment facilities (MTFs) found 16 later died from their wounds; proximal injuries and a high injury severity score (ISS) or NISS were associated with poor prognosis (117; 193). An additional study of UK military bilateral amputees identified that 88% involved at least one trans-femoral amputation, demonstrating the power of modern IEDs (214). Casualties with multiple amputations are now surviving to MTF due to advances in pre-hospital care and extraction; up to 31% in recent conflicts sustained multiple amputations (94). Tourniquets were applied in 80% of cases, and the number of amputations positively correlated with development of complications such as renal failure, wound infections and deep vein thrombus (DVT) or pulmonary embolism (PE) (94).

Blast injury is known to affect multiple systems within the body through an interplay of local, systemic and cerebral responses. Systemic inflammation can occur from the activation-priming of the innate immune system by endogenous signals activated during tissue or cell damage. Localized tissue damage can produce systemic cytokines which direct cell migration and recruit other immune cells. Secondary injury following trauma refers to the effects of the self-propagating dysregulated immunological and physiological response that can arise following severe injury, leading to both local

and systemic damage of viable and healthy tissue. Failure of an early compensatory anti-inflammatory response mechanism that counters the initial pro-inflammatory innate immune response may lead to heightened and persistent overexpression of genes and production of immune response mediators that are the main causes of immunosuppression and multiple organ dysfunction syndrome (MODS); progressive dysfunction and damage of two or more organ systems (11) that is present in a third of trauma patients (279). Multiple organ dysfunction syndrome is associated with a 6-fold increase in mortality when compared to no organ failure, and quadruples the risks of requiring assistance in the activities of daily living at 2 years post-injury compared to uncomplicated or single organ failure trauma patients (279).

After head injuries, lower extremity injuries were most associated with delayed death following blast-related trauma, representing 24% of all delayed mortality in the United Kingdom (UK) military during operations in Afghanistan (133). Approximately half died within 7 hr, and the remainder died of acute respiratory distress syndrome (ARDS) or MODS at least a week post injury (133). Early identification of the risk of organ dysfunction is important to enable secondary sorting, allocation of health resources, and appropriate escalation or transfer to higher levels of care to improve survival. Complications following polytrauma are common and may result from direct effects such as primary blast lung injury (PBLI), or secondary to the systemic immune response such as acute kidney injury (AKI), acute lung injury (ALI), or heterotopic ossification (HO). The latter condition of ectopic bone formation now occurs in up to 65% of military personnel with blast associated amputations and leads to pain, delayed healing and reduced limb function (4; 30; 31; 45; 73). Similarly, in a retrospective study

of patients where blast exposure was the predominant mechanism (71%), 25% developed rhabdomyolysis, leading to AKI in 20.7% of patients when utilizing the KDIGO classification of AKI (73), despite poor correlation with creatine kinase (CK) ($r=0.27$ $p<0.05$), implying that the development of secondary remote organ injury is multifactorial and not solely due to the toxicity of myoglobin from the breakdown of muscle.

COMBAT VASCULAR INJURIES

Trauma remains the leading cause of death both globally and in the USA between the ages of 1 and 45 (144; 246; 275). Hemorrhage is the most prevalent mechanism of death following both civilian and battlefield trauma, varying from 39% in the civilian setting to 85% on the battlefield (134; 236). Over a third of potentially survivable military casualties have hemorrhages amenable to external tourniquets, with the remainder of hemorrhages being truncal or at non-tourniquetable sites such as the neck or groin (134). Wars in Iraq and Afghanistan have been associated with a 5-fold increase in vascular injuries compared to previous conflicts (300), most likely as a result of the complexity of injuries sustained following blast exposure compared to gunshot wounds (GSW). A recent UK retrospective review of blast versus non-blast vascular injury repair outcomes ($n=115$; 80 blast vs 35 non-blast patients) concluded that despite blast injured patients being more likely to require blood products and have more regions injured, complications were not statistically increased by mechanism of injury. Thrombosis of vascular repairs appeared more prevalent in blast injured patients (22% vs 6% RR 3.94 $p=0.154$), and limb salvage rates were lower in the blast injured group, with amputation twice as likely (53 vs 76% $p=0.056$) (246), suggesting that there may be an effect which this study failed to identify due to the sample size, resulting in a Type II error. Sharrock

et al concluded that blast did not affect vascular repair thrombosis, although the 95% confidence interval of the risk ratio (RR) when calculated is 0.54-28.9 which suggests this study sample size may be insufficient to detect a true difference; the size and skew of the RR is more consistent with a likely undetected effect from blast exposure. Other work by the same authors showed that blast exposure was associated with higher primary and secondary amputation rates due to the influences of soft tissue loss, associated fractures and multiple arterial injuries on limb salvage, rather than limb revascularization (247). Other studies have identified that blast injured patients are more prone to acute coagulopathy of trauma, and present more tachycardic and shocked than a GSW injury, all of which predisposes to multi-organ dysfunction and increased mortality (250). Collectively, these retrospective clinical studies confirm a need for further evaluation of how blast exposure may negatively impact on vascular injuries and their associated pathophysiology to aid in minimizing the complications and secondary injuries of blast-associated combat wounds.

TOURNIQUETS

A tourniquet is a device primarily designed to control bleeding in the extremities, usually achieved by providing circumferential pressure around a limb to occlude the underlying blood vessels. They are used in elective surgeries to provide a relative bloodless operating field as well as in the emergency management of uncontrolled, potentially catastrophic, hemorrhage. The concept of tourniquets in battlefield medicine is not new; however the popularity of the device has waxed and waned in history before coming to the fore again in recent times during conflicts in Iraq and Afghanistan, as a result of the increased prevalence of acute vascular injuries from penetrating trauma.

Tourniquets similar to modern devices can be traced back as far as the Franco-Dutch wars of the late 15th century, and their use became more extensive during the 1800s (297). Prolonged field care (PFC), the delivery of medical care under field or austere conditions beyond the doctrinal planning guidelines, came to attention during the World Wars. Increased numbers of mass casualties as a result of advances in weaponry, combined with prolonged evacuation times from the point of wounding to field hospitals, frequently meant that tourniquets were applied for protracted amounts of time. This often exceeded 6 hours, resulting in complications such as ischemia reperfusion injury, compartment syndrome, nerve injury, limb loss and ultimately death. This led to mistrust in their overall benefit and so their use in civilian practice fell over the coming decades (23). They remained valuable to the military throughout the 20th century where shorter treatment timelines from point of wounding to surgical care prevented many of the associated complications.

While the primary indication for tourniquet application in the pre-hospital environment should be uncontrolled bleeding, their use is often wider. A review of tourniquet utilization during the Iraq conflict showed the primary indication for application was open fracture (61%), vascular injury (22%) or amputation (13%), with an overall survival of 96% during Iraq (63). Tourniquet application in the military context is only recommended for 2 hr before reassessment to prevent deleterious effects. Prospective studies of tourniquet use in Iraq showed >91% had application times of <2 hr; increased time was associated with amputation and fasciotomies (143). Similar application times are used in elective surgery, where pneumatic tourniquets are used to reduce intra-operative bleeding. In a remote environment or care-under-fire setting, when

extraction to definitive care is delayed, these timings may well be exceeded. The concept of a haemostatic ladder has been proposed for non-hostile situations, escalating from direct wound pressure and elevation through to advanced haemostatic dressings designed to promote clotting, prior to the application of a tourniquet (108). However, during care under fire in combat, the immediate escalation to tourniquet application to obtain hemorrhage control is advised, with subsequent review and downgrading once the firefight has ended (192). Prolonged application increases risk of complications from ischemia and reperfusion injury, leading to greater risk of local and systemic complications that include organ dysfunction. The need to understand any synergistic effect that mechanism of injury, such as blast, may have on safe tourniquet time is important to inform both civilian and military clinical guidelines: the success of tourniquet application following blast has led to increasing use in civilian care, for example after the 2013 Boston Marathon bombing (111). Despite evidence to suggest that blast exposure negatively affects hemorrhage resuscitation strategies and coagulopathy when compared to non-blast injuries (88; 250), the effect of blast wave exposure has not been examined with respect to tourniquet usage.

ISCHEMIA REPERFUSION INJURY

Restoration of blood-flow to an injured limb following vascular injury repair or the removal of a tourniquet is not without consequence: the influx of oxygenated blood can lead to an ischemia reperfusion injury (IRI). The mechanisms by which this occurs are not fully elicited. A diversity of mechanisms and tissue specific factors capable of causing IRI make direct comparison difficult. The cause can be iatrogenic (e.g. tourniquets), thrombotic or embolic relating to specific organs (myocardial infarction,

mesenteric infarction, stroke, transplant), or generalized following severe hypotension. All stem from the failure of arterial perfusion to distal tissues, resulting in insufficient oxygen and nutrient delivery, and consequently a hypoxic cellular environment. The duration of injury and previous health of the tissues influence the viability of cells, with skeletal muscle one of the most resistant (126). Anaerobic metabolism occurs in cells, developing an oxygen debt and causing the cellular environment to become increasingly acidic from lactate accumulation. Hydrogen ions are excreted via sodium-coupled pumps, depleting ATP and causing overload of intracellular calcium ions. This causes further muscle damage by altering cellular permeability and producing edema.

Molecular mechanisms underlying IRI involve the production of reactive oxygen species (ROS). Excessive reactive oxygen species (ROS) production can damage cell membranes, DNA or proteins and lead to cell death. Glycolysis and inflammation are endogenous sources of ROS generation, with prolonged hypoxia and hypoxia-inducible factor 1- α (HIF-1 α) stabilization promoting cell glycolysis as a source of ATP. Neutrophils and their oxidative burst mechanism also act as an alternative source of ROS during reperfusion. Mitochondrial dysfunction has been identified as a potential factor in tourniquet-related IRI, with studies demonstrating a role in excess ROS and superoxide production (273; 274). Mitochondrial dysfunction after blast injury has also shown reduced ATP production (13).

Bilateral hind-limb IRI has been shown to significantly affect survival in mice, with bilateral tourniquet times of 2.5-3 hr leading to mortality of >80% at 48 hr, compared to 0% in a 4 hr unilateral model (132). mRNA expression of interleukin-6 (IL-6) was unaffected by ischemia but upregulated after 12 hr of reperfusion, while inducible

nitric oxide synthase (iNOS) was reduced by 2 hr of reperfusion. Knockout of iNOS led to earlier upregulation of IL-6 (132). Administration of sodium nitrate treatment led to improved survival in the bilateral hindlimb IRI model, reducing inflammation as well as local and lung myeloperoxidase (MPO) activity (139). Early elevation of oxidative stress markers has been shown to precede increased *Hsp70* and *c-fos* gene expression in remote organs at 3 hr after reperfusion, highlighting that responses occur in remote organs long before histological changes occur (203).

Necrosis is a cellular process which can occur following cell injury and leads to non-programmed cell death. Cell death arises from either the loss of cell membrane integrity, dysfunction of cell homeostasis resulting in an influx of extracellular ions and water with swelling of intracellular organelles, or ATP depletion. This results in cell rupture and the release of the intracellular content into the extracellular space which generates an inflammatory response with phagocytosis occurring by neutrophils and macrophages. Disruption of cells by the process of necrosis following IRI may cause local and systemic effects through the release of DAMPs. These endogenous particles are typically proinflammatory in nature, promoting a pro-coagulopathic state and further neutrophil recruitment.

Remote organ injury following limb IRI is not uncommon, with lung and kidney more frequently affected than the liver (174; 202; 309). Investigation of mitochondrial dysfunction following lower-limb IRI and how it may affect ATP and ROS generation demonstrated impaired lung mitochondrial capacity due to complex I dysfunction, with no negative effect on liver or kidney (174). This sensitivity of lung mitochondria to remote injury may predispose them to early damage following concurrent injury.

A study also established that during reperfusion, but not ischemia, chemokines for both neutrophils and monocytes are produced. C-X-C motif chemokine ligand 2 (CXCL2), also referred to as macrophage inflammatory protein 2-alpha (MIP-2 α), and C-X-C motif chemokine ligand 1 (CXCL1), previously referred to as GRO1 or KC were elevated (110). Both serum and tissue levels of CXCL1 and tissue CXCL2 continued to rise at 24 hr which was the end of the study period (110). This is in keeping with the concept that reperfusion is associated with worsening injury through a pro-inflammatory response. Other studies have shown that deletion of neutrophils in hind-limb ischemia was associated with reduced granulocyte and MPO levels, but had no impact on muscle fiber injury or adenosine triphosphate (ATP) levels (9).

Impaired oxygenation from a concurrent lung injury could impair the resolution phase, particularly in a delayed extraction or mass casualty event where medical support may be limited. Recovery from blast lung exposure correlated with resolution of inflammation and activation of anti-oxidant enzymes up to 192 hr post injury (36). A later study showed that anti-oxidants facilitated lung recovery within 48 hr (35). No models of ischemia-reperfusion in the context of concurrent blast injury exist in the literature. Hind-limb IRI in the absence of blast has been shown to cause muscle infarction, dysfunction of mitochondrial electron transport chains, up-regulation of superoxide production and decreased manganese superoxide dismutase (MnSOD) scavenging, suggesting failure to compensate for oxidative stress (274). Cell death following IRI has been shown to be a combination of apoptosis and necrosis, with contribution from superoxides and activation of calcium channels (273). In a non-human primate model of hind limb IRI, ischemia and reperfusion phases produced different

patterns of gene expression. Upregulation of IL-6, CXCL1/3, and IL-8 were seen during ischemia and rose further during reperfusion, while IL-1 β was only significantly elevated during reperfusion. Alpha-1-antichymotrypsin, IL-1 β and TNF α induced proteins were most elevated during reperfusion compared to ischemia, while aldehyde dehydrogenase 1 family and nuclear receptor subfamily 4 were increasingly depressed by extending reperfusion time. Prolonged bilateral hind-limb IRI demonstrated activation of nonspecific proteinases both locally, systemically and in remote organs at 24 hr, with IL-1 β , IL-6 and TNF α elevated by between 10 and 40-fold (145). Neutrophils have frequently been implicated as the main protagonist of inflammation following IRI; however depletion does not protect against muscle fiber damage, despite reducing MPO and neutrophil extracellular traps (NETs) (9) which consist of matrices of extracellular DNA, chromatin, and antibacterial proteins released by neutrophils that mediate extracellular killing of microorganisms. Use of exogenous DNase-1 to reduce NETs did not prevent fibre damage or ATP reduction, and did not reduce inflammatory markers (9). These findings highlight the complex multi-cellular response to inflammation that is incompletely understood in IRI.

OXIDATIVE STRESS

Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen and nitrogen species (ROS and RNS respectively) and the antioxidants which counter them. Reactive oxygen species are typically generated secondary to the mitochondrial electron transport chain which is responsible for generation of ATP via a transmembrane gradient resulting from a series of redox reactions. The reduction of oxygen by the addition of electrons generates superoxide anions ($\cdot\text{O}_2^-$), peroxide ($\cdot\text{O}_2^{-2}$),

hydroxyl radical ($\cdot\text{OH}$) and nitric oxide. Antioxidant enzymes exist to catalyze these ROS to less harmful molecules. Superoxide dismutase (SOD) converts superoxide anions in a two-step process to water via hydrogen peroxide intermediaries. Glutathione (GSSG) is oxidized by ROS; the ratio of this as well as its reduced state (GSH) ratio can act as a marker of oxidative stress. Many markers of oxidative stress are short-lived within cells, making direct detection problematic. Other markers detect the effects of excessive ROS, such as DNA/RNA damage, lipid peroxidation and protein oxidation (85), which are often more stable than direct measures of ROS or antioxidants and represent evidence of oxidative damage.

Reactive oxygen species are associated with IRI pathophysiology, and treatment with ROS scavengers has been demonstrated to limit the extent of injury following IRI (96; 204). Reactive oxygen species are also generated by neutrophils and the oxidative burst mechanism by which they clear bacteria (41). Mitochondrial derived ROS have also be shown to have chemoattractant properties towards neutrophils (287). The use of tourniquets, with associated ischemia and reperfusion injury, has been shown to generate ROS following orthopaedic surgery (42; 101). Reactive oxygen species have an ability to promote inflammation and initial wound healing, but this can become dysregulated from an exaggerated inflammatory response and associated endothelial dysfunction (189), leading to secondary tissue injury. ROS such as hydrogen peroxide can have multiple cytosolic effects, including activation of HIF-1 α (172; 251), NF- κ B (34) and the NLRP3 inflammasome (1; 318), promoting proinflammatory cascades.

Blast wave exposure has been shown to increase ROS levels in rodent models of traumatic brain injury (TBI) (124; 223) and lead to transient mitochondrial dysfunction,

resulting in decreased ATP reduction at 6 hr which recovered by 24 hr (13). This dysfunction could lead to increased ROS presence during the initial reperfusion injury window potentiating an inflammatory response.

THE INFLAMMATORY RESPONSE TO ACUTE TRAUMATIC INJURY

Acute traumatic injury creates a complex process of physiological and immunological interactions. The level of activation from damaged tissues and pathogens after poly-trauma over stimulates the innate immune response that in milder trauma would have promoted haemostasis and healing. An imbalance between the pro- and counter-inflammatory responses that the body mounts leads to secondary injury. Moore et al proposed a 'two hit hypothesis': a traumatic injury can prime the immune system, such that a secondary insult such as surgery, infection or ventilation (254) precipitates an exaggerated response (190). Severe trauma alone can also cause a huge immunological prime that has been termed 'the one hit' hypothesis (90; 190). Secondary injury in trauma refers to the self-propagating dysregulated immunological and physiological response that can arise following injury, leading to both local and systemic damage of viable and healthy tissue. Failure of compensatory mechanisms that should counter the initial pro-inflammatory innate immune response may lead to either a persistent pro-inflammatory state, or an the development of immunosuppression. Anti-inflammatory cytokines which provide the compensatory mechanism to dampen inflammation may be produced in excess, leading to an impaired hypoinflammatory immune state, which can predispose to infection and sepsis (24; 294). With modern advances in hemorrhage control and resuscitation strategies, patients survive injury patterns which evolutionarily would not have been possible due to exsanguination at or shortly after their injury. These new

survivors are at increased risk of immune complications leading to organ dysfunction from pro-inflammatory cytokine cascades, or become susceptible to secondary infection if they develop immunosuppression.

Our immune response which in moderate injury evolved to conserve and return organ systems to haemostasis and increase survival, becomes overstimulated with a failure of negative feedback pathways. Following severe trauma, Systemic Inflammatory Response Syndrome (SIRS) can develop. It is defined by two or more abnormal physiological or biochemical variables and is independent of source of infection: temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$, heart rate >90 beats per minute, respiratory rate >20 breaths per minute, or a blood white cell count (WCC) >12 or $<4 \times 10^3/\text{ul}$ (25). SIRS can be sterile, as in the case of ischemia, burns, pancreatitis or trauma, or related to infection, where if the above criteria are met and a source of infection is known or suspected, it is termed sepsis (25).

Traumatic injury activates the innate immune system, the part of the immune system that provides our first-line defense typically against infection. Pattern recognition receptors (PRR) usually detect evidence of exogenous pathogens such as bacteria or viruses, collectively termed pathogen-associated molecular patterns (PAMPs), activating inflammatory cascades through Toll- and NOD-like receptors (TLRS and NLRs respectively), which upregulate the NF- κ B signaling pathway, leading to initiation of the inflammatory response and priming of the adaptive immune system. The SIRS response to severe trauma mirrors many of the clinical characteristics usually associated with sepsis. Matzinger proposed in 1994 that the widely held view that the immune system functioned to discriminate between self and non-self, detecting exogenous pathogens or

insults, was flawed; she put forward her own theory known as the ‘Danger model’ (180; 181). This model proposed that the immune system discriminates by recognition of pathogen or danger signals from affected cells. These endogenous ‘danger signals’, now collectively termed danger-associated molecular patterns (DAMPs), are released by cells undergoing damage or stress, activated neutrophils, or following cell necrosis; they act via PRRs in a similar manner to PAMPs to activate the inflammatory cascade.

Development of MODS has been attributed to an excessive systemic immune response induced by DAMPs (167). These endogenous signaling molecules are in part responsible for the activation of the innate immune system following trauma, either directly or via complement pathways, leading to the recruitment of neutrophils and macrophages and the ensuing inflammatory response. Numerous DAMPs have been proposed, including high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), S100 molecules, free mitochondrial DNA (mtDNA), and cold-inducible RNA-binding protein (CIRP) (86; 167; 197). HMGB1 is released by necrotic cells and can activate Kupffer cells to a pro-inflammatory phenotype (40). Release of HMGB1 occurs following non-programmed cell death and is detectable following trauma within 30 minutes, with plasma levels correlating with injury severity, adverse outcome and organ dysfunction (46). HMGB1 has a pro-inflammatory effect, mediated through TLRs, on neutrophils (268), monocytes (265), dendritic cells (62; 233), as well as on lymphoid cells (154), acting via the nuclear transcription factor (NF- κ B) pathway (148). Other trauma studies have shown that the magnitude of early HMGB1 is associated with development of sepsis and a more exaggerated cytokine response, involving both the innate and adaptive response (198). Presence of mtDNA, which can be released by cell

necrosis or bone fracture, increases SIRS, organ dysfunction and death (249). MtDNA have also been shown to induce pro-inflammatory signaling in lymphocytes (243). This response to necrotic cells is likely to be more exaggerated as mtDNA originates ancestrally from intracellular bacteria (175), which is not normally presented to the innate immune system when cells undergo apoptosis. Other mitochondrial associated DAMPs such as ATP and cytochrome C have been associated with inflammatory signaling and SIRS (97).

Genomic studies in blunt trauma have shown that the early leukocyte response leads to upregulation of systemic inflammation and a decrease in compensatory pathways, including the early adaptive immune response, with no genomic evidence to support a 'second hit' to cause dysfunction, only the duration and severity of the genomic response effecting outcome. Pathways most upregulated by trauma were integrin, IL-10, TLRs, Ephrin, IL-6 and TREM-1 signaling (306). Recent clinical data has shown that trauma patients who develop organ dysfunction following predominantly blunt trauma had early lymphocyte population changes with an increased population of natural killer (NK) CD56 dim cells and reduced T-cell sub-sets early in admission based on flow cytometry, and that failure to mount a lymphocytic response was associated with 45% mortality. IFN- γ , IL-8 and IL-1 β were also associated with organ dysfunction (173). Other studies confirm that presentation with low Th1 lymphocytes also have a negative effect on outcome (258).

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a member of the immunoglobulin (Ig) superfamily (28), expressed on innate immune cells including monocytes, neutrophils and dendritic cells, with expression being upregulated under

hypoxic conditions (27; 188). TREM-1 is emerging as a significant factor in both sepsis-driven (163; 312; 320) and sterile injury, where its blockade has been shown to reduce injury (164; 267; 312) by limiting recruitment of neutrophils, macrophages and pro-inflammatory cytokines. Signaling has been implicated via NF- κ B and Nod-like receptor protein-3 (NLRP3) inflammasomes (163), with expression of TREM-1 having been shown to correlate with ischemia related injuries such as myocardial infarction (118), acute mesenteric ischemia (150), and renal IRI (80). TREM-1 presence in distal tissue following a primary injury highlights its potential role in mediating distal organ injury (310; 311), and TREM-1 has been identified as a prognostic biomarker in sterile systemic inflammatory response syndrome (SIRS), which is an inflammatory state affecting the whole body (262). Urinary sTREM-1 has been shown to predict AKI and intensive care unit (ICU) mortality in cohorts of septic ICU patients (77; 248), and to be an independent predictor of death following myocardial infarction (47; 118). As a marker of chronic disease, TREM-1 has been shown to correlate with disease severity and functional outcome in rheumatoid arthritis patients (69), and with development of osteoarthritis (60). After lung contusion, bronchoalveolar lavage fluid (BALF)-derived sTREM-1 correlates at 16 hr with disease extent (22), making it a potential biomarker for the assessment of trauma injury. Inhibition of TREM-1 signaling in vivo has been shown to be protective against organ dysfunction in a rat hemorrhagic shock model (93), and in vitro studies in human monocytes demonstrated reduced cytokine production following DAMPs stimulation, with the blockade of TREM-1 and TLR4 completely abolishing pro-inflammatory cytokine production (70). Both TREM-1 and hypoxia-inducible factor 1-alpha (HIF-1 α) expression have been shown to be increased in examples of abnormal

healing such as hypertrophic scars and decubitus ulcers (216), suggesting that hypoxia and inflammation affect successful tissue healing. HIF-1 α has also been shown to be a key transcription factor which promotes HO formation (4), but the role of TREM-1 has not been previously studied in this disease process.

S100 proteins are a group of intracellular calcium binding proteins whose intracellular functions include calcium homeostasis, regulation of transcription factors and cell cycle. S100A8 and S100A9 (also known as MRP8 and MRP14 respectively) have been shown to have extracellular actions as proinflammatory molecules (231), and are present in myeloid cells. These proteins frequently exist as either hetero- or homodimers of S100A8 and S100A9, the heterodimer being known as Calprotectin, and they upregulate chemokines and adhesion molecules which leads to further inflammation. The significance of these markers is well established in chronic inflammatory diseases with S100A8/A9 being proposed as potential monitor of both inflammatory disease progression and therapeutic response (92; 207). More recent work has addressed S100A8/A9 complex and its receptor sRAGE (soluble receptor for advanced glycation end products) as an early biomarker of organ damage risk following polytrauma, with S100A8/A9 expression increasing over time ($p=0.001$), and increased admission levels of sRAGE associated with mortality ($p=0.015$) and renal injury ($p=0.005$) (120). Serum and urine concentrations of S100A8/A9 have been shown to be elevated following ischemia reperfusion in multiple tissue types, including colon and kidney, where they have been suggested as an alternative marker of renal tubular injury (65; 99; 270). S100A8 and A9 have both been shown to activate the Toll-like receptor 4 (TLR4), a well-established

receptor in sterile inflammation which has been shown to be upregulated under IRI conditions, and to signal through both NF- κ B and the NLRP3 inflammasome (264).

ENDOTHELIAL DYSFUNCTION

The endothelium represents the inner layer of blood vessels and consists of a monolayer of endothelial cells. These vasoactive cells are involved in physiological homeostasis, inflammation, platelet activation, cell transport and vascular permeability and angiogenesis. Due to their diverse nature and function, it has been argued that it should be treated as an organ system in its own right (8).

Damage to blood vessels following trauma disrupts the endothelial barrier, exposing the underlying matrix which activates platelets through von Willebrand factor (vWF), and promotes expression of adhesion molecules such as ICAM-1, VCAM-1 and both E- and P-selectin. The attraction of circulating neutrophils and macrophages to the site of injury is a key component of the early inflammatory response: adhesion molecules increase recruitment of cells and their extravasation into injured tissues (152; 291). Exposure to blast injury has been shown to damage the endothelial barrier, with upregulation of tissue mRNA and circulating endothelial markers seen at 6 hr post isolated blast injury to the limb in a rabbit model (259).

Endothelial dysfunction has been implicated as one of the possible mechanisms which potentiates ischemia reperfusion injury and the 'no reflow' phenomena which can occur following ischemia. Small capillaries become obstructed by platelets and circulating leukocytes, with endothelial swelling reducing the luminal diameter further (224). Ischemia reperfusion injury can compound endothelial dysfunction through oxidative stress secondary to ROS production or through localized pH changes (308).

Proinflammatory cytokine TNF- α has been shown to upregulate arginase expression in endothelial cells, causing O₂⁻ production and counteracting nitric oxide vasodilation of vessels; this causes endothelial dysfunction and predisposes to increased extravasation of inflammatory cells (87). Combined with evidence that blast wave exposure is sufficient to induce endothelial dysfunction, the combination of blast wave exposure and ischemia reperfusion could predispose to more exaggerated dysfunction and decreased end organ perfusion.

EXTREMITY INJURIES

Bilateral femoral fractures are associated with organ dysfunction (140; 147). Logistic regression from clinical data identified bilateral versus unilateral femoral fractures to be associated with higher rates of lung failure but not MODS, despite overall rates of MODS and mortality being higher; additional regression factors associated with MODS were thoracic injury and massive transfusion requirement (140), mirroring other studies (147). A recent large retrospective study assessed mortality and MODS following femoral fracture in relation to soft tissue injuries, demonstrating organ failure was greater in open femur fractures and when stratified for increasing extent of soft tissue injury. Regression analysis identified transfusion, other region injuries and age, but not fracture type or soft tissue injury as predictors of MODS (296). Several studies have shown both local and systemic cytokine changes following femur fracture (103), demonstrating that macrophages have a biphasic responses over 7 days with initial suppression of cytokine function and antigen presentation (182); the effects of these were greater in combined injury (301). Fracture-hemorrhage with a late secondary sepsis insult has also been shown to increase neutrophil adhesion after 96 hr in the lungs and increase mortality

(282), supporting the ‘secondary hit’ theory. Remote organ injury can occur, bilateral femoral fractures having been shown to cause systemic inflammation and liver injury in a TLR4 dependent manner (151). Both immunosuppression and systemic inflammation following musculoskeletal trauma are described; persistent IL-6 and IL-8 expression were associated with poor prognosis (225).

Delayed or incomplete fasciotomies of combat casualties are associated with increased mortality, suggesting that continued ischemia following blast injury is a significant factor in organ dysfunction and death (142; 228). Compartment syndrome following blast can occur due to fractures, crush injuries and ischemia, though it has also been reported in limbs with minimal evidence of trauma (276), highlighting the potential importance of the primary blast wave in damaging cellular structures. Muscle loss, which can occur in complex lower limb injury, has been shown to impair fracture healing, with prolonged myeloid and lymphocyte presence in the tissue. DAMPs release from injured soft tissue is a possible cause, acting via TLRs and NLRP3 to elevate IL-1 β , IL-6 and TNF α . The effects of ischemia on bone healing has led to mixed results, with no effect (131), delayed union (168), and enhanced BMP-2 mediated repair described (278). Hypoxia might be thought to favor chondrocyte differentiation but this was not always seen (168). Periosteal hypertrophy and hyperplasia were shown to significantly rise with ischemic duration (>3 hr), correlating with muscle necrosis (252).

BLAST RELATED LOWER LIMB TRAUMA

The understanding of the immune response following blast injury is incomplete. Variations in both model design and the techniques used in generating blast waves makes direct comparison difficult. Additionally, scaling of injuries between species and

differences in inter-species immune systems add further limitations to the generalizability of experimental findings. Much of the current literature has focused on the immune response to blast related traumatic brain injury. Research focused on the systemic inflammatory response to blast injury has predominantly evaluated the early neutrophil and monocyte response (17; 18; 68). Several studies have assessed the effects of blast induced traumatic hind-limb amputation, showing it can cause remote injury at 6 hr to the lungs (202), liver and kidney (316), with systemic rises in cytokines, oxidative stress markers and reduced ROS scavengers (199; 200; 202). Activation of neutrophils and macrophages were inferred by rises in MPO and $\text{TNF}\alpha$ respectively. The same study group went on to show that hypothermia of the limb was protective against remote organ injury (199; 201; 316), most likely through reduced mitochondrial activity, and that exogenous dinitrogen-sulphide (N_2S) donors could attenuate ALI through reducing oxidative stress, neutrophil action and increased Nrf2 signaling (200). A confounder in their work was that the explosives used had the potential to induce a primary blast lung injury (PBLI), rather than this being a remote injury effect. The study time was limited to 6 hr but suggests that in the absence of hemorrhage and secondary penetrating trauma, a traumatic limb amputation could cause distal organ dysfunction.

A study assessing the early immune response in PBLI (138 kPa/20 psi) in a rat model showed neutrophils and classically-activated (M1) monocytes-macrophages, which are monocytes-macrophages polarized to produce pro-inflammatory cytokines and nitric oxide (NO)(176), were associated with an acute inflammatory phenotype increased both systemically and in bronchoalveolar lavage fluid (BALF), but other immune cells and organs were unaffected at 6 hr (18). Isolated hind-limb blast injury from a shock-

wave tube has been shown to initiate an acute inflammatory response in the absence of histological changes, characterized by elevated neutrophils, classical monocytes and serum cytokines. A longer blast wave duration (10 ms), rather than peak overpressure correlated with inflammation (68). Blast wave duration greater than 14 ms has also been shown to change microglial gene expression (129), and low intensity 20-120 kPa blast overpressure (BOP) alters mRNA expression levels, with greater upregulation at higher peak pressures in genes associated with DNA repair or lymphocyte recruitment and activation (16). Localized blast injury activates the endothelium in an overpressure dependent manner. High blast overpressures are required to cause endothelial damage which was associated with histological change and elevated tissue markers, including HIF-1 α at 12 hr, implying tissue hypoxia or the creation of oxidative stress (259). Lack of systemic effects may be due to the focused nature of the injury, short time frame or absence of concomitant injury.

A study of temporal changes following low intensity BOP (132 kPa/19 psi) showed oxidative stress markers were consistently elevated from 2 hr until 1 month post injury (HIF-1 α , 4-HNE, Ceruloplasmin), while vascular markers were more variable (AQP1, VEGF, vWF) (6). Multiple blast exposures have also been shown to elevate hypoxic and oxidative stress factors such as HIF-1 α , 4-HNE and Ceruloplasmin (7). Isolated blast waves are associated with increased expression of HIF-1 α and iNOS in both tissue and serum, implying that hypoxia and oxidative stress are resultant effects of blast exposure on cells (161). Low intensity BOP has been shown to be sufficient to deplete antioxidants and cause structural changes in the lung (72). Higher peak BOP of 145 kPa (21 psi) are sufficient to cause an early transient rise in serum liver and skeletal

tissue markers, with evidence of liver necrosis at 24 hr but not in muscle. Blast overpressures of 103 kPa (15 psi) were sufficient to induce CK release, while higher peak BOP were required for solid organ injury markers (14), or to produce evidence of blast induced oxidative stress and upregulate both mRNA and protein expression of pro-inflammatory mediators (43).

The detrimental effects of primary blast injury on the body have been shown in a swine model of hemorrhagic shock combined with blast, where the use of hypotensive resuscitation, a well-recognized technique in poly-trauma, resulted in significantly worse survival times (88). This was despite other evidence to suggest hypotensive resuscitation alone does not significantly impair organ perfusion (240). This highlights the need to incorporate all factors associated with mechanism of injury when assessing subsequent treatments, to ensure that no unintended consequences occur. Despite the high incidence of lower extremity injuries following blast, few studies have specifically addressed the role of extremity injury and the immune inflammatory response in developing secondary injury. In the models that have been performed, none have accounted for the effects of tourniquet use which has become almost ubiquitous in the care of these patients. Given the effects of IRI that can arise from prolonged application without blast injury, there is a need to assess the effects of tourniquet application following blast injury to establish any secondary effects of their use on the immune response to trauma.

MARKERS OF SECONDARY INJURY AND REMOTE ORGAN DYSFUNCTION

Organ dysfunction can be defined as the impairment of an organ to conduct its normal physiological functions. The use of dysfunction rather than ‘failure’ in guidelines and literature has served to highlight that injury to an organ is often reversible, and that

performance and function can be modified. Multiple organ dysfunction syndrome is defined by reduced function in 2 or more organs, such that the performance of these organs need to be supported in order to prevent deterioration and death (11). MODS is the most common mechanism for delayed trauma associated mortality, present in nearly 50% of trauma patients on ICU, and associated with a 6-fold increase in risk of death (279).

Organ dysfunction can arise from the systemic immune response that occurs following trauma. Lower limb IRI has been shown to cause systemic cytokine upregulation and to induce organ dysfunction in the lungs, kidneys and liver (309). Epidemiological studies have shown that organ dysfunction usually occurs within 3 days, apart from liver failure which was usually a week post injury (235), with the lungs most commonly affected. Evidence is also emerging that there may be different patterns of organ dysfunction dependent on timing, varying between early (<7 days) and prolonged (90). Lung tissue is particularly sensitive to remote injury and this can lead to impaired gas exchange. Mitochondrial dysfunction can occur remotely in the lungs as well as in skeletal muscle after hind limb IRI (174).

Existing injury severity scores do not fully categorize injury burden, particularly with extremity trauma, and provide only crude markers of risk. More recently, tools to predict organ failure such as the Denver Emergency Department Trauma Organ Failure Score (TOF) have been developed to predict organ failure at 4 hr (286). Factors from the multi-variant regression model of over 4,000 patients identified age, early intubation, hemocrit, hypotension, blood urea nitrogen (BUN) and white blood cell count (WBC) as correlates of organ dysfunction (285). Prognostic tools exist to predict mortality such as

the Sequential Organ Failure Assessment (SOFA) tool (121). This utilizes parameters based on organ function, support and overall physiology to predict mortality. Many of these markers could be considered to act as proxies for injury severity and impaired organ function but tell us little about temporal changes that could identify patients who are failing to respond to treatment until organ dysfunction has already occurred. A systematic review of serum biomarkers after trauma identified only IL-6 and IL-10 to be consistently elevated following post-traumatic surgery. CXCL8 had conflicting evidence, while other cytokines showed no detectable change. Early surgery (<3 days) increased IL-6 and CXCL8 levels, predisposing to MODS (64).

Acute lung injury (ALI) results from acute inflammation causing loss of integrity of the endothelial and epithelial barriers of the lung, leading to increased lung permeability, edema and ultimately mortality. Biomarkers such as IL-6, CXCL8, soluble intercellular adhesion molecule-1 (sICAM-1) and Von Willebrand factor (vWF) are all increased in the condition (20; 119; 183). The recommended methods for experimental assessment of ALI include neutrophil count, histology and measuring the integrity of the alveolar capillary barrier (178; 179). Acute kidney injury (AKI), previously referred to as acute renal failure, represents an acute deterioration in kidney function resulting in waste-product build-up, metabolic acidosis and further secondary injury to the body. It is usually detected clinically by an increased level of serum creatinine, reduced glomerular filtration rate (GFR), or by a sudden persistent reduction in urine production, oliguria or anuria. Multiple criteria have been proposed for AKI and have been amalgamated into the KDIGO criteria, where a greater than 1.5-fold increase in baseline or predicted serum creatinine (100) or 25% GFR decrease represents an AKI risk. Creatinine level changes

may lag timewise following injury. An alternative measurement is the BUN (blood urea nitrogen test), which can also be expressed as a ratio with creatinine. In trauma, the most common cause is pre-renal failure, where renal perfusion is decreased. The inflammatory response driven by neutrophils and M1-phenotype macrophages can worsen renal injury (303). Both serum creatinine and BUN assess the function of the kidneys by their ability to excrete bioproducts, but both are slow to change following injury and have poor sensitivity. Creatinine rises may not manifest until 24-48 hr post injury, making it a poor candidate for the early prediction of organ injury or to stratify extent of renal injury, and can be affected by other factors such as muscle mass, metabolism and fluid status. In healthy individuals, kidneys have considerable reserve capacity and therefore absent or minimal increase in serum creatinine may mask a significant structural renal injury. To better address this the 10th Acute Dialysis Quality Initiative (ADQI) Consensus statement recommended the use of markers of both functional and structural injury to more fully characterize AKI and optimize patient care (194). The combination of functional and structural markers enables a continuum of injury to be assessed from no functional or structural impact of an insult, through to damage to the kidney but with maintained function, or loss of function but maintained structure, through to worse AKI encompassing both structural and functional loss. Biomarkers have been proposed using either serum or urine to detect and monitor renal injury. An alternative marker of glomerular filtration function to creatinine is Cystatin C (78; 260), while markers of renal tubular damage include kidney injury molecule-1 (KIM-1) (102; 232), a type-1 transmembrane protein, β_2 microglobulin (β_2 M) (12; 237), a 12kDa light chain of the major histocompatibility class (MHC) I molecule, and neutrophil gelatinase-associated

lipocalin (NGAL) (57; 171), an iron-transport protein. Urinary calprotectin, sTREM1 and IL-18, which are recognized DAMPs associated with inflammation and myeloid cell activity, have been proposed as markers of renal inflammation and repair (209).

Acute liver failure represents the global dysfunction of the liver, usually secondary to toxins or drug overdoses, but it can occur following trauma when it arises from ischemia secondary to hypoperfusion following prolonged hypovolemic shock. If persistent this leads to ATP depletion and necrosis of hepatocytes. In a heightened immune state, tissue metabolic demand may be increased, compounding injury. Functional effects include coagulopathy (INR >1.5), predisposition to infection, loss of fluid homeostasis and AKI development (19). Activated Kupffer cells, which are a specialized form of tissue resident macrophages in the liver in the walls of the sinusoids, increase cytokine production and formation of ROS, furthering local liver tissue injury (59; 162). Generalized liver dysfunction can be detected by elevated hepatic enzymes in the blood. Alkaline phosphatase (ALP) is present in both bone and the liver, with elevated levels associated with liver injury or an obstruction of the hepatic ducts. Alanine aminotransferase (ALT) metabolizes protein, but the damage of hepatocytes can lead to increased serum levels. Aspartate transaminase (AST) metabolizes alanine, and serum levels can rise either as a result of liver injury or of cardiac and skeletal muscle damage. Lactate dehydrogenase (LDH) can also be raised following liver injury, but as its presence is almost ubiquitous throughout the body, any elevation in levels is less specific than other liver function markers. Muscle damage markers include creatine kinase (CK), an enzyme that catalyzes the formation of creatine in an ATP dependent manner, which is released following muscle fiber damage, as well as LDH and AST (29).

LOCAL TISSUE DYSFUNCTION – HETEROTOPIC OSSIFICATION

Heterotopic ossification (HO) is a common complication of both civilian and combat blast-related amputation (30; 67), characterized by the development of abnormal ectopic bone in connective tissues within muscle, tendons and joints. There are both genetic and acquired forms of the condition, the latter of which is typically associated with traumatic injury. Fibrodysplasia ossificans progressiva (FOP) is a progressive autosomal dominant genetic condition where muscle and connective tissue including tendons and ligaments are gradually replaced by bone following mild trauma or inflammation. The mutation is typically a R206H hyperactivity mutation in the activin A type I receptor (*ACVR1*). *ACVR1* is responsible for the activin-like kinase 2 protein (ALK2) which forms a receptor for bone morphogenetic protein (BMP) signaling. In the mutated state the receptor is hyperactive to its ligand BMP-2 and Activin A. This leads to upregulation of phosphorylation of SMAD1/5 proteins, promoting chondrogenesis and osteogenesis of tissue-derived multipotent mesenchymal osteo-progenitor populations.

Traumatic HO formation has been shown to correlate with traumatic brain injury, spinal cord injury, soft-tissue/long-bone disruption, joint arthroplasty and burn injury. Combat associated HO frequently occurs following blast related traumatic amputation; the heterogeneous nature of combat polytrauma means many of these HO associated injury patterns may occur together. A systematic review identified age, blast injury, high ISS, and through zone of injury (ZOI) amputation as predisposing factors (66; 67). It also highlighted that the pathogenesis and contribution of individual variables in the formation of HO has yet to be elucidated (66). While blast alone does not appear to cause HO, civilian data suggests that enclosed blast, which can lead to higher peak pressures and

energy transfer, results in greater HO formation despite less contamination from an outside environment (67).

Endochondral ossification has been shown to be the main mechanism by which HO formation occurs after trauma (3; 4; 51; 75; 212; 213; 217). It requires hyaline cartilage as a precursor, which typically comes from tissue-derived multipotent mesenchymal osteo-progenitor populations. These cells proliferate to form mesenchymal condensations, and undergo differentiation into chondrocytes under the control of Sox9. Following differentiation and activation, other genes promote proliferation and hypertrophy (*Runx2*, *Alp*), resulting in mature chondrocytes. In trauma models, *Hif-1 α* and *Runx2* are highly associated with ectopic bone formation (75; 221). Proliferation of chondrocytes at the ZOI amputation site occurs as early as Day 5 post traumatic injury, increasing by Day 10. Overexpression of osteo- and chondrogenic genes and proteins (*Runx-2*, *OCN*, *NOG*, *SP-1*) when compared to contralateral limb samples, suggests that HO pathogenesis involves endochondral ossification (221). Abnormal dystrophic calcification in major organs has also been shown to occur following ischemia reperfusion injury. Liver transplants can develop dystrophic calcification deposits and this has been linked to myofibroblasts in the affected areas, exhibiting increased TGF β -1 and BMP2 expression, along with genes such as *Runx2* and *MSX2* (125); the process of dystrophic calcification has also been suggested to be part of a pathologic continuum with HO (191). Cellular apoptosis and necrosis could have acted as a trigger under these conditions to induce early upregulation of osteogenic gene transcription, but this was not assessed in either study.

Hypoxia-inducible factor 1 is a master transcription factor which controls multiple genes and forms part of the cellular response to hypoxic states; its actions include promoting angiogenesis via VEGF (54), shifting cell metabolism to anaerobic state(211), Sox9 mediated chondrogenesis (10; 313), and enabling the innate immune response including invasion and aggregation (48). Inhibition of HIF-1 α has been shown to significantly reduce ectopic bone formation in both trauma and genetically induced HO, suggesting that HIF-1 α is a common target between the two forms (4). HIF-1 α is required for chondrocyte survival and differentiation, and cellular hypoxia has been shown to increase HO formation by upregulating BMP signaling through persistence of the *ACVR1* receptor leading to ectopic bone formation in FOP models (292). Tourniquets induce a global hypoxic environment in the limb during the ischemia phase, and prolonged application may induce a reperfusion injury that further aggravates local tissue injury. Both mechanisms would have a deleterious effect on a HIF-1 α dependent pathway and potentially stabilize BMP signaling and increase HO formation. Single cell level studies of overall RNA production under hypoxic conditions have shown that synthesis is upregulated within 90 minutes and that the magnitude increases with duration of exposure (21), a time period well within current tourniquet application guidelines.

The effects of ischemia reperfusion following tourniquet application on HO formation has not been previously examined. Immobilization-manipulation studies in a rabbit model, mimicking IRI and neurogenic HO, demonstrated that free radical scavengers significantly reduced HO formation. This suggests that ROS formation may play a significant role in the pathogenesis of HO (283). Hypoxanthine increases with ATP hydrolysis and superoxide ions are produced during the ischemic phase. Re-

introduction of oxygen during reperfusion acts as an electron acceptor generating O_2^- and H_2O_2 species. The metabolic rate of tissues, which would be expected to be higher in younger populations, could also affect the rate of these changes and may explain why young age is a risk factor for HO. Elective orthopaedic surgery has shown rates of HO ranging from 15-39% following joint surgery, but less than 1% were significantly impaired (49; 272). These operations had average tourniquet times of less than an hour and were performed in older patients whose skeletal muscle would be less metabolically active.

The ischemic and hypoxic effects of a tourniquet can be rationalized to support increased HO formation. Hypoxia is a driver for angiogenesis, chondrogenesis and osteogenesis; stabilization of HIF-1 α induces VEGF and other factors that promote this (239). Connective tissue progenitor cells from injured service personnel who developed HO demonstrated increased COL10A1, COL4A3, COMP, FGFR2, FLT1, IGF2, ITGAM, MMP9, PHEX, SCARB1, SOX9, and VEGFA, but no increase in BMP2 or BMP4 expression (51). Hypoxia stabilizes HIF, and HIF-1 α has been shown to be elevated in both traumatic and genetic HO (4). Similarly, ROS and nitrates can also stabilize HIF-1 α (123; 219; 222). This link between hypoxic stress, HIF, VEGF and osteogenesis may represent a key factor in local tissue secondary injury. Hypoxia also affects human MSC differentiation, promoting osteogenesis (288).

Heightened and prolonged pro-inflammatory signaling has been identified as one of the mechanisms that promote osteo-chondrogenesis (74); however the early inflammatory changes are not fully characterized. Assessment of systemic and local cytokines has shown that blast injured patients have a prolonged systemic inflammatory

phase, with IL-3, IL-12(p70) and IL-13 identified as cytokines associated with HO (81). IL-13 is considered anti-inflammatory and promotes monocyte survival, while IL-3 is involved in differentiation of hematopoietic stem cells into myeloid cell lines. Cytokine studies have also shown that serum IL-6, IL-10 and CCL2 and wound effluent CXCL10 and CCL3 are associated with combat related HO formation (74), highlighting that systemic inflammation is a driver in addition to local inflammation. TREM-1 has also been shown to act as a negative regulator of osteoclast differentiation (149). In FOP, it is small inflammatory flares, from either mild trauma or infection in soft tissues, that can lead to ossification. Development of laboratory models to examine the molecular and cellular mechanisms of HO have shown that the addition of a systemic blast overpressure (120 kPa/17 psi) to a rodent model of soft-tissue crush injury, fracture and through zone of injury amputation significantly increased the incidence of ectopic bone formation, highlighting the importance of blast waves in changing the tissue microenvironment to predispose to ectopic bone formation (217). The mechanism by which this occurs is not fully understood. Concurrent wound infection with MRSA in the same model was also shown to further increase rate and volume of ectopic bone formation, demonstrating the importance of bioburden on impaired healing, likely through both local and systemic inflammation (212).

Heterotopic bone marrow has been shown to contain neutrophils, monocytes and lymphocytes in addition to mesenchymal stromal cells (MSCs) and endothelial cells. Neurogenic HO has been shown to contain macrophages, whose ablation reduced HO formation by 90% (89), supporting the role of inflammation and particularly phagocytic macrophages in the condition. The role of M1 inflammatory macrophages which respond

to Th1 lymphocyte derived IFN- γ has been implicated, and this phenotype is likely to be maintained by prolonged systemic inflammation, resulting in a failure to progress to normal healing. Macrophages are known to be vital to both inflammation and the switch to healing. Macrophages are involved in bone formation and produce osteogenic stimuli such as BMP-2, and can form osteoclasts in response to the osteoprotegerin ligand (OPGL) called RANKL (33). Osteoclastogenesis is upregulated by pro-inflammatory cytokines acting on macrophages. The presence of macrophages early in bone formation and their persistence through all stages of wound remodeling highlights their importance, and how the milieu of inflammatory mediators present may affect the timely phenotypical switching that leads to wound healing.

While bone morphogenetic protein (BMP) signaling has been shown to be a key pathway in the formation of HO, other pathways have also been implicated. Hedgehog, Wnt/ β -catenin and fibroblast growth factor (FGF) signaling have all been shown to contribute, in addition to inflammatory upregulation through NF- κ B, on HO formation. Hedgehog signaling, both Indian and Sonic variants, influence chondrocyte differentiation and osteoblast formation respectively (128; 261; 307). Wnt signaling is responsible for chondrocyte maturation (44) and can also upregulate BMP2 expression (196; 314), leading to further osteogenic signaling which drives bone marrow derived MSCs towards osteogenic phenotypes. FGF released from mast cells under hypoxic conditions has been shown to increase fibroblast proliferation, matrix deposition and ultimately lead to abnormal bone formation, with the transition from normal healing to abnormal being associated with increased Bcl-2 signaling (83).

The presence of oxidative stress as a result of localized inflammation increases fibroplastic change to fibrocartilage, supporting endochondral ossification and increased HO formation. Assessment of clinical tissues has also demonstrated increased evidence of ROS, through presence of MPO and other markers of ROS damage, as well as increased expression of oxidative stress genes (84). Consistent with the premise that inflammation and persistent tissue hypoxia and oxidative stress contributes to the maladaptive microenvironment predisposing to abnormal bone formation, inhibition of NF- κ B signaling through pharmacologic inhibition reduces HO formation (122). The immune response following injury has been shown to be a significant driver in combat associated HO, with both local and systemic cytokine patterns being elicited. Given the duration of endochondral ossification to occur, both the innate and adaptive immune system have been implicated in HO formation (141).

Heterotopic ossification represents a debilitating secondary injury following lower limb extremity trauma and blast. Formation of ectopic bone appears to be associated with hypoxic signaling pathways and persistence of both local and systemic inflammation. Tourniquets are a common adjunct in the early management of severe limb injury, but to date mechanisms by which their application could contribute to HO formation, through either prolonged ischemia or the consequent reperfusion injury, has not been assessed. Assessment of any contribution made by tourniquet application may help further the understanding of the early pathophysiology of HO or enable more accurate models of combat related HO trauma to be constructed for testing prophylactic strategies.

CLINICAL RELEVANCE AT A TIME OF CHANGING OPERATIONAL DEMANDS

Prolonged field care (PFC) is the delivery of field medical care beyond current doctrinal planning guidelines and has been identified during a US Army capabilities analysis as a significant gap in the US Army's operating capabilities; strategies to mitigate these risks are therefore critical to the advancement of military medicine. Conflict in Afghanistan and Iraq has led to the longest periods of sustained operations for both UK and US military personnel in recent history. Logistical support to these established operations has enabled rapid evacuation from point of wounding to military treatment facilities (MTFs), mainly due to airspace dominance and freedom of maneuver for air and ground assets to extract casualties over a relatively small geographical area. Looking forward, it is likely that future contingency operations will fall outside current doctrinal timelines for medical care, either due to geography, limited logistical support or enemy interference. United States PFC cases during 41 recent missions showed durations between 4-120 hr of care with active bleeding (43%), shock (29%), burns (17%) and fracture (11%) the most common injury patterns managed(55). Despite a prolonged field care (PFC) provider being present at time of injury or illness in 40% of cases, the remote nature (96%) and weather or enemy activity (37%) may have contributed to the 9.3% of casualties dying prior to transfer to a higher level of care (55).

A key focus to PFC is reducing morbidity as well as mortality. Since PFC extends from hours through to days, there is a need to address the prevention, identification and treatment of conditions typically encountered during the advanced stages of medical care. Uncontrolled catastrophic hemorrhage remains the leading cause of preventable death following trauma, and tourniquets are a highly effective adjunct at managing extremity bleeding. If tourniquet use is to continue in order to prevent

avoidable combat deaths, there is a need to understand how combat tourniquet use and the development of IRI may occur under these more challenging timelines in austere environments. To achieve this, we need to address questions regarding the use of tourniquets in an era of blast associated combat injuries.

RESEARCH OBJECTIVES AND STATEMENT OF HYPOTHESIS

The objective of the proposed research was to determine the effects of tourniquet induced hind-limb ischemia reperfusion injury on the development of organ dysfunction and heterotopic ossification in a blast-related model of combat related lower limb extremity injury and amputation. Despite evidence to support blast exposure upregulating pathways associated with the development of IRI such as oxidative stress, endothelial dysfunction and inflammation, no work has previously addressed whether blast exposure could exaggerate or hasten the onset of tourniquet induced IRI, or whether tourniquets may have a synergistic effect on blast related lower extremity injury and amputation by compounding existing cellular damage.

These two questions can be related to two distinct injury patterns borne out in the literature and trauma registries from recent conflicts. The former is applicable to an isolation vascular injury, treated initially with a tourniquet to control hemorrhage, sustained from close proximity to a blast device but not at point of detonation. There is conflicting published literature as to whether blast exposure increases the risk of vascular injury complications; development of a model to address whether blast injury potentiates the onset of an IRI would help to support this question and provide guidance to clinical planning. The second is the more common injury pattern of recent conflict, that of traumatic amputation. Traumatic amputation leads to extensive soft tissue, bone and

vascular disruption, with tourniquet application in 80% of cases. The additional insult of reperfusion injury could result in semi-viable tissue undergoing delayed necrosis or apoptosis, contributing to any systemic inflammatory response.

The overarching hypothesis of this proposal is that tourniquet-induced ischemia-reperfusion injury plays a critical role in exacerbating the early inflammatory response following blast-related lower extremity injury/amputation, by compounding any pre-existing cellular damage or ROS-mediated injury.

Tourniquet induced ischemia reperfusion injury is of paramount importance in escalating multiple immune activation cascade pathways which drive local and systemic inflammation, resulting in secondary end-organ injury and increased heterotopic ossification.

The objectives of this research and the hypothesis are addressed by considering the two distinct clinical patterns of blast related injury, 1) blast-associated complex limb injury with traumatic amputation (primary blast injury), versus 2) a blast associated isolated vascular injury from debris (secondary blast injury), in the context of two specific aims:

Specific Aim 1: Determine the effect of blast wave exposure on the duration of time required to induce an ischemia reperfusion injury following application of a temporary hind-limb tourniquet, and the magnitude of any inflammatory response.

Exposure to a blast overpressure wave is known to induce oxidative stress in tissues, which may lead to mitochondrial dysfunction, endothelial damage and impaired function. Tourniquets are used to control hemorrhage at the expense of inducing global ischemia and oxidative stress. If application time is prolonged, a reperfusion injury or

permanent tissue damage may occur; when this arises is tissue dependent and considered to occur after 2 hr in skeletal muscle. Pre-existing oxidative stress and tissue damage following blast injury may therefore predispose to an earlier or exaggerated reperfusion injury, exposing the casualty to an increased risk of secondary injury. No studies to our knowledge have previously examined the effects of tourniquet application in either blast injury alone or in a model of extremity poly-trauma. Understanding how these two mechanisms of injury may affect both the safe application time and the inflammatory response of this commonly used life-saving medical device could help inform clinical guidelines as well as highlight biomarkers and therapeutic targets for future investigation.

Specific Aim 2: Determine if hind-limb ischemia reperfusion injury from tourniquet application increases heterotopic bone formation in a model of blast-related hind-limb trauma.

The use of tourniquets following blast-related lower limb injury in recent conflicts to counteract catastrophic hemorrhage has become widespread. During this time, ectopic bone formation in injured limbs has become more prevalent. Heterotopic ossification has been identified in 60-80% of traumatic amputations in both civilian and military patients in recent decades (67; 218). Whilst a life-saving intervention, the possible effects that tourniquets may have in potentiating the formation of HO in blast associated lower-limb injury has not been assessed in vivo. A consequence of tourniquet application is ischemia to the limb, and previous animal studies have identified HIF-1 α as a key transcription factor in both genetic and trauma-induced HO formation (4; 292). Determining if HO formation is increased with tourniquet associated ischemia reperfusion injury would identify this as a previously unaccounted for variable in HO

models, and understanding the mechanisms by which this occurs could help create prophylactic strategies.

CHAPTER 2: Materials and Methodology

EXPERIMENTAL MODELS AND PROCEDURES

IACUC institutional approval.

All experiments and animal care procedures for this research were approved by the Naval Medical Research Center (NMRC) and Walter Reed Army Institute of Research (WRAIR) Institutional Animal and Care and Use Committee (IACUC) under the protocol numbers 15-OUMD-19S, 17-OUMD-31S and 18-OUMD-10S. All activities were conducted in accordance with all applicable regulations and best practices pertaining to the use of animals in research.

Animals and husbandry.

Adult male (11-12-week-old (350-450 gm) specific pathogen free Sprague Dawley rats (*Rattus norvegicus*) were obtained from Taconic Biosciences (Germantown, NY). Animals were housed for a minimum of 7 days for acclimatization and quarantine purposes in the vivarium located at the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD. All rats were housed in clean clear plastic cages and exposed to a 12 hr light/dark cycle, with free access to food (standard rodent chow) and water, under veterinary care and supervision. Animal weights were assessed prior to surgery and whilst on protocol.

Systemic blast overpressure exposure.

Induction of general anesthesia was achieved by placing the rat in an induction chamber connected to a 5% isoflurane source for 3 minutes. Analgesia was administered

(0.05 mg/kg Buprenex SC) to minimize any discomfort prior to the rat being secured in a tapered plastic film DecapiCone tube (Braintree Scientific Inc, Braintree, MA) which prevents secondary injury to the limb or tail from blast wave exposure. The rat is then secured using three straps into a holding device fixed at the end of the shock tube apparatus. The shock tube used for all of these experiments is located at WRAIR/NMRC and is pneumatically driven using compressed air. The tube's overall dimensions are 5.94 m long with an internal diameter of 30.5 cm. The tube consists of two parts, a 0.76 m compression section and a 5.18 m expansion section (5) (Fig. 1). These chambers are separated by a Mylar membrane held in place by hydraulic rams to maintain an airtight seal, which ruptures when the compressed air pressure in the compression section exceeds the membrane limit. The thickness of the chosen membrane dictates the characteristics and peak overpressures of the blast wave created at the end of the expansion section. Previous calibrations of this blast tube have shown that the thickness of Mylar membrane used in these experiments generates a typical peak overpressure of 120 ± 7 kPa at the point where the animal and the blast wave interact, and that the waveform generated by this blast tube is known to exhibit Friedlander wave characteristics (5; 36; 37; 71; 165).



Figure 1. The WRAIR/NMRC shock tube.

(a) the WRAIR/NMRC shock tube, consisting of, (b) compression section, (c) membrane holder and hydraulic rams, (d) expansion section, (e) rat restraint apparatus, (f) membrane in situ pre-blast, (g, h) membrane ruptured post blast, (i) membrane with linear split representing successful pressurization and blast wave initiation.

Analgesia and anesthesia.

All subjects received anesthesia and/or analgesia prior to any procedures (blast exposure, tourniquet application, surgical procedures) to reduce any discomfort experienced and to minimize confounders from stress. For blast overpressure (BOP) exposure, rats were anesthetized with isoflurane (2-5%) inhalation by using an induction chamber box as outlined above and received prophylactic analgesia (Buprenex, rat dose: 0.05 mg/kg IP). Following BOP exposure, rats were recovered from anesthesia prior to transfer to the surgical suite. For the induction of anesthesia for the either the sole

application of tourniquet, or any group requiring creation of a complex lower limb injury, anesthesia was induced by intraperitoneal injection (ketamine 80 mg/kg and xylazine 10 mg/kg). Additional boluses of ketamine and/or xylazine (10-20/1-3 mg/kg) were administered to maintain a plane of general anesthesia. All animals undergoing prolonged anesthesia as part of their surgical procedure were placed on a warming blanket (Hot Dog, Eden Prairie, MN), to aid with thermoregulation with a target temperature of 37°C and received a 5 ml subcutaneous (SC) injection of sterile normal saline (0.9% sodium chloride solution) into the loose skin over the neck and shoulders to help reduce dehydration and hypovolemia due to prolonged anesthesia. To provide prolonged analgesic coverage, buprenorphine SR (1.2 mg/kg SC) was administered to the same location in applicable groups at the end of the surgical procedure and repeated at 48-72 hr, with further doses given as indicated based on animal assessment.

Hind limb complex extremity injury model.

The complex lower extremity model employed has been described previously with the exception of the tourniquet phase (212; 217; 221; 244). In summary the steps to create the model include (i) creation of a comminuted femur fracture, (ii) creation of a soft tissue crush injury, (iii) application of tourniquet if applicable, (iv) completion of the trans-femoral amputation through the zone of injury and wound closure.

A custom lateral long bone ballistic system based on previous designs (26; 153) utilizing a drop weight apparatus was used to create a comminuted fracture. A 581gm weight dropped from a height of 88 cm drives the guillotine ramming system, creating the femoral diaphysis fracture. A crush injury was then created immediately post fracture by rotating the limb between two support anvils to a force of 138 kPa (20 psi), calibrated

against a digital force gauge (DFE II, AMETEK, FL), for a duration of 1 minute.

Dependent on group allocation, subjects were either then transferred to the thermoregulated heating blanket and had their tourniquet applied or proceeded directly to amputation.

Trans-femoral amputation was performed through the zone of injury using a separate sterile field and dissection kit for each animal. The amputation was performed by a lateral incision exposing the distal femur and tibia. Hemostasis was achieved by cautery or ligation of the femoral vessels proximal to the injury site. The sciatic nerve was cut under tension, resulting in retraction away from the amputation site. The amputation was completed through the zone of injury, with removal of any bone fragments and irrigation of the wound. Any sharp, protruding spurs of the femur were removed to prevent discomfort on weight bearing. A myoplasty was created covering the fracture site using the biceps femoris, vastus lateralis and gastrocnemius. Closure was in a layered fashion; muscle layers were closed using interrupted 3-0 sutures (Viscorb, CP Medical, GA), subcutaneous layer with a continuous 4-0 suture (Monoswift, CP Medical, GA), with interrupted 2-0 nylon mattress sutures (Monomid, CP Medical, GA) providing additional protection from early wound opening by self-interference. Topical tissue adhesive (Gluture, Zoetis, MI) was then applied to the wound to provide a waterproof, clear dressing that reduced infection risk while enabling the wound to be observed.

Hind-limb tourniquet application.

Following induction of general anesthesia, hind limb ischemia was achieved by the application of a pneumatic tourniquet (UDC1.6™, Hokanson, WA) to the proximal aspect of the limb, as previously described (136; 290; 302). Elevation of the limb prior to

tourniquet application was performed to reduce venous pooling, mimic exsanguination associated with limb trauma and reduce likelihood of thrombotic complications following tourniquet release. Tourniquets were inflated and controlled using a segmental cuff selector enabling rapid and independent inflation and deflation (MV10, Hokanson, WA), connected to a aneroid sphygmomanometer (DS400, Hokanson, WA), with inflation pressures maintained at 280-300 mmHg, which exceeded the 220 mmHg which has been reported to stop microvascular flow in a rat limb (290; 302). Tourniquet duration based on group assignment was from 90-150 minutes, to mirror clinical timelines from point of injury to surgical intervention.

Post-operative monitoring and blood sampling.

Animals were observed twice daily for evidence of pain or distress, with additional buprenorphine SR administered as required. Wound inspections were performed at least twice daily; any animal with an open wound was anesthetized using isoflurane (2-5%), and the wound inspected. The wound was irrigated, any debris or necrotic tissue debrided, and the wound closed in the manner previously described, with additional short and long acting analgesics administered. Animals were limited to a maximum of two openings before being euthanized.

Blood was obtained at necropsy by cardiac puncture performed at either 6 hr, 24 hr, Day 3, Day 7 or Day 10, while blood draws obtained via a tail bleed were performed at either 3 hr, 9 hr or Day 3 post-surgery dependent on group allocation. Blood collected was standardized at a limit of 10% of total body weight. Phlebotomy was performed using a butterfly needle. The rats were placed in a restraining device, their tail cleaned and warmed prior to lidocaine hydrochloride topical preparation being applied to

minimize discomfort from the procedure. Blood from tail vein bleeds was collected in either serum 1.3 ml micro separator tubes (SST) or plasma-EDTA tubes (Sarstedt Ag & Co, Numbrecht, Germany), while necropsy samples used larger 9 ml SST and 3 ml EDTA tubes (Vacuette, Greiner Bio-One, Monroe, USA). Blood for CBC and serum chemistries was run the same day and the remaining serum stored at -80°C prior to use.

In vivo micro computed tomography (μ CT).

In vivo μ CT imaging was recorded using a SkyScan 1176 in vivo high-resolution μ CT (Bruker, Kontich, Belgium). This μ CT administers ultra-low dose radiation (<10 mGy per scan at 35 μ m pixel size), enabling serial imaging during observational studies without causing radiation-induced effects. Rats in the observational study arms were imaged while under anesthesia from 2-5% isoflurane. Imaging was conducted at post-operative days 14, 28, 56 and 84. Scans were conducted with the same settings as previous studies; 89-kV polychromatic x-ray beam, 256 μ A current and an exposure time of 81 milliseconds per 180° rotation (212; 221). Images were processed using NRecon Reconstruction software (Bruker, Kontich, Belgium) to align scan images and generate reconstructed images and cross-sectional images.

Two investigators reviewed the serial slices using CTAn software (Bruker, Kontich, Belgium); the region of interest was set as the injured femur and surrounding soft tissue. On every 3-5 slices, regions of interest were drawn encompassing all new bone formation, including bone in continuity with the cortex, and a second review to capture ectopic bone, defined as bone not in continuity with the cortex. Analysis of 2D slices and 3D reconstructions at earlier timepoints enabled the identification of displaced

bone fragments from the original fracture which were excluded from the measurements of either total or ectopic bone.

Euthanasia and tissue collection and storage

Rats were euthanized in accordance with the 2013 American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. This was performed by the IP injection of an FDA approved euthanasia solution (Beuthanasia-D Special, Merck Animal Health, Madison, NJ) in accordance with the manufacturer's instructions based on the weight of the rat. Deep anesthesia was confirmed by assessing the pedal reflex prior to a midline laparotomy being performed, exposing the diaphragm, which was incised to visualize the heart. A terminal bleed was then performed using a 14-gauge needle and 10 ml syringe, with blood collected into both EDTA and serum separator tubes. Following the cardiac blood draw, a bronchial alveolar lavage was performed of the left lung, prior to the organs and muscle tissue being collected and processed as outlined below.

Bronchoalveolar lavage (BAL)

This was performed immediately following the cardiac blood draw. The sternohyoid muscle, sternocleidomastoid muscle and thyroid tissues were divided to expose the trachea. The incision was extended caudally to the level of the tracheal bifurcation. Two fine right-angled forceps were used to occlude the right branch of the trachea. A 14-gauge venous catheter was then inserted into the main trachea and advanced towards the bifurcation. 5 ml of chilled 1x PBS solution (Invitrogen, Rockville, MD) was then used to inflate the left lung. The fluid was lavaged in and out of the lung a total of two times per 5 ml wash and transferred to a 15 ml conical tube, which was kept on ice (257). This process was repeated twice, so a total of 10 ml of PBS was

infused into each rat lung. Samples were placed on ice and processed immediately following necropsies, with the total volume of BAL wash recovered from the lung recorded. The BAL fluid was centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was collected and stored at -80°C before processing for protein concentration. The BAL cell pellet was resuspended in 0.5 ml of chilled PBS.

Tissue handling and storage, including histopathology

At necropsy, tissue was prepared and stored for histology, protein and gene expression analysis. Tissues collected were from the lung, liver, kidney and injured muscle. For histology, samples were stored in 10% buffered formalin prior to paraffin embedding and sectioning. For histology, the middle lobe of the right lung was inflated with 1 ml of buffered formalin to maintain airway architecture. The median lobe was prepared for histology, while the whole left kidney was removed for renal histology. Muscle was dissected as a 1-2 cm block of tissue from the vastus lateralis which would have been immediate distal and underlying the tourniquet site and proximal to the amputation site. The site of muscle removal was consistent between amputated and non-amputated animals. Formalin fixed samples were processed at either USUHS, WRAIR/NMRC or Histoserv Inc. (Germantown, MD). Formalin fixed tissues were embedded in paraffin and 5 µm thick sections were cut. These sections were then prepared and stained with hematoxylin and eosin (H&E). A board-certified veterinary pathologist, blinded to group and injury pattern evaluated the slides using a light microscope (BX-41, Olympus America Inc., Center Valley, PA) and scored the slides based on evidence of tissue injury on multiple criteria for each tissue type (Table 1). Findings were graded as follows: normal (0), minimal (1), mild (2), moderate (3), marked

(4), or severe (5). The only exception to this was for the radiating portal hepatocytes, which were rated as normal (0), mild (1), moderate (2), moderate to severe with mild cytoplasmic inclusions (3), moderate to severe with moderate cytoplasmic inclusions (4), or moderate to severe with marked cytoplasmic inclusions (5).

Protein samples were collected, placed in cryotubes and immediately flash frozen in liquid nitrogen, prior to long-term storage at -80°C. The post caval lobe of the right lung, left lateral lobe of the liver, right kidney and vastus lateralis and biceps femoris immediately adjacent to the femur and/or zone of injury was collected.

Samples to be used for RNA extraction were prepared using RNase AWAY™ (Thermo Scientific 7002, Waltham, MA) treated surfaces and instruments. Dissected tissues were prepared (tissue samples ≤0.5 cm in any direction) and stored in 1ml of RNAlater™ stabilization solution (Invitrogen, Waltham, MA), initially at 4°C for 72 hr to allow full penetration of the solution through the tissues, before removing the tissue from the RNAlater™ solution and transferring to -80°C storage prior to RNA extraction. Samples for RNA extraction came from the adjacent tissues used for protein with the exception of lung tissue which was taken from the right upper lobe.

Table 1. Histological scoring criteria for different tissue types

Lung	Liver	Kidney	Muscle
Hemorrhage	Hepatocyte apoptosis	Tubular necrosis/ apoptosis, cortex	Cellular infiltrate, mononuclear
Edema, intra- alveolar	Hepatocyte necrosis	Tubular necrosis/ apoptosis, medulla	Cellular infiltrate, mixed
Cellular infiltrates	Portal hepatocyte radiating	Tubular proteinosis	Edema
Alveolar histiocytosis	Hepatic cord dissociation	Cellular infiltrate	Hemorrhage

Thickened alveolar septa	Increased hepatocyte mitosis	Tubular basophilia	Fibroplasia
Crystals	Increased multinucleate hepatocyte		Perivascular inflammation
	Lipid change		Myocyte degeneration/necrosis
	Extramedullary hematopoiesis (EMH)		

Experimental Groups

This research incorporates three distinct study groups, each with multiple groups. The effects of blast exposure on whole limb tourniquet induced ischemia reperfusion injury were assessed for up to 24 hr of reperfusion. Tourniquet durations of hind limb ischemia (HLI) or 90 and 150 minutes were examined. Tourniquet only groups (HLI 90 and HLI 150 respectively) were compared to blast and tourniquet exposed animals (blast HLI 90 and blast HLI 150). Additional controls were performed, an age matched normal non-injured group (naïve) and a blast only group, to account for the effects of blast overpressure exposure in isolation. Two timepoints were examined at 6 hr and 24 hr of reperfusion time (Table 2).

To examine the effects of tourniquet on a blast complex injured limb, a further set of experiments were performed. These rats received either the standard injury pattern of blast exposure, drop-weight femoral fracture, 1 min crush injury and amputation through zone of injury and wound closure (complex control), or the standard injury pattern but with a 90- or 150-minute tourniquet application following the crush injury but preceding the amputation phase (complex HLI 90 and complex HLI 150). These groups had an extended follow up with necropsy timepoints at 6hr, 24hr, Day 3 and Day 7 post injury (Table 2).

The third study addressed the formation of heterotopic ossification in a complex injured limb. To assess this outcome, complex HLI 150 group animals were observed to postoperative Day 84 (12-weeks) using μ CT. These images were compared to existing scans of the standard model (complex control) at this timepoint to establish if there was a difference in bone volume. This was then supported with an examination of the kinetics in the early response phase (6hr to Day 10 post injury) to identify the likely mechanistic effects of the tourniquet.

Table 2. Study groups and necropsy timepoints.

Table indicates the overall group design and the total number per group available for analysis

Study group	Number available per endpoint						
	Total	6hr	24hr	Day 3	Day 7	Day 10	Day 84
Naive control	5	-	-	-	-	-	-
Blast overpressure only	26	8	8	5	5	-	-
HLI 90 min	14	7	7	-	-	-	-
HLI 150 min	16	8	8	-	-	-	-
Blast + HLI 90 min	16	8	8	-	-		-
Blast + HLI 150 min	16	8	8	-	-		-
Complex Control	35	7	8	7	8	5	-
Complex HLI 90	23	7	8	-	8	-	-
Complex HLI 150	50	7	9	6	9	5	14
Total animals	201						

EXPERIMENTAL ASSAYS AND PROCEDURES PERFORMED

Blood analysis for CBC and Chemistry

Whole blood samples were run for CBC analysis at 3, 6, 9, 24, 72 and 168 hr post-surgery using a Sysmex XT-2000i Automated Hematology Analyzer (Sysmex America Inc, Mundelein, IL). Blood chemistry panels (Chem19) were run at necropsy timepoints and included a renal and electrolyte panel (BUN, Creat, Na⁺, K⁺, Cl⁻, CO₂, Phos, Ca²⁺), a liver panel (AST, ALT, LDH, ALKP, BILI) and a metabolic panel

(Albumin, Total protein, Glucose, Cholesterol, Triglycerides) and creatine kinase (CK) and were performed on a Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ).

BAL total leukocyte count

The resuspended BAL cell pellet was processed immediately following resuspension and held on ice. A sample (25 μ l) of the cell suspension was combined 1:1 with 25 μ l of acridine orange/propidium iodide (AO/PI) viability stain (Nexcelom Biosciences, Lawrence, MA). Twenty microliters (20 μ l) of this mixture was then pipetted onto a counter chamber slide (SD100 Slides, Nexcelom Biosciences, MA) in duplicate. Total cell count and percent cell viability was determined using an automated fluorescent viability cellometer using the immune cell, low RBC program (Cellometer Auto 2000, Nexcelom Biosciences, MA). Total cells, live cells, dead cells and viability were recorded in duplicate for each sample, with additional repeats performed if there was a large disparity between chamber readings. Total leukocyte count was then calculated, adjusted for dilution factor, to give both the total leukocyte count and an adjusted count relative to BAL volume recorded at necropsy.

Lung wet/dry ratio

The lower lobe of the right lung was used to calculate the wet/dry ratio of the lung, a marker of increased lung permeability. Following excision, the lower lobe was blotted to remove any excess blood or fluid from the outside of the lung. It was then placed on a small weigh boat, which had already been weighed, and the wet weight of the lung calculated. The lung was then placed in a drying oven at 60°C and weighed daily until then weight stabilized across at least a 24-hour period. This weight was recorded as

the dry weight. The weigh boat was then remeasured to ensure there had been no effect of the drying process on its weight and the wet/dry ratio calculated on the basis of the following equation:

$$\frac{Wet\ Lung\ Weight - Weigh\ Boat}{Dry\ Lung\ Weight - Weigh\ Boat} = Wet\ Dry\ Ratio$$

BAL protein Content

The protein concentration of the BAL supernatant was calculated using the Pierce™ BCA Protein Assay (cat# 23225 Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. In summary, 25 µl of BAL supernatant was assayed undiluted in duplicate or triplicate with a sample to working reagent ratio of 1:8, giving a working range of the assay of 20-2000 µg/ml, over a 30-minute incubation period. Optical density of the wells was determined using a microplate reader set to a 560 nm absorbance measurement. The average absorbance of the blank standard replicates was subtracted from all wells and a standard curve calculated using the adjusted values. Curve fitting algorithms utilized by Prism 8 (GraphPad Software, San Diego, CA) was used to interpolate the protein concentrations of the BAL samples assayed.

Acute kidney injury (AKI) biomarkers

Serum levels of known AKI biomarkers were assayed to detect structural damage to the kidneys as a result of the injury patterns. Three different markers were chosen, neutrophil gelatinase-associated lipocalin (NGAL), Kidney Injury Molecule-1 (KIM-1) and Cystatin C (CysC) all of which have been validated for the detection of AKI (52; 160; 229; 245; 281; 299). ELISA kits were purchased and performed according to the manufacturer's instructions (cat#s ab119602, ab201281, ab223858, ab239422, Abcam,

Boston, MA). Relative O.D. values were calculated and used to generate 4-PL standard curves to interpolate the concentrations of the samples, which was then adjusted by the appropriate dilution factor.

Endothelial dysfunction marker

To assess expression of serum soluble markers of endothelial damage and dysfunction, sandwich enzyme-linked immunosorbent assays (ELISAs) were performed to detect ICAM1 (CD54) (cat# ab100763, Abcam, Boston, MA). ELISA kits were performed according to the manufacturer's standard protocol and were assayed with a dilution factor of 1:20. The relative optical density (O.D.) at 450 nm (O.D. of each well minus the mean O.D. of the blank wells) was used to interpolate the concentration of the unknown samples from the standard curve and then adjusted for the dilution factor.

Damage associated molecular patterns (DAMPs) markers

Detection of DAMPs markers was performed using ELISAs. TREM-1 was assessed in serum using a sandwich ELISA (MTRM10, R&D Systems, Minneapolis, MA). Plates were performed according to manufacturer's recommendation with sample dilutions from undiluted to 1:4. The microplate was read at 450 nm and 560 nm and the O.D. determined as O.D.450 minus O.D.560, to correct for plate optical imperfections per the protocol. The relative O.D. was then calculated and standard curves created using a four-parameter logistic (4-PL) curve. Quantitative detection of HMGB1 (cat# LS-F11640, LifeSpan BioSciences, Seattle, WA) and Calprotectin (S100A8/S100A9) (cat# LS-F22540, LifeSpan BioSciences, Seattle, WA) were performed on plasma samples according to the manufacturer's instructions. Microplates were read at 450 nm and

relative O.D. calculated before interpolating unknown concentrations from the standard curve using a 4-PL curve.

Oxidative stress markers

Analysis for evidence of oxidative stress damage was performed on extracted RNA, serum and muscle tissue. Oxidative damage markers, 8-hydroxy-2'-deoxyguanosine, 8-hydroxyguanosine, and 8-hydroxyguanine were detected in extracted RNA by high sensitivity ELISA (cat# 589320, Cayman Chemicals, Ann Arbor, MI). RNA was extracted as described previously and nucleic acid concentration quantified by NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). RNA was then digested using nuclease P1 (cat# N8630, Sigma Aldrich, St Louis, MO) according to manufacturer's instructions and adjusted to a pH of 7.5-8.5 using 1M Tris. 1 unit of alkaline phosphatase (cat# 10713023001, Sigma) per 100 µg of RNA was added before incubation at 37°C for 30 minutes prior to boiling for 10 minutes. The RNA solution was then tested and analyzed according to the ELISA protocol.

Lipid peroxidation was determined indirectly by assessing the decomposition product, malondialdehyde (MDA). This was measured by a thiobarbituric acid reactive substances (TBARS) assay (cat# 700870, Cayman Chemicals). Serum was measured directly without further preparation steps, tissue was homogenized in RIPA buffer (cat# R0278, Sigma) including a protease inhibitor cocktail (cat# P8340, Sigma) before centrifuging at 1,600 x g for 10 minutes at 4°C and stored on ice. The assay was performed according to the manufacturer's protocol.

Inflammatory mediator immunoassays

Cytokine and chemokine expression were determined in serum samples using a multiplexing approach. Bio-Plex Pro™ Rat Cytokine 23-Plex assays (cat# 12005641, Bio-Rad, Hercules, CA) were performed on a Bio-Plex 200 Luminex system with high-throughput fluidics (cat# 171000205, Bio-Rad, Hercules, CA) which had undergone both validation (Bio-Plex Validation Kit 4.0, Hercules, CA) and daily calibration prior to use (Bio-Plex Calibration Kit, Hercules, CA) to standardize signal output and sensitivity between runs. Assay working ranges were determined using Bio-Plex Manager 6.1 software (Bio-Rad, Hercules, CA) and used for the automatic identification and removal of outliers and optimization of 5-PL standard curves to interpolate the concentrations of analytes in the unknown samples.

TISSUE BASED ASSAYS AND PROCEDURES

Quantitative reverse transcription PCR (RT-qPCR) gene analysis

RNA was isolated from tissues previously prepared in RNAlater™. RNA was extracted using RNeasy Lipid Tissue Mini kits and protocols (Qiagen, Germantown, MD). Samples were weighed and approximately 50 mg of lung, liver or kidney, or 90 mg of muscle tissue was homogenized using an Omni Tissue homogenizer (TH) with disposable hard tissue Omni Tip™ plastic homogenizing probes (Omni International, Kennesaw, GA) in 1ml of QIAzol lysis reagent (Qiagen, Germantown, MD). The manufacturer's standards protocol was followed, with 350 µl of the upper aqueous phase being combined with an equal volume of 70% ethanol. Extracted RNA was suspended in 50 µl (30 µl for muscle) of RNase-free water and held on ice.

Following 20 minutes held on ice, the extracted RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific™, Waltham, MA) for nucleic acid quantification using a 1 µl sample. Total RNA concentration was recorded as well as spectral data ratios indicating sample purity (260/230 and 260/280). For the 260/280 ratio a target of ~2.0 was used, with samples below 1.8 undergoing re-extraction. 260/230 ratio target was 2.0-2.2, with lower ratios being repeated if suggestive of contamination. Based off the initial concentration measurement, the stock extracted RNA was used to create diluted working samples with a target concentration of 150 ng/µL and stored at -80°C.

Samples passing quantification by spectrophotometry underwent further quality analysis using electrophoresis to determine the RNA integrity number (RIN) by assessing the 28S:18S ratio of ribosomal RNA. All samples were analyzed using the Agilent 2100 Bioanalyzer system and RNA 6000 Nano kits (Agilent, Santa Clara, CA) to determine RIN values using the integrated algorithms. Samples were prepared and processed according to the manufacturer's instructions. A threshold of a RIN of 7 was set following review of the literature (116), with all samples used in these experiments clearly exceeding this threshold.

Prior to reactions for creating complementary DNA (cDNA), the diluted RNA samples underwent thorough mixing prior to at least two measurements using the NanoDrop 2000 to confirm the nucleic acid concentration following dilution; an agreement of within 2 ng/µl on repeat measures was used as the threshold. These measurements were then averaged, and this concentration used to establish the amount of RNA to be included in the cDNA reaction which was performed using the iScript

Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA) following the manufacturer's instructions. The reaction generated sufficient cDNA to enable each well on a plate had 8.7 ng of cDNA per well reaction, with the cDNA being stored at -20°C prior to use.

RT-qPCR was performed using 8.7 ng of cDNA per well and a SYBR Green supermix (SsoAdvanced™ Universal SYBR Green Supermix, Bio-Rad, Hercules, CA) and nuclease-free water to a total well volume of 10 µl. Two 384-well custom plates were designed (IRI plate and HO plate), the first targets 29 individual ischemia reperfusion injury (IRI) associated genes (12 samples per plate), and the second targets 87 a mixture of individual osteogenic, chondrogenic and angiogenic genes (4 samples per plate) (Bio-Rad, Hercules, CA). The genes of interest and the corresponding amplicon sequences are included in Appendix 2 (Tables 14 and 15). The plates were optimized for use with the QuantStudio real-time PCR System (QuantStudio 7 Flex, Applied Biosystems, Waltham, MA). The SYBR Green supermix incorporates a blend of passive reference dyes (including ROX) which are validated for use with the real-time PCR machine. Plate designs incorporated housekeeping genes for the normalization of target gene expression, 2 were included on the IRI plate, *Gapdh* and *Hprt1*, and 5 were included on the HO plate, *Gapdh*, *B2m*, *Actb*, *Hprt1*, *Rplp0*. The thermal cycling protocol for the reaction commenced with a first stage of 90 seconds at 50°C, followed by 10 minutes at 95°C. The second stage consisted of 40 amplification cycles of 15 seconds denaturation at 95°C, followed by 30 seconds at 60°C for annealing and extension. The final stage consisted of a melt curve analysis was then performed at the end of the protocol between 60-95°C using 0.5°C increments at 2-5 sec/step.

Data was exported and analyzed using Qbase+ data analysis software, version 3.2 (Biogazelle, Zwijnaarde, Belgium). Analysis was performed on a per experiment basis with normalization performed against reference genes selected using the geNorm algorithm to determine reference gene expression stability across groups and select the optimal number of reference genes (284). Scaling was then performed against naïve tissue expression. Amplification efficiency was adjusted to target specific amplification efficiencies for each target which was calculated based on performed standard curves. Data exclusion criteria included wells with a quantification cycle (Cq) of >36. Normalization factor, fraction of detected targets and Mean Cq value were reviewed as part of sample quality control to identify erroneous sample runs. Statistical analysis was performed comparing the mean of gene expressions using the unpaired t-test with two-sided significance.

Quantification of protein expression

Three genes identified as being differentially expressed on RT-qPCR in injured muscle when comparing tourniquet and non-tourniquet amputation groups were selected for quantification. Muscle samples obtained at Day 1, 3 and 7 post injury were extracted. Protein concentration of the extract was determined using a BCA protein assay (cat# 23225, Pierce Biotechnology, Rockford, IL). Quantification of protein content in the tissue extracts were determined by ELISAs against runt-related transcription factor 2 (RUNX2), platelet-derived growth factor subunit A (PDGFA), (cat#s LS-F53973, LS-F519, LifeSpan BioSciences, Seattle, WA) and integrin binding sialoprotein (IBSP) (cat# MBp2-70022, Novus Biologicals, Centennial, CO).

STATISTICAL ANALYSIS

The criteria for statistical significance was defined as $p < 0.05$, and tests performed for two-sided significance. Data was assessed for gaussian distribution using D'Agostino-Pearson normality testing, or Shapiro-Wilk testing for smaller sample sizes and outlier analysis was performed using the ROUT method. Parametric data is presented as a mean with standard deviation (SD) unless otherwise stated. Non-parametric data is presented as a median and inter-quartile range (IQR). Comparison of 2 parameters was conducted using either an unpaired t test with Welch's correction for parametric data or Mann-Whitney for non-parametric data. Multiple variables with Gaussian distribution were compared using the Brown-Forsythe and Welch ANOVA tests, not assuming equal SDs, or by Kruskal-Wallis test. Follow-up testing of means or mean ranks and corrections for multiple testing when performed if appropriate are defined in the relevant section of results. 2-way ANOVA with Tukey correction was performed for inflammatory mediator data. Correlation tests when performed were Pearson's for parametric and Spearman's rank for non-parametric data. Gene expression analysis was performed using Qbase+ data analysis software, version 3.2 (Biogazelle, Zwijnaarde, Belgium), with results reported as mean values with standard error of the mean (SEM) based on an unpaired t-test. An intra-class correlation coefficient (ICC) for assess inter-rater agreement between calculations of bone volumes. Statistical analysis was performed with the exception of gene expression data using Prism 8.1.2 for Mac (GraphPad Software, La Jolla, CA) and SPSS 25 for Mac (IBM Corp, Armonk, NY) software.

CHAPTER 3: Results

THE EFFECTS OF BLAST OVERPRESSURE EXPOSURE ON THE DEVELOPMENT OF HIND LIMB ISCHEMIA REPERFUSION INJURY AND SECONDARY INJURY

Fragments generated from the detonation of a nearby explosive device can cause an isolated extremity vascular injury with minimal tissue disruption in a similar manner to an isolated gunshot wound. These injuries, generated by the debris from an explosion, are termed secondary blast injuries and account for many of the injuries sustained following an explosion. Frequently improvised explosion devices (IEDs) are specifically constructed with nails, steel ball bearing or metal fragments, to increase the number of secondary blast injuries sustained by those in the proximity of the device. To model this injury pattern, we developed a rat model which includes blast wave overpressure exposure and then a period of unilateral hindlimb tourniquet application to simulate the application of a combat tourniquet following an isolated extremity vascular injury at point of wounding. Tourniquet durations were chosen to cover a range spanning the current 120-minute doctrinal guidance on time to damage control surgery, with tourniquet times of 90 and 150 minutes used. These durations are considerably shorter than the published literature for unilateral tourniquet induced IRI in a rat model of 3 to 4hr.

Blast wave exposure results in increased biochemical evidence of both local tissue damage and remote organ dysfunction

To our knowledge, no research has previously addressed if blast wave exposure either compresses the time window for a tourniquet to induce an ischemia reperfusion injury following application of a temporary hind-limb tourniquet or augments the magnitude of any systemic immune response. By assessing serum markers at 6 and 24 hr

post tourniquet removal, the reperfusion time, we were able to assess systemic evidence of organ dysfunction based on established biochemical markers associated with renal, liver, lung and muscle injury.

Blast overpressure exposure leads to increased renal injury when compared to tourniquet alone

Blood urea nitrogen (BUN) and creatinine (Cr) have become established markers of renal function, used in clinical practice for over 100 years. Both are increased in the serum when renal injury leads to decreased function and clearance of end products of metabolism. Normality testing confirmed a non-Gaussian distribution for both serum BUN and Cr. Kruskal-Wallis one-way analysis of variance (ANOVA) was performed assessing for a significant variation between groups' median values at both 6 and 24 hr (Fig. 2A, 2B). At 6 hr, serum BUN varied significantly between groups ($p < 0.001$), which was not seen with serum Cr ($p = 0.17$). A post hoc uncorrected Dunn's test was then performed between pairings of clinical interest; variation from naïve, tourniquet duration with or without blast, and between tourniquet durations. These demonstrated at 6 hr post tourniquet release that BUN was elevated in all groups compared to naïve (16.7-71.4% increase, $p = < 0.001-0.04$), with the exception of blast only exposure (4.7% increase $p = 0.81$), confirming that 120 kPa blast overpressure alone is insufficient to induce renal injury. Blast significantly increased serum BUN 47.0% over HLI 150 alone (36.0 (IQR 29.5-39.75) vs 24.5 (23.0-28.0) mg/dL $p = 0.04$). This result supports the hypothesis that blast wave exposure results in an increased level of renal injury following prolonged tourniquet use as early as 6 hr post injury.

Analysis of renal markers after 24 hr of reperfusion confirmed a smaller variation between groups for serum BUN ($p = 0.04$), and no variation in serum Cr ($p = 0.38$) (Fig.

2C, 2D). The variation in serum BUN between blast HLI 150 and HLI 150 groups continues at 24 hr with a smaller 17.9% increase (23.0 (21.25-41.25) vs 19.5 (19.0-21.75) mg/dL, $p=0.03$). In blast exposed 90- and 150-minute treatment groups, longer tourniquet time resulted in a 21.1% increase in median serum BUN levels (23.0 (21.25-41.25) vs 19.0 (19.0-19.0) mg/dL, $p=0.002$). No significant change in serum BUN was detected in non-blast exposed tourniquet groups (19.5 (19.0-21.75) vs 20.0 (16.0-24.75) mg/dL, $p>0.99$).

The BUN to Cr ratio (BCR) has been used as a diagnostic measurement to assess renal function and differentiate between pre-renal acute kidney injury (PR-AKI) and intra-renal AKI (I-AKI) injury. An increase in the ratio is associated with pre-renal causes of injury, such as hypoperfusion of the kidney, while a failing ratio can represent intra-renal damage and reduced resorption. Analysis at the 6 hr timepoint showed overall variation between the groups ($p=0.001$), tourniquet use was associated with an increased median BCR compared to uninjured age-matched normal controls (naïve) (47-54 vs 42, $p=0.003-0.19$), with the effect more exaggerated with the inclusion of blast exposure (53.3-60.0 vs 42.0, $p<0.001-0.009$), despite blast only exposure having no effect on the median ratio (43.25 vs 42.0, $p=0.62$). Median BCR ratios increased 27.7% between HLI 150 and blast HLI 150 treatment groups (47.0 vs 60.0, $p=0.04$), further supporting the hypothesis that blast wave exposure has a detrimental effect on renal function compared to tourniquet alone, through a pre-renal cause of hypoperfusion (Fig. 2E). Analysis at 24 hr showed the effect between groups to be less significant ($p=0.03$). Both 90- and 150-minute tourniquet application show a difference between blast and non-blast exposed groups, with the 90 minute group having a 12% reduction in median BCR in those which

were blast exposed ($p=0.04$), while the 150 minute groups exhibited a 24% increase in median BCR value ($p=0.04$), demonstrating the effect seen at 6 hr (27.7%) was not transient(Fig. 2F). This was consistent with the difference in median BCR observed between the blast HLI 90 and blast HLI 150 treatment groups, with the additional 60 minutes of tourniquet increasing the BCR by 32.1% (34.83 vs 46, $p=0.004$), supporting that a small increase in tourniquet time accompanied with blast exposure lead to a significant increase in renal injury from hypoperfusion, which was not seen in their respective non-blast exposed groups.

A correlation analysis was performed using Spearman's rank test between all serum BUN and creatinine results from the groups (r_s 0.384, $p=0.0002$), with sub-analysis at 6 hr (r_s 0.428 $p=0.007$) and 24 hr (r_s 0.385, $p=0.01$), confirming a weak positive monotonic relationship between the two renal markers. While creatinine can be used as a marker to determine the severity of AKI at a later stage post injury, its value as an early AKI marker has been debated in the literature, as it lacks sensitivity to act as an early AKI marker. Creatinine is used to determine the glomerular filtration rate of the kidneys, where functional reserve may mask a renal injury, or only establish an injury at a late stage, greater than 24 hr post injury. In developing sensitive panels to early renal injury, it will be necessary to substitute creatinine with other newer biomarkers of AKI to detect early onset secondary renal injury.

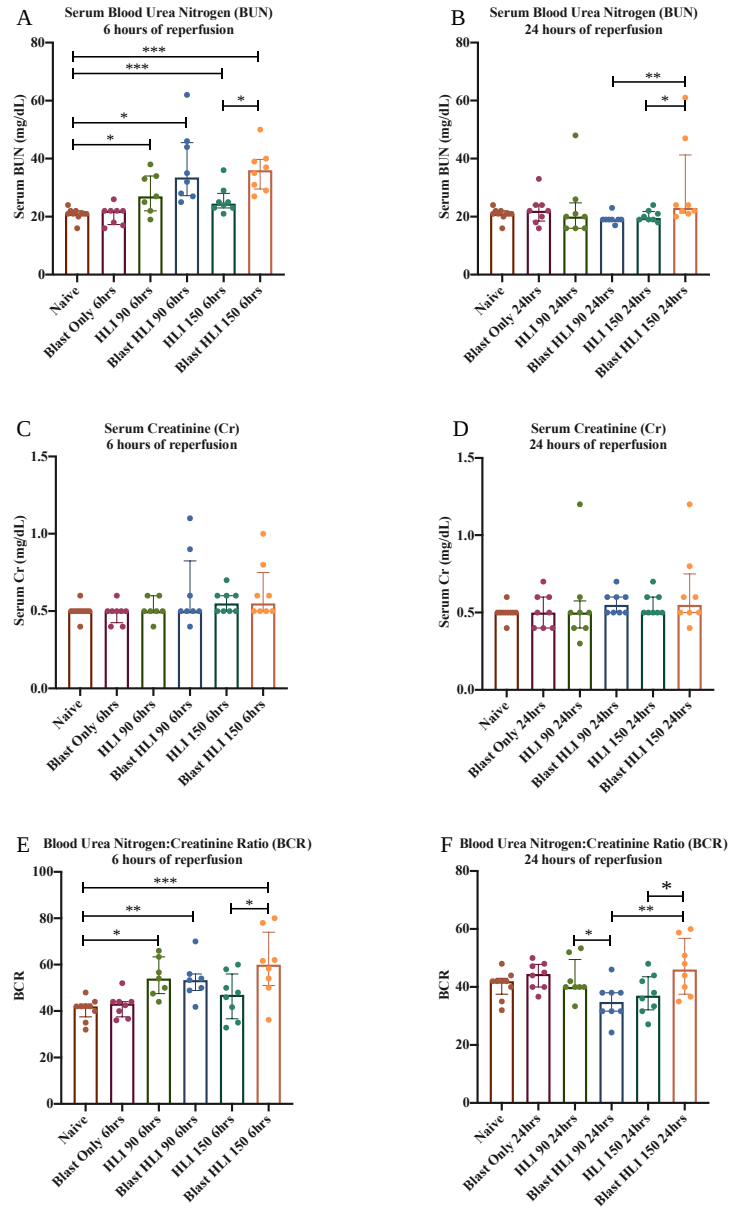


Figure 2. Tourniquet induced Renal Injury is exacerbated by blast overpressure exposure Serum blood urea nitrogen (BUN) and creatinine (Cr) levels at 6 and 24 hr post tourniquet removal. (A,B) Serum BUN levels after 6 hr (A) and 24 hr (B) of limb reperfusion, (C,D) Serum Cr levels after 6 hr (C) and 24 hr (D) of limb reperfusion, (E,F) BUN:Cr ratio (BCR) after 6 hr (E) and 24 hr (F) of limb reperfusion. Graphs show median and 95% CI with Kruskal-Wallis test with post-hoc uncorrected Dunn's test as non-parametric data. Significance shown as *P≤0.05, **P≤0.01, ***P≤0.001.

Collectively these results support the hypothesis that blast exposure has a synergistic effect on IRI induced renal injury, increasing both the magnitude of renal injury measured by serum BUN, and by 24 hr, a difference between blast exposed 90 and 150 minute tourniquet groups, an observation which is not present without blast exposure, further supporting an earlier onset of IRI and systemic injury. Changes in the BCR between blast and non-blast groups support a pre-renal cause, in this context likely from hypoperfusion of the kidney as a response to a more established ischemia reperfusion injury. Creatinine, due to its low sensitivity and delayed response to injury is a poor biomarker to assess renal injury in either the acute setting immediately post injury, where it would be used to risk stratify patients for AKI risk and early evacuation, or as part of patient monitoring in a prolonged field care setting.

Increased hepatocellular damage increased by the effects of blast on tourniquet induced ischemia reperfusion injury

As the largest solid organ in the body, responsible for multiple processes relating to homeostasis including metabolism, immune activation and detoxifying the blood, the liver's extensive vascular supply from both the portal vein and hepatic artery leave it exposed to remote organ injury following IRI.

Liver dysfunction was assessed using serum alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). ALT, a marker of hepatocellular damage showed early elevation with variation between groups present at 6 hr post injury (ANOVA, $p < 0.001$) (Fig. 3A). Tourniquet use, but not blast only exposure, caused a significant increase in mean serum ALT concentrations compared to naïve (HLI 90 100% increase, HLI 150 138% increase, $p = 0.04$ and $p < 0.001$), with higher levels of injury detected in blast exposed groups (blast HLI 90

157% increase, blast HLI 150 368% increase, $p=0.002$ and $p<0.001$). When comparing between blast and non-blast groups, blast exposure significantly elevated ALT levels at both HLI 90 and 150 minutes (HLI 90 129.6 ± 23.6 vs 145.3 ± 17.0 U/L $p=0.009$, HLI 150 132.3 ± 5.4 vs 253.0 ± 29.5 $p=0.004$) at 6 hr, confirming blast exposure has a deleterious effect on liver injury following tourniquet use, increasing the severity of hepatocellular damage. The variation in ALT persisted at 24 hr ($p<0.001$) with all groups except blast only showing an increase in ALT ($p<0.001-0.08$) (Fig. 3B). Consistent with the 6 hr timepoint, serum ALT levels in HLI 90, HLI 150 and blast HLI 150 treatment groups were increased, 97%, 112% and 182% above baseline values. By 24 hr post tourniquet removal the difference in serum ALT measurements between blast and non-blast at 90 and 150 minutes was no longer significant, however a difference was observed between blast HLI 90 and 150 levels (100.8 ± 14.8 vs 188.5 ± 30.7 $p=0.03$); this is likely due to a lower than expected blast HLI 90 serum ALT levels.

Lactate dehydrogenase (LDH) levels trended towards elevation after 6 hr of reperfusion. Mean LDH levels in HLI 90, HLI 150, blast HLI 90 and blast HLI 150 treatment groups increased 43.7%, 61.1%, 56.8% and 115.7% respectively, when compared to naïve. Due to individual results within groups having a large variance and the subsequent effect on the SD, only naïve vs blast HLI 150 reached significance ($p=0.01$), with no statistical difference between blast and no blast tourniquet groups (Fig. 3E).

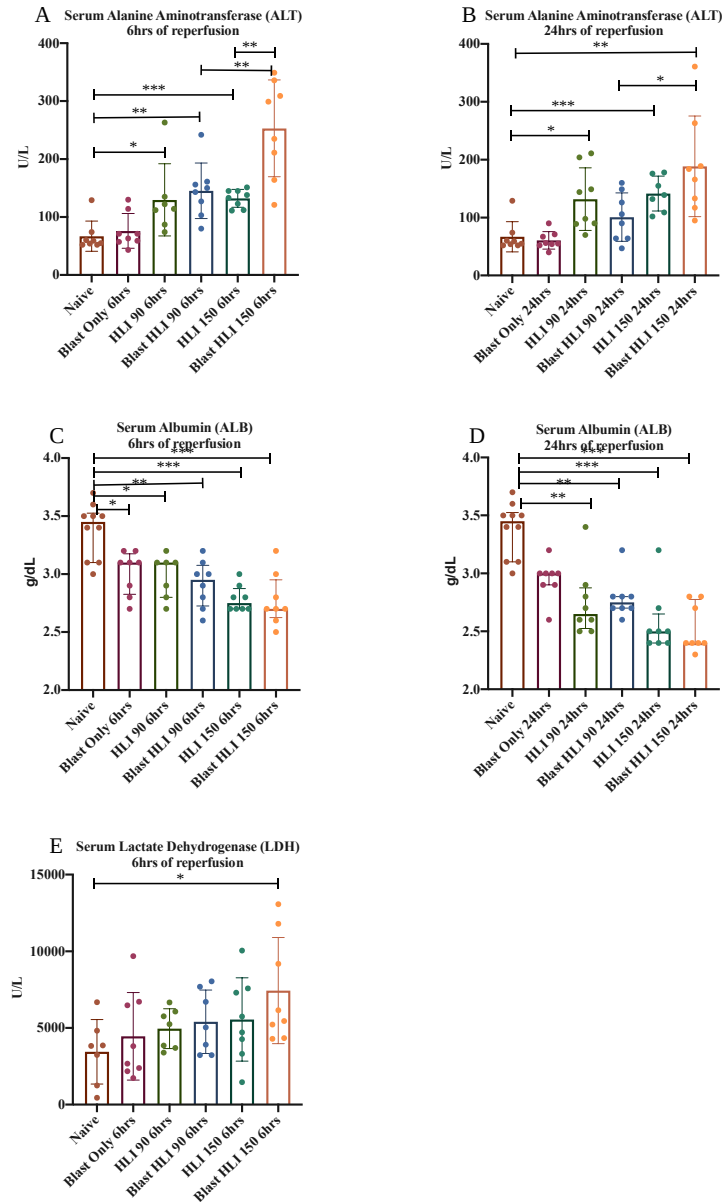


Figure 3. Tourniquet induced liver injury exacerbated by blast overpressure exposure Serum alanine aminotransferase (ALT), albumin (ALB) and lactate dehydrogenase (LDH) levels at 6 and 24 hr post tourniquet removal. (A,B) Serum ALT levels after 6 hr (A) and 24 hr (B) of limb reperfusion, (C,D) Serum ALB levels after 6 hr (C) and 24 hr (D) of limb reperfusion, (E) Serum LDH after 6 hr of limb reperfusion. Graphs demonstrate mean and 95% CI for parametric data with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction (A,B,E), or median and 95% CI with Kruskal-Wallis test with post-hoc uncorrected Dunn's test for non-parametric data (C,D). Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Variation of mean serum ALP (data not shown) at both 6 and 24 hr was not significant across all treatment groups ($p=0.71$ and $p=0.18$), consistent with the liver dysfunction being due to hepatocellular inflammation or damage from IRI, rather than an obstructive biliary source.

Serum albumin is the most abundant circulating protein, whose synthesis occurs in the liver, and production has been shown to be affected by trauma and ischemia, with low levels associated with poor prognosis(38). Serum albumin is also recognized as an antioxidant, and metabolism is via catabolic, renal and gastrointestinal clearance. Albumin was consistently lower at both 6 and 24 hr post-tourniquet removal. Results did not follow a normal Gaussian distribution. Variation was present between injury groups and naïve state at 6 hr ($p<0.001$) (Fig. 3C), with post hoc analysis showing all tourniquet groups and the blast only group to have lower median serum albumin concentrations (10.1-21.7% reduction $p<0.001$ -0.03 and 10.1% reduction $p=0.05$, respectively) compared with naïve. Blast exposure did not significantly alter tourniquet time effect on serum albumin levels at 6 hr. HLI and blast HLI treatment groups both had significant reductions in median serum albumin values (20.3-30.4% reduction, $p<0.001$ -0.005) (Fig. 3D) at 24 hr. A trend of larger reductions was observed with increasing tourniquet time but failed to reach significance (blast HLI 90 vs blast HLI 150, 2.75 vs 2.40 g/dL, $p=0.08$). These reductions in serum albumin may support either reduced liver synthesis, increased renal loss due to AKI associated reduced resorption, increased ROS mediated catabolic clearance, or loss from the circulation due to increase vascular permeability due to endothelial dysfunction.

Blast exposure increases evidence of muscle damage following extended tourniquet use

Assessment of biochemical markers of muscle injury was performed using creatine kinase (CK) and aspartate aminotransferase (AST). Elevated levels of AST can also be produced by the liver following inflammation and/or damage the liver. The AST/ALT ratio can be used to infer the source of AST from either muscle or liver origin. Analysis of the AST:ALT ratio demonstrated as increase in mean ratios at 6 hr ($p=0.01$) (Fig. 4A), where the increase was significant in the HLI 150 and blast HLI 90 treatment groups in comparison to naïve controls (33.7% $p=0.001$, 36.8% $p=0.04$). These increases were more pronounced in tourniquet groups, both blast and non-blast at 24 hr (Fig. 4B), all reaching significance (57.5-70.3% increase, $p<0.001$). The notable exception at both timepoints is blast HLI 150, whose AST:ALT ratio remained lower, suggesting that the rise in AST in this group was accompanied with more a more established ALT rise from an IRI induced liver injury.

Analysis of AST in isolation as a marker of muscle injury was performed on the basis of the AST:ALT ratio data. Variation between groups were detectable at both 6 and 24 hr timepoints ($p<0.001$) (Fig. 4C and 4D). At 6 hr, no change in AST was seen in the blast only group, further supporting that a systemic blast overpressure exposure of 120 kPa in isolation does not induce muscle injury, while HLI 90 and HLI 150 treatment groups exhibited increased median serum AST levels (HLI 90 89% increase $p=0.03$, HLI 150 189% increase $p=0.003$). Median AST level increases were greater in blast injured groups compared to naïve (blast HLI 90 198% $p=0.002$, blast HLI 150 232% $p<0.001$), but failed to reach significance between blast and non-blast tourniquet times ($p=0.37$ - 0.55). A similar pattern was present at 24 hr, with serum AST levels in all tourniquet-

treated groups remaining elevated compared to naïve ($p<0.001$ - 0.02) but with no statistical difference between blast and non-blast tourniquet groups ($p=0.39$ - 0.58).

After 6 hr of reperfusion, median CK levels were significantly elevated in the blast HLI 150 group compared to both naïve (668% $p<0.001$), HLI 150 (569% $p<0.003$) and blast HLI 90 (509% $p=0.02$) treatment groups (Fig. 4E), supporting the hypothesis that blast exposure increased tourniquet induced muscle injury and that increasing tourniquet time from 90 to 150 minutes is sufficient under blast exposed conditions to have a detrimental effect on tourniquet induced muscle damage and breakdown. This effect was transient with resolution by 24 hr; however, creatine kinase is used as the diagnostic marker of rhabdomyolysis and is known to have a short half-life.

Rhabdomyolysis can cause AKI due to direct effect of accumulation of myoglobin, obstructing the renal tubules, through heme associated ROS, or through hypoperfusion of the kidney.

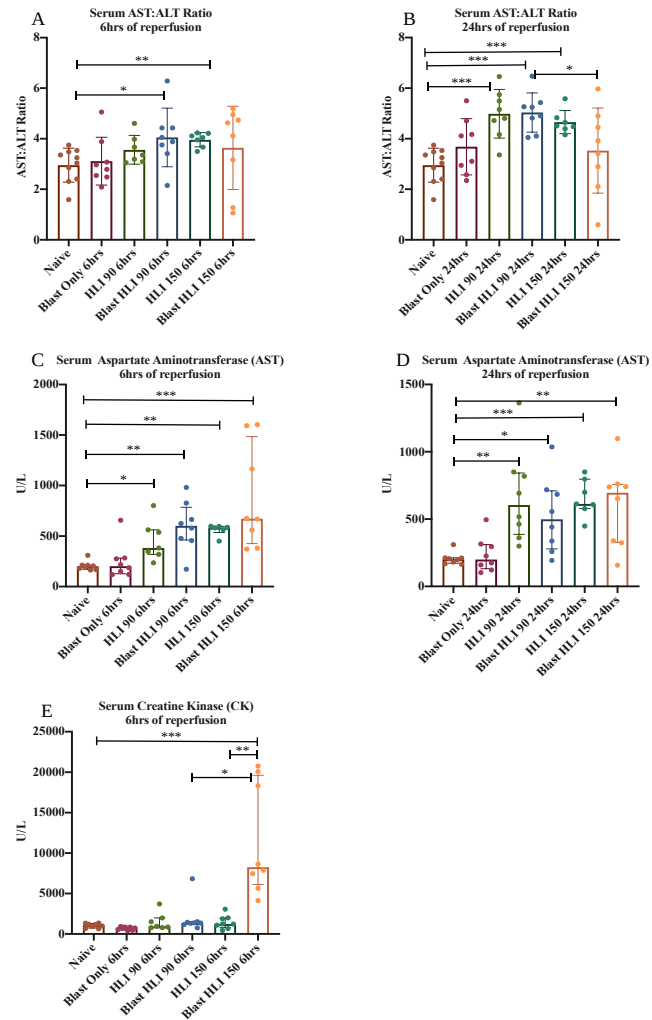


Figure 4. Tourniquet induced muscle injury exacerbated by blast overpressure exposure. Serum aspartate aminotransferase (AST):alanine aminotransferase (ALT) ratio, aspartate aminotransferase (AST) and creatine kinase (CK) levels at 6 and 24 hr post tourniquet removal. (A,B) Serum AST:ALT ratio after 6 hr (A) and 24 hr (B) of limb reperfusion, (C,D) Serum AST levels after 6 hr (C) and 24 hr (D) of limb reperfusion, (E) Serum CK after 6 hr of limb reperfusion. Graphs demonstrate mean and 95% CI for parametric data with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction (A,B), or median and 95% CI with Kruskal-Wallis test with post-hoc uncorrected Dunn's test for non-parametric data (C,D,E). Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Tourniquet associated reperfusion injury causes greater lung injury than 120 kPa primary blast wave

The lungs are at risk of a primary blast injury following blast associated trauma through the direct pressure effect of the blast overpressure wave on the alveolar space, or as an immune mediated secondary injury where SIRS can lead to increased neutrophil infiltration, increased lung permeability and reduced oxygen exchange.

Analysis of the right lower lobe of the lung was performed at necropsy. Calculation of a wet-to-dry lung weight ratio acts as a marker of increased lung permeability and fluid accumulation as a result of lung injury. At 6 hr, analysis of variance showed an association ($p=0.03$), with all tourniquet groups showing a small increase in mean ratio values compared to naïve (4.70-4.92 vs 4.67), with only blast HLI 90 reaching individual significance (4.92 vs 4.67, $p=0.01$). Blast only exposure did not induce an effect ($p=0.14$), suggesting that 120 kPa BOP does not directly cause increased lung permeability (Fig. 5A). Analysis of lungs from the 24 hr groups showed no variation from naïve ($p=0.30$), with all groups having a small increase in ratios which was not significant (4.70-4.80 vs 4.67, $p=0.09$ -0.66), with longer tourniquet time and blast exposure trending towards higher ratios.

Protein concentration in lung BALF was determined by BCA protein assay. At 6 hr of reperfusion, there was a small but statistically significant increase in BALF protein concentration compared to naïve in all tourniquet groups ($p=0.03$ -0.05), but not the blast only group (Fig. 5B). At 24 hr, while mean BALF protein concentrations were elevated over naïve levels, these failed to reach significance, suggesting any lung injury resulting in increased permeability of proteins into the alveolar space is mild and transient within 24 hr of reperfusion.

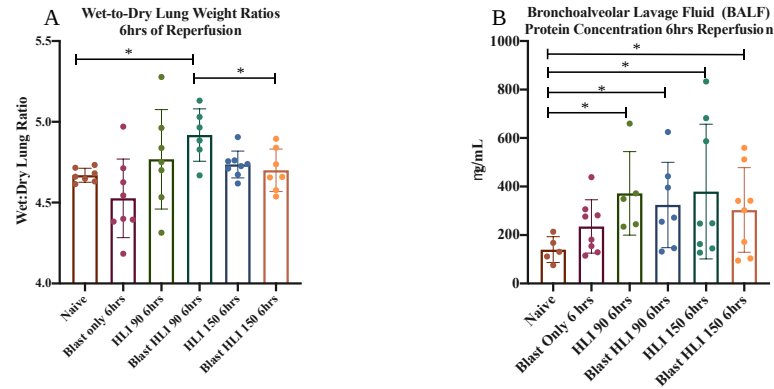


Figure 5. Tourniquets induce greater lung injury than primary blast wave exposure. Markers of increased lung permeability are elevated at 6 hr of reperfusion. (A) Wet-to-Dry lung ratio after 6 hr of reperfusion and (B) protein concentration measured by BCA protein assay in lung bronchoalveolar lavage fluid (BALF) at 6hr of reperfusion. Graphs demonstrate mean and SD for parametric data with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$.

Whole blood analysis demonstrates neutrophilic response to IRI with greater increases in blast injured groups

Whole blood was evaluated using for total WBC, and the number and percentage of neutrophils, monocytes and lymphocytes. 6 hours of reperfusion resulted in an increase in total WBC in all tourniquet groups, but these changes did not reach significance between groups (Fig. 6A). The increase was attributable to a rise in neutrophils, which was significant in all tourniquet groups ($p < 0.001-0.02$) (Fig. 6B). When assessed as percentage of the total leukocyte populations, the neutrophil count increased from 12.6% in the naïve group to 27.9% in the blast only group ($p = 0.01$), and to 66% and 72% in the HLI and blast HLI groups ($p < 0.001$). This is consistent with an early innate response to injury and demonstrates that exposure to systemic blast overpressure causes immune cell activation in the absence of visible injury. The presence of a neutrophilia persisted at 24 hr of reperfusion in all tourniquet groups ($p < 0.001-0.01$) and in the blast only group ($p = 0.02$), when compared to naive. Neutrophil counts were higher in both blast HLI 90

and blast HLI 150 than non-blast groups but failed to reach significance (2.5 vs 2.1 $\times 10^3/\mu\text{l}$, $p=0.59$ and 2.4 vs 1.9 $\times 10^3/\mu\text{l}$, $p=0.43$). This demonstrates an innate immune response is generated by the 90 or 150 minutes of tourniquet but while there was a trend towards a greater neutrophilia with blast, this did not reach significance.

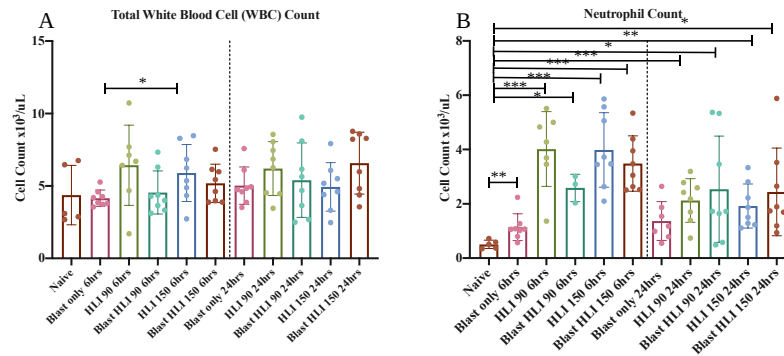


Figure 6. Whole blood analysis demonstrates neutrophilia as early response
Whole blood analysis of white cell count (WCC) and neutrophil assessed at 6 hr and 24 hr of reperfusion. The graph shows mean and SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$.

Blast exposure exacerbates IRI induced endothelial dysfunction as early as 3 hr post tourniquet removal

To establish if endothelial dysfunction contributed to the effects seen in the analysis of biochemical markers of organ injury, serum levels of Intercellular Adhesion Molecule-1 (ICAM-1) and Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) were analyzed. The analysis of TREM-1 is reported in detail in the DAMPs section (See below).

Intercellular Adhesion Molecule-1 (ICAM-1), also known as CD54, is expressed on endothelial cells and leukocytes. In its soluble form, it acts as a biomarker for inflammation and injury to the endothelium. Analysis at 6 hr of reperfusion showed a small 24% elevation in mean serum levels compared to blast only, reaching significance in the blast HLI 150 group ($p=0.03$). Serum analysis was performed on samples obtained

at 3 hr of reperfusion to establish if soluble ICAM-1 could be an early biomarker of injury extent (Fig. 7). It was found that serum ICAM-1 levels were higher at 3 hr post reperfusion than at 6 hr. At 3 hr, serum ICAM-1 in the blast HLI 150 group was significantly elevated over that measured in naïve animals (34%, $p=0.04$) and blast only treatment group (92%, $p=0.002$). Moreover, a significant difference in circulating ICAM-1 measured between HLI 150 and blast HLI 150 treatment groups (54%, $p=0.01$) was observed, suggesting blast exposure increases early endothelial damage following reperfusion. Greater circulating ICAM-1 was detected following blast HLI 90 treatment versus blast only (66%, $p=0.02$) and HLI 90 treatment (19%, $p=0.04$) suggesting blast effects even short periods of tourniquet use. Analysis performed at 24 hr of reperfusion demonstrated a similar trend of increased circulating ICAM-1 in HLI 150 (27%, $p=.004$), blast HLI 90 (18%, $p=0.03$) and blast HLI 150 (42%, $p=0.03$) treatment groups in comparison to levels detected in animals that received only blast exposure. Collectively this data supports the hypothesis that blast exacerbates endothelial dysfunction, a key component of IRI, with differences detectable as early as 3 hr post-injury.

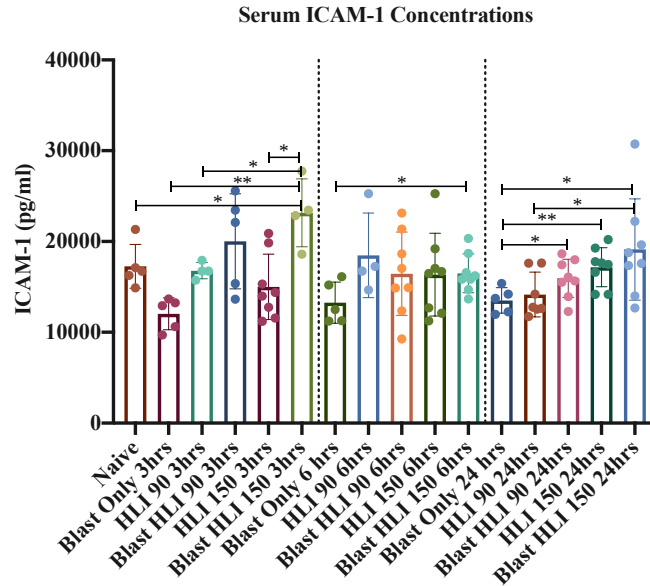


Figure 7. Blast exposure increases expression of endothelial dysfunction marker ICAM-1 (CD54) at 3hr post injury
 Marker endothelial dysfunction ICAM-1 were assessed at 3 hr, 6 hr and 24 hr of reperfusion. The graph shows mean and SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$.

Novel serum AKI markers confirm that increased tourniquet duration associated with acute kidney injury onset

To further assess the degree of renal ischemia reperfusion injury, several newer more sensitive biomarkers of AKI were chosen to be assessed to identify if they were detectable in serum. These were Cystatin C, neutrophil gelatinase–associated lipocalin (NGAL), and kidney injury molecule-1 (KIM-1).

Serum Cystatin C levels at 6 hr of reperfusion were increased in all groups with the highest increases observed in HLI 150 (29%, 1371 ± 790 vs 1060 ± 396 ng/ml, $p = 0.46$) and Blast HLI 150 (37%, 1457 ± 534 ng/ml, $p = 0.17$) when compared to naïve (Fig. 8A). At 24 hr, all groups involving tourniquet injury exhibited further increases in serum Cystatin C levels compared to 6 hr, wherein Cystatin C serum levels were elevated in all tourniquet treatment groups when compared to blast only controls, reaching significance

in the HLI 150 treatment group (76%, $p<0.001$), but not in the blast HLI 150 treatment group (37%, $p=0.07$) (Fig. 8B). The findings of early increases in serum Cystatin C against the finding of unchanged creatinine supports its use as an early biomarker of renal injury and is consistent with previous research (78; 195).

Neutrophil gelatinase-associated lipocalin (NGAL) has been shown to have a high sensitivity in both urine and plasma for the detection of AKI (187), with the ability to detect subclinical AKI exhibiting minimal elevational in traditional functional markers. At 6 hr of reperfusion, serum NGAL levels varied significantly between treatment groups ($p=0.002$), with post hoc testing showing the HLI 150 and blast HLI 150 treatment groups exhibited elevated NGAL compared to naïve controls and the blast only treatment groups. HLI 90 or blast HLI 90 treatment did not cause a significant rise in circulating NGAL when compared to naïve control levels. There were significant differences between HLI 90 and HLI 150 (282 ± 119 vs 1008 ± 780 ng/ml, $p=0.05$) and blast HLI 90 and blast HLI 150 treatments (472 ± 351 vs 1066 ± 500 ng/ml, $p=0.02$), suggesting that the difference between 90 and 150 minutes of tourniquet is sufficient to upregulate renal injury (Fig. 8C). After 24 hr of tourniquet-induced ischemia reperfusion injury, serum NGAL was significantly elevated in all treatment groups when compared to both naïve controls ($p=0.001-0.04$) and blast only treatment groups ($p=0.001-0.03$). Differences in serum NGAL levels were also significantly between HLI 150 and blast HLI 150 (1657 ± 804 vs 2565 ± 1677 ng/ml, $p=0.004$), confirming an additional blast effect (Fig. 8D). A difference between blast HLI 90 and blast HLI 150 groups (503 ± 349 vs 2565 ± 1677 ng/ml, $p=0.01$) was also detected but this may be skewed by a low blast HLI 90 mean compared to HLI 90 alone. This demonstrates that severity of AKI increases

with duration of reperfusion during the first 24 hr, that longer tourniquet times increase NGAL, and that blast exposure significantly increases serum NGAL levels, while blast exposure alone had no effect.

Analysis of serum KIM-1 showed no significant differences at either 6 or 24 hr between treatment groups or naïve controls. This finding could be consistent with functional but not renal tubular structural injury; KIM-1 is shed into the urine under MAPK signaling, and subtle increases in urinary concentrations may not have been detectable using serum.

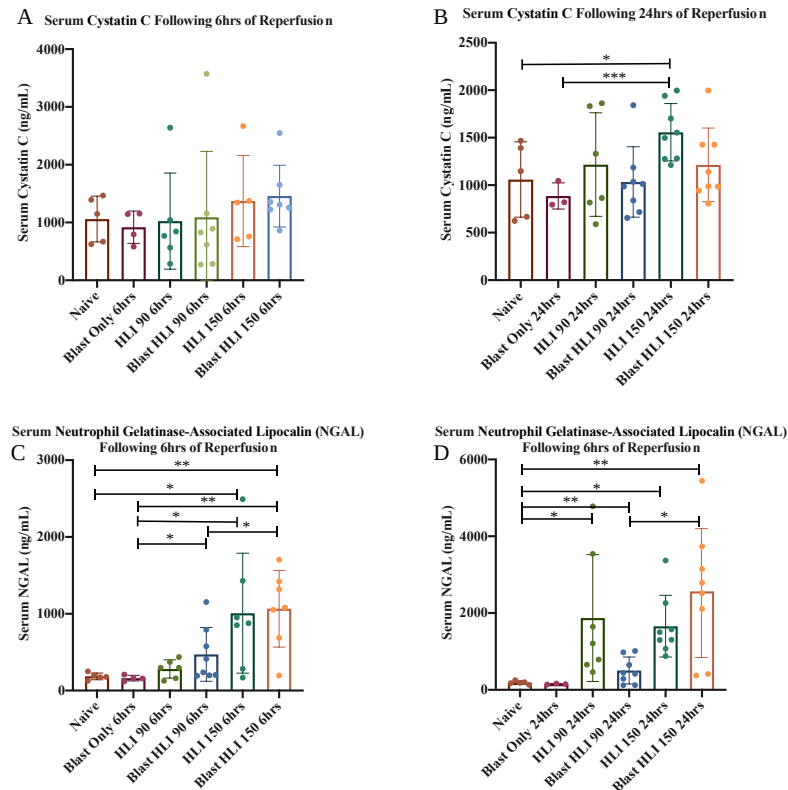


Figure 8. Serum Cystatin C and NGAL confirm that acute kidney injury increases with tourniquet duration

Novel AKI markers were assessed at 6 hr and 24 hr of reperfusion. The graphs show mean and SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as *P≤0.05, **P≤0.01, ***P<0.001.

Damage associated molecular patterns detectable in circulation after only 3 hr of reperfusion

Damage-associated molecular patterns (DAMPs) are endogenous molecules that are constitutively expressed and released upon tissue injury and damage, resulting in the activation of the innate immune system. In this study, we assessed the systemic accumulation in serum of three well characterized DAMPs (key proinflammatory danger signals; HMGB1, Calprotectin-S100A8/A9, and TREM1) which are either secreted by innate immune cells and/or released passively by injured or damaged cells in response to injury and non-programmed cell death. Two were chosen based on existing literature relating to trauma and ischemia reperfusion injury (HMGB1 and TREM-1), with the third based on early gene expression results from the models performed (S100A8/A9).

High mobility group box-1 (HMGB1) was assessed at both 6 and 24 hr of reperfusion but no statistically significant changes were noted. Small increases in plasma concentrations were seen early after 6 hr of reperfusion in injured groups, which ranged from 17-45% compared to naïve (376-467 vs 322 pg/ml $p=n.s.$), but no further increases were seen at 24 hr of reperfusion.

Plasma concentrations of calprotectin (S100A8/A9) were assessed at 6 and 24 hr of reperfusion. After 6 hr of reperfusion injury, serum calprotectin levels in all groups were significantly elevated compared to blast only treatment (19-44%, $p=0.004-0.02$), with higher levels of calprotectin detected in the HLI 150 and blast HLI treatment groups. At 24 hr of IRI, serum calprotectin levels remained modestly elevated over naïve or blast only controls but were not statistically significant (Fig. 9A).

The serum level of TREM-1, a DAMP associated with sterile inflammation and a myocardial IRI marker, but also now being recognized as an early marker of endothelial

dysfunction, was investigated (Fig. 9B). At 6 hr of reperfusion there was variation between groups ($p=0.004$). Post hoc analysis confirmed increased mean TREM-1 serum levels in the HLI 150 (73-86%) and blast HLI 150 treatment groups (52-63%), compared to both naïve ($p=0.001-0.02$) and blast only controls ($p<0.001-0.01$). No significant differences were detectable between the blast only treatment group and naïve controls (653 ± 123 vs 703 ± 104 pg/mL $p=0.14$). With the exception of the blast only treatment group (895 ± 228 vs 703 ± 104 pg/ml, $p=0.14$), serum levels of TREM-1 significantly increased in all treatment groups ($p<0.001-0.008$) following 24 hr of reperfusion. The magnitude of serum TREM-1 elevation was most evident in the blast HLI 150 treatment group when compared to naïve (111%, $p<0.001$). Further analysis confirmed that TREM-1 was detectable in serum within 3 hours of injury, with the serum concentration in the blast HLI 150 group significant after 3 hr of reperfusion against naïve (1228 ± 180 vs 702 ± 104 pg/ml, $p<0.001$) and blast only groups (1228 ± 180 vs 664 ± 117 pg/ml, $p<0.001$). Elevated concentrations of serum TREM-1 were present in all the other treatment groups and were approaching significance, suggesting that TREM-1 has potential as an early marker of injury.

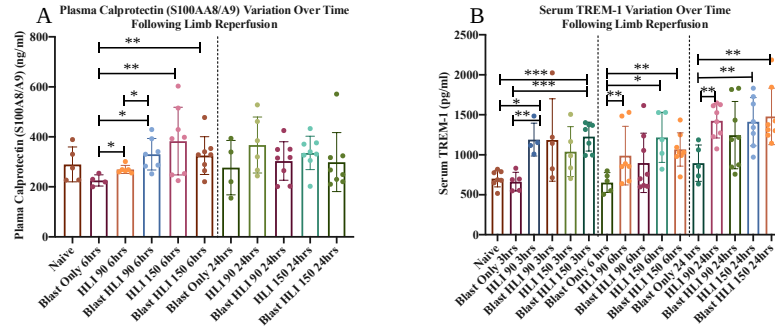


Figure 9. Plasma calprotectin and serum TREM-1 show early systemic evidence of tissue injury following whole limb reperfusion
Circulating DAMPs were assessed at 3 hr, 6 hr and 24 hr of reperfusion. The graphs show mean and SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$.

Blast significantly increases systemic markers of oxidative stress over tourniquet use alone

Lipid peroxidation was assessed systemically in plasma using the TBARS assay, which detects levels of malondialdehyde (MDA). Analysis using ANOVA demonstrated significant differences between both HLI 150 (2.8 vs. 2.2 μM , $p=0.04$) and blast HLI 150 (3.59 vs 2.2 μM , $p<0.001$) compared to naïve at 6 hr of reperfusion. Blast only exposure showed an increase, not significant against naïve baseline, with blast HLI 150 remaining significant against blast only (3.6 vs 2.5 μM , $p=0.01$). Of particular note the rise in TBARS was significant between HLI 150 and blast HLI 150 group (3.6 vs 2.8 μM , $p=0.03$), demonstrating that blast wave exposure significantly increases systemic markers of oxidative stress above tourniquet use alone. At 24 hr of reperfusion, both HLI 150 and blast HLI 150 were significantly elevated above naïve ($p<0.001$) and blast only ($p=0.02-0.04$) (Fig. 10A). The blast only group was again elevated but not significantly compared to naïve ($p=0.24$) and the significant difference between HLI 150 and blast HLI 150 had resolved. Analysis of MDA levels in homogenized injured muscle, adjusted for protein

concentration, demonstrated a 43% increase in the blast HLI 150 group compared to naïve, blast only and HLI 150, but none reached significance.

DNA and RNA can be damaged by oxidative stress. The base guanine is most susceptible to oxidation and metabolites of the repair process can be detected as an indicator of damage. 8-hydroxyguanosine (8-OHG), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-hydroxyguanine were collectively assayed in plasma and RNA extracted from injured muscle samples. No significant differences were detected in plasma at either 6 or 24 hr of reperfusion between groups. After 24 hr of reperfusion, levels in HLI 150 and blast HLI 150 groups were increased 66% compared to naïve baseline, but this was not significant ($p=0.19-0.25$). RNA damage at 6 hr of reperfusion was not significantly elevated, but by 24 hr of reperfusion expression of 8-OHdG had increased significantly against naïve levels in both the HLI 150 group (1069 vs 816 pg/ml, $p=0.02$) and blast HLI 150 group (1103 vs 816 pg/ml, $p=0.008$) with lesser, non-significant increases also present in HLI 90 (970 pg/ml) and blast HLI 90 (938 pg/ml) groups (Fig. 10B). This suggests that a tourniquet time of 150 minutes is sufficient to contribute to RNA oxidation.

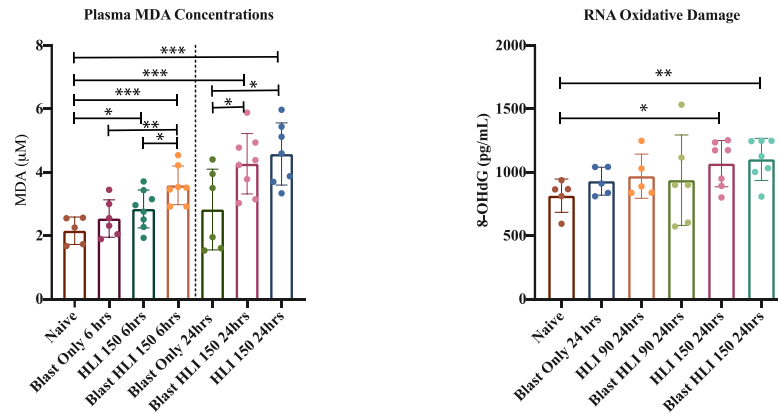


Figure 10. Blast exposure increases evidence of circulating and tissue oxidative stress during reperfusion.

Plasma MDA concentrations and RNA 8-OHdG following whole limb reperfusion. The graphs show mean and SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Gene expression analysis confirms blast exposure upregulates ischemia reperfusion associated genes leading to greater pro-inflammatory, oxidative stress and apoptotic signaling

Gene expression analysis of targeted gene activity following tourniquet-induced ischemia reperfusion injury (IRI) was performed on lung, liver, kidney and injured muscle. Tissue samples were obtained after either 6 or 24 hr of whole limb reperfusion from HLI and blast HLI groups following either 90 or 150 minutes of tourniquet application. Extracted mRNA was quantified using qRT-PCR analysis and the relative gene expression is described, normalized to aged matched naïve tissue ($n=5$). Genes with a mean fold change of less than ± 2 from the naïve baseline were considered not to be significantly affected.

General trends in the remote organs showed a more pronounced effect in the blast HLI groups, at both 90 and 150 minutes, when compared to their respective HLI only groups. Upregulation of gene transcripts occurred early, with more genes and higher fold changes being present at 6 hr of reperfusion when compared to 24 hr. There was limited

evidence any late-phase upregulation, suggesting that this subset of genes is upregulated early after reperfusion.

In lung tissue, *Nos2* was the most upregulated gene, with this finding being consistent across all groups. Expression was increased in both blast injured groups at 24 hr and at 6 hr in the HLI 150 group compared to HLI alone, suggesting blast potentiates the expression of *Nos2*, which encodes nitric oxide synthase, the protein responsible for nitric oxide production. Pro-inflammatory genes such as *Il-1 β* and *Il-6* were also significantly elevated. Elevation of *Il-1 β* gene expression was most consistent in lung tissue across all timepoints when compared with other tissues types. This may represent evidence of an additional local pro-inflammatory insult resulting from the direct effect of the blast wave on the lung parenchyma. *Bid* expression, a cell death agonist, was upregulated at 6 hr with the exception of the HLI 90 group, with higher expression levels present in blast injured groups, demonstrating longer tourniquet times and or the addition of blast wave exposure increases pro-apoptotic behaviour. *S100a8*, which encode for a subunit of calprotectin, was increased with longer tourniquet time and blast exposure, providing a greater DAMP stimulus to the innate immune system following injury. *Lcn2* demonstrated a similar pattern of distribution and is expressed by neutrophils, with the differences between blast and non-blast groups reaching statistical significance with the exception of the HLI 90 6 hr timepoint. This data, combined with *Cxcl2*, a potent chemokine of monocytes and neutrophils, being elevated in the blast HLI 150 6 hr group only, is consistent with the greater expression of *Socs3*, a cytokine activity inhibitor, in blast injured groups compared to HLI alone. These findings collectively show an increase in lung injury associated gene expression, with evidence of proinflammatory and

apoptosis genes upregulation in blast injured groups compared to tourniquet alone. This upregulation more evident with longer tourniquet time and the inclusion of blast exposure leading to a more pronounced and persistent effect when observed at 24 hr of reperfusion.

Liver exhibited the greatest variation in gene expression of the remote organs. Blast wave exposure had a deleterious effect on the liver, causing significant increases in gene expression compared to HLI 150 alone at 6 hr and 24 hr of reperfusion. Increases in *Nos2*, *Atf3*, *Bcl2*, *Bcl3*, *Ccl20* and *Cxcl1* suggests a more aggerated cellular stress response, apoptosis, promotion of innate and adaptive immune response and greater chemotactic activity. These increases were apparent between the HLI 150 and blast HLI 150 groups, and with similar trends in the HLI 90 groups (Appendix 1, Tables 8 and 9). Increases in gene regulation reached significance between blast and non-blast HLI 150 groups, with the additional insult being statistically significant for *Serpine*, *Nr4a1* and *Adm* at 6 hr, and *Il-6* at 24 hr. The relative effect on blast injured groups following 90 minutes of tourniquet was less conclusive, with only small increases at 24 hr of reperfusion being present in chemoattractant genes. *Serpine1*, *Hspa1b* and *Ccl20* were the only genes consistently upregulated by the addition of blast wave exposure in the HLI 90 groups. *Cxcl1*, *Il-6*, *Lcn2*, and *Nos2* had the greatest individual fold changes with the HLI 90 groups, but addition of blast exposure did not potentiate that effect. Apoptosis associated genes were elevated by the inclusion of blast exposure; *Bid*, *Casp8*, *Bcl2*, *Myc* all had increases in mean fold change when comparing blast and non-blast groups, with *Casp8* being significantly elevated at 24 hr between HLI 150 and blast HLI 150.

Blast wave exposure resulted in more genes being differentially regulated at 6 hr of reperfusion than tourniquet use alone in kidney samples at both 90 and 150 minutes of

tourniquet. In the 90-minute groups, inclusion of blast lead to upregulation of *Bcl2*, *Bcl3*, *Bid* and *Myc*, consistent with increased cell death through apoptosis. *Casp8* also increased but fell short of the 2-fold threshold. *Nfkbiz*, *Bcl3* and *Il-6* upregulation supports blast exposure upregulation of proinflammatory signaling through the NF- κ B pathway. Increased gene expression of *S100a8*, whose protein heterodimer calprotectin signals as a DAMP through TLR4 and RAGE, and *Socs3*, a negative feedback to IL-6 signaling are also consistent. Expression of *Lcn2*, a known biomarker of AKI supports that increasing tourniquet time or blast overpressure exposure induces a functional decline in kidney performance. Evidence of oxidative stress in the kidneys is present across all groups with increased expression of *Nos2* and *S100a8*, which also has a role as an oxidant scavenger, occurring with the addition of blast exposure except with HLI 90 6 hr. A blast effect on the expression of chemoattractants such as *Cxcl1* and *Cxcl2* and cytokine *Il-6* is observed with both 90- and 150-minute tourniquet durations. As with other remote tissues, *Serpine1* is upregulated following blast. As an inhibitor of fibrinolysis, this suggests blast and tourniquet use predisposes to a prothrombotic state in the absence of a vascular injury in this model.

Gene expression in injured muscle can be used to assess the effect on both increasing tourniquet duration and blast exposure on the development of IRI. At 6 hr of reperfusion 150 minutes of tourniquet results in an increase in *Nos2* expression compared to 90 minutes, as well as upregulation of *Adm*, associated with angiogenesis. The increase from baseline to *Serpine1* is more persistent, remaining at 24 hr of reperfusion, in addition to *Il6*, *Lcn2*, *Hspa1b* and *Maff*. Most upregulated genes in the HLI 90 only group return to baseline by 24 hr, however blast injury results in in increased upregulation of

IRI-associated genes at 24 hr, with increased expression of apoptosis associated genes such as *Bcl3*, *Bid* and *Casp8*. Increased cytokine and chemokine gene expression is present with the upregulation being greater and more persistent in *Ccl2*, *Ccl20*, *Cxcl1*, *Cxcl2*, *Il1 β* and *Il6*, supporting a greater production and activity of immune mediators with blast HLI 150. At 24 hr of reperfusion, increases in *Atf3*, *Bcl2*, *Bcl3*, *Casp8*, *Cxcl2*, *Il1 β* , *Il6*, *Lcn2*, *Nfkbiz* and *Serpine1* are all significant compared to HLI 90 24 hr as a baseline.

The effect of blast exposure on gene regulation occurred earlier in the HLI 150 group, with the greatest increases being observed at 6 hr of reperfusion, rather than the 24 hr timepoint for blast HLI 90 group (Appendix 1, tables 8 and 9). Blast exposure led to upregulation of nearly all IRI-associated genes examined compared to HLI 150 alone, including increased expression of *Hif1a*, *Ccl2* and *Jun*. *HIF1a* encodes for a protein which is the master transcription factor for the cellular adaptive response to hypoxia, while *Ccl2* is chemotactic to monocytes. *Jun* is part of the AP-1 early response transcription factor. Comparison of expression levels between Blast HLI 150 and HLI 150 at 6 hr of reperfusion showed statistical significance in *Bcl2*, *Bcl3*, *Bid*, *Cxcl2*, *Gapdh*, *Hif1a*, *Il6*, *Jun*, *Lcn2*, *Myc*, *Nfkbiz*, *Nr4a1*, *S100a8*, *Serpine1*, *Socs3* and *Sod2*. Many of these significantly upregulated genes are consistent with the blast effect observed at 24 hr in the HLI 90 group, suggesting blast exposure may lead to increased tissue hypoxia (*Gapdh* and *Hif1a*), increased cellular stress and apoptosis (*Bcl2*, *Bid* and *Myc*), inflammatory response (*Bcl3*, *Cxcl2*, *Il6*, *Lcn2*, *Nfkbiz*, *S100a8* and *Socs3*) and prothrombotic state (*Serpine1*). While GAPDH is frequently used as a housekeeping gene (HKG) in qRT-PCR, there is evidence to show that GAPDH mRNA is upregulated

under hypoxic conditions(106; 317); this makes if a poor choice of HKG in trauma models, confirmed by our experimental findings and subsequent GeNorm results. Expression following 24 hr of reperfusion showed blast exposure caused an increase in apoptotic genes (*Bid*, *Myc*), oxidative stress (*Nos2*), pro inflammatory cytokines (*Il1 β* and *Il6*) and evidence of increased NF-kB signaling (*Maff* and *Nfkbiz*). Collectively this data supports that blast wave exposure increases proinflammatory signaling, combined with increased apoptosis and oxidative stress and the upregulation of known DAMPs. The effect of blast exposure is greater in the HLI 150 than HLI 90 groups, with the gene response typically occurring early post injury. Blast exposure increases the duration IRI associated genes are upregulated in muscle; in remote organ such as liver, kidney and muscle the effect on gene upregulation is limited to 6 hr with 90 minutes of tourniquet application, while with 150 minutes of application the increased effect persists to 24 hr of reperfusion.

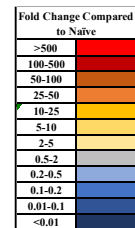
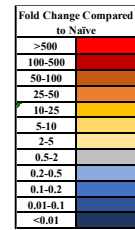
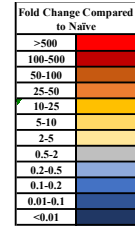
Table 3. Blast exposure increases expression of ischemia reperfusion associated genes. The expression level of 29 ischemia reperfusion associated gene targets normalized to uninjured, age-matched tissues. A heat map shows fold change, yellow through to red for upregulated genes, with light blue to dark blue for downregulated genes. Genes which were not differentially regulated ± 2 -fold expression are shaded gray.

Lung		HLI 90 6hrs	HLI 90 24hrs		Blast HLI 90 6hrs	Blast HLI 90 24hrs
Adm			0.03			0.03
Atf3			0.03		0.04	
Bcl2						
Bcl3		0.05			0.03	
Bid					0.05	
Casp8						
Ccl2						
Ccl20						
Cxcl1					0.04	
Cxcl2						
Fos						
Gapdh					0.04	
Hif1a						
Hspa1b		0.03			0.007	
Hspd1						
Il1b		<0.001	0.01		0.007	0.001
Il6						0.005
Jun						
Len2		0.007	0.02		0.04	
Maff		0.007				
Myc		0.001			0.009	0.04
Nfkbiz		<0.001	0.009		<0.001	<0.001
Nos2		<0.001	0.009		<0.001	<0.001
Nr4a1						
S100a8		<0.001	0.02		0.004	0.01
Serpine1		0.001			<0.001	0.003
Socs3		0.02			0.01	0.05
Sod2						

	HLI 150 6hrs	HLI 150 24hrs		Blast HLI 150 6hrs	Blast HLI 150 24hrs
Adm	0.03				0.03
Atf3		0.03			
Bcl2				0.04	
Bcl3	0.01			0.003	
Bid				0.02	
Casp8					0.04
Ccl2					
Ccl20					
Cxcl1					
Cxcl2				0.01	
Fos		0.03			
Gapdh	0.03			0.002	
Hif1a					
Hspa1b	0.001				
Hspd1					
Il1b	<0.001	0.03		<0.001	0.02
Il6	0.03			0.004	0.02
Jun				0.02	
Len2	0.002			<0.001	0.05
Maff	0.03			0.003	
Myc	<0.001			0.004	
Nfkbiz					
Nos2	<0.001	0.001		<0.001	<0.001
Nr4a1					
S100a8	<0.001			<0.001	0.02
Serpine1	<0.001			<0.001	
Socs3	0.02			0.001	
Sod2	0.01			0.001	

Fold Change Compared to Naive	
>500	Red
100-500	Dark Red
50-100	Orange
25-50	Yellow
10-25	Light Yellow
5-10	Light Green
2-5	Green
0.5-2	Light Blue
0.2-0.5	Blue
0.1-0.2	Dark Blue
0.01-0.1	Very Dark Blue
<0.01	Black

Liver		HLI 90 6hrs	HLI 90 24hrs		Blast HLI 90 6hrs	Blast HLI 90 24hrs
	Adm					
	Atf3					
	Bcl2					0.03
	Bcl3	<0.001	0.01		<0.001	0.05
	Bid					0.04
	Casp8					
	Ccl2					
	Ccl20				0.04	0.001
	Cxcl1	<0.001	<0.001		<0.001	0.02
	Cxcl2					
	Fos		0.02			0.03
	Gapdh				0.05	0.004
	Hif1a	0.01				
	Hspa1b	0.001			0.007	0.04
	Hspd1					
	Il1b					
	Il6		0.007			0.005
	Jun				0.02	
	Lcn2	<0.001	<0.001		<0.001	<0.001
	Maff	0.002				
	Myc					
	Nfkbiz	<0.001			<0.001	0.05
	Nos2	0.001	0.002		<0.001	0.002
	Nr4a1					
	S100a8	<0.001	0.001		<0.001	0.02
	Serpine1	0.01			0.009	0.02
	Socs3	<0.001	0.004		<0.001	0.01
	Sod2					
Kidney		HLI 90 6hrs	HLI 90 24hrs		Blast HLI 90 6hrs	Blast HLI 90 24hrs
	Adm					
	Atf3				0.02	
	Bcl2	0.03			0.001	
	Bcl3				0.03	
	Bid				0.004	
	Casp8				0.009	0.05
	Ccl2				0.003	
	Ccl20	0.02			0.02	
	Cxcl1					
	Cxcl2	0.03			0.02	
	Fos					
	Gapdh	0.03	0.03			0.005
	Hif1a					
	Hspa1b	0.006				
	Hspd1					
	Il1b					
	Il6	0.04			0.006	0.002
	Jun				0.05	
	Lcn2	0.002			0.007	0.01
	Maff	0.04			0.05	
	Myc				0.02	
	Nfkbiz	0.04			0.03	0.02
	Nos2	<0.001	0.005		<0.001	0.001
	Nr4a1				0.04	
	S100a8	<0.001	0.001		<0.001	0.001
	Serpine1	0.007	0.03		0.001	0.001
	Socs3	0.002			0.002	0.005
	Sod2					
Muscle		HLI 90 6hrs	HLI 90 24hrs		Blast HLI 90 6hrs	Blast HLI 90 24hrs
	Adm					0.01
	Atf3		0.03			
	Bcl2		<0.001			
	Bcl3					
	Bid	0.01				0.002
	Casp8					0.009
	Ccl2					
	Ccl20	0.04				0.02
	Cxcl1					0.04
	Cxcl2					
	Fos					
	Gapdh				0.010	
	Hif1a	0.02				
	Hspa1b					
	Hspd1		0.04			0.002
	Il1b	0.02				0.01
	Il6	0.04				0.001
	Jun				0.03	
	Lcn2	0.01			0.05	0.008
	Maff	0.003			0.03	
	Myc	0.03	0.02			
	Nfkbiz	0.01				0.011
	Nos2	<0.001	NaN		0.01	0.001
	Nr4a1	<0.001	0.006		0.020	0.005
	S100a8	<0.001	0.01		0.001	0.001
	Serpine1	<0.001			0.02	0.001
	Socs3	0.04				
	Sod2					
		HLI 150 6hrs	HLI 150 24hrs		Blast HLI 150 6hrs	Blast HLI 150 24hrs
	Adm				0.02	
	Atf3		0.04		0.02	
	Bcl2				0.004	0.03
	Bcl3	<0.001	0.02		<0.001	0.006
	Bid		0.03			0.003
	Casp8					0.001
	Ccl2					
	Ccl20				<0.001	0.03
	Cxcl1	<0.001	0.01		<0.001	0.01
	Cxcl2				0.001	
	Fos	0.04	0.02			0.015
	Gapdh		0.000		0.001	0.003
	Hif1a					
	Hspa1b	0.001			0.002	0.03
	Hspd1	0.002	0.04			
	Il1b					
	Il6				0.03	
	Jun		0.003		0.05	
	Lcn2	<0.001	<0.001		<0.001	0.001
	Maff	0.04			<0.001	
	Myc				0.03	
	Nfkbiz	0.005			<0.001	
	Nos2	0.001	0.001		<0.001	0.004
	Nr4a1				0.004	
	S100a8	0.000	0.002		0.004	0.01
	Serpine1	0.01			<0.001	
	Socs3	<0.001	0.007		<0.001	0.003
	Sod2				0.01	
		HLI 150 6hrs	HLI 150 24hrs		Blast HLI 150 6hrs	Blast HLI 150 24hrs
	Adm				0.007	
	Atf3					
	Bcl2					
	Bcl3					
	Bid					
	Casp8					0.001
	Ccl2				0.01	
	Ccl20	0.01				
	Cxcl1					
	Cxcl2	0.03			0.02	0.05
	Fos					
	Gapdh		0.03		0.01	
	Hif1a					
	Hspa1b	0.01			0.02	0.03
	Hspd1				0.04	
	Il1b	0.03				
	Il6	0.05			0.002	0.008
	Jun		0.04			
	Lcn2	0.003	0.02		0.001	
	Maff		0.04		0.008	
	Myc				0.006	0.02
	Nfkbiz				0.001	0.05
	Nos2	0.002	<0.001		<0.001	0.004
	Nr4a1				0.001	
	S100a8	<0.001	<0.001		<0.001	0.008
	Serpine1	0.002	0.04		<0.001	0.002
	Socs3	0.01			<0.001	0.03
	Sod2					
		HLI 150 6hrs	HLI 150 24hrs		Blast HLI 150 6hrs	Blast HLI 150 24hrs
	Adm	0.006			<0.001	
	Atf3				0.009	
	Bcl2				0.01	
	Bcl3				<0.001	
	Bid		0.04		0.001	0.02
	Casp8				0.02	0.02
	Ccl2					
	Ccl20	0.001			0.02	0.04
	Cxcl1	0.03			0.005	
	Cxcl2				0.003	
	Fos					
	Gapdh				0.006	
	Hif1a		0.03		0.003	
	Hspa1b				0.01	
	Hspd1					0.01
	Il1b	0.05			0.007	0.03
	Il6	0.04			0.001	0.05
	Jun	0.01	0.02		<0.001	0.007
	Lcn2	0.03	0.03		0.002	0.04
	Maff	0.009			<0.001	0.009
	Myc		0.05		<0.001	0.04
	Nfkbiz	0.002	0.001		<0.001	0.004
	Nr4a1	0.02	0.003		<0.001	0.001
	S100a8	0.000	0.009		<0.001	0.002
	Serpine1	0.009	0.005		<0.001	0.03
	Socs3	0.02			<0.001	
	Sod2					



Blast exposure increased cytokine profile during reperfusion injury

The inflammatory mediator profile was analyzed in serum at 6 and 24 hr of reperfusion. A Bio-Plex suspension array system was used to simultaneously analyze 23

proteins in samples using multiplex assays. The cytokines and chemokines tested were IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p70), IL-13, IL-17, IL-18, G-CSF (CSF3), GM-CSF (CSF2), GRO/KC, IFN- γ , M-CSF (CSF1), MIP-1 α , MIP-3 α , RANTES (CCL5), TNF- α , VEGF and MCP-1 (CCL2).

Following 6 hr of reperfusion, all analytes with the exception of RANTES (CCL5) showed a statistically significant increase from baseline (naïve) levels in tourniquet and blast tourniquet groups ($p < 0.001$). The effect of blast exposure alone had an overall significant ($p = 0.004-0.04$) but less pronounced systemic effect on proinflammatory mediator production than that produced by tourniquet application alone, with no change seen in RANTES (CCL5) and MIP-3 α compared to baseline levels. The majority of the inflammatory mediators studied that peaked at 6 hr following blast alone injury showed a similar pattern of progressive decrease over time, whereas GM-CSF (CSF2), GRO, MCP-1 (CCL2), MIP-3 α and RANTES (CCL5) were not significantly elevated in comparison to baseline levels at 24 hr. When comparing HLI and blast HLI groups with the same duration of tourniquet, no significant effect was observed in any analyte in the 90-minute tourniquet groups. Prolonged tourniquet application with blast exposure (blast HLI 150 group) resulted in a significant increase in the serum concentration of IL-2, IL-13, IL-17, IL-18, G-CSF (CSF3) and IFN- γ ($p = 0.003-0.03$) following 6 hr of reperfusion compared to HLI 150, demonstrating an early effect in upregulating cytokine expression as a result of the addition of blast injury.

Following 24 hours of reperfusion, circulating levels of nearly all of the 23 measured inflammatory mediator biomarkers were significantly higher in the blast HLI 150 group when compared to the HLI 150 without blast injury. Overall percentage

increases between serum concentration in the blast HLI 90 and non-blast HLI 90 groups were lower at 24 hr than their 150-minute counterparts, with percentage changes within $\pm 11\%$ compared to a consistent upregulation of expression in the 150-minute groups of 1-41%. Statistical testing of analytes confirmed significant increases in IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-10, IL12(p70), IL-13, IL-17, IL-18, GM-CSF (CSF2), IFN- γ , M-CSF (CSF1), MIP-1 α , MIP-3 α , TNF α and VEGF (Appendix 1 Table 6). The early excessive inflammatory cytokine production of these proinflammatory mediators/biomarkers in the blast HLI 150 group at 24 hr is consistent with the gene expression findings, showing increased expression of mRNA for the IL1 β , IL6 and CCL20 (MIP-3 α). Systemic production of MCP-1 (CCL2) was not significantly affected by blast, and this is also consistent with gene expression findings in the whole limb reperfusion models. Collectively, this data supports the development of a severe systemic inflammatory response syndrome (SIRS) arising from the addition of blast following 150 minutes of tourniquet, an effect which is not observed following the addition to 90 minutes of tourniquet application. Results in Figure 11 illustrates the systemic inflammatory response and the systemic profiles for several key inflammatory mediators/biomarkers. Full details of blast effect changes and statistical significance can be found in Appendix 1 Table 6.

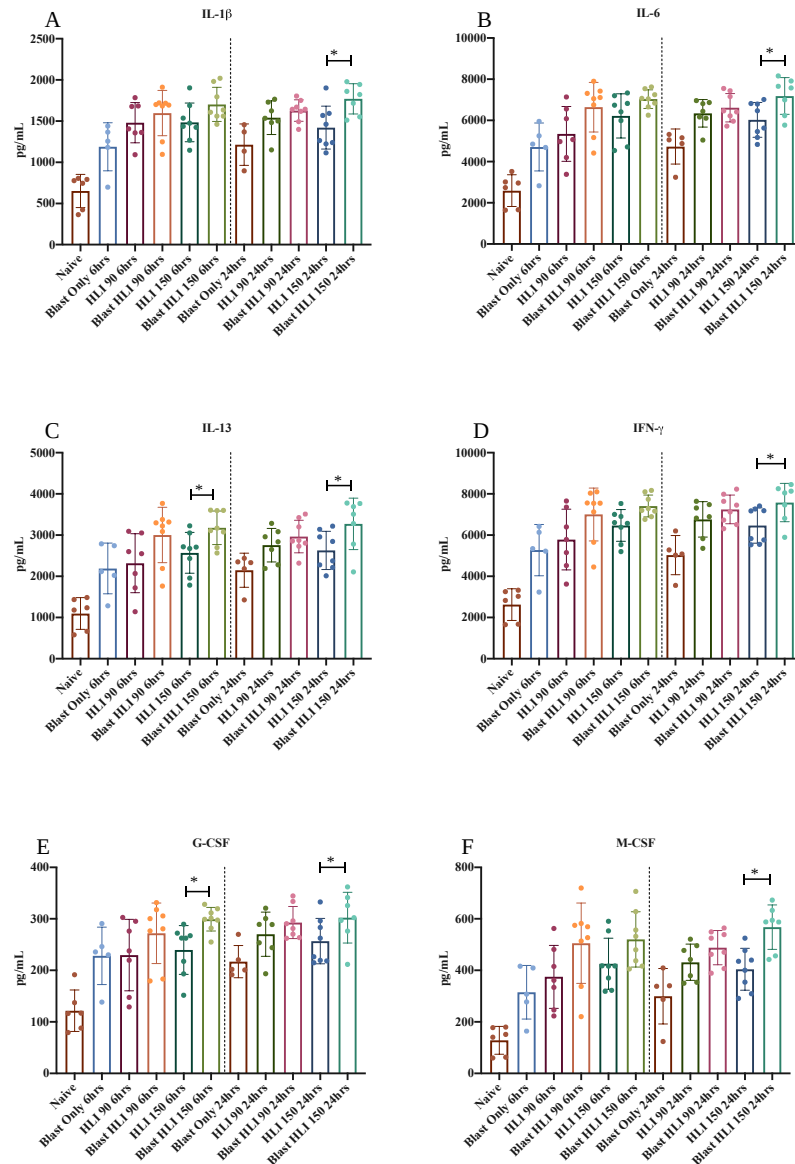


Figure 11. Blast exposure results in increased cytokine and chemokine signaling following 150 minutes of tourniquet. Serum expression of key inflammatory mediators detected by multiplex assay. Graphs show mean±SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as *P≤0.05, **P≤0.01, ***P<0.001.

Histological findings support biochemical evidence of increased organ injury

Histological assessment was performed on H&E stained tissues samples (n=5 per group) by a veterinary pathologist blinded to group and injury pattern. Assessment was

performed on lung, liver, kidney and injured skeletal muscle. Due to insufficient formalin inflation of lung tissue at time of necropsy in several groups, the accurate structural assessment of lung injury was not possible using H&E staining and was therefore excluded.

Assessment of liver tissue in the whole limb reperfusion model using non-parametric testing as scoring systems are ordinal, demonstrated that when injury scores were combined to assess the overall impact on the organ, blast only exposure had no effect on injury scores, while all other groups exhibited a statistically significant change from naïve controls in their median scores ($p < 0.001-0.05$) (Fig. 12A). When cumulative injury scores were compared, no difference between blast and non-blast groups scores at 6 hr or 24 hr of reperfusion was observed histologically, though blast injured typically exhibited higher median scores. Hepatic apoptosis levels were elevated in all blast HLI groups compared to naïve, with greater evidence at 6 hr in both blast HLI groups, but this effect was not significant against HLI only groups (Fig. 12B). Portal hepatocyte radiation was increased 6 hr post-injury compared to naïve ($p < 0.001-0.02$), with this effect greater in both blast HLI 90 and 150 groups compared to HLI only ($p = 0.14$ and $p = 0.02$ respectively) (Fig. 12C). Combined with the hepatocyte apoptosis in the portal region, portal hepatocytes underwent elongation, where Periodic acid–Schiff (PAS; stain for polysaccharides) negative intracytoplasmic pseudo-inclusions were more abundant in blast exposed animals that had higher histology scores at 6 hr post-injury. By transmission electron microscopy (TEM) these cytoplasmic pseudo-inclusions appear to contain cytoplasm, loose smooth endoplasmic reticulum and are devoid of other organelles (Fig. 13). Hepatic cord dissociation was significant in all groups compared to

naïve controls at 6 hr of reperfusion ($p<0.001-0.02$), and was less pronounced by 24 hr, and showed no significant differences with the addition of blast to HLI groups.

Significant increases in multinucleate hepatocytes were observed in all treatment groups at 6 hr post injury, compared to naïve control ($p=0.001-0.05$).

High magnification examination of kidney tissue from all treatment groups revealed apoptosis/necrosis of renal tubular epithelial cells, commonly in the outer stripe of the medulla (OSOM), compared to the cortex. After 6 hr of reperfusion, only blast HLI groups showed differences in the cortical region of the kidney when compared to tissue from naïve controls ($p=0.03$), with increases in renal cell apoptosis delayed and occurring later at 24 hr post injury in HLI only groups ($p=0.003-0.02$) (Fig. 12D). Differences in grading for the number of apoptotic cells between blast and non-blast HLI at 6 hr fell short of significance in the 90-minute group (2 vs 0.5, $p=0.08$) and at 150-minutes (2 vs 0, $p=0.29$). With respect to the OSOM, mean ranks were higher at 6 hr similar to the cortex, however there was no significance compared to naïve, this is in part due to mild apoptosis being a normal clinical finding. Assessment of tubular proteinosis, cellular infiltration and tubular basophilia showed no significant variation across reperfusion groups.

Comparison of combined skeletal muscle injury scores did not show any additional injury effect of blast over tourniquet use alone. Individual scores post-ischemia reperfusion injury showed limited variation in edema, cellular infiltrates, hemorrhage and fibroplasia. Perivascular inflammation was present, but no differences were noted between groups. Myocyte degeneration and necrosis was the most common

finding, with all groups showing evidence of injury with score significantly elevated from naïve control, but no significant differences between blast and non-blast HLI groups.

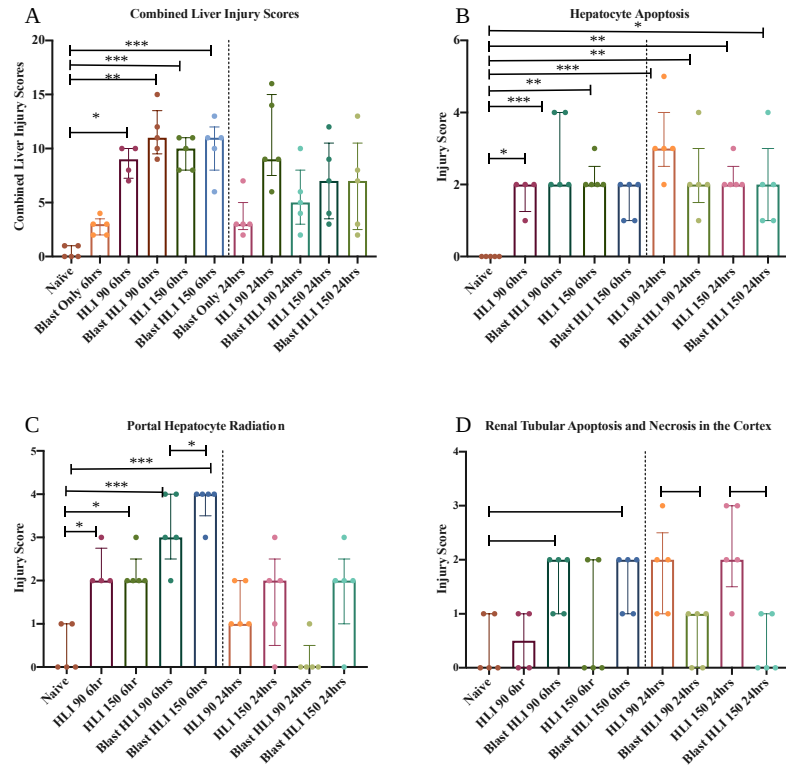


Figure 12. Variation in histological injury scores indicate blast exposure increases structural damage effects of tourniquet IRI. Histological injury scores based on H&E staining. Graphs show medium $\pm$ IQR with statistical analysis using Krushal-Wallis test and post-hoc uncorrected Dunn's test. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$.

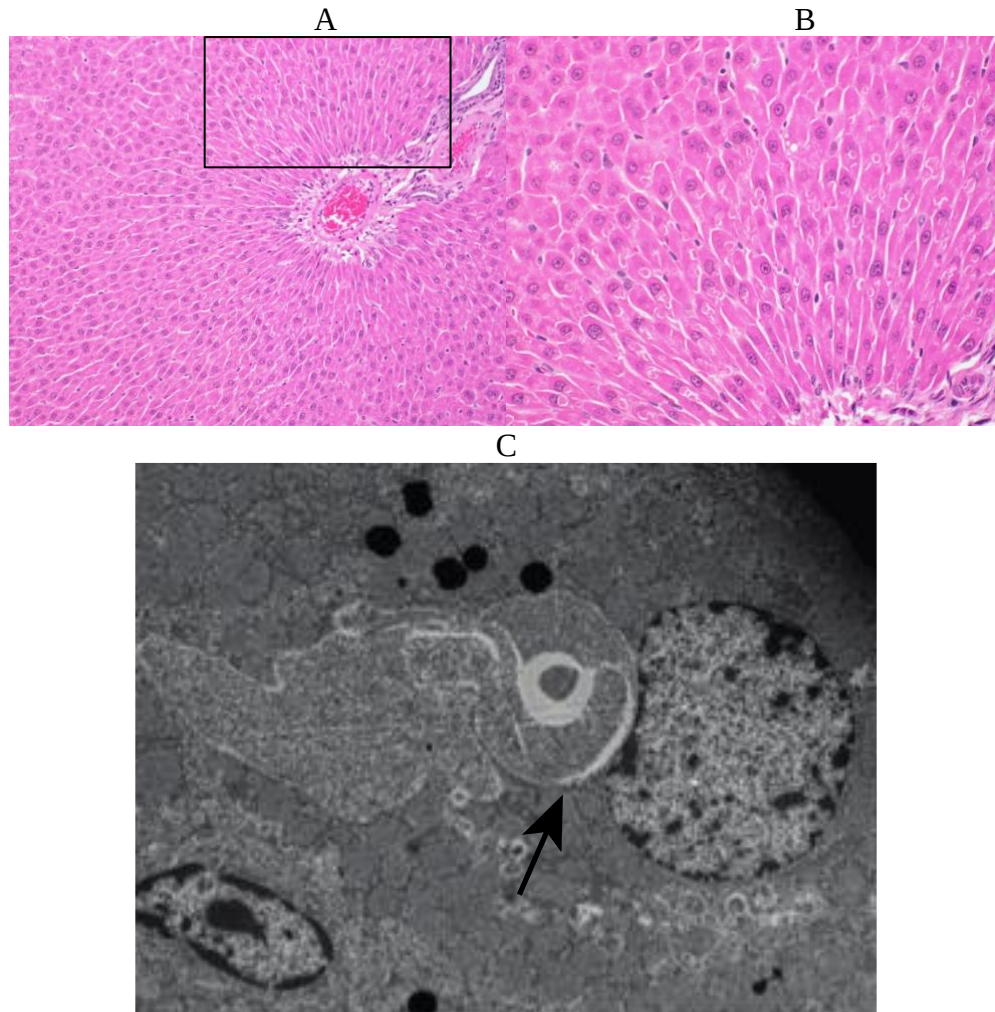


Figure 13. Blast injury combined with whole limb reperfusion following tourniquet induced ischemia-reperfusion injury results in hepatocyte radiation and development of hepatocyte pseudo-cytoplasmic inclusions. (A) Liver, portal region, elongating/ spindling/radiating hepatocytes with intracytoplasmic inclusions, H&E, 20X. (B) Same region, H&E, 40x; inclusions were PAS negative. (C) Liver, hepatocyte containing an intracytoplasmic pseudo-inclusion causing compression of the nucleus TEM, 3000x.

THE EFFECTS OF TOURNIQUET INDUCED HIND LIMB ISCHEMIA ON A BLAST COMPLEX INJURED LIMB AND THE DEVELOPMENT OF SECONDARY INJURY

Having investigated the effects of blast wave exposure on whole limb reperfusion injury, simulating an isolation vascular injury, it was necessary to address the effect tourniquets may have on a complex injured limb. Blast-associated traumatic amputation has become a signature in pattern of the past two decades, with trans-femoral amputations and extensive soft tissue damage a common injury pattern. Using our established rodent model of blast-associated complex lower limb trauma and amputation we assessed the effect of 90- or 150-minutes of tourniquet on the development of systemic inflammatory and remote organ injury.

Blast wave exposure results in increased biochemical evidence of both local tissue damage and remote organ dysfunction

Tourniquet use is associated with a limited but persisting elevation of renal injury markers

Serum BUN was assessed for variation between groups using Kruskal-Wallis ANOVA at 6 hr, 24 hr, Day 3 (Day 3) and Day 7 (D7) following tourniquet removal and compared against levels obtained from uninjured naïve control rats and rats subjected to blast injury, femoral fracture, soft-tissue crush injury, and limb amputation through the zone of injury (complex control injury model) without tourniquet use. At 6 hr post injury, serum BUN varied significantly between groups ($p < 0.001$), with injured groups exhibiting elevated median BUN levels by 57-129% compared to uninjured naïve controls (33.0-48.0 mg/dL vs 21.0 mg/dL, $p < 0.001$), but with only a 18-33% increase between complex controls and complex HLI 90 or 150 treatment groups ($p = 0.46-0.57$) (Fig. 14A). At 24 hr BUN remained elevated above baseline values in the treatment groups ($p = 0.02$), with only complex HLI 90 showing a difference on post hoc testing

against naïve ($p=0.001$) and blast only controls ($p=0.1$) (Fig. 14B). The increased BUN levels seen with complex HLI 90 fell short of significance against the complex control group ($p=0.11$). At Day 3 post injury, the complex HLI 150 treatment group had higher BUN values overall compared to the naïve ($p=0.05$), blast only ($p=0.06$), and complex injury control group ($p=0.59$).

Changes in serum creatinine were more pronounced in the complex injured group than the complex HLI groups (90 or 150 minutes). At 6 hr of reperfusion, only serum creatine levels in the complex control group were elevated above naïve baseline ($p=0.03$), with both complex control and complex HLI 150 elevated against blast only control ($p=0.02$ and $p=0.05$ respectively) (Fig. 14C). At 24 hr post injury, all injured groups had increased serum creatinine levels compared to controls, with both complex control ($p=0.003$ and $p=0.01$) and complex HLI 90 ($p<0.001$ and $p=0.002$) having significantly elevated serum creatinine levels compared to both naïve and blast only groups (Fig. 14D).

Correlation analysis using Spearman's rank test between all serum BUN and creatinine results from the complex injured groups (r_s 0.679, $p<0.0001$), with sub-analysis at 6 hr (r_s 0.228 $p=0.41$), 24 hr (r_s 0.402, $p=0.05$), Day 3 (r_s 0.457, $p=0.07$) and Day 7 (r_s 0.512, $p=0.006$) showing that the correlation between BUN and creatinine improves with time, further supporting that notion that creatinine is an inappropriate renal marker to predict or detect early onset AKI in the context of traumatic injury or ischemia reperfusion.

Analysis of BCR ratios (Figs 14E,F) showed all complex groups demonstrated a statistically significant shift to higher ratios at all timepoints ($p=0.008$ - 0.02), except 24 hr

($p=0.08$), with a trend to higher ratios with tourniquet use, suggesting that tourniquet use and IRI further increased hypoperfusion of the kidney. Median values were higher in tourniquet groups compared to complex control group at all timepoints but failed to reach significance, likely due to group sizes ($n=7-8$).

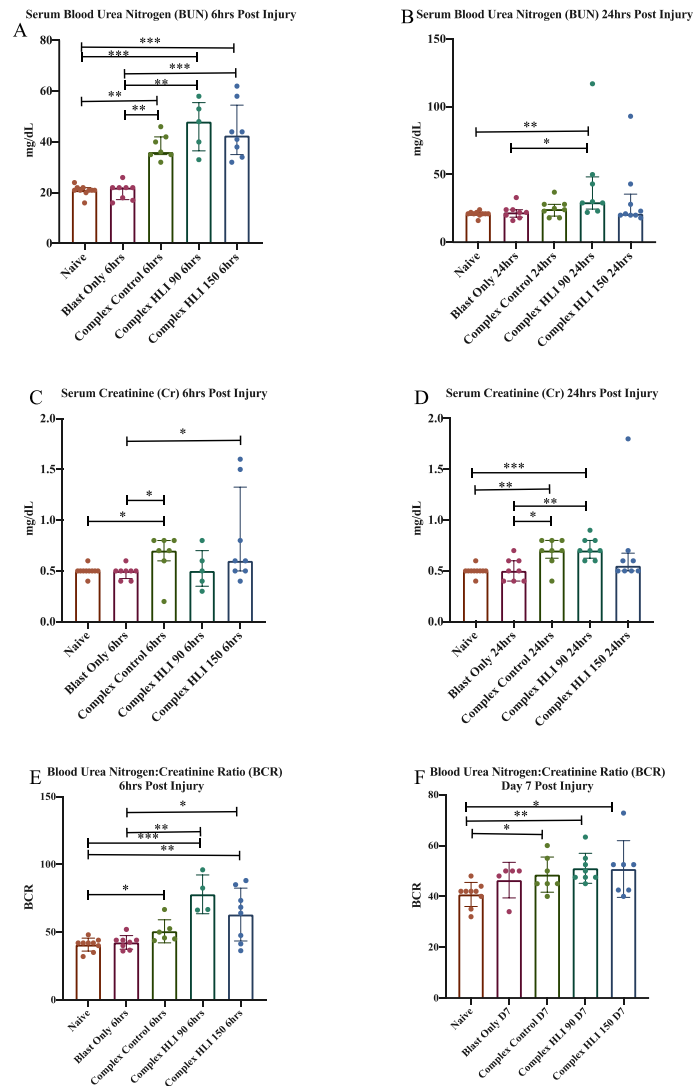


Figure 14. Application of tourniquets to a complex injured limb results in a small increase in AKI markers at 6 hr Serum blood urea nitrogen (BUN) and creatinine (Cr) levels. (A,B) Serum BUN levels after 6 hr (A) and 24 hr (B) post injury, (C,D) Serum Cr levels after 6 hr (C) and 24 hr (D) post injury, (E,F) BUN:Cr ratio (BCR) after 6 hr (E) and Day 7 (F) post injury. Graphs show median and 95% CI with Kruskal-Wallis test

with post-hoc uncorrected Dunn's test as non-parametric data. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Tourniquet application causes a trend to greater early hepatocellular damage than a complex injury pattern alone

Hepatocellular injury as measured by ALT was elevated at 6 hr in complex control and complex tourniquet injured groups when compared to naïve control values. Mean ALT in the complex control group was increased 91% (110.6 ± 10.4 units/mL, $p=0.002$) compared to naïve (58.0 ± 2.9 units/mL), with a 178% increase seen in the complex HLI 150 group (186.0 ± 50.5 units/mL, $p=0.04$), however post hoc comparison between these complex HLI 150 and complex control groups did not reach significance ($p=0.18$). At 24 hr, mean ALT levels increased 139% in the complex control group and 136-157% in the tourniquet groups compared to naïve and blast only groups ($p < 0.001$) (Fig. 15A,B). By postoperative Day 3 and 7 ALT levels had normalized across all injury groups.

Higher levels of serum LDH were detected at 6 hr in the complex tourniquet groups compared with the control group. LDH serum levels increased 90% ($p=0.09$), 72% ($p=0.03$), and 36% ($p=0.11$) in the complex HLI 90, complex HLI 150, and complex control injury groups, respectively relative to naïve control levels. At 24 hr post injury, the tourniquet induced augmentation of serum LDH production had resolved.

Serum ALP showed no variation between groups at 6 hr post injury ($p=0.42$), however at 24 hr ($p=0.002$) through to Day 3 ($p=0.001$) and Day 7 ($p < 0.001$), ALP levels in the injured groups were comparatively lower than those measured in naïve controls or blast only groups at the respective timepoints (Fig. 15E,F). Post hoc testing confirmed that this effect was present in all treatment groups when compared to naïve and blast only

groups, supporting that this is a true effect and not as a result of an elevated baseline.

These decreases in mean serum ALP levels varied from 13-48% from 24 hr to Day 7 post injury.

In comparison to baseline and naïve control values, serum albumin levels were statistically lower at all timepoints from 6 hr to Day 7 in the complex control and tourniquet groups ($p < 0.001-0.04$). Differences failed to reach statistical difference on post hoc testing between the median values of complex control and the complex HLI groups at 6 hr, 24 hr and Day 7, but bordered significance at Day 3 between complex control and complex HLI 150 (2.9 vs 2.6 g/dL, $p = 0.05$) (Fig. 15C,D).

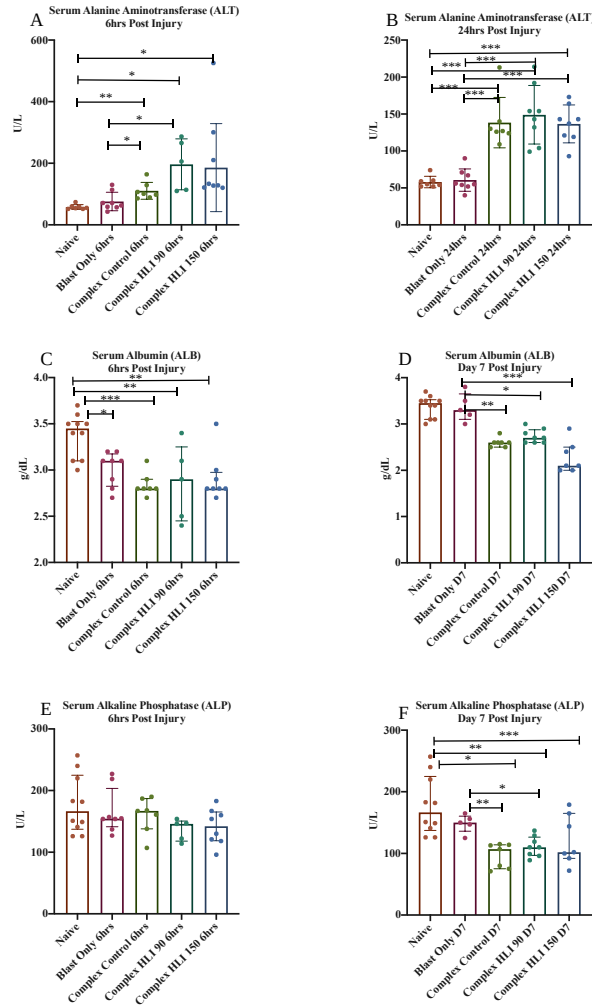


Figure 15. Tourniquets increase early markers of hepatocellular injury compared to traumatic amputation alone (A,B) Serum ALT levels after 6 hr (A) and 24 hr (B) post injury, (C,D) Serum ALB levels after 6 hr (C) and Day 7 (D) post injury, (E, F) Serum ALP after 6 hr (E) and Day 7 (F) post injury. Graphs demonstrate mean and 95% CI for parametric data with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction (A,B), or median and 95% CI with Kruskal-Wallis test with post-hoc uncorrected Dunn's test for non-parametric data (C-F). Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Tourniquets are associated with greater evidence of muscle damage in the first 24 hr post injury

Comparison of the AST:ALT ratio showed no variation at 6 hr between treatment groups ($p=0.26$), however at later timepoints variation between treatment groups were observed ($p<0.001-0.01$). At 24 hr, complex tourniquet injured groups ratios had

increased significantly against both naïve and blast only groups ($p<0.001-0.003$), with complex HLI 90 being markedly increased compared to complex control (5.68 ± 0.34 vs. 7.22 ± 0.48 , $p=0.02$), suggesting greater muscle damage. By Day 3, the rise in AST:ALT ratio persisted in complex control and tourniquet groups compared to naïve ($p=0.002-0.009$), and this continued at Day 7 ($p<0.001-0.02$), with no inter-group difference detected (Fig. 16A,B).

Analysis of AST levels at 6 hr demonstrated increases in both complex control and complex tourniquet groups compared to naïve ($p<0.001-0.01$) and blast only ($p=0.002-0.03$). While median complex HLI 150 levels had a greater rise at 109% compared to 80% in the complex control group, this difference failed to reach significance ($p=0.56$). Variation in groups continued at 24 hr to be different from both naïve and blast only ($p<0.001-0.002$), reaffirming that a 120 kPa blast overpressure exposure alone has negligible effect on muscle without a secondary injury, but with no differences detected between injured groups (Fig. 16C,D). At Day 3 and 7 post injury there was no variation between groups detected ($p=0.66$ and $p=0.20$ respectively). This supports that muscle damage and release of AST is an early phenomenon occurring in the first 24 hr post injury and that levels trended towards higher expression in complex tourniquet groups over complex control.

Serum creatinine kinase (CK) was significantly elevated in complex control (523%, $p=0.002$) and complex HLI 150 (364%, $p=0.02$) treatment groups at 6 hr when compared to values from uninjured naïve rats, and the blast only treatment group (821% and 586%, $p<0.001$), though the difference between the two injured groups was not significant ($p=0.38$). At 24 hr post injury, there was no significance difference in CK

levels between injured and blast only rats, with complex HLI 150 being lower compared to naïve ($p=0.01$) which is likely to be artifact given the absence of difference to blast only 6 hr group. At Day 3 and Day 7 amputated groups exhibited lower CK levels than either naïve or blast only groups (Day 3 $p=0.02$), which may represent a latent effect from blast only exposure.

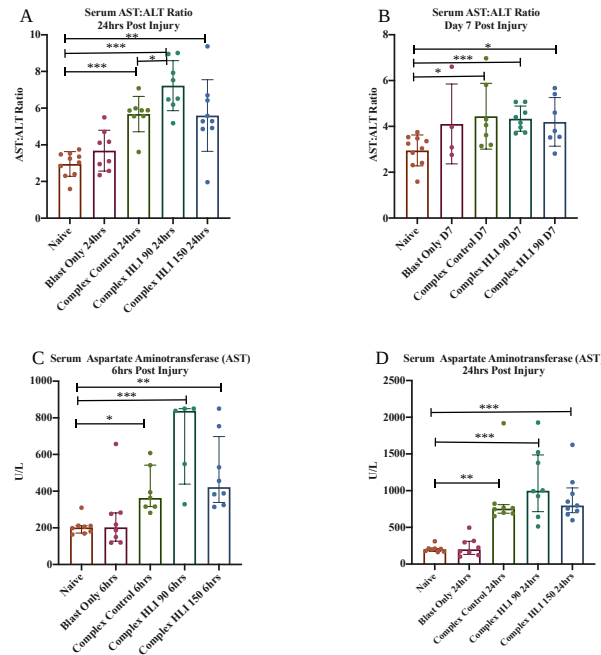


Figure 16. Tourniquets exacerbate early muscle damage in the first 24 hr post traumatic amputation (A,B) Serum AST:ALT ratio after 24 hr (A) and Day 7 (B) post injury, (C,D) Serum AST levels after 6 hr (C) and 24 hr (D) post injury. Graphs demonstrate mean $\pm$ SD for parametric data with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction (A,B), or median and IQR with Kruskal-Wallis test with post-hoc uncorrected Dunn's test for non-parametric data (C,D). Significance shown as * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$.

Tourniquets cause a greater and more prolonged lung injury evidenced by markers of increased lung permeability

Analysis of wet-to-dry lung weight ratios showed no significance differences at 6 or 24 hr between groups ($p=0.08$ and 0.45), with mean ratios increasing across all groups

slightly higher than naïve control measurements. At Day 3 post injury overall variation between groups was not significant ($p=0.12$), potentially in part due to this timepoint having a smaller sample size ($n=5$) compared to other timepoints ($n=7-8$), however direct comparison of complex HLI 150 to naïve was significant (4.80 ± 0.14 vs 4.58 ± 0.15 , $p=0.03$) while the complex control group was not (4.67 ± 0.24 , $p=0.53$). At Day 7 post injury, the effect was more pronounced between injured groups ($p<0.001$), with the wet-to-dry lung weight ratios of both complex HLI 90 (4.91 ± 0.14) and complex HLI 150 (4.95 ± 0.11) remaining significantly elevated compared to naïve (4.59 ± 0.15 , $p<0.001-0.002$), blast only (4.66 ± 0.05 , $p<0.001-0.001$) and complex control (4.72 ± 0.19 , $p=0.01-0.04$). Blast only groups and complex control groups showed no significant differences between themselves ($p=0.39$) or naïve ($p=0.16-0.32$). This result demonstrates that increased fluid accumulation and infiltration persists in the lungs longer following tourniquet use than in the standard complex injury model, supporting either increased initial injury from IRI, or greater persistence and/or magnitude of systemic inflammation leading to increased lung permeability.

Total leukocyte analysis was performed on BALF. At 6 and 24 hr post injury there were significant variations between results ($p<0.001$). Both timepoints were associated with a fall in BALF total leukocyte count in all complex injury $\pm$ tourniquet groups compared to naïve and blast only controls. At 6 hr post injury a significant rise in total leukocyte count (61%, $p=0.05$) was measured in the blast only group, while counts in the complex control and complex HLI 90 and 150 were reduced 32-48% compared to uninjured naïve controls ($p<0.001-0.01$) and 58-68% compared to the blast only injured group ($p=0.003-0.008$), with no intra-complex injured group variation. This effect may be

as a result of the injured limb acting as a larger stimulus to recruit circulating neutrophils in the immediate post injury phase prior to any systemic or secondary injury effect. By 24 hr, mean BALF total leukocyte levels had begun to recover but were still mildly depressed in the complex injured groups. Further increases in total counts were seen by Day 3 leading to a positive increase of 10-45%. This continued through to reaching significance in the complex HLI 90 tourniquet group compared to naïve at Day 7 ($p=0.03$). Adjustment of total BALF results relative to returned lung wash volume demonstrated similar findings with a reduction in total leukocytes in the injured groups at 6 hr against both naïve and blast only. Recovery in leukocyte numbers at 24 hr again was confirmed, persisting through Day 3 with Day 7 seeing a slight elevation of total leukocytes, reaching significance in the complex HLI 90 group (84% increase, $p=0.05$).

Total protein concentration in lung BALF significantly increased in complex controls (135%, $p=0.004$), complex HLI 90 (119%, $p=0.004$) and complex HLI 150 (89% $p=0.05$) groups at 6 hr post injury compared to baseline values (naïve uninjured controls). At 24 hr, all groups had an increased BALF protein concentration relative to baseline values, however total protein concentration only remained significantly increased in the complex HLI 90 group (83%, $p=0.02$). At Day 3 post injury total protein concentration was elevated 79% ($p=0.07$) and 19% ($p=0.44$) in the complex HLI 150 group and complex injury groups, respectively compared to naïve controls. By Day 7 there was no measurable differences in the total protein concentration in the BALF in any of the groups. Collectively this data supports that while initial elevations in lung injury within the first 6 hr may be similar, tourniquet associated groups developed a greater and more prolonged lung injury evidenced by markers of increased lung permeability.

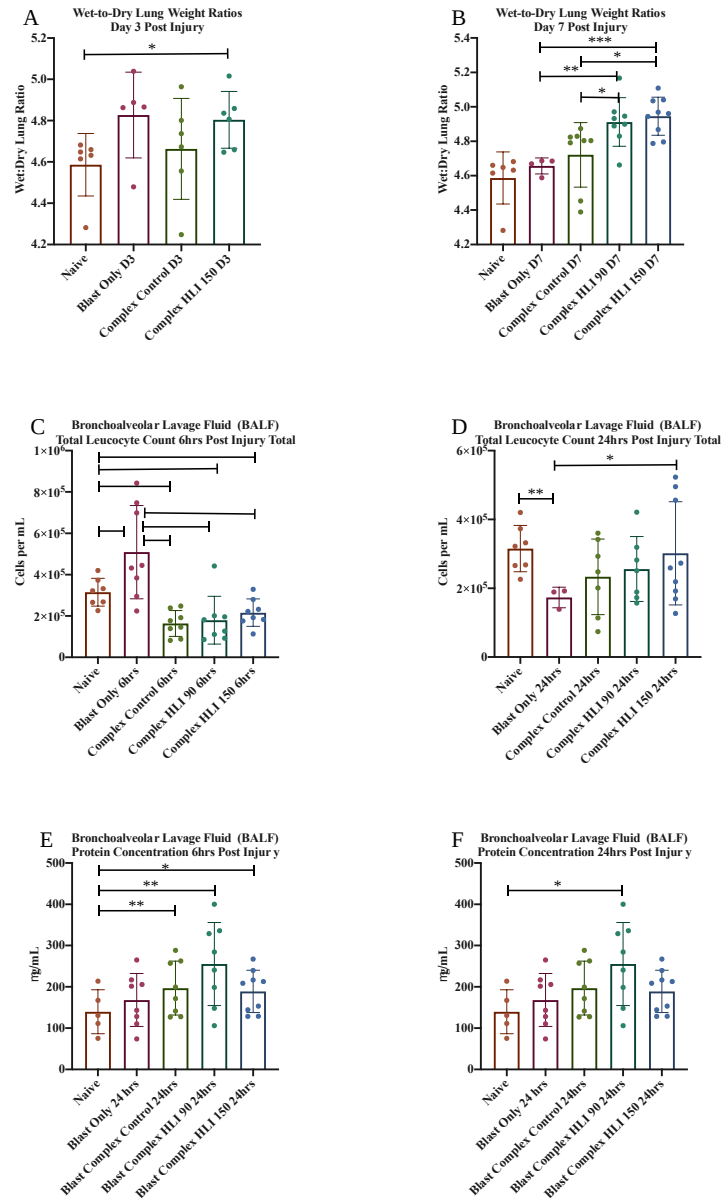


Figure 17. Tourniquet use in a complex injured limb leads to increased lung permeability (A,B) Wet-to-dry ratios at Day 3 (A) and Day 7 (B) post injury. (C,D) show BALF total cell counts at (C) 6 hr, and (D) 24 hr post injury. (E,F) BALF protein concentration at (E) 6 hr and (F) 24 hr post injury. Graphs demonstrate mean $\pm$ SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Tourniquets increase total WBC due to a greater and more sustained neutrophilia

Whole blood was evaluated using for total WBC, and the number and percentage of neutrophils, monocytes and lymphocytes. This confirmed an early neutrophilia in the

complex injured groups against both naïve and blast only controls ($p<0.001-0.004$). While all complex injured groups were elevated against naïve (42-49% vs 12.6, $p<0.001$), only complex HLI 90 and HLI 150 were significant against blast only ($p=0.03-0.008$), with no difference between complex injured groups observed ($p=0.36$). Total monocyte counts increased earlier than in HLI models, with both complex control and complex HLI 90 elevated ($p=0.03-0.003$) (Fig. 18A).

The early neutrophilia seen at 6 hr continued at later time points with tourniquet use increasing neutrophil count at 24 hr compared to complex control (2.8-3.0 vs $1.9 \times 10^3/\mu\text{l}$, $p=0.17-0.22$). By Day 3 post-injury, total WBC was increased in the complex HLI groups against naïve (7.6-9.8 vs $4.4 \times 10^3/\mu\text{l}$, $p=0.002-0.02$), but not in the complex control group (6.9 vs $4.4 \times 10^3/\mu\text{l}$, $p=0.06$); complex HLI 90 was also significantly elevated compared to the complex control ($p=0.005$) (Fig. 18B). This suggests that the inflammatory response in the tourniquet exposed complex injury groups reaches a greater magnitude and persists longer, with more inflammatory cells present systemically. This was due to increases in monocytes and lymphocytes, with lymphocytes increased in complex HLI 90 and HLI 150 groups against blast only ($p<0.001-0.02$), with the difference also significant against complex control in the complex HLI 90 group (7.1 vs 4.5, $p<0.002$), suggestive of a larger early adaptive response in complex HLI groups compared to complex control.

At 7 days post injury the difference in WBCs was most distinct. Total WBC levels in complex HLI groups remained elevated against naïve and blast only (7.6-7.8 vs $4.4 \times 10^3/\mu\text{l}$, $p=0.05-0.10$ and 7.6-7.8 vs $3.5 \times 10^3/\mu\text{l}$, $p=0.04-p=0.007$), with complex control WBC elevation no longer significant against naïve or blast only ($4.9 \times 10^3/\mu\text{l}$,

p=0.65 and p=0.08). Differences between complex control and complex HLI groups fell short of significance (p=0.06-0.14). Neutrophil counts were greater in complex HLI groups than complex control (2.6-2.8 vs 1.6 $\times 10^3/\mu\text{l}$, p=0.02-0.07) confirming a persisting effect at 7 days post injury of tourniquet application on the innate immune response to a complex injured limb.

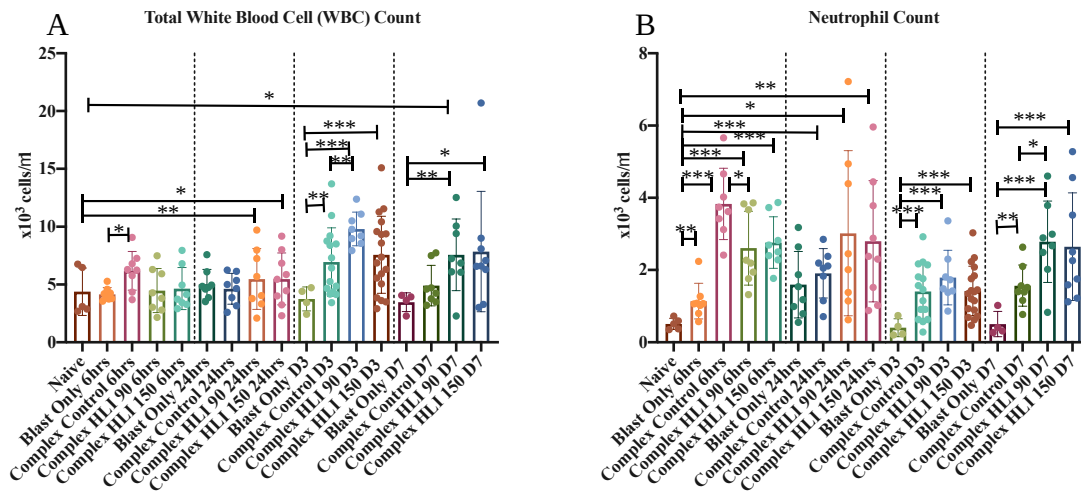


Figure 18. Tourniquet use in a complex injured limb increases total white cell and neutrophil counts at Day 3 and 7 post injury
Whole blood analysis of white cell count (WCC) and neutrophil assessed between 6 hr and Day 7 post injury. The graph shows mean and SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$.

Endothelial dysfunction is greater in tourniquet groups evidenced by prolonged ICAM-1 elevation

Assessment of serum ICAM-1 in the complex injury model at 6 hr post injury showed no difference between groups (p=0.11). At 24 hr post injury there was variation between groups (p=.02), wherein serum ICAM-1 levels were increased in blast complex HLI 90 (32%, p=0.004) and HLI 150 (22%, p=0.03) compared to blast only, while serum level of ICAM-1 levels in the blast complex control group were only modestly increased (18%, p=0.10). Serum ICAM-1 levels remained significantly elevated at Day 3 post

injury in all groups versus blast only ($p<0.001-0.01$), with complex HLI 90 increased 42% compared to complex control ($p=0.007$). This finding persisted at Day 7 when there was a 29% difference between complex HLI 90 and complex control ($p=0.03$), demonstrating a persistence of the endothelial dysfunction marker ICAM-1 systemically in tourniquet groups beyond complex injury alone (Fig. 19).

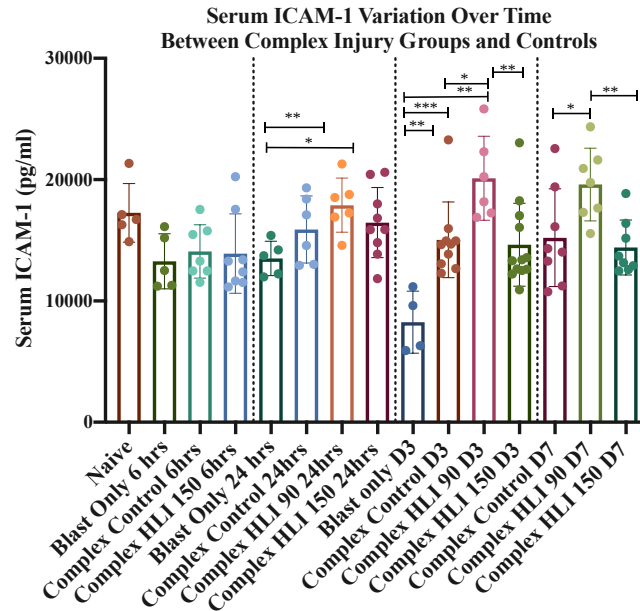


Figure 19. Prolonged expression of endothelial dysfunction marker ICAM-1 (CD54) seen with tourniquet use

Marker of endothelial dysfunction ICAM-1 was assessed from 6 hr to Day 7 post injury. The graph shows mean $\pm$ SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Prolonged tourniquet use increases serum AKI biomarkers with both a greater expression and duration

Serum Cystatin C levels rose at 6 hr post injury in all groups compared to naïve, except blast only, with only complex control (62%, $p=0.02$) and complex HLI 90 (111%, $p=0.04$) reaching significance compared to blast only controls. At 24 hr, serum Cystatin C levels continued to rise with all groups significant against blast only controls by 64-

137% ($p=0.002-0.02$). Serum levels continued to be significantly elevated through to Day 7 post injury compared to blast only controls; at Day 3, complex control was increased 79% ($p=0.007$) and complex HLI 150 53% ($p=0.04$), while Day 7 showed greater persistence of Cystatin C in complex HLI 150 (54%, $p=0.05$) compared to blast only controls, relative to complex control (42%, $p=0.005$) (Figure 20A).

Analysis of serum KIM-1 levels from 6 hr to Day 7 in the complex injured groups showed no variation from naïve or blast only control levels consistent with findings in the tourniquet models and suggesting that this is not an appropriate blood biomarker and its use may be limited to urine detection only.

Serum NGAL levels in the complex injury models showed significant changes across the time course of the study, whereas the amount of serum NGAL was similar in blast only groups as compared to naïve baseline controls at all timepoints ($p=0.05-0.41$). At 6 hr post injury, serum NGAL levels were significantly different between tourniquet and complex control groups (complex HLI 90 493% increase, $p=0.009$ and complex HLI 150 184% increase, $p=0.008$). At 24 hr, a further significant rise in serum NGAL in the complex HLI 150 group was detected in comparison to serum NGAL levels in the complex control group (85%, $5,743 \pm 1,159$ vs $3,112 \pm 1,863$ ng/ml, $p=0.009$) as well as against complex HLI 90 (70%, $5,743 \pm 1,159$ vs $3,369 \pm 887$ ng/ml, $p<0.001$) group suggesting that inclusion of a tourniquet time of 150 minutes causes a renal injury significantly greater than traumatic amputation alone. At Day 3 post injury serum NGAL remained significantly elevated in both complex control and complex HLI 150 groups against naïve control ($p=0.004$) and blast only ($p=0.003$), wherein levels in the complex HLI 150 group were 16% higher than complex control but this difference was not

significance ($3,030 \pm 1,355$ vs $2,604 \pm 1,165$ ng/ml, $p=0.57$). At Day 7 serum NGAL levels in all complex injury groups were significantly elevated over naïve and blast only controls ($p<0.001-0.05$), however serum NGAL concentrations in the complex control group were closer to baseline ($1,499 \pm 1,403$ vs 186 ± 44 ng/ml, $p=0.05$), compared with complex HLI groups ($1,642 \pm 710$ vs 186 ± 44 ng/ml, $p=0.004$). Increased serum NGAL activity in the complex HLI 150 group persisted through to Day 7 ($4,512 \pm 1,858$ ng/ml, $p<0.001$) and nearly 200% and 175% higher as compared with that in the complex control ($p=0.006$) and complex HLI 90 ($p=0.005$) groups (Fig. 20B). This data supports the hypothesis that prolonged tourniquet use increases AKI in a blast associated complex limb injury model, with both a greater magnitude and duration of serum AKI biomarkers detectable when compared to either shorter tourniquet durations or traumatic amputation alone.

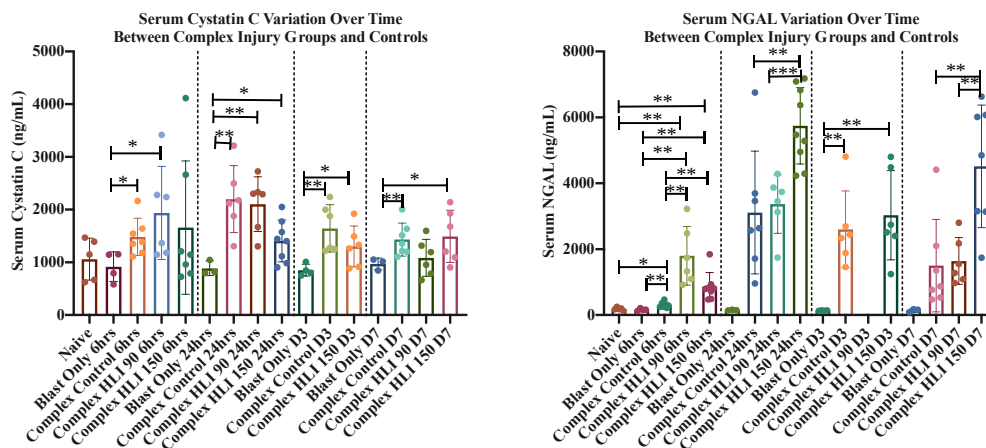


Figure 20. Serum AKI markers are more persistent following tourniquet application. The graph shows mean $\pm$ SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$.

Damage associated molecular patterns (DAMPs) expression are increased by tourniquet use

Analysis of HMGB1 in the complex injured groups demonstrated significant rises as early as 6 hr post injury. At the timepoint, plasma concentrations in the complex HLI 150 (1.08 ± 0.19 ng/ml) were significantly elevated compared to naïve (0.32 ± 0.06 ng/ml, $p=0.006$), blast only (0.36 ± 0.04 ng/ml, $p=0.008$) and the complex control groups (0.43 ± 0.07 ng/ml, $p=0.01$). Complex HLI 90 reached significance against naïve only (0.54 ± 0.07 vs 0.32 ± 0.06 ng/ml $p=0.05$). At 24 hr post injury, this trend continued with complex HLI 150 remaining significantly elevated compared to naïve, blast only and both complex control and HLI 90 levels ($p<0.001-0.002$). At Day 3 post injury, the complex HLI 150 group remained significantly elevated against naïve levels (0.66 ± 0.09 ng/ml $p=0.02$), though no longer compared to complex control (0.50 ± 0.09 ng/ml, $p=0.28$). Seven days post injury, levels detected of HMGB1 were still significantly elevated in complex control (0.75 ± 0.09 ng/ml, $p<0.001$ and 0.003), complex HLI 90 (0.99 ± 0.17 ng/ml, $p=0.005$ and 0.003) and complex HLI 150 groups (0.59 ± 0.08 ng/ml, $p=0.05$ and $p=0.009$) compared to naïve and blast only, but despite increased concentration in the tourniquets groups, were not significant against complex control ($p=0.13-0.22$) (Fig. 21A).

In both the complex HLI 90 and HLI 150 groups, plasma calprotectin levels were elevated at 6 hr post complex injury when compared to uninjured naïve control (29-42%, 375.2 ± 119.6 and 410.4 ± 77.2 vs 290.0 ± 69.7 ng/ml, $p=0.21$ and 0.02) and blast only control (66-82%, 226.1 ± 22.4 ng/ml, $p=0.05$ and $p<0.001$) values. Moreover, the complex HLI 150 group exhibited significantly higher levels of serum calprotectin than

the complex control group (40%, $p=0.01$). At 24 hr post injury, the amount of circulating calprotectin in the complex HLI groups remained elevated in comparison to the uninjured control and complex control group (complex HLI 90 449.0 ± 63.6 and complex HLI 150 374.1 ± 77.9 vs complex control 329.7 ± 52.3 ng/ml, $p=0.005$ and $p=0.06$). At Day 7, serum calprotectin values remained significantly elevated in all injury groups when compared to uninjured control values ($p<0.001-0.01$), furthermore serum calprotectin values in complex HLI groups remained higher than in the complex control group, and was significant for complex HLI 90 (34%, 566.3 ± 99.1 vs 422.5 ± 79.2 ng/ml, $p=0.007$)) (Fig. 21B). Calprotectin is associated with inflammation and this data supports that tourniquet use compound the inflammatory response following a complex lower limb injury, with the effect still detectable at 7 days post injury.

Analysis of TREM-1 in the complex injured models demonstrated rises in serum expression at 6 hr in both complex control and complex tourniquet groups when compared to naïve or blast only ($p=0.002-0.05$). The complex HLI 150 group had 20% higher mean serum TREM-1 levels than the complex control group but this did not reach significance ($1,113\pm281$ vs 927 ± 139 pg/ml, $p=0.12$). As observed in the HLI models, serum TREM-1 levels continued to rise at 24 hr, with all group values significantly different to naïve or blast only controls ($p<0.001-0.04$). Expression of TREM-1 was 24% higher in the complex HLI 150 group as compared to the complex control group, narrowly failing to reach significance at 24 hr post injury ($1,572\pm406$ vs $1,271\pm235$ pg/mL, $p=0.08$) (Fig. 19C). At Day 3, further significant increases in TREM-1 in the serum were detected in the tourniquet groups with a smaller increase in the complex control group, resulting in both tourniquet groups being significantly elevated (46-79%)

compared to complex control (complex HLI 90 (2,185±297 vs 1,224±244 pg/mL, $p<0.001$) and complex HLI 150 (1,781±464 vs 1,224±244 pg/mL, $p=0.002$). Day 7 data demonstrated all complex injured groups continued to be elevated compared to blast only and naïve controls ($p<0.001$), but the inter-group difference between complex control and complex HLI 150 now only 14% (1820±456 vs 2079±686 pg/ml, $p=0.38$). An additional analysis was performed using serum collected at 3 hr post injured confirmed early expression of TREM-1 in serum, as was found in the HLI whole limb reperfusion model. Both complex control and complex HLI 150 models were elevated from naïve and blast only controls at 3 hr ($p=0.001-0.008$) supporting the use of TREM-1 as an early marker of injury burden.

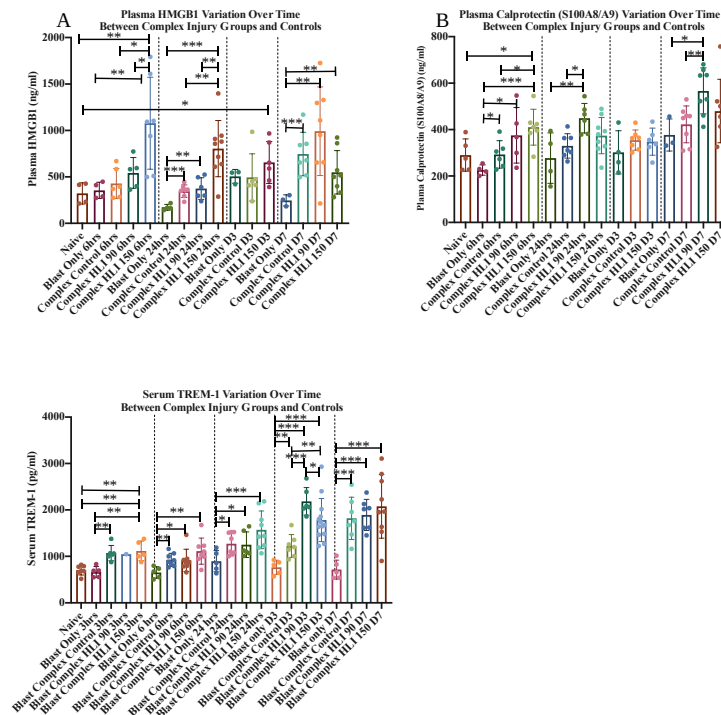


Figure 21. Damage associated molecular patterns (DAMPs) are increased by tourniquet application compared to traumatic amputation alone
 The graphs show mean±SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P\leq0.05$, ** $P\leq0.01$, *** $P<0.001$.

Tourniquet use is associated with evidence of increased oxidative stress both systemically and at site of injury

Analysis of lipid peroxidation as a marker of oxidative stress and damage at 6 hr post injury showed both complex control and complex HLI 150 groups to have elevated levels compared to naïve ($p=0.002-0.005$) and blast only ($p=0.02-0.06$) with plasma levels similar to that induced by whole limb reperfusion in the blast HLI 150 group (3.2-3.6 vs 3.6 μM). At 24 hr post injury, plasma levels continued to increase and remained significant against naïve (4.2-4.3 vs 2.2 μM , $p<0.001-0.002$) and bordered significance against blast only (4.2-4.3 vs 2.8 μM , $p=0.05-0.06$) (Fig. 22A). There was no difference detected between complex control and complex HLI 150 at either timepoint. Analysis of MDA in homogenized muscle adjusted for protein concentration at 6 hr post injury showed a trend to higher levels in the complex HLI 150 group by 40%, with the difference falling short of significance. This trend persisted at 24 hr, with levels 80% higher than naïve and 23% higher than the complex control group, demonstrating a consistent, non-significant trend to higher levels of oxidative stress in the tourniquet affected injured muscle.

Analysis of plasma and muscle tissue for evidence of DNA or RNA oxidation in complex injured groups demonstrated no changes at 6 hr post injury, with no elevation from baseline at this timepoint, similar to the whole limb HLI models. At 24 hr post injury mean levels were elevated in the complex HLI 150 group significantly against both naïve (3473 vs 1590 pg/ml, $p=0.01$) and complex control groups (3473 vs 1877 pg/ml, $p=0.04$), suggesting that tourniquet associated IRI increases oxidative stress above that of complex injury and amputation (Fig. 22B). Analysis of 8-OHdG expression in extracted RNA was increased at 6 hr post injury and was significant between complex HLI 150 and

the blast only group at this timepoint (917 vs 640 pg/ml, $p=0.004$). At 24 hr post injury, the complex HLI 150 group continued to express higher levels of 8-OHdG but did not reach significance against any other group (Fig. 22C).

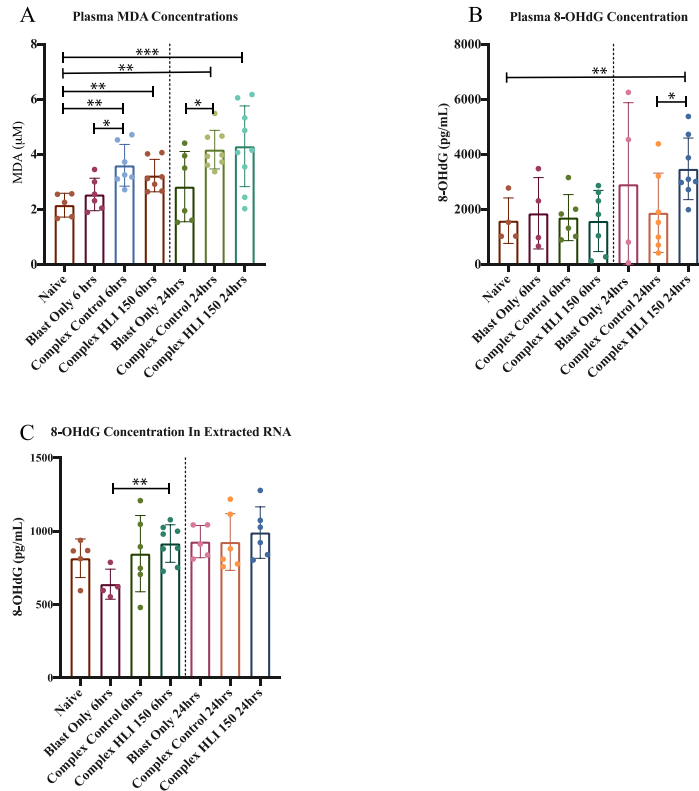


Figure 22. Tourniquet application leads to increased evidence of oxidative stress in plasma and RNA compared to complex injury alone
The graph shows mean±SD with statistical analysis using ANOVA and post-hoc unpaired T-test with Welch's correction. Significance shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Tourniquet use further upregulates ischemia reperfusion injury associated genes predisposing to a proinflammatory state both locally and systemically

Analysis of IRI gene expression in lung tissue from complex control and complex HLI 150 groups demonstrated the most upregulation at 6 hr post injury with significant changes compared to normal uninjured tissue (naïve) in both groups (Table 4). Relative expression between complex HLI 150 and complex control demonstrated predominantly increased expression in the complex HLI 150 group, the only notable exception being

Lcn2 (p=0.02) (Appendix 1 Tables 10 and 11). *Lcn2* and *Nfkbiz* (p=0.04) were the only genes that reached significance between groups at 6 hr. Similar to the HLI whole limb models, *Nos2* saw the both the biggest absolute and relative increase in gene expression between the complex models, consistent with increases oxidative stress (Appendix 1 Tables 10 and 11). As both models were exposed to blast overpressure, this likely to represent evidence of a remote injury effect from tourniquet use. Gene expression was otherwise consistent with an early proinflammatory response (*Il1 β* , *Il6*, *Cxcl2*, *S100a8*, *Nfkbiz*), with apoptotic and cellular stress mechanisms being upregulated (*Atf3*, *Bcl3*, *Bid*). Evidence of increased DAMPs genes with high expression of both *S100a8* and *Hspa1b* support their release being associated with increased inflammatory response and both were expressing higher levels in the complex HLI 150 group. Both *Cxcl2* and *Fos* had some of the higher fold change differences between groups, supportive of early AP-1 upregulation and monocyte and neutrophil activation. Differences between the complex HLI 150 and complex control group were predominantly confined to the early 6 hr period. At 24 hr to Day 7 *Nos2*, *S100a8* remained the most significantly elevated in comparison to normal healthy lung tissue (naïve), the levels of which persisted longer in the complex HLI 150 group.

Liver tissue demonstrated more prolonged upregulation of IRI associated genes when comparing complex HLI 150 to complex control than seen in the lung tissue. While both groups exhibited high levels of upregulation of *Il6*, *Lcn2*, *Nos2*, *S100a8* and *Serpine1*, the magnitude and persistent of these signals were significantly greater in the complex HLI 150. Expression of *Il6* was consistently greater from 24 hr to Day 7 (p=0.009-0.04). *Maff*, which can be induced by proinflammatory cytokines was also

upregulated. Persistence of gene increases between the complex control and complex HLI 150 group were more consistent and significant than in lung tissue. *Ccl2*, *Ccl20*, *Cxcl1* and *Cxcl2* were consistently upregulated in both groups across all timepoints, with the exception of *Ccl2* at 6 hr. Chemokines are known to be upregulated following acute liver injury and cell death and all of these chemokines are associated with liver IRI injury (234). Their production can arise from injury to both hepatocytes and kupffer cells, promoting macrophage and neutrophil infiltration into the liver. *Ccl2*, *Ccl20* and *Cxcl2* expression was greater in the complex HLI 150 models than complex control at both Day 3 ($p=0.003-0.03$) and Day 7 ($p=0.06-0.48$), supportive of a greater acute liver injury following tourniquet use in the complex injured models. *Serpine1*, whose protein plasminogen activator inhibitor-1 (PAI-1) acts as an acute phase protein and is elevated in liver injury, was elevated compared to complex control at 6 hr post injury ($p=0.02$). This trend continued through to Day 7, further supporting a persistence of an increased acute response within the liver.

Evidence of remote effects in the kidney demonstrated similar distribution of upregulated genes at 6 hr post injury in both complex control and complex HLI 150 groups when compared to the naïve state, with minimal differences in absolute fold change seen at this timepoint between groups (Appendix 1 Table 11). The most notable trend was seen in *S100a8* and *Nos2* where over the time course the relative and absolute difference in expression between these groups remained elevated, supporting of a prolonged insult leading to oxidative stress and oxidant scavenging. *Cxcl1*, *Cxcl2* and *Il6* expression remained higher in the complex HLI 150 group than control consistent with prolonged inflammation in the renal parenchyma at later timepoints.

As the site of direct injury, muscle exhibited the greatest changes in gene expression of all tissue types. All genes were upregulated with the exception of *Sod2*. Comparison of the complex HLI 150 group against complex control showed further upregulation in the complex HLI 150 group, with significance reached for *Adm*, *Hsp1ab* and *Jun*. *Adm* is associated with angiogenesis, while *Hsp1ab* encodes for HSP70, a known DAMP which is upregulated in response to thermal or oxidative stress. *Nos2* and *S100a8* experienced the highest absolute levels of increase between injured groups but didn't reach significance to the large confidence intervals in these injured groups. Inflammatory mediator genes such as *Cxcl1*, *Cxcl2*, *Il1 β* and *Il6* also demonstrated increase over the complex control group, with *Cxcl1* returning to baseline by Day 3 but remaining elevated in the tourniquet groups. Apoptosis genes were more highly expressed in the complex HLI 150 group (*Bcl3*, *Bid*) at all timepoints with the inclusion of a tourniquet. Both *Fos* and *Jun* were significantly elevated at 6 hr compared to naïve in only the complex HLI 150 group; both are AP-1 subunits and the heterodimer in this structure is associated with apoptosis. Collectively this is consistent with increased cellular stress with increased oxidative stress responses and cellular apoptosis in the tourniquet injured groups, with persistence of chemotactic signaling and inflammation locally in the muscle tissue despite early amputation leading to increased muscle tissue dysfunction.

Table 4. Ischemia reperfusion genes are upregulated by tourniquet use in a complex traumatic amputation

The expression level of 29 IRI associated gene targets normalized to uninjured, age-matched tissues. A heat map shows fold change, yellow through to red for upregulated genes, with light blue to dark blue for downregulated genes. Genes which were not differentially regulated ± 2 -fold expression are shaded gray.

Lung		Complex Control 6hrs	Complex Control 24hrs	Complex Control Day 3	Complex Control Day 7
Adm			0.002		
Atf3	0.001				0.05
Bcl2				0.010	
Bcl3	0.003	0.03	0.009	0.02	
Bid	0.03		0.01	0.009	
Casp8			0.03		
Ccl2					
Cd20					
Cxcl1					
Cxcl2	0.001				
Fos					
Gapdh	0.03		0.005	0.01	
Hif1a					
Hspa1b	0.02		0.04		
Hspd1					
Il1b	<0.001	<0.001	0.01	0.005	
Il6	<0.001	<0.001	0.007		
Jun	0.04		0.03		
Lcn2	<0.001		0.009		
Maff	0.005		0.03		
Myc	<0.001		0.003	0.01	
Nfkbiz	0.02		0.02	0.05	
Nos2	<0.001	<0.001	<0.001	<0.001	
Nr4a1					
S100a8	<0.001	<0.001	<0.001	0.006	
Serpine1	<0.001		<0.001	0.02	
Socs3	0.001	0.004	0.006	0.005	
Sod2	0.007		0.05	0.03	
Liver		Complex Control 6hrs	Complex Control 24hrs	Complex Control Day 3	Complex Control Day 7
Adm	0.003				
Atf3	0.01	0.03			
Bcl2		0.001	0.02	0.003	
Bcl3	<0.001	<0.001	<0.001	<0.001	
Bid		0.02	0.009	0.009	
Casp8	0.008		0.012	0.003	
Ccl2		0.05		0.04	
Cd20	0.003	<0.001	0.002	0.001	
Cxcl1	<0.001	<0.001	<0.001	<0.001	
Cxcl2	<0.001	0.001			
Fos					
Gapdh					
Hif1a					
Hspa1b	0.003	0.01	0.04		
Hspd1	0.006				
Il1b			0.02	0.002	
Il6	0.01	0.002	0.04		
Jun					
Lcn2	<0.001	<0.001	<0.001	<0.001	
Maff	<0.001			0.04	
Myc					
Nfkbiz		0.005	<0.001	<0.001	
Nos2	<0.001	<0.001	0.003	<0.001	
Nr4a1	0.004				
S100a8	<0.001	<0.001	<0.001	<0.001	
Serpine1	0.001	0.008			
Socs3	<0.001	<0.001	<0.001	<0.001	
Sod2					

	Complex HLI 150 6hrs	Complex HLI 150 24hrs	Complex HLI 150 Day 3	Complex HLI 150 Day 7
Adm		0.001		0.03
Atf3	<0.001			
Bcl2	0.03			
Bcl3	0.002			
Bid	0.022			
Casp8				
Ccl2				
Cd20				
Cxcl1		0.05		
Cxcl2	0.001			
Fos	0.03			
Gapdh	0.02			
Hif1a		0.01		0.009
Hspa1b	0.007			
Hspd1				
Il1b	0.007			0.003
Il6	0.002	0.02	0.02	0.003
Jun	0.003			
Lcn2	0.001			
Maff	0.002			
Myc	<0.001			
Nfkbiz	0.002			
Nos2	<0.001	<0.001	0.001	<0.001
Nr4a1				
S100a8	<0.001	0.02	<0.001	<0.001
Serpine1	<0.001			0.05
Socs3	<0.001		0.05	
Sod2	0.001			

	Complex HLI 150 6hrs	Complex HLI 150 24hrs	Complex HLI 150 Day 3	Complex HLI 150 Day 7
Adm		0.03		
Atf3	0.01			
Bcl2	0.05	0.05	0.001	0.04
Bcl3	<0.001	0.001	0.04	0.002
Bid				
Casp8	0.02			
Ccl2		0.05	0.001	0.02
Cd20	0.04	0.01	0.002	0.009
Cxcl1	<0.001	0.001	<0.001	<0.001
Cxcl2	<0.001	0.04	0.02	0.002
Fos				
Gapdh	0.008			
Hif1a				
Hspa1b	0.01	0.02		
Hspd1	0.009			
Il1b				0.02
Il6	0.03	0.001	0.002	0.004
Jun	0.006			
Lcn2	<0.001	<0.001	0.001	<0.001
Maff	<0.001	0.03		0.009
Myc	0.002			
Nfkbiz	<0.001	0.006	0.04	<0.001
Nos2	<0.001	0.003	0.005	0.001
Nr4a1	0.001			
S100a8	<0.001	<0.001	<0.001	<0.001
Serpine1	<0.001	0.003	0.03	0.02
Socs3	<0.001	<0.001	0.002	<0.001
Sod2				

Fold Change Compared to Naive	
>500	
100-500	
50-100	
25-50	
10-25	
5-10	
2-5	
0.5-2	
0.2-0.5	
0.1-0.2	
0.01-0.1	
<0.01	

Fold Change Compared to Naive	
>500	
100-500	
50-100	
25-50	
10-25	
5-10	
2-5	
0.5-2	
0.2-0.5	
0.1-0.2	
0.01-0.1	
<0.01	

Kidney		Complex Control 6hrs	Complex Control 24hrs	Complex Control Day 3	Complex Control Day 7
Adm					
Atf3	0.003	0.04	0.006	0.001	
Bcl2				<0.001	
Bcl3	<0.001		0.04	<0.001	
Bid			0.01	0.001	
Casp8			0.001	<0.001	
Ccl2	0.008			0.03	
Cd20	0.04	0.04			
Cxcl1		0.01		0.04	
Cxcl2	0.02	0.01	0.05		
Fos					
Gapdh	0.002		0.001	0.008	
Hif1a				0.004	
Hspa1b	0.005			0.02	
Hspd1	0.003				
Il1b		0.05		0.002	
Il6	0.002	0.001	0.008	0.006	
Jun					
Lcn2	0.006	0.03		0.004	
Maff	0.004				
Myc	<0.001				
Nfkbiz	<0.001	0.002	0.03	0.007	
Nos2	<0.001	0.001	0.003	0.001	
Nr4a1	0.003			0.03	
S100a8	<0.001	<0.001	<0.001	<0.001	
Serpine1	<0.001	0.001	0.004	0.002	
Socs3	<0.001	<0.001	0.01	<0.001	
Sod2					
Muscle		Complex Control 6hrs	Complex Control 24hrs	Complex Control Day 3	Complex Control Day 7
Adm		<0.001	0.06	0.009	0.008
Atf3					
Bcl2	0.001			0.001	
Bcl3	<0.001	0.02	<0.001	0.001	
Bid	0.001	0.03	<0.001	<0.001	
Casp8			<0.001	0.002	
Ccl2	0.007	0.04			
Cd20	0.004	<0.001	0.001	0.009	
Cxcl1	0.004	0.02			
Cxcl2	0.001	0.02		0.005	
Fos					
Gapdh					
Hif1a	0.005	0.04	<0.001	0.02	
Hspa1b					
Hspd1		0.003		0.02	
Il1b	0.002	0.02	0.008	0.004	
Il6	<0.001	0.002	0.02	0.04	
Jun					
Lcn2	0.002	<0.001	0.002	0.004	
Maff	<0.001	0.001	0.008		
Myc	<0.001	0.001	0.001	0.006	
Nfkbiz	<0.001	0.002	0.004	0.01	
Nos2	<0.001	0.005	0.002	<0.001	
Nr4a1	0.03	0.006	0.003		
S100a8	<0.001	0.003	<0.001	<0.001	
Serpine1	<0.001	0.005	0.02		
Socs3	<0.001	0.01	0.002	0.004	
Sod2				0.02	

	Complex HLI 150 6hrs	Complex HLI 150 24hrs	Complex HLI 150 Day 3	Complex HLI 150 Day 7
Adm				
Atf3		0.03	0.05	
Bcl2		0.03	0.05	
Bcl3		0.04	0.04	
Bid				
Casp8		0.02		
Ccl2	0.002			
Cd20		0.008		
Cxcl1		0.02		
Cxcl2	0.01	0.02	0.03	
Fos				
Gapdh			0.05	
Hif1a				
Hspa1b	0.05	0.02	0.03	
Hspd1	0.03			
Il1b		0.03		
Il6	0.002	0.003	0.02	
Jun	0.02			
Lcn2	0.007	<0.001	0.01	
Maff	0.001			
Myc	0.02	0.007		
Nfkbiz	0.001	0.02	0.01	
Nos2	<0.001	0.003	0.02	
Nr4a1	0.01	0.05		
S100a8	<0.001	<0.001	<0.001	<0.001
Serpine1	<0.001	<0.001		
Socs3	<0.001	0.001	0.009	
Sod2				

Fold Change Compared to Naive	
>500	
100-500	
50-100	
25-50	
10-25	
5-10	
2-5	
0.5-2	
0.2-0.5	
0.1-0.2	
0.01-0.1	
<0.01	

Fold Change Compared to Naive	
>500	
100-500	
50-100	
25-50	
10-25	
5-10	
2-5	
0.5-2	
0.2-0.5	
0.1-0.2	
0.01-0.1	
<0.01	

Application of tourniquet to a complex injured limb results in a heightened and prolonged systemic inflammatory response

Multiple inflammatory mediators/biomarkers were assessed in the serum collected from serial blood samples as previously described in the whole limb reperfusion model. Comparison was made between the complex control group and both complex HLI 90 and complex HLI 150 between 6 hr and 7 days post injury. A 2-way ANOVA model with correction for multiple testing using Tukey test was performed to assess variation by group and time.

At 6 hr post injury, complex HLI 90 and 150 models exhibited higher serum concentrations of all the assayed inflammatory biomarkers with the exception of VEGF.

A greater number of biomarkers were significantly elevated in the complex HLI 90 group relative to complex control (IL-1 α , IL-2, IL-4, IL-5, IL-7, IL-10, IL-12(p70), IL-17, IL-18, G-CSF (CSF3), GM-CSF (CSF2), GRO/KC, RANTES (CCL5) and MCP-1 (CCL2) compared to only IL-7, IL-10, GM-CSF (CSF2), M-CSF (CSF1) and MCP-1 (CCL2) in the complex HLI 150 group. This finding may be explained by greater variance of serum concentrations in the complex HLI 150 group affecting statistical power compared to complex HLI 90, as the overall mean values of both groups are similar. At 24 hr post-injury, serum biomarker concentrations had continued to rise in all complex injured groups. Statistically significant variations between the complex control and complex HLI groups became more notable. In addition to the cytokines and chemokines upregulated after 6 hr, significant further elevations in IL-1 β , IL-6, IL-13, IFN- γ , M-CSF (CSF1) and TNF- α were detected in the complex HLI 90 group in addition to the increases in serum IL-1 α , IL-1 β , IL-5, IL12(p70), IL-18, G-CSF (CSF3), IFN- γ and TNF- α in the complex HLI 150 group. These findings suggest a more significant early core proinflammatory response. With the exception of IL-2, IL-4, IL-6 and IL-13, the pattern of increased gene expression was similar across both tourniquet groups when compared to complex control serum concentrations. MCP-1 (CCL2), which was significantly elevated compared to control in both tourniquet groups, also demonstrated a significant difference between complex HLI 90 and HLI 150 (HLI 90 vs HLI 150, 5200 vs 6343, $p < 0.001$).

By Day 3 post injury, while complex HLI 90 typically exhibited increased concentration of serum analytes compared to the complex control group, most differences no longer reached statistical significance, with the exception of IL-1 β , IL-5 and TNF- α

($p=0.006-0.03$). Persistence of these cytokines is suggestive of increased pro-inflammatory signaling, with $\text{TNF-}\alpha$ known to activate the inflammasome, which leads to $\text{IL-1}\beta$ maturation and release. The complex HLI 150 group continued to exhibit higher serum concentrations than the complex control group at Day 3 in all analytes assessed ($p<0.001-0.03$). All these increases were statistically significant with the exception of GRO/KC , $\text{MIP-3}\alpha$, RANTES (CCL5) and VEGF ($p=0.24-0.60$).

Differences were also observed between complex HLI 90 and HLI 150 groups in $\text{IL-1}\alpha$, IL-2 , IL-5 , IL-7 , IL-10 , IL-12(p70) , IL-17 , G-CSF (CSF3) , GM-CSF (CSF2) , $\text{MIP-1}\alpha$, VEGFA and MCP-1 (CCL2) ($p<0.001-0.04$).

At Day 7 post-injury most biomarkers in the complex HLI 90 group had resolved to levels in the complex control group. IL-4 ($p=0.003$), G-CSF (CSF3) ($p=0.04$) and $\text{IFN-}\gamma$ ($p=0.04$) remained elevated by 35-44% compared to controls. $\text{TNF-}\alpha$ and IL-6 remained elevated by 28-41% but fell short of significance on adjusted p -value modelling ($p=0.06-0.13$). In the complex HLI 150 group all analytes except GRO/KC , RANTES (CCL5) and VEGF were still significantly elevated compared to complex control ($p<0.001-0.02$) 7 days post injury, with average increases in serum analyte concentrations ranging from 16-94%. Inter-group differences between complex HLI 90 and 150 groups were statistically significant ($p<0.001-0.04$) for all analytes except, IL-4 , IL-6 , IL-12(p70) and G-CSF (CSF3) , which were the analytes still or near being significantly increased in the complex HLI 90 group. The only exception to this is $\text{IFN-}\gamma$ which was elevated 42% compared to control in complex HLI 90 ($p=0.04$) and 88% with 150 minutes of tourniquet ($p<0.001$), with the difference between tourniquet groups remaining significant ($p=0.01$). This data demonstrates that in a complex injured model,

tourniquet application leads to an increased and prolonged inflammatory response systemically. The additional systemic inflammatory response generated by 90 minutes of tourniquet had resolved by 7 days post-injury, while 150 minutes of tourniquet results in further prolongation of the systemic inflammatory response beyond 7 days post injury. Results in Figure 23 and Appendix 1 Table 7 illustrate the systemic heightened and prolonged systemic inflammatory response/profile in the complex HLI 150 group for several key inflammatory mediators/biomarkers.

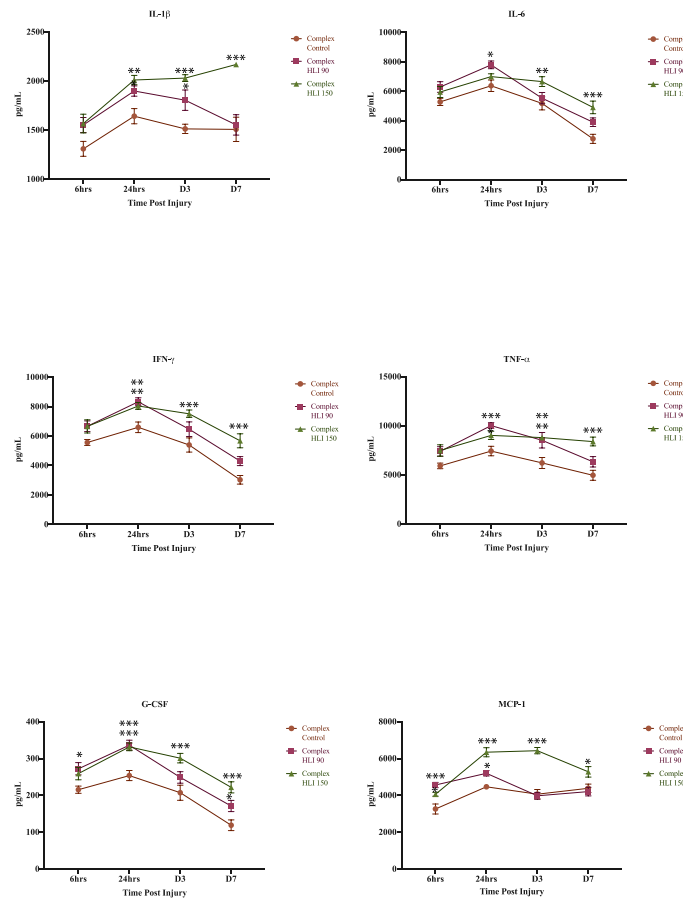


Figure 23. An increased and prolonged systemic inflammatory response occurs following tourniquet application to a complex injured limb. Serum concentrations (pg/mL) of IL-1 β , IL-6, IFN- γ , TNF- α , G-CSF (CSF3) and MCP-1 (CCL2) between 6 hours and Day 7 post injury in the complex control and complex HLI 90- and 150-minute groups. Graphs show mean and SEM with statistical testing using 2-way ANOVA with Tukey corrected for multiple testing. Significance shown as *P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001.

Histology confirmed evidence of increased remote organ injury from tourniquet use a complex injured limb

Based on cumulative mean histological assessment liver injury scores from 6 hr to Day 7 post injury, no differences in median scores were noted between the complex control and complex HLI groups. We detected elevated levels of hepatocyte apoptosis at Day 3 ($p=0.09$) and Day 7 ($p=0.09$) post injury between complex control and either complex HLI 90 or 150. Portal radiation which was a feature of the whole limb reperfusion models, and also showed differences between complex control and complex HLI models at 24 hr post-injury; complex HLI 90 elevated (3.0 vs 1.0, $p=0.002$) and HLI 150 (2.0 vs 1.0, $p=0.06$), with the difference resolved by Day 3. Cord dissociation was unaffected by addition of tourniquet, however increased hepatocyte mitosis was seen in both the complex HLI 90 ($p=0.03$) and HLI 150 ($p=0.04$) groups at 6 hr post injury when compared to complex control liver slides. A significant increase in multinucleate hepatocytes was noted at Day 1 post injury in the complex HLI 150 ($p=0.03$) group. Lipid changes within hepatocytes occurred earlier in the complex control group than complex HLI groups (2.0 vs 0.5-0.0, $p=0.08-0.006$) at 6 hr, this increase resolving by 24 hr post injury.

Kidney injury cumulative scores were higher in both complex HLI 90 and complex HLI 150 compared to complex controls at 6 hr post injury ($p=0.02$). Median injury scores remained elevated in the tourniquet groups at 24 hr (complex HLI 150 4 vs 1 $p=0.16$, complex HLI 90 3 vs 1 $p=0.33$) and at Day 3 post-injury (complex HLI 150 6 vs 3, $p=0.11$). Tourniquet application resulted in an increased evidence of apoptosis and necrosis in the renal cortex at 6 hr compared to complex control for both complex HLI 90 and 150 ($p=0.02$), with the effect persisting at 24 hr ($p=0.02$) and Day 3 ($p=0.03$) post-

injury in the complex HLI 150 group. A similar increase in cellular apoptosis was observed by 6 hr of reperfusion in the medulla of the kidney after 90 minutes ($p=0.002$) and 150 minutes ($p=0.02$) of tourniquet time. Later timepoints trended to greater apoptosis and necrosis in tourniquet groups but were not significant. An early intra-tissue basophil infiltration was also observed with increases at 6 hr in the tourniquet groups bordering significance in the complex HLI 150 group ($p=0.05$).

Skeletal muscle taken from the quadriceps around the zone of injury showed no significant differences in cumulative injury scores between treatment groups at any timepoint. Edema was increased in complex control groups at 6 hr compared to complex HLI 90 ($p=0.02$) and HLI 150 ($p=0.11$), but edema persisted longer in the complex HLI 150 group at 24 hr ($p=0.07$). Fibroplasia at early timepoints were similar in all groups, with a trend towards greater fibroplasia in the complex HLI 150 group with time, just failing to reach significance at Day 7 ($p=0.06$). Myocyte degeneration and necrosis levels trended towards higher levels in the tourniquet groups at 6 hr with complex HLI 150 ($p=0.16$) and again at 24 hr post injury ($p=0.09$). At 24 hr post injury complex HLI 150 myocyte necrosis reached significance compared to complex HLI 90 ($p=0.03$), a trend which persisted through 7 days post injury.

THE EFFECTS OF TOURNIQUET APPLICATION ON THE DEVELOPMENT OF BONE FORMATION IN A MODEL OF BLAST-RELATED COMPLEX HIND-LIMB TRAUMA.

150 minutes of tourniquet increases total new bone formation in a model of blast associated complex lower limb traumatic amputation.

Non-invasive μ CT imaging was used to determine whether tourniquet application increases the volume of total new bone formation/heterotopic ossification formed in our model of complex lower limb trauma. Of the 15 animals used, 1 failed to recover from the general anesthesia following the 2 weeks post injury μ CT scan. Of the 14 scans that were performed at the 12-week endpoint, 3 scans had likely movement artefact following reconstruction and formatting which could not be eliminated and which the reviewers felt made interpretation more difficult. Volumetric assessment of the remaining microCT scans (n=11) performed at 12 weeks post injury demonstrated a 70% increase in total bone formation in the complex HLI 150 group compared to existing μ CT scans in a complex control group (n=14) ($17.8 \pm 7.0 \text{ mm}^3$ (95% CI 13.1-22.5 mm^3) vs $10.4 \pm 4.1 \text{ mm}^3$ (95% CI 8.0-12.8 mm^3), mean $\pm$ SD, p=0.007) (Fig. 24). Inclusion of all the complex HLI 150 12-week scans (n=14) led to a greater difference being observed ($19.3 \pm 8.5 \text{ mm}^3$ (95% CI 14.3-24.1-24.2 mm^3), p=0.01). An intraclass correlation coefficient (ICC) was calculated for the reviewers which was 0.94 (95% CI 0.87-0.97, p<0.001), showing very good correlation. Whole limb sectioning and H&E staining was performed to confirm that μ CT findings were consistent with histological evidence of chondro-osteogenesis; samples showed evidence of fibroblastic tissue at the zone of injury, and evidence of chondrogenic differentiation and heterotopic endochondral ossification. Given these findings, a kinetic analysis was performed to assess the early osteo-chondrogenic gene expression patterns between the two groups from 6 hr to 10 days post injury.

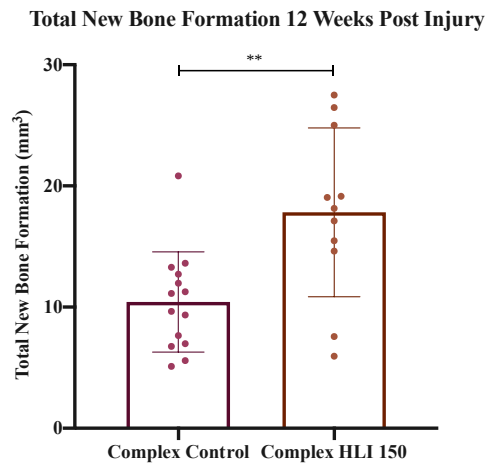


Figure 24. 150 minutes of tourniquet application increases new bone deposition in a model of blast-associated traumatic limb amputation
Volumetric assessment of total new bone formation 12 weeks post injury calculated from microCT imaging. Graphs show mean $\pm$ SD. Statistical analysis performed using an unpaired t-test with Welch's correction. ** p<0.01.

150 minutes of tourniquet application significantly upregulates angiogenic, osteogenic and inflammatory gene pathways

Total RNA was isolated from injured muscle at the zone of injury at 6 hr, 24 hr, Day 3, Day 7 and Day 10 post injury. We used a custom-made low-density array, each measuring the expression of 87 individual genes involved in early osteogenesis, chondrogenesis, angiogenesis and inflammation, key pathways found to be associated with early endochondral bone development and ectopic bone formation-heterotopic ossification (HO). Expression was normalized against age matched normal (uninjured naïve) muscle tissue using multiple stable reference genes selected using the geNorm algorithm (284). Amplification efficiencies were calculated for all gene sets, with the specific amplification taken into account in the quantification model, along with error propagation ($\pm$ SEM) (104). Expression is described as normalized relative quantities (NRQ). Table 5 below shows fold change as the ratio between two quantities (e.g. B/A),

the injured group expressed relative to normal aged matched tissue (naïve). Additional tables are included in Appendix 1 showing fold change between injured groups both as a fold change ratio (B/A) (Appendix 1 Table 12) and also as an absolute difference between groups (B minus A) (Appendix 1 Table 13), which highlights more clearly differentially expressed genes that have large fold changes, but when expressed as a ratio of each appear small, which can occur with a high denominator expression value.

Upregulated expression of gene transcripts for inflammatory chemokines and cytokines *Ccl2* (MCP-1), *Ccl3* (MIP-1 α), *Cxcl1* (GRO/KC), *Cxcl5*, *Cxcl10*, *Cxcl12* and *Csf3* (G-CSF), *Il1 β* , *Il6* and *Tnfa* were most pronounced earlier and for a longer timeframe (24 hr to Day 7) in the complex HLI 150 model. For example, tissue *Tnfa* gene transcript expression in the complex control group rose ~2-fold by Day 10, whereas in the complex HLI 150 models a 2-3-fold increase was detectable as early as 6 hr post-injury. Consistent with increased localized production of these cytokine/chemokines at the site of inflammation, gene expression of the anti-inflammatory cytokine IL-10 was consistently higher at all timepoints in the complex HLI 150 samples with only early upregulation detected in tissue collected from the complex control group.

Prolonged tissue hypoxia is a known driver of this inflammation. Hypoxia plays an important role in endochondral ossification (4; 107; 292; 319), Ischemia-reperfusion induced increased expression of hypoxia-inducible factor 1 α (HIF-1 α), the master transcription factor regulated by hypoxia was upregulated longer and more consistently in the complex HLI 150 group, with changes at Day 3 and Day 7 remaining statistically significant compared to naïve (p=0.02 and p=0.006 respectively), the fold difference between groups being significant at Day 7 (p=0.004).

consistently higher in the tourniquet model from 24 hr to Day 7 post injury, reaching statistical significance at 24 hr ($p=0.03$) and Day 7 ($p=0.02$).

Consistent with increased chemokine signaling to promote recruitment, migration and functional activity of innate immune effector cells (primarily neutrophils and macrophages) immediately post injury (82; 227; 256; 295), elevated expression of the gene *Itgam*, which enables neutrophil and macrophage adherence to endothelium, was detected in the complex HLI 150, the expression levels greater and more prolonged than in the complex control model. *Itga1*, *Itga2* and *Itgav* are genes which encode for the subunits of integrin receptors, which are adhesion molecules required for inflammation and fibrosis, forming part of the extracellular matrix (ECM), and are also increased in the complex HLI 150 model compared to complex control across the timeframe, with the increases between the two models reaching significance for *Itga1* and *Itgav* at 24 hr and 7 days post-injury. *Has2*, which encode for a hyaluronan synthase whose product hyaluronic acid (HA) is also part of the ECM, also exhibited higher expression during the first 7 days. CD44 is a cell surface protein which is a receptor for HA, and whose gene expression was greater and more consistent in the complex HLI 150 groups than complex control during the first 7 days.

At 6 hr post injury, bone morphogenetic protein (BMP) signaling was upregulated in the complex HLI 150 group compared to complex control with increased and more prolonged gene expression of *Bmp2*, -4 and -6, most notably in *Bmp2*. Fold differences between groups reached significance at 6 hr and Day 7 for *Bmp2* ($p=0.02$ and $p=0.001$) and for BMP6 ($p=0.009$) at 24 hr. BMPs are TGF- β superfamily proteins and have been shown to play a critical early role in cartilage and endochondral bone development, as

well their ability to induce ectopic bone formation (226). BMP2 is able to promote osteoblast differentiation through canonical BMP pathway signaling through the phosphorylation of SMAD 1/5/8 (39; 293). Expression of *Smad3* was higher in the complex HLI 150 group particularly at Day 7. *Bglap* encodes for a protein secreted by osteoblasts and is upregulated longer and with higher abundance in the early phase post injury in the complex HLI 150 group compared to control. Upregulation of *Rhoa* is seen in the complex HLI 150 model from 6 hr post injury versus Day 10 in the complex control models, the differences being significant between injured groups at 24 hr ($p=0.04$) and Day 7 ($p=0.002$). BMP2 signaling through the RhoA/ROCK pathway has been shown to influence the differentiation of multipotent mesenchymal stem/stromal cells (MSCs) towards osteogenesis. *Igf2* expression in complex HLI 150 also reaches significance against naïve sooner than in the complex control group, with expression between the groups being consistently higher in the complex HLI 150 group. Insulin-like growth factor 2 promotes osteogenic cell differentiation through MAPK pathways and has angiogenic properties. The *Mmp9* gene encodes for matrix metalloproteinase 9 (MMP9), the pathological elevation of which leads to reorganization and calcification of the ECM, an initial precursor to HO formation and therefore a potential biomarker of early HO formation (230). In these models *Mmp9* levels are consistently upregulated across all timepoints compared to both naïve and complex control, highly suggestive of increased and earlier chondrogenic and osteogenic activity. *Runx2*'s protein is essential for osteoblastic differentiation and maturation during endochondral ossification. Expression of this gene is significantly elevated early in the complex HLI 150 model from 6 hr post injury compared to naïve, with upregulation occurring later in the complex

control model. Along with increased and more prolonged upregulation of SPP1, which encodes for osteopontin, a protein that is integral to matrix mineralization, these genes further support evidence of earlier osteogenic gene activation in the complex HLI 150 injured model. Osteopontin also has a cytokine function, increasing IFN- γ and IL-12 production, whose serum concentrations were earlier shown to be elevated in the complex HLI 90 and HLI 150 models compared to complex control during the first 7 days post injury.

Statistically significant differences in fold change between complex control and complex HLI 150 were focused predominantly at 24 hr and 7 days post injury, where nearly all genes were upregulated significantly in the complex HLI 150 group. At 6 hr significant differences were limited to *Pdgfa* (p=0.04) and *Bmp2* (p=0.02), both early signaling molecules of angiogenesis and the BMP/SMAD pathway respectively, whose early inhibition have been shown to reduce HO (138; 298). At 24 hr post injury significant upregulation in the complex HLI 150 gene were related to angiogenesis (*Vegfa*, *Pdgfa*, *Eng*, *Flt1*), inflammatory signaling (*Cxcl10*) and early cartilage and bone differentiation (*Igf2*, *Scarb1*), with the genes associated with BMP signaling (*Bmp6*, *Gusb*, *Igf2*, *Rhoa*, *Smurf1*), Wnt signaling (*Lrp5*) and hedgehog signaling (*Smo*, *Gli2*) pathways. Other upregulated genes are associated with the formation of the ECM (*Itga1*, *Itgav*), and its increased mineralization (*Alpl*). Day 3 changes saw the persistence of *Pdgfa* upregulation and the increased expression of *Hat1*, a gene associated with chondrocyte development. Day 7 post injury saw extensive differences in most genes analyzed covering inflammatory (*Ccl3*, *Cxcl10*, *Cxcl12*, *Csf3*, *Il1 β* , *Il6*, *Il10*, *Tnf*), angiogenic (*Angpt2*, *Eng*, *Flt1*, *Hif1a*, *Pdgfa*, *Vegf*) chondrogenic (*Comp*, *Gli2*, *Hat1*),

extracellular matrix (*Has1, Has2, Itga1, Itga2, Itgam, Itgav, Itgax*) and osteogenic (*Alpl, Bmp2, Col11a1, Col1a1, Fgf10, Gusb, Hdac1, Hnf1a, Igf2, Mmp9, Phex, Ptch1, Rhoa, Runx2, Scarb1, Smo, Smurf1, Sox9, Sp1, Sparc, Spp1, Tgfb1, Tgfb3, Twist, Wnt5a*) processes. By 10 days post-injury there was minimal differences observed between the injured groups with any persistence of inflammation resolved or slightly greater in the complex control group (*Cxcl1* $p=0.03$, *Il1b* $p=0.02$, *Il6* $p=0.04$).

To further support these findings that the addition of 150 minutes of tourniquet increased the kinetics of osteo-chondrogenesis, injured muscle samples available from the complex HLI 90 groups at 6 hr, 24 hr and 7 days post injury were assessed for markers of early osteogenesis. Gene transcript expression results from injured tissue obtained from the HLI 90 group show a similar profile pattern of regulation at 6 hr as the complex HLI 150 group, with increased inflammatory signaling (*Ccl2, Ccl3, Csf3, Cxcl1, Cxcl5, Il1b, Il6, Tnf*) compared to both naïve and complex control. Upregulation of ECM associated genes was also seen (*Itga1, Itgam, Itgav, Itgax*) compared to complex control. Chondrogenic genes (*Acan, Gli2, Hat1, Jag1, Sox9*) were mildly increased. *Fgf2, Has1* and *Ptk2* were all significantly upregulated compared to complex control, all known to influence MSC development and differentiation. When comparing complex HLI 150 to complex HLI 90 expression levels, *Bmp2, Hif1a, Runx2, Rhoa, Pdgfa* and ECM associated genes were increased with longer tourniquet time. Comparison at 24 hr post injury showed no genes to be significantly increased when compared to complex control. Genes that showed a trend to higher expression were inflammatory (*Ccl3, Cxcl1, Cxcl5*) with expression levels otherwise very similar between the groups. Compared to complex HLI 150, the additional effects on gene expression of increased tourniquet time were

apparent with most of the genes assessed increased. These increases were significant in adipogenic (*Adipor1* p=0.01), osteogenic (*Smad3* p=0.02, *Smo* p=0.01, *Smurf1* p=0.009, *Sp1* p=0.02, *Sparc* p=0.01, *Tgfb1* p=0.01, *Tgfb3* p=0.02), MSC differentiation (*Col11a1* p=0.03, *Jag1* p=0.04, *Notch1* p=0.007, *Rhoa* p=0.03) and angiogenic associated genes (*Eng* p=0.03, *Fgf1* p=0.03, *Flt1* p=0.03).

Analysis of gene transcript expression results obtained from the HLI 90 group at 7 days post injury showed several similar trends seen in the complex HLI 150 group with persistent elevation of *Hif1a*, *Igf2*, *Itgam*, *Mmp9*, *Runx2* and *Spp1* compared to naïve and complex control. Comparing between complex HLI 90 and complex control, genes were again overwhelming significantly increased compared to control (Appendix 1 Table 11 and 12, p<0.001-0.05). The largest differences in expression between complex HLI 90 and control were seen in *Igf2*, *Itgam* and *Spp1*. Comparing the fold change between complex HLI 90 and HLI 150 helps identify which genes are further upregulated by tourniquet and also provides verification independent of the complex control Day 7 group of any upregulation from tourniquet use. This confirmed that gene upregulation at Day 7 in the complex HLI 150 group is a true effect with multiple genes being significantly upregulated compared to complex HLI 90 and the overall trend of continued higher expression was preserved. Angiogenic (*Angpt2* p=0.04, *Pdgfa* p=0.01), osteogenic (*Bmp2* p=0.05, *Fgf10* p=0.003, *Gusb* p=0.05, *Itgam* p=0.03, *Itgax* p=0.03, *Scarb1* p=0.03, *Sp1* 0.02, *Spp1* p=0.05, *Tgfb1* p=0.05), chondrogenic (*Hat1* p=0.02), inflammatory (*Ccl3* p=0.05, *Il1b* p=0.04, *Il6* p=0.03), adipogenesis (*Fadp4* p=0.005) and MSC differentiation (*Pparg* p=0.04) genes were all significantly upregulated between complex HLI 150 and complex HLI 90 at 7 days post injury. The greatest difference in

fold expression normalized to naïve between the complex HLI groups were *Spp1* (187.9, p=0.05), *Mmp9* (25.0, p=0.08), *Itgam* (23.1, p=0.03), *Itgax* (14.9, p=0.03) and *Ccl3* (11.9, p=0.05). Collectively this gene expression data supports the μ CT observations of increased evidence of osteo-chondrogenesis and bone formation when a complex injured limb undergoes 150 minutes of tourniquet application prior to limb amputation.

The additional effects of 150 minutes of tourniquet application on inflammatory mediators in a complex injured model persists for 7 days post injury

Earlier it was shown that the systemic inflammatory response associated with complex limb injury and amputation remained statistically elevated at 7 days post-injury when comparing 150 minutes of tourniquet to complex control and that 90 minutes of tourniquet increased the magnitude of inflammatory response but not necessarily the duration. As part of this study, we the assessed serum biomarker profiles to include Day 10 post injury. As a whole, all biomarker levels trended significantly higher in the complex HLI 150 group from 6 hr to Day 7 but were similar at Day 10 post injury (detected differences were not statistically significance using two-way ANOVA with correction for multiple comparisons) (Fig. 25 and Appendix 1 Table 7).

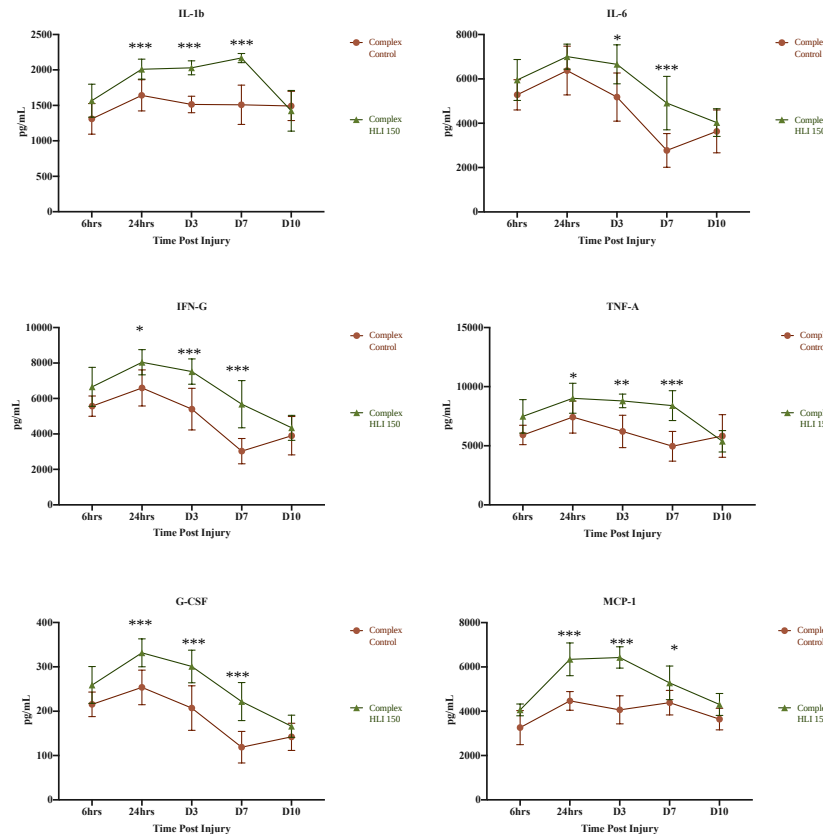


Figure 25. Additional effects of 150 minutes of tourniquet application on inflammatory mediators in a complex injured limb resolve by 10 days post injury. Serum concentration of cytokines and chemokines remain significantly elevated above those of the complex injured model up to 7 days post injury. Graphs show mean values $\pm$ SD. Statistically analysis performed using two-way ANOVA with correction for multiple comparisons. * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$

mRNA upregulation translates to increased protein expression in injured muscle tissue

Expression analysis of proteins involved in osteogenic regulation to include IBSP, RUNX2 and PDGF was determined by ELISA from homogenized muscle samples from injured muscle from both complex HLI 150 and complex control samples at Day 1 to Day 10 post injury, with expression normalized to total protein concentration using a BCA protein assay. IBSP trended towards higher expression from 3 days through Day 10 post injury, reaching significance at Day 7 (1.05 ± 0.52 vs 0.54 ± 0.11 ng per mg, $p=0.03$)

(Fig. 26B). RUNX2, a master transcription factor for osteogenesis, was elevated as at Day 7 post injury (269.8 ± 273.2 vs 44.2 ± 21.9 ng per mg, $p=0.03$), with higher mean expression present from Day 1 post injury in the complex HLI 150 group (Fig. 26A). PDGF expression varied less with no consistent pattern of elevation observed, with elevation at Day 1 favoring complex HLI 150 (0.17 ± 0.03 vs 0.12 ± 0.01 ng per mg, $p=0.04$) while at Day 7 complex control was higher (0.08 ± 0.02 vs 0.14 ± 0.04 ng per mg, $p=0.02$) (Fig. 26C). Overall expression of PDGF was lower than the other measured osteogenic proteins, which combined with the small sample sizes ($n=3-7$) may contribute to these findings. The small sample sizes also effect the likelihood of statistical significance being reached at a given timepoint.

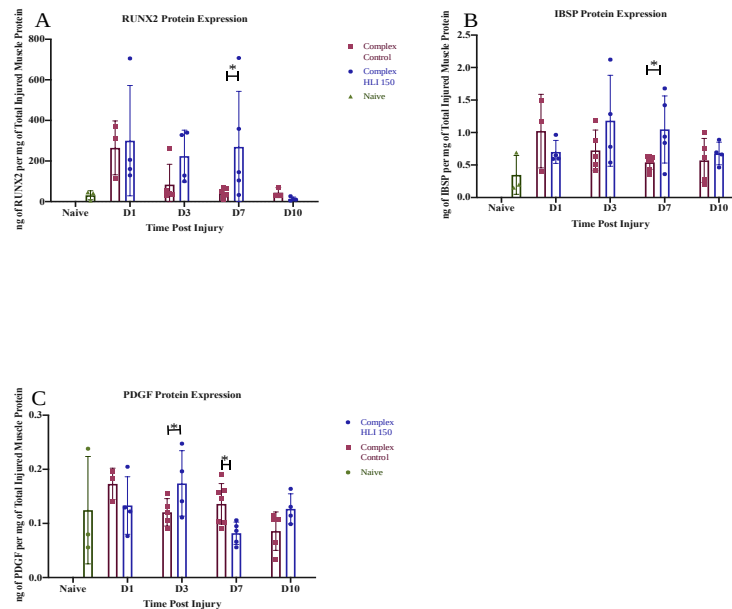


Figure 24. Protein expression in injured muscle tissue homogenates is consistent with upregulated mRNA of HO associated genes
 Graphs show comparison of the protein expression of (A) RUNX2, (B) IBSP, (C) PDGF between complex HLI 150 and complex control at Day 1 to Day 10 post injury. Protein concentrations normalized and expressed in relation to total injured muscle protein levels. Charts show mean $\pm$ SD. Statistical comparison performed using multiple t-tests. * $p<0.02$ ** $p<0.01$, *** $p<0.001$

CHAPTER 4: Discussion

Tourniquet use has become ubiquitous in the care of catastrophic hemorrhage, both in the military and increasingly in the civilian environment. Campaigns such as ‘Stop the Bleed’ are championed by the American College of Surgeons and offer education on hemorrhage control in a similar way to cardio-pulmonary resuscitation (CPR) is taught to the general public, aiming to turn bystanders into first responders (79; 114). This program is designed to improve survival from mass casualty incidents or following an active shooter: over 500,000 people have completed a 90-minute class on the principles of direct pressure application, wound packing and use of tourniquets. This initiative was a response to the Sandy Hook elementary school shooting in 2012 (115), which saw 29 casualties, 27 of whom died. Many active shooter incidents in recent years have involved AR-15 style semi-automatic rifles, bringing battlefield wounds to a civilian environment (53); the soft tissue destruction associated with their 5.56 caliber round is much greater than that from a 9mm handgun, increasing the likelihood of catastrophic bleeding and tourniquet use (280). Blast injury remains the most common mechanism of injury for deployed military personnel. Whilst a less common mechanism of injury in mainland USA, between 1987 and 1997 there were still over 17,500 bombings resulting in 4,063 injuries and 427 deaths due to explosive devices (205). Global terrorism has seen explosive devices and blast injuries used against civilian targets such as the Boston marathon 2013, London Underground 2005, Madrid 2004 and the September 11th 2001 attacks in the USA. The frequency of terrorist bombings has increased, with these incidents causing more injuries per attack (177; 304). Future terrorist attacks or industrial accidents may therefore see civilian first responders managing blast associated

catastrophic hemorrhage during a mass casualty incident, with the potential for prolonged tourniquet use as a result. By addressing the question of whether blast exposure affects the development of tourniquet induced IRI, we are adding to the body of evidence used to train for and manage such catastrophes.

WHOLE LIMB REPERFUSION MODEL DISCUSSION

In our model of whole limb reperfusion we demonstrate that exposure to a 120 kPa blast overpressure is capable of increasing serum BUN, a marker of renal dysfunction by 46% ($p=0.04$) between blast and non-blast groups as early as 6 hr into reperfusion following 150 minutes of tourniquet, with a lesser effect occurring with a shorter 90 minutes tourniquet time (24%, $p=0.14$). This increased effect persisted to the study endpoint at 24 hr in the blast HLI 150 group when compared to both HLI 150 ($p=0.03$) and also blast HLI 90 ($p=0.002$). This suggests that there is a threshold above which renal dysfunction persists beyond 6 hr in our model, and that inclusion of blast significantly increases and prolongs renal injury following 150 minutes of tourniquet. Serum creatinine was shown to be a poor early marker of renal injury, and though it has historically been used in AKI scores, the utility of creatinine as an early marker of renal injury is limited. This is consistent with conclusions in the wider literature (253) and the emergence of more novel and sensitive AKI markers. Defining renal dysfunction and setting a binary threshold is difficult in animal models; current gold-standard guideline scores in humans require urinary output measurements or an increase in serum creatinine of 1.5-2 times baseline or ≥ 0.3 mg/dl based on human standards (166). The interpretation of the blood urea nitrogen (BUN): creatinine ratios (BCRs) ratios demonstrated a change between groups at both 6 hr and 24 hr suggestive of a pre-renal cause which would be

consistent with hypoperfusion from IRI or endothelial dysfunction. A limitation of our current model is that it does not include a hemorrhage phase prior to tourniquet application as would occur in trauma, and therefore renal effects from hypoperfusion are likely to be magnified by a real-world injury requiring tourniquet and subject to the increasingly common strategy of hypotensive resuscitation. Having identified evidence of renal injury using traditional biochemical markers, analysis of novel AKI markers such as Cystatin C, KIM-1 and NGAL was performed. Serum KIM-1 was unaffected; most evidence existed for the use of this as a urinary biomarker as it is excreted into the urine and therefore absence of detection in serum is to be expected. Cystatin C has been shown to have good correlation with AKI and is detectable earlier than serum creatinine (195); our data shows a trend towards higher levels with longer tourniquet times and with blast at 6 hr, consistent with our BUN data. The most striking support for renal effects from more novel AKI marker occurred though with NGAL. The early upregulation at 6 hr showed that 150 minutes caused significantly more renal injury than at 90 minutes, with differences between blast ($p=0.02$) and non-blast ($p=0.05$) both significant. Blast further increased serum NGAL concentrations in all tourniquet times but not sufficiently to reach significance. At 24 hr, serum NGAL was 55% higher in the blast HLI 150 group than HLI 150 alone ($p=0.20$); this failed to reach significance due to two outliers which were outside the lower 99% CI but not sufficiently to be removed by conservative outlier analysis (ROUT technique, $Q=1\%$). Given the changes in BUN and NGAL between blast and non-blast HLI 150 groups, it is highly likely that blast injury compounds and prolongs renal injury following 150 minutes of tourniquet, but that any IRI generated by 90 minutes, with or without blast injury, is insufficient to cause prolonged renal

dysfunction. Thus, with under 2 hours of tourniquet application, blast is unlikely to induce any further remote injury. This conclusion would be consistent with the renal gene expression data in these models which showed *Lcn2* expression, the gene which encodes for NGAL, had higher expression 6 hr post injury in the blast HLI 150 than the non-blast group.

The liver and its extensive vascular supply predispose it to injury from IRI and systemic inflammation. Similar to evidence of renal dysfunction, blast exposure in isolation was not sufficient to upregulate markers of liver injury. Serum ALT is an established clinical marker of liver health and is released into the circulation following acute hepatocellular injury. Addition of blast exposure increased ALT by 91% compared to 150 minutes of tourniquet alone at 6 hr ($p=0.004$), with blast HLI 150 also being significantly elevated compared to blast HLI 90 (74%, $p=0.009$). This clearly demonstrates that blast exposure increases early acute hepatocellular injury; furthermore, the difference between blast groups shows that this blast effect is tourniquet duration dependent. The additional 60 minutes of tourniquet results in an exaggerated increase in liver injury under blast conditions that is not seen in tourniquet only groups, suggesting that a synergistic response occurs with blast exposure and prolonged tourniquet time. Gene expression analysis of liver tissue showed that blast exposed groups had upregulation of inflammatory and oxidative stress gene signaling, frequently reaching statistical significance in the 150-minute groups adding further support to this finding. The timeframe of this study was short for the development of structural changes but histological analysis of the liver showed that all tourniquet groups had increased liver injury scores compared to naïve groups ($p<0.001-0.02$), but it was only HLI 150, blast

HLI 90 and blast HLI 150 which were increased compared to blast only controls ($p=0.005-0.04$). Evidence of increased hepatocyte apoptosis, as well as portal hepatocyte radiation, pseudo-cytoplasmic inclusion development and increased multinucleate hepatocytes was seen with the addition of blast exposure, further supporting the premise that blast exposure worsens tourniquet induced IRI leading to greater liver secondary injury.

Less evidence of remote lung injury was identified, part of which may be attributable to experimental design. Wet-to-dry ratios, a marker of increased lung permeability, are prone to technical error and the use of only a single lung lobe may have contributed to greater sampling error masking any effect. Histological examination of lung tissue for markers of injury was also not possible due to insufficient inflation of the lungs with formalin in most samples at time of necropsy. Gene expression demonstrated that blast exposed groups had high expression of IRI genes, with a greater up-regulatory effect in the 150-minute groups where expression of oxidative stress and apoptosis both increased. *Lcn2* was the most consistently upregulated, reaching statistical significance between blast and no-blast groups at both 6 and 24 hr ($p=0.03$ and $p=0.01$), and also being significantly increased in the 90-minute groups at 24 hr ($p=0.009$). Blast increased expression of chemoattractants *Cxcl1*, *Cxcl2* and *Ccl2* at 24 hr following blast exposure with both tourniquet durations; collectively they are chemoattractant to monocytes and neutrophils, suggesting that the non-significant trends towards increased lung permeability observed with the wet-to-dry ratios and BALF protein concentrations may result from an increased recruitment of leukocytes in blast injured groups.

Limb skeletal muscle tissue is directly exposed to the effects of hypoxia by tourniquet application. It is also physiologically one of the best tissue types in the body at withstanding such stresses(126; 127). Biochemical assessment using the AST/ALT ratio to infer the source of AST as either from muscle or liver demonstrated increasing mean ratios with more exaggerated injury from blast or tourniquet time, supportive of a skeletal cause. A future refinement would be to run assays for the AST muscle isoenzyme, which is more specific for skeletal muscle AST. Serum AST trended towards increased concentrations with greater tourniquet time but there were no significant differences. Creatine kinase (CK), an enzyme released into the blood following muscle damage, was significantly elevated at 6 hr compared to blast only controls for HLI 150 ($p=0.04$) and blast HLI 90 ($p=0.007$), but the most marked rise in serum concentrations occurred in the blast HLI 150 group which was elevated compared to blast only ($p<0.001$), HLI 150 ($p=0.003$) and blast HLI 90 groups (0.02). This suggests that 150 minutes of extended tourniquet use can cause muscle breakdown, and that blast injury significantly potentiates this effect resulting in a markedly increased level of muscle breakdown compared to tourniquet alone, particularly with extended tourniquet use. The evidence that this serum marker provides of a more exaggerated IRI and muscle damage with the addition of blast exposure becomes much clearer when considered in context of the IRI gene expression data. This showed that at 6 hr of reperfusion, the fold change of nearly every IRI gene is increased when comparing between blast HLI 150 and HLI 150 alone. These fold change differences reached statistical significance in over half, which given the comparison of two groups with marked biological variation post injury attests to the blast exposure significantly increasing IRI in skeletal muscle. With 90 minutes of tourniquet, blast

trended at 6 hr to higher IRI gene expression, but the blast effect was not as marked or statistically significant. An interesting finding which was not anticipated is that at 24 hr in the HLI 90 group, blast exposure resulted in IRI genes being more upregulated. Multiple apoptosis and proinflammatory genes were significantly elevated in blast HLI 90 compared to HLI 90 including *Il1b*, *Il6*, *Cxcl1*, *Bcl2*, *Bcl3* and *Casp8*, with hypoxia and oxidative stress genes trending towards higher expression. This suggests that blast prolongs the effect of 90 minutes of tourniquet, with persistence of oxidative stress, apoptosis and proinflammatory signaling at 24 hr where it would otherwise have resolved in muscle.

Having established compelling biochemical evidence to support the hypothesis that blast exposure worsens IRI and subsequent remote organ injury following tourniquet application, we sought to identify the mechanisms by which this occurred. Endothelial dysfunction, oxidative stress, increased DAMPs and SIRS have all been shown previously as ways in which IRI exerts effects systemically. Increased expression of endothelial markers such as adhesion molecule ICAM-1 is known to occur in IRI(91; 169; 186). Blast HLI 150 was shown to be the only group significantly upregulated against controls at 3 hr post injury but also against HLI 150 3 hr (57% increase, $p=0.01$). Blast HLI 150 continued at both 6 and 24 hr to show a trend of higher expression levels than other groups and blast only controls. Blast exposure increased serum ICAM-1 expression in the 90-minute group (3 hr 19%, $p=0.24$, 24 hr 13%, $p=0.16$), demonstrating that blast appears to cause an increase in ICAM-1 expression and early endothelial dysfunction with both tourniquet durations. These findings are consistent with previous work showing that isolated blast wave exposure to the extremities causes activation of the

endothelium, elevating both circulating endothelial cells and mRNA tissue expression (259).

Triggering receptor expressed on myeloid-1 (TREM-1) has been described as both a DAMP and endothelial injury marker; this protein showed early upregulation at 3 hr post injury in all tourniquet groups. Expression was highest again in the blast HLI 150 group, reaching significance against controls ($p < 0.001$) and with an 18% increase compared to HLI 150 ($p = 0.27$), despite no increase seen between blast only and naïve controls. This further suggests a synergistic effect of blast overpressure exposure with longer tourniquet durations on IRI development.

The S100A8 subunit of calprotectin (S100A8/A9) was upregulated on gene expression consistently in all tissue types compared to naïve, with blast increasing the expression of the gene in all tissue types following 150 minutes of tourniquet at 6 hr, most notably in muscle ($p = 0.04$). Acting as an endogenous ligand to TLR4 and RAGE (238), calprotectin has been shown to be significantly upregulated during IRI (56) and is associated with mitochondrial dysfunction and increased IRI in cardiac muscle (156). Plasma analysis of calprotectin demonstrated that increased mRNA expression translated to significant elevations in circulating protein compared to blast only controls in all tourniquet groups at 6 hr post injury ($p = 0.004-0.02$). These changes were not significant against the naïve group due to a higher mean and variance in this control. S100A8 has been shown to also act as a ROS scavenger (157; 266), and ROS generation and oxidative stress is a major factor in IRI (155). Direct markers of oxidative stress are by their very nature unstable, and therefore it is often easier to detect markers of oxidative damage as a surrogate. Lipid peroxidation is a common effect of increased ROS presence, and

malondialdehyde (MDA) is a biproduct of this reaction. Plasma samples showed increased levels of MDA as early as 6 hr post injury with total plasma concentrations continuing to rise at 24 hr of reperfusion, suggestive of continued oxidative damage. At 6 hr of reperfusion, blast had significantly increased the levels of MDA detected compared to HLI 150 alone (26% increase, $p=0.03$), confirming that blast increases the magnitude of IRI and oxidative stress experienced systemically compared to tourniquet alone during the first 6 hr. Further evidence to support this was seen when assessing oxidative damage in the muscle tissue; a longer 150-minute tourniquet time was required to cause increased RNA oxidation (Fig. 10C), and muscle MDA trended to greater expression in the blast HLI 150 group compared to HLI 150 alone, although not reaching statistical significance (figure not shown). These findings are consistent with wider research into blast TBI effects which have shown that blast overpressure alone can increase MDA expression in brain tissue as well as systemic markers of inflammation (185; 271).

Cumulatively these results, from increased biochemical evidence of remote organ dysfunction and gene expression markers, through to circulating evidence of increased oxidative stress and DAMPs expression, were supportive of blast injury increasing the IRI effect of 150 minutes of tourniquet, with limited or no evidence to confirm a similar effect in the 90 minute groups. The use of multiplex assay technology enabled an extensive examination of the effect that blast had on IRI through defining the systemic cytokine and chemokine profile. This confirmed the gene expression findings of an upregulated proinflammatory response. Blast increased IL-2, IL-13, IL-17, G-CSF (CSF3) and IFN- γ by statistically significant levels at 6 hr hours of reperfusion between

150-minute groups. By 24 hr more analytes reached significance including pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α by 24 hr of reperfusion as well as other analytes. Several of the early elevated analytes, IL-2, G-CSF (CSF3) and IFN- γ , remained elevated but fell short of statistical significance when adjusted for multiple comparisons at 24hr. This provides credence to both datasets and indicates that the changes seen in mRNA expression lead to corresponding increases in protein expression and systemic inflammation.

Summarizing the findings in this model, the data supports the hypothesis that blast overpressure exposure increases ischemia reperfusion injury in a model of whole limb reperfusion. This is most likely due to increased systemic proinflammatory signaling, with evidence of early endothelial dysfunction and oxidative stress. Remote organ injury effect was variable with more conclusive evidence to support liver and kidney secondary injury compared to lung. Blast appears to increase muscle breakdown in the first 6 hours of reperfusion following 150 minutes of tourniquet application, evidenced by high serum creatinine kinase levels and evidence of increased upregulation of oxidative stress and apoptosis associated genes. These effects are tourniquet time dependent; there was neither biochemical nor histological evidence to support remote organ injury following 90 minutes of tourniquet. There is good evidence that blast wave exposure increases the duration of upregulation of IRI genes beyond 6 hr in a 90-minute model, the effects most notably in muscle, but also in lung and kidney. It has not been demonstrated however these changes equate to functional or structural organ impairment with 90 minutes of tourniquet.

COMPLEX INJURY MODEL DISCUSSION

The second model performed was a complex injury model which incorporates several of the key mechanisms and injury patterns associated with blast-related extremity trauma, namely blast overpressure exposure, femoral fracture, soft tissue crush injury and amputation. The use of tourniquets has become almost ubiquitous in these injury patterns, frequently due to the associated catastrophic hemorrhage or to obtain hemorrhage control while under fire. The effect of isolated tourniquet application on the systemic inflammatory response to this complex injury pattern is unknown and therefore we sought to establish if tourniquet application, either 90- or 150- minutes duration resulted in an increase in the SIRS response or development of remote injury.

Addressing the evidence for remote injury, our assessment of biochemical markers of organ function or acute injury demonstrated that early renal markers of injury such as BUN were more elevated in the complex HLI groups than in the complex control group with median concentrations increased 18-33% in tourniquet groups ($p=0.46-0.57$) at 6 hr post injury with a trend of higher expression to Day 7. Similar trends were seen with serum creatinine and BCR ratios; however no statistically significant inter-group differences were demonstrated between complex tourniquet and complex control groups, likely as a result of the larger variance in results with complex injury patterns, and the relatively small sample size ($n=7-8$ per group). Histological analysis did support evidence of increased renal injury compared to complex control in tourniquet groups, with 90 minutes having a short effect noted at 6 hr while 150 minutes resulted in increased renal apoptosis and necrosis damage up to Day 3 post-injury. These structural changes suggest increased renal injury following tourniquet use, with the novel AKI marker NGAL also demonstrating the same pattern of expression at 6 hr, 24 hr and Day 7 post injury as the

apoptosis and necrosis changes. Statistically higher levels of expression between complex control and both tourniquet groups were seen at 6 hr, with complex HLI 150 remaining significantly elevated compared to control at 24 hr and 7 days post injury. The combination of histological and novel AKI marker findings makes it highly likely that renal function is further impaired by tourniquet use of 150 minutes, which is prolonged compared to short tourniquet duration. It also highlights further the insensitive and delayed response of serum creatinine as a renal marker of acute injury, and that it therefore has limited value in the clinical decision-making process at point of wounding. Further investigation and correlation of NGAL in combat casualties may confirm this biomarker as a potential early marker of impending renal dysfunction and need for organ support.

Serum markers of acute hepatocellular damage were higher in tourniquet groups at 6 hr post injury but not reaching significance; these differences were resolved by 24 hr. The early rise observed may have resulted from the presence of IRI in the complex injured limb, with the liver previously shown to be susceptible to secondary injury from IRI due to increased cytokine expression as early as 6 hr post injury (95; 135). This would be consistent with the later histological findings of increased portal radiation and multinucleate hepatocytes seen at 24 hr post injury, and the earlier lipid changes seen at 6 hr, which all reached significance compared to complex control samples.

Establishing evidence of remote lung injury was hampered for the similar experimental reasons as identified in the whole limb HLI models. Increased lung permeability was observed in the complex HLI 150 group as early as Day 3 post-injury when compared to normal lung tissue. At Day 7 both complex HLI groups exhibited

significant differences in wet-to-dry ratios compared to both complex control and naïve groups. This demonstrates that tourniquets can induce an additional remote lung injury compared to a complex injured limb alone; the effect being greater and detectable at an earlier timepoint with longer tourniquet durations.

Muscle injury resulted in the most exaggerated increases in AST in tourniquet groups compared to complex control during the first 3 days post-injury, but no statistical difference was shown between tourniquet and complex control groups. Differences in CK expression and AST/ALT ratios between groups were similar, reflecting the extent of local tissue damage and that any additional effect would be difficult to detect given the complexity of the injury pattern. Histologically tourniquets resulted in increased edema during the first 6-24 hr post injury, the effect longer lasting with 150 minutes of tourniquet; similarly with myocyte degeneration and necrosis. These findings imply more early damage resulting from tourniquet use which could manifest as greater DAMPs release and endogenous priming of the inflammatory cascade.

Analysis of circulating DAMPs demonstrated elevated plasma concentrations of HMGB1 in the complex HLI 150 group compared to complex control at 6 hr ($p=0.01$) and 24 hr ($p=0.002$), as well as elevation compared to 90 minutes of tourniquet. HMGB1 is an intracellular chromatin protein, whose release is known to contribute to IRI through endogenous TLR2 and TLR4 signaling, and through increasing IL-6, MCP-1 (CCL2) and TNF- α expression (58; 305), thus driving an increased inflammatory cascade in tourniquet groups compared to control. Calprotectin expression was significantly higher in the tourniquet groups at 6 hr and 24 hr and also at Day 7. TREM-1 expression was also higher in complex HLI 150 group compared to complex control at all timepoints, falling

short of significance during the first 24 hr ($p=0.12$ and $p=0.08$) becoming significant at Day 3 ($p=0.002$). Collectively these endogenous markers activate many of the key pro-inflammatory pathways such as TLR4/NF- κ B/MAPK, NLRP3 or RAGE, providing a mechanism by which tourniquet use and IRI in a complex injured limb primes the immune system for a more exaggerated inflammatory response and increases the risk of secondary injury. Gene expression data correlated with this with IL-6 mRNA expression in the liver significantly elevated between complex HLI 150 and complex control from 24 hr to Day 7 ($p=0.009-0.03$). Expression of *S100a8* was consistently higher in complex HLI 150 versus control in all tissues and timepoints, but this did not reach statistical significance. The greatest fold changes seen due to the effect of the addition of 150 minutes of tourniquet were seen in the liver and injured muscle, with cytokine and chemokine signaling, oxidative stress and DAMPs markers being the most affected. The difference in fold change often became more pronounced after Day 3 (Appendix 1 Table 9) in the liver and muscle, demonstrating that the complex HLI 150 group has persistence in IRI gene upregulation while the complex control group genes were returning back towards homeostasis. In lung and kidney tissue this effect was smaller with *S100a8*, *Nos2*, *Il6* and *Cxcl2* remaining upregulated.

Further confirmation of this prolonged inflammatory state, likely driven by increased and persistent DAMPs expression, was seen on cytokine and chemokine profiling. Cytokine and chemokines assessed were nearly all increased from as early as 6 hr post injury when comparing complex tourniquet groups to complex control. During the first 24 hr post injury, the increased expression of analytes relative to complex control was consistent between both 90 and 150 minutes of tourniquet times, peaking for most

analytes at 24 hr. From 24 hr post injury, the prolonged inflammatory effects of longer tourniquet duration and IRI become apparent. By Day 3 the percentage increase between complex HLI 90 and complex control had fallen to less than 20%, with the notable exception of TNF- α (37.1% increase, $p=0.006$). With 150 minutes of tourniquet, most analytes continued to show statistically significant increases in cytokine and chemokine expression against both complex control and complex HLI 90 groups at Day 7 post injury. Resolution of this sustained pro-inflammatory effect occurred by Day 10 post-injury, with analyte levels converging towards serum concentrations similar to the complex control group.

In total these findings suggest that there is a threshold between 90 minutes and 150 minutes of tourniquet application, which when reached leads to an exaggerated pro-inflammatory cascade in a complex injured limb. This effect is more surprising and noteworthy when considering that the majority of ischemic muscle tissue distal to the fracture site is amputated at time of surgery. Further investigation is needed to confirm whether semi-viable muscle tissue is more likely to undergo necrosis rather than apoptosis, releasing endogenous signaling molecules with extended tourniquet time. Given the increased DAMPs expression and histological findings, this may represent part of the cellular mechanism by which the systemic pro-inflammatory effect is exerted. Limited data in the wider literature exists for the combined effects of complex limb injury and tourniquet on the cytokine profiling. In civilian lower limb orthopaedic trauma surgery, the decision to use a tourniquet has frequently been debated. Systematic reviews and meta-analysis performed in both 2010 and 2019 collectively concluded that avoidance of tourniquets was associated with less postoperative pain, shorter hospital

stays and reduced limb swelling (220; 255). Similarly, tourniquet has been shown to be associated with higher wound complication (263) in orthopaedic surgery, as well as more thrombotic events (315). While none of these studies addressed the pathophysiology of how tourniquets worsened outcome in fracture surgery, our findings of an increased pro-inflammatory, pro-thrombotic state would provide an explanation for many of these clinical findings.

HETEROTOPIC OSSIFICATION MODEL DISCUSSION

Having addressed our hypothesis in respect to remote organ injury, it was necessary to establish if tourniquet use leads to greater local tissue dysfunction and results in increased bone formation in our combat applicable model of blast lower limb trauma. A high percentage (~65%) of blast associated traumatic amputations develop heterotopic ossification; by assessing the effect tourniquet application has on the development of osteo-chondrogenesis and heterotopic ossification (HO), we sought to further understand the influence that injury patterns have on the development of this debilitating secondary injury. Using *in vivo* μ CT imaging we demonstrated at least a 70% increase in total bone formation in limbs subjected to 150 minutes of tourniquet prior to amputation. This finding supports previous narratives in the published literature which have provided plausible rationales why hypoxia or IRI induced by tourniquets may be associated with higher occurrences of HO (113), but prior to these results we are aware of no published work presenting conclusive evidence confirming the link. Demonstrating that tourniquets are a factor in HO development in a rodent model of traumatic amputation helps to advance our understanding of factors that contribute to this pathological process. Model development had already demonstrated the importance of

blast and bioburden in increasing HO formation (212; 217); 150 minutes of tourniquet can now be considered a significant variable when modelling traumatic HO. Like a wound bioburden, prolonged tourniquet use and IRI exerts local tissue effects through increased and prolonged inflammation.

The molecular pathways of early HO formation are complicated and have not yet been fully elicited. The role of hypoxia in aberrant bone deposition has been well described (83; 208; 292) with HIF-1 α situated at the molecular crossroads between both hypoxic and inflammatory signaling (4; 112; 159; 210), affecting neutrophil and macrophage function by controlling proinflammatory gene signaling, upregulating phagocytic activity and reducing neutrophil apoptosis (289). Our gene expression data demonstrated that *Hif1a* expression was significantly upregulated compared to the naïve state in the complex HLI 150 group until Day 7 post-injury, versus only 24 hr post injury in the complex control group. This establishes that even with the majority of ischemic tissue removed by amputation, tourniquet application resulted in greater and prolonged hypoxia of the zone of injury. *Pdgfa*, another angiogenic gene, was the most consistently upregulated gene across the time course when comparing injured groups ($p < 0.001-0.05$). Given the critical importance of angiogenesis in the recruitment of mesenchymal stem/stromal cells (MSCs) chondro-angio-osteogenic progenitor cells in early HO formation, the significant upregulation of these gene groups in the tourniquet applied group adds further credence to the observational findings and highlights some of the molecular mechanisms which we sought to elicit. Both PDGF and HIF-1 α inhibitors have been shown to significantly reduce HO volume in rodent Achilles tenotomy models (4; 158; 298), suggesting that these therapies may also help inhibit the angiogenic

promoting effects of prolonged tourniquet use in a combat applicable model of traumatic HO.

Local and systemic inflammation have been shown clinically to be key drivers of HO in injured service personnel, with expression of IL-3, IL-6, IL-10, IL-12(p70), ILP-10 (CXCL10), MIP-1 α (CCL3) and MCP-1 (CCL2), either systemically in serum or locally in wound effluent (74; 81). Our gene expression data demonstrates prolonged and upregulated expression of many of the genes associated with these proteins in the complex HLI 150 group compared to complex control, with our multiplex data confirming translation and increased expression of these proteins systemically in serum. Collectively this data supports the hypothesis of the existence of a more exaggerated and persistent pro-inflammatory state following tourniquet use in injured muscle tissue, potentially as a result of continuing tissue hypoxia, cellular apoptosis/necrosis or more exaggerated tissue injury following IRI.

Bone morphogenic proteins (BMPs) control cell proliferation and differentiation, with their role in HO well described. Early upregulation occurred with tourniquet use compared to controls as early as 6 hr post-injury, priming the tissue environment for osteoblastic differentiation, with hypoxia shown previously to induce BMP-2 expression (277). Osteoblastic differentiation is controlled by a range of factors that influence bone formation. Runx2, involved in osteoblast differentiation from precursors, acts as a key transcription factor for the production of osteoblasts, and sits downstream of BMP and Wnt signaling. Key downstream genes of Runx2 involved in osteoblast differentiation were all elevated in the complex HLI 150 (*Bglap*, *Ibsp*, *Mmp9*, *Spp1*) group based on our

qRT-PCR (Table 12), demonstrating a clear upregulation of the pathway consistent with our observational μ CT findings.

The analysis of muscle samples from groups subjected to the complex injury pattern plus 90 minutes of tourniquet were also analyzed to address two questions. The first was to ensure that the lower expressions seen in the complex control Day 7 group were not due to sampling error, and the second was to attempt to establish how a shorter tourniquet time may affect the angio-, chondro- and osteogenic gene profile. When conducting these experiments, every effort was made to ensure that muscle samples harvested for gene expression and protein analysis were taken from the same area at the zone of injury at distal end of the femur, with each group consisting of approximately 7 to 8 animals. Complex injury patterns result in a wide variation of response, often with high degrees of variance between samples and groups due to the complex and localized nature of tissue repair and regeneration. Tissue changes are not visible macroscopically at this stage, and as such sampling variation could contribute to any anomaly found in any group or timepoint. Sample size with large baseline variances make it difficult to exclude samples from injured groups compared to naïve baselines, which typically have much narrower physiologic ranges of gene expression. Analysis confirmed very similar trends to the complex HLI 150 group, with comparable or greater increases in early angiogenic, inflammatory and osteogenic signaling at 6 hr. At 24 hr post injury, while some angiogenic and osteogenic genes (*Alpl*, *Bglap*, *Ibsp*) were increased, it was only chemokines such as *Ccl3* and *Cxcl1* that had clear fold differences between complex HLI 90 and complex control. At Day 7 post-injury there were significant differences in expression across angiogenic, inflammatory and osteogenic genes when compared to

complex control, the pattern of which is consistent with other models describing the early gene characterization of HO (221). The suggestion that 90 minutes of tourniquet could increase HO formation remains speculative without an observational study to demonstrate it. The complex HLI 90 HO gene data also provides a secondary validation dataset to confirm the effects seen in the complex HLI 150 group are accurate. When comparing complex HLI 150 against complex HLI 90 gene expression (Appendix 1 Tables 12 and 13), the relative ratio and absolute difference at Day 7 in fold change between the two groups follows the same pattern as observed against the complex control Day 7 group. Of even greater note, is that of the many genes associated with angiogenesis, extracellular matrix, proinflammatory signaling and osteogenesis were upregulated, a high proportion of these continue to reach statistical significance when comparing between 150 and 90 minutes of tourniquet. This data supports the concept that increasing tourniquet time is associated with worsening tissue injury and that minimization of tourniquet time, even at the early clinical timeframe of around 2 hours post-injury, is sufficient to result in less inflammatory and osteogenic signaling.

The interpretation and statistical analysis of gene expression data in this research uses normalization with multiple stable reference genes, gene specific amplification efficiencies which are expressed as normalized relative quantities (NRQs). This method adjusts for PCR inefficiencies through the use of individual standard curves, providing an individual efficiency and a standard error. By including these adjustments, propagation of these errors occurs through the statistical testing process, compounding the biological variance already present. A significant challenge of establishing statistical proof in relation to gene expression occurs due to the wide range of confidence intervals

generated as a result of the complexity and variation in biological response to a polytraumatic injury. While a sample size of $n=8$ can provide clear deviation from the physiological naïve state with statistical weighting, this was not usually possible between complex injured groups, even with large differences in mean fold change. This should be a key consideration in future studies to ensure that they are not underpowered to detect a true effect.

The observational study demonstrated a 70% increase in bone formation; to understand the relevance of this when modeling HO it is necessary to consider other drivers of inflammation which have been shown to increase bone formation. Wound contamination is a common complication of traumatic wounds. Previous work using the same complex injury model has shown that inoculation of the wound with a gram positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) increased HO formation by over 320%, while *Acinetobacter baumannii*, a gram-negative bacteria, only led to a 28% increase (212). Toll-like receptors 2 and 4 are known to detect gram-positive bacteria such as MRSA (269), and different proinflammatory cytokine profiles of leukocytes have been shown to occur between different bacteria types (76; 105), highlighting the subtleties of inflammatory pathway activation that relate to the development of secondary injury.

Similar to the way in which a more persistent or different bacterial strain may lead to greater inflammation and HO formation, the construct of our HO model could also influence the development of secondary injury. The steps of the complex injury model were blast overpressure, femoral fracture, crush injury, $\pm$ tourniquet application, followed by amputation through the zone of injury and layered wound closure. While our model

incorporates the patterns of injuries frequently associated with blast, in combat these all occur simultaneously while in our model they are created sequentially; the exception to this is the application of the tourniquet, which in both instances is applied post-injury. This models early amputation at the equivalent of a Role 2 forward MTF, with the early surgical removal of a non-salvageable limb. It also closely approximates immediate traumatic amputation at point of wounding with tourniquet application then debridement and dressing of the wound at an MTF. An increasingly common treatment modality is the attempt at early limb salvage when soft tissue loss is not too extensive. The choice of amputation versus limb salvage is a complex and challenging decision for a surgeon which extends beyond the scope of this research and discussion. Consideration should be paid though to the effects of increasing tourniquet time as early amputation is a recognized strategy to avoid revascularization and IRI in prolonged tourniquet use, which this work has demonstrated to increase the risk of local tissue dysfunction and HO formation. Recent conflicts have typically seen shorter tourniquet durations than those modeled in these experiments; however a study has cautiously shown that service members who undergo amputation have a better functional outcomes than those with limb salvage (61).

FUTURE DIRECTION

Our research supports the hypothesis that mechanism of injury is a critical variable which can significantly affect the nature and course of the systemic inflammatory response and ultimately outcome following injury. The duration of tourniquet use in our experiments was conservative compared to other unilateral models in the existing literature; this was intentional given the unknown effects that blast

exposure has on tourniquet effect, or how inclusion of a tourniquet would affect a complex limb injury. Further investigation into the inclusion of other injury patterns applicable to combat trauma would help improve the fidelity of our pre-clinical models of combat applicable lower extremity trauma and ischemia reperfusion injury. Prolonged field care (PFC) is recognized as one of the greatest capability gaps facing US and UK military forces. The development of models that replicate the complexity of blast limb trauma and seek to replicate the inflammatory milieu that arises provide a platform for pre-clinical investigation to enable bench-to-bedside-to-battlefield innovation.

This research addresses one small part in the complex interplay of injury pattern, mechanisms and life-saving interventions. Having established that IRI can be potentiated by blast exposure, and that even with early amputation reperfusion injury can occur in the remaining tissue sufficient to cause an increased systemic inflammatory response, other factors need to be considered in model development. Recent US PFC cases highlighted that 17% of cases involved burns (55), an injury known to trigger a significant SIRS, cause metabolic disturbances and drive the development of HO. Bilateral lower limb amputation is a common pattern of blast-related lower limb extremity trauma (214); existing pre-clinical models show that mortality occurs with much shorter tourniquet durations when comparing bilateral to unilateral application, and therefore understanding the effect of multiple tourniquets on the systemic inflammatory response is important.

By further understanding this newly identified effect of blast on IRI, and thus how different combat injuries may affect safe tourniquet use, we seek to prevent avoidable IRI and secondary injury. Predicting, preventing or treating IRI in austere environments to prevent secondary injury would significantly reduce the logistical and medical burden of

injured personnel and has the potential to reduce both morbidity and mortality following traumatic injury. Serum proteomic profiling of inflammatory mediators and DAMPS, combined with gene expression analysis (preferably by RNA-Seq given the reducing costs of this platform) would allow a whole genome approach to examining the transcriptomic profile of these injury patterns, identifying key molecular pathways related to IRI that may be trauma specific variables. This data could then inform either future targets for therapeutic manipulation to mitigate IRI injury or be used with advanced modelling and bioinformatic techniques to construct clinical decision support tools for forward deployed care to improve the prediction of patients at risk of IRI.

CONCLUDING REMARKS

The study design and models in the research were chosen in an attempt to replicate the injury patterns that have become commonplace following blast injury in our service personnel. In doing so they build on existing clinical and pre-clinical data that has contributed to improving survival following combat injury, despite evidence of increasing injury burden (215). The lifesaving benefits of tourniquets with catastrophic hemorrhage remain clear and should continue to be championed both within the military and civilian environment. Hemorrhage remains the primary cause of preventable death following trauma; early point of wounding ‘buddy-buddy’ care with hemorrhage control, damage control resuscitation with blood products and correction of coagulopathy alongside selective surgical intervention are all required to ensure casualties survive long enough for secondary injury complication to be a consideration. That said, a recognition of the additional burden that blast exposure has on the development of IRI and secondary injury as demonstrated by this work must be noted. Clinical timelines to definitive

surgery need to reflect this in a blast complex injured limb, where we have demonstrated that tourniquets increase proinflammatory signaling which can lead to more exaggerated and sustained SIRS response with risk of both local and remote organ dysfunction.

The potential gains to be made by the prevention of secondary injury cannot be understated. Multiple organ dysfunction increases mortality 6-fold when compared to no organ dysfunction; for those surviving it results in increased morbidity with a 4-fold jump in requiring assistance with daily living at 2 years post injury (279). Reducing this burden by identifying patients at risk of secondary injury has both social and financial benefits. Consideration should be given to avoiding tourniquet use in semi-elective lower limb orthopaedic fracture surgery, where the risk of catastrophic hemorrhage is minimal. In the combat environment where blast-associated trauma is more likely to result in large soft tissue loss and catastrophic hemorrhage, minimizing tourniquet duration is paramount in reducing the magnitude and duration of a SIRS response. Current North Atlantic Treaty Organization (NATO) Allied Joint Medical Doctrine (206) defines damage control surgery (DCS) as the ability to save life, limb or function and that it should be provided within 1-2 hours of wounding, requiring the forward deployment of surgical teams. While in Iraq and Afghanistan conflicts, these timelines were predominantly achieved, they present a logistical challenge in the maritime environment and may be exceeded in future conflicts. A delay of 2.5 hours has been shown in our rat model of combat applicable lower limb trauma to result in a more exaggerated inflammatory response capable of increasing both systemic and local secondary injury. In more austere environments or where geography or hostile forces prohibit a time to DCS of under two hours, regular reassessment of the absolute need for tourniquets is

recommended, as published literature has shown that less than a third are applied for arterial bleeding. Furthermore, it should be understood that a mechanism of injury such as blast may reduce the safe duration of tourniquet application, lowering the threshold for replacement of tourniquets with hemostatic dressings and other forms of hemorrhage control if clinical indications allow to prevent the early onset of ischemia reperfusion injury and to reduce the risk of remote organ dysfunction.

APPENDIX 1

Table 6. Summary data of multiplex cytokine and chemokine expression in a whole limb reperfusion model comparing the effect of blast and tourniquet on expression

	6 hours of Reperfusion									
	Naïve	Blast Only	HLI 90	Blast HLI 90	Blast HLI 90 as a % of HLI 90	Adjusted p-value	HLI 150	Blast HLI 150	Blast HLI 150 as a % of HLI 150	Adjusted p-value
IL-1a	1065	1900	2184	2522	115.5%	0.41	2294	2661	116.0%	0.16
IL-1b	652	1189	1481	1597	107.8%	0.63	1485	1703	114.7%	0.13
IL-2	9589	18331	20474	24745	120.9%	0.16	21167	26140	123.5%	0.007
IL-4	762	1274	1461	1642	112.4%	0.44	1641	1683	102.6%	0.81
IL-5	1690	2194	2674	2609	97.6%	0.72	2595	2671	102.9%	0.56
IL-6	2592	4714	5344	6644	124.3%	0.14	6221	7036	113.1%	0.15
IL-7	568	1087	1398	1613	115.4%	0.12	1386	1617	116.7%	0.11
IL-10	758	1262	1722	1779	103.3%	0.85	1753	1861	106.2%	0.26
IL-12(p70)	2835	4395	5963	6252	104.8%	0.86	6027	6652	110.4%	0.35
IL-13	1098	2192	2318	3004	129.6%	0.15	2569	3178	123.7%	0.04
IL-17	300	451	516	565	109.5%	0.45	526	604	114.8%	0.02
IL-18	9028	15232	18665	20539	110.0%	0.30	18905	21304	112.7%	0.06
G-CSF	122	228	230	272	118.3%	0.40	240	299	124.6%	0.02
GM-CSF	562	936	1137	1276	112.2%	0.16	1152	1277	110.9%	0.15
GRO/KC (CXCL1)	374	602	1145	1193	104.2%	0.94	1830	1431	78.2%	0.36
IFN-g	2628	5273	5786	7004	121.1%	0.21	6469	7416	114.6%	0.03
M-CSF	129	315	375	506	134.9%	0.17	426	520	122.1%	0.16
MIP-1a (CCL3)	161	235	283	293	103.5%	0.90	349	328	94.0%	0.83
MIP-3a (CCL20)	104	166	227	279	122.9%	0.20	291	270	92.8%	0.79
RANTES (CCL5)	1878	1526	1856	1690	91.1%	0.45	1977	1928	97.5%	0.94
TNF-a	2633	6069	6575	7837	119.2%	0.20	7047	8086	114.7%	0.17
VEGF	1266	2483	2136	2313	108.3%	0.33	2147	2382	110.9%	0.13
MCP-1 (CCL2)	2587	3532	4677	4359	93.2%	0.31	4907	4803	97.9%	0.83

	24 hours of Reperfusion									
	Naïve	Blast Only	HLI 90	B HLI 90	Blast HLI 90 as a % of HLI 90	Adjusted p-value	HLI 150	Blast HLI 150	Blast HLI 150 as a % of HLI 150	Adjusted p-value
IL-1a	1065	1854	2359	2520	106.8%	0.48	2200	2792	126.9%	0.008
IL-1b	652	1214	1541	1628	105.6%	0.58	1421	1770	124.6%	0.02
IL-2	9589	16644	21955	23347	106.3%	0.30	21532	25146	116.8%	0.09
IL-4	762	1266	1599	1746	109.2%	0.37	1550	1854	119.6%	0.05
IL-5	1690	2109	2698	2732	101.3%	0.84	2590	2791	107.8%	0.07
IL-6	2592	4732	6346	6618	104.3%	0.69	6026	7189	119.3%	0.04
IL-7	568	1368	1425	1530	107.4%	0.42	1338	1690	126.3%	0.01
IL-10	758	1250	1820	1843	101.3%	0.92	1659	1924	116.0%	0.02
IL-12(p70)	2835	4650	5805	6436	110.9%	0.42	5601	6984	124.7%	0.04
IL-13	1098	2148	2756	2966	107.6%	0.33	2630	3274	124.5%	0.05
IL-17	300	471	558	610	109.3%	0.13	551	632	114.7%	0.04
IL-18	9028	16033	19564	20425	104.4%	0.49	18723	21317	113.9%	0.03
G-CSF	122	217	270	293	108.5%	0.46	257	302	117.5%	0.16
GM-CSF	562	1159	1176	1257	106.9%	0.43	1098	1343	122.3%	0.02
GRO/KC (CXCL1)	374	554	812	760	93.6%	0.86	659	715	108.5%	0.66
IFN-g	2628	5031	6765	7250	107.2%	0.44	6467	7581	117.2%	0.06
M-CSF	129	300	432	488	113.0%	0.25	404	568	140.6%	0.005
MIP-1a (CCL3)	161	268	294	312	106.1%	0.55	279	333	119.4%	0.02
MIP-3a (CCL20)	104	157	286	263	92.0%	0.77	228	296	129.8%	0.09
RANTES (CCL5)	1878	1506	1839	1782	96.9%	0.93	1648	1666	101.1%	>0.99
TNF-a	2633	5965	7204	7641	106.1%	0.57	6717	8555	127.4%	0.02
VEGF	1266	2299	2145	2326	108.4%	0.23	2067	2471	119.5%	0.03
MCP-1 (CCL2)	2587	4094	5149	4838	94.0%	0.71	4934	5135	104.1%	0.52

Table 7. Summary data of multiplex cytokine and chemokine expression in a complex injured model between 6 hours and Day 10 post injury comparing the complex control group with complex HLI 90 and 150 groups

	6 hours Post Injury									
	Naïve	Complex Control	Complex HLI 90	Complex HLI 90 as % of Control	Adjusted p-value	Complex HLI 150	Complex HLI 150 as % of Control	Adjusted p-value	Complex HLI 150 as % of Complex HLI 90	Adjusted p-value between Complex HLI 90 and 150
IL-1a	1065	1934	2400	124.1%	0.02	2338	120.9%	0.09	97.4%	0.94
IL-1b	652	1309	1552	118.6%	0.05	1566	119.6%	0.06	100.9%	>0.99
IL-2	9589	18208	22636	124.3%	0.02	21202	116.4%	0.22	93.7%	0.68
IL-4	762	1302	1577	121.1%	0.01	1309	100.5%	>0.99	83.0%	0.02
IL-5	1690	2118	2593	122.4%	<0.001	2252	106.3%	0.18	86.8%	<0.001
IL-6	2592	5282	6290	119.1%	0.08	5952	112.7%	0.37	94.6%	0.77
IL-7	568	1181	1448	122.6%	0.02	1523	129.0%	0.005	105.2%	0.74
IL-10	758	1366	1761	128.9%	<0.001	1657	121.3%	0.008	94.1%	0.52
IL-12(p70)	2835	4730	5892	124.6%	0.01	5667	119.8%	0.08	96.2%	0.86
IL-13	1098	2410	2806	116.4%	0.26	2819	117.0%	0.29	100.5%	>0.99
IL-17	300	483	558	115.5%	0.05	529	109.5%	0.36	94.8%	0.66
IL-18	9028	16373	19173	117.1%	0.009	18724	114.4%	0.05	97.7%	0.89
G-CSF	122	216	272	125.9%	0.01	259	119.9%	0.11	95.2%	0.81
GM-CSF	562	941	1184	125.8%	0.002	1128	119.9%	0.03	95.3%	0.73
GRO/KC (CXCL1)	374	1236	2077	168.0%	<0.001	1475	119.3%	0.34	71.0%	0.002
IFN-g	2628	5566	6666	119.8%	0.06	6658	119.6%	0.10	99.9%	>.99
M-CSF	129	351	431	122.8%	0.19	482	137.3%	0.03	111.8%	0.57
MIP-1a (CCL3)	161	250	300	120.0%	0.34	282	112.8%	0.69	94.0%	0.88
MIP-3a (CCL20)	104	207	259	125.1%	0.11	248	119.8%	0.29	95.8%	0.92
RANTES (CCL5)	1878	1254	2109	168.2%	<0.001	1275	101.7%	>.99	101.4%	0.003
TNF-a	2633	5913	7385	124.9%	0.07	7485	126.6%	0.07	101.4%	0.99
VEGF	1266	2380	2338	98.2%	0.95	2169	91.1%	0.36	92.8%	0.52
MCP-1 (CCL2)	2587	3262	4563	139.9%	<0.001	4061	124.5%	0.03	89.0%	0.25

	24 hours Post Injury									
	Naïve	Complex Control	Complex HLI 90	Complex HLI 90 as % of Control	Adjusted p-value	Complex HLI 150	Complex HLI 150 as % of Control	Adjusted p-value	Complex HLI 150 as % of Complex HLI 90	Adjusted p-value between Complex HLI 90 and 150
IL-1a	1065	2440	3043	124.7%	0.003	2876	117.9%	0.03	94.5%	0.59
IL-1b	652	1642	1899	115.7%	0.04	2010	122.4%	0.001	105.8%	0.50
IL-2	9589	22072	28998	131.4%	<0.001	25264	114.5%	0.10	87.1%	0.05
IL-4	762	1549	1889	121.9%	0.001	1685	108.8%	0.28	89.2%	0.06
IL-5	1690	2304	2815	122.2%	<0.001	2592	112.5%	<0.001	92.1%	0.004
IL-6	2592	6378	7795	122.2%	0.01	7003	109.8%	0.34	89.8%	0.18
IL-7	568	1602	1754	109.5%	0.25	1946	121.5%	<0.001	110.9%	0.10
IL-10	758	1617	2021	125.0%	<0.001	2004	123.9%	<0.001	99.2%	0.98
IL-12(p70)	2835	6051	7293	120.5%	0.01	7246	119.7%	0.01	99.4%	0.99
IL-13	1098	3044	3747	123.1%	0.02	3418	112.3%	0.28	91.2%	0.37
IL-17	300	580	654	112.8%	0.05	636	109.7%	0.16	97.2%	0.83
IL-18	9028	18756	23800	126.9%	<0.001	21834	116.4%	0.003	91.7%	0.08
G-CSF	122	254	336	132.3%	<0.001	332	130.7%	<0.001	98.8%	0.97
GM-CSF	562	1145	1408	123.0%	<0.001	1403	122.5%	<0.001	99.6%	>.99
GRO/KC (CXCL1)	374	1060	1115	105.2%	0.93	1083	102.2%	0.99	97.1%	0.98
IFN-g	2628	6595	8329	126.3%	0.002	8045	122.0%	0.01	96.6%	0.82
M-CSF	129	446	656	147.1%	<0.001	569	127.6%	0.02	86.7%	0.13
MIP-1a (CCL3)	161	305	339	111.1%	0.59	342	112.1%	0.54	100.9%	>.99
MIP-3a (CCL20)	104	360	391	108.6%	0.45	359	99.7%	>.99	91.8%	0.41
RANTES (CCL5)	1878	1384	1343	97.0%	0.98	1386	100.1%	>.99	103.2%	>.99
TNF-a	2633	7424	10014	134.9%	<0.001	9020	121.5%	0.03	90.1%	0.26
VEGF	1266	2792	2506	89.8%	0.11	2722	97.5%	0.87	108.6%	0.26
MCP-1 (CCL2)	2587	4468	5200	116.4%	0.03	6343	142.0%	<0.001	122.0%	<0.001

	3 Days Post Injury									
	Naïve	Complex Control	Complex HLI 90	Complex HLI 90 as % of Control	Adjusted p-value	Complex HLI 150	Complex HLI 150 as % of Control	Adjusted p-value	Complex HLI 150 as % of Complex HLI 90	Adjusted p-value between Complex HLI 90 and 150
IL-1a	1065	2272	2492	109.7%	0.50	2975	130.9%	0.001	119.4%	0.02
IL-1b	652	1513	1805	119.3%	0.03	2030	134.2%	<0.001	112.5%	0.09
IL-2	9589	17446	14596	83.7%	0.25	23477	134.6%	0.002	160.8%	<0.001
IL-4	762	1367	1537	112.4%	0.22	1732	126.7%	0.001	112.7%	0.11
IL-5	1690	2179	2402	110.2%	0.01	2606	119.6%	<0.001	108.5%	0.02
IL-6	2592	5180	5534	106.8%	0.76	6657	128.5%	0.01	120.3%	0.06
IL-7	568	1428	1529	107.1%	0.6	2016	141.2%	<0.001	131.9%	<0.001
IL-10	758	1449	1441	99.4%	>.99	2008	138.6%	<0.001	139.3%	<0.001
IL-12(p70)	2835	5755	6249	108.6%	0.51	7293	126.7%	0.002	116.7%	0.04
IL-13	1098	2375	2683	113.0%	0.51	3154	132.8%	0.01	117.6%	0.17
IL-17	300	506	510	100.8%	0.99	623	123.1%	0.002	122.2%	0.002
IL-18	9028	17531	18687	106.6%	0.50	20704	118.1%	0.006	110.8%	0.09
G-CSF	122	207	249	120.3%	0.15	301	145.4%	<0.001	120.9%	0.03
GM-CSF	562	1067	1220	114.3%	0.11	1462	137.0%	<0.001	119.8%	0.003
GRO/KC (CXCL1)	374	750	810	108.0%	0.94	920	122.7%	0.60	113.6%	0.79
IFN-g	2628	5396	6469	119.9%	0.12	7516	139.3%	<0.001	116.2%	0.10
M-CSF	129	394	451	114.5%	0.52	565	143.4%	0.003	125.3%	0.05
MIP-1a (CCL3)	161	311	333	107.1%	0.84	408	131.2%	0.03	122.5%	0.08
MIP-3a (CCL20)	104	272	231	84.9%	0.32	300	110.3%	0.57	129.9%	0.03
RANTES (CCL5)	1878	1663	2086	125.4%	0.22	2059	123.8%	0.24	98.7%	0.99
TNF-a	2633	6211	8518	137.1%	0.006	8793	141.6%	0.001	103.2%	0.91
VEGF	1266	2666	2226	83.5%	0.02	2871	107.7%	0.38	129.0%	<0.001
MCP-1 (CCL2)	2587	4062	3980	98.0%	0.96	6428	158.2%	<0.001	161.5%	<0.001

	7 Days Post Injury									
	Naive	Complex Control	Complex HLI 90	Complex HLI 90 as % of Control	Adjusted p-value	Complex HLI 150	Complex HLI 150 as % of Control	Adjusted p-value	Complex HLI 150 as % of Complex HLI 90	Adjusted p-value between Complex HLI 90 and 150
IL-1a	1065	2024	2203	108.8%	0.61	2884	142.5%	<0.001	130.9%	<0.001
IL-1b	652	1508	1553	103.0%	0.92	2168	143.8%	<0.001	139.6%	<0.001
IL-2	9589	13270	14265	107.5%	0.83	20746	156.3%	<0.001	145.4%	<0.001
IL-4	762	955	1288	134.9%	0.003	1732	181.4%	<0.001	134.5%	0.170
IL-5	1690	2179	2346	107.7%	0.08	2524	115.8%	<0.001	107.6%	0.030
IL-6	2592	2774	3904	140.7%	0.06	4909	177.0%	<0.001	125.7%	0.080
IL-7	568	1357	1361	100.3%	>.99	2089	153.9%	<0.001	153.5%	<0.001
IL-10	758	1327	1259	94.9%	0.71	1857	139.9%	<0.001	147.5%	0.001
IL-12(p70)	2835	5049	5392	106.8%	0.12	6860	135.9%	<0.001	127.2%	0.070
IL-13	1098	1161	1694	145.9%	0.98	2249	193.7%	0.001	132.8%	<0.001
IL-17	300	410	403	98.3%	0.98	537	131.0%	0.001	133.3%	<0.001
IL-18	9028	16912	17238	101.9%	0.94	21150	125.1%	<0.001	122.7%	<0.001
G-CSF	122	119	171	143.7%	0.04	222	186.5%	<0.001	129.8%	0.300
GM-CSF	562	1053	1102	104.7%	0.78	1504	142.8%	<0.001	136.5%	<0.001
GRO/KC (CXCL1)	374	700	635	90.7%	0.92	740	105.7%	0.970	116.5%	0.770
IFN-g	2628	3029	4304	142.1%	0.04	5680	187.5%	<0.001	132.0%	0.010
M-CSF	129	312	373	119.6%	0.44	546	175.0%	<0.001	146.4%	<0.001
MIP-1a (CCL3)	161	319	328	102.8%	0.97	426	133.5%	0.01	129.9%	0.01
MIP-3a (CCL20)	104	193	203	105.2%	0.94	276	143.0%	<0.001	136.0%	0.02
RANTES (CCL5)	1878	2813	2803	99.6%	>.99	2065	73.4%	0.009	73.7%	0.005
TNF-a	2633	4956	6329	127.7%	0.13	8393	169.4%	<0.001	132.6%	0.006
VEGF	1266	2690	2619	97.4%	0.89	2985	111.0%	0.15	114.0%	0.04
MCP-1 (CCL2)	2587	4384	4204	95.9%	0.83	5279	120.4%	0.02	125.6%	0.002

10 Days Post Injury				
	Complex Control	Complex HLI 150	% increase	Adjusted p-value
IL-1a	2081	1890	90.8%	0.56
IL-1b	1492	1424	95.4%	0.69
IL-2	15451	14843	96.1%	0.77
IL-4	1173	1190	101.4%	0.89
IL-5	2151	2263	105.2%	0.29
IL-6	3632	4028	110.9%	0.50
IL-7	1421	1351	95.1%	0.70
IL-10	1345	1396	103.8%	0.74
IL-12(p70)	4587	5181	112.9%	0.39
IL-13	1641	1587	96.7%	0.86
IL-17	434	432	99.5%	0.93
IL-18	16436	14178	86.3%	0.07
G-CSF	142	166	116.9%	0.25
GM-CSF	1057	1050	99.3%	0.95
GRO/KC (CXCL1)	551	525	95.3%	0.75
IFN-g	3901	4344	111.4%	0.50
M-CSF	328	302	92.1%	0.73
MIP-1a (CCL3)	309	278	90.0%	0.41
MIP-3a (CCL20)	184	156	84.8%	0.36
RANTES (CCL5)	1865	2056	110.2%	0.17
TNF-a	5824	5380	92.4%	0.67
VEGF	2598	2155	82.9%	0.09
MCP-1 (CCL2)	3646	4305	118.1%	0.09

Table 8. Relative fold difference between blast and non-blast HLI groups
Ischemia reperfusion injury gene expression data displayed as a ratio of fold change between blast and non-blast HLI groups. P-values of significance shown between groups using unpaired t-test

Lung

	HLI 90 6hrs	HLI 90 24hrs		HLI 150 6hrs	HLI 150 24hrs
Adm			Adm	0.03	
Atf3			Atf3		
Bcl2			Bcl2		
Bcl3			Bcl3		
Bid			Bid		
Casp8			Casp8		0.03
Ccl2			Ccl2		0.04
Ccl20			Ccl20		
Cxcl1			Cxcl1		
Cxcl2			Cxcl2		
Fos			Fos		
Gapdh			Gapdh	0.04	
Hif1a			Hif1a		
Hspa1b	0.03		Hspa1b	0.02	
Hspd1			Hspd1	0.05	
Il1b			Il1b		
Il6		0.05	Il6		
Jun			Jun		
Len2		0.009	Len2	0.03	0.01
Maff			Maff		
Myc			Myc		
Nfkbiz			Nfkbiz		
Nos2			Nos2		
Nr4a1			Nr4a1		
S100a8			S100a8		
Serpine1			Serpine1		
Soes3	0.04		Soes3		
Sod2		0.04	Sod2		

Fold Change between Blast and Non-Blast Group	
50+	
25-50	
5 - 10	
2 - 5	
1.5 - 2	
1.5-2	
1.25-1.5	
1-1.25	
0.75-1	
0.5-0.75	
0.2-0.5	
0.1-0.2	
<0.1	

Liver

	HLI 90 6hrs	HLI 90 24hrs		HLI 150 6hrs	HLI 150 24hrs
Adm			Adm	0.01	0.05
Atf3			Atf3		0.05
Bcl2			Bcl2		
Bcl3			Bcl3		
Bid			Bid		
Casp8			Casp8		0.02
Ccl2			Ccl2		
Ccl20			Ccl20		
Cxcl1			Cxcl1		
Cxcl2			Cxcl2		
Fos			Fos		
Gapdh			Gapdh	0.01	
Hif1a			Hif1a		
Hspa1b			Hspa1b		
Hspd1			Hspd1		
Il1b			Il1b		
Il6			Il6		0.02
Jun	0.01		Jun		
Len2			Len2		
Maff			Maff		0.02
Myc			Myc		
Nfkbiz			Nfkbiz		
Nos2			Nos2		
Nr4a1			Nr4a1	0.002	
S100a8			S100a8		
Serpine1			Serpine1	0.04	
Soes3			Soes3		
Sod2			Sod2		

Fold Change between Blast and Non-Blast Group	
50+	
25-50	
5 - 10	
2 - 5	
1.5 - 2	
1.5-2	
1.25-1.5	
1-1.25	
0.75-1	
0.5-0.75	
0.2-0.5	
0.1-0.2	
<0.1	

Kidney

	HLI 90 6hrs	HLI 90 24hrs		HLI 150 6hrs	HLI 150 24hrs
Adm			Adm		
Atf3			Atf3		
Bcl2	0.01		Bcl2		
Bcl3			Bcl3		
Bid	0.01		Bid		
Casp8			Casp8		0.006
Ccl2	0.009		Ccl2		
Ccl20			Ccl20		
Cxcl1			Cxcl1		0.04
Cxcl2		0.05	Cxcl2		
Fos			Fos		
Gapdh			Gapdh		
Hif1a			Hif1a		
Hspa1b			Hspa1b		0.02
Hspd1			Hspd1		
Il1b			Il1b		
Il6			Il6		
Jun	0.02		Jun		
Len2			Len2		
Maff			Maff		
Myc			Myc	0.009	0.05
Nfkbiz			Nfkbiz	0.03	
Nos2		0.03	Nos2		
Nr4a1			Nr4a1	0.04	0.02
S100a8			S100a8		
Serpine1		0.04	Serpine1		0.02
Soes3			Soes3		
Sod2			Sod2		

Fold Change between Blast and Non-Blast Group	
50+	
25-50	
5 - 10	
2 - 5	
1.5 - 2	
1.5-2	
1.25-1.5	
1-1.25	
0.75-1	
0.5-0.75	
0.2-0.5	
0.1-0.2	
<0.1	

Muscle		HLI 90 6hrs	HLI 90 24hrs		HLI 150 6hrs	HLI 150 24hrs
	Adm		0.009	Adm		
	Atf3		0.05	Atf3		
	Bcl2		0.003	Bcl2	0.02	
	Bcl3		0.01	Bcl3	0.03	
	Bid			Bid	0.04	
	Casp8		0.004	Casp8		
	Ccl2			Ccl2		
	Ccl20			Ccl20		
	Cxcl1		0.03	Cxcl1		
	Cxcl2			Cxcl2	0.03	
	Fos			Fos		
	Gapdh			Gapdh	0.007	
	Hif1a			Hif1a		
	Hspa1b			Hspa1b		
	Hspd1			Hspd1		
	Il1b		0.005	Il1b		0.04
	Il6		0.04	Il6	0.03	
	Jun			Jun	0.008	
	Lcn2		0.04	Lcn2	0.05	
	Maff			Maff		
	Myc			Myc	0.004	
	Nfkbiz		0.02	Nfkbiz	0.004	
	Nos2			Nos2		
	Nr4a1			Nr4a1	0.02	
	S100a8			S100a8	0.04	
	Serpine1		0.003	Serpine1	0.007	
	Socs3			Socs3	0.03	
	Sod2			Sod2	0.04	

Fold Change between Blast and Non-Blast Group	
50+	
25-50	
5 - 10	
2 - 5	
1.5 - 2	
1.5-2	
1.25-1.5	
1-1.25	
0.75-1	
0.5-0.75	
0.2-0.5	
0.1-0.2	
<0.1	

Table 9. Fold difference between blast and non-blast HLI groups expressed as absolute fold difference by subtraction of groups

Lung		HLI 90 6hrs	HLI 90 24hrs		HLI 150 6hrs	HLI 150 24hrs
	Adm			Adm	0.03	
	Atf3			Atf3		
	Bcl2			Bcl2		
	Bcl3			Bcl3		
	Bid			Bid		
	Casp8			Casp8		0.03
	Ccl2			Ccl2		0.04
	Ccl20			Ccl20		
	Cxcl1			Cxcl1		
	Cxcl2			Cxcl2		
	Fos			Fos		
	Gapdh			Gapdh	0.04	
	Hif1a			Hif1a		
	Hspa1b	0.03		Hspa1b	0.02	
	Hspd1			Hspd1	0.05	
	Il1b			Il1b		
	Il6		0.05	Il6		
	Jun			Jun		
	Lcn2		0.009	Lcn2	0.03	0.01
	Maff			Maff		
	Myc			Myc		
	Nfkbiz			Nfkbiz		
	Nos2			Nos2		
	Nr4a1			Nr4a1		
	S100a8			S100a8		
	Serpine1			Serpine1		
	Socs3	0.04		Socs3		
	Sod2		0.04	Sod2		

Fold change of blast minus non blast group	
500+	
100 - 500	
50 - 100	
25 - 50	
10-25	
5 - 10	
1 - 5	
-1 - 1	
-1 - -5	
-10 - -5	
-25 - -10	
-50 - -25	
-100 - -50	
-500 - -100	
<-500	

Liver

	HLI 90 6hrs	HLI 90 24hrs
Adm		
Atf3		
Bcl2		
Bcl3		
Bid		
Casp8		
Ccl2		
Ccl20		
Cxcl1		
Cxcl2		
Fos		
Gapdh		
Hif1a		
Hspa1b		
Hspd1		
Il1b		
Il6		
Jun	0.01	
Lcn2		
Maff		
Myc		
Nfkbiz		
Nos2		
Nr4a1		
S100a8		
Serpine1		
Socs3		
Sod2		

	HLI 150 6hrs	HLI 150 24hrs
Adm	0.01	0.05
Atf3		
Bcl2		
Bcl3		
Bid		
Casp8		0.02
Ccl2		
Ccl20		
Cxcl1		
Cxcl2		
Fos		
Gapdh	0.01	
Hif1a		
Hspa1b		
Hspd1		
Il1b		
Il6		0.02
Jun		
Lcn2		
Maff		0.02
Myc		
Nfkbiz		
Nos2		
Nr4a1	0.002	
S100a8		
Serpine1	0.04	
Socs3		
Sod2		

Fold change of blast minus non blast group	
500+	
100 - 500	
50 - 100	
25 - 50	
10-25	
5 - 10	
1 - 5	
-1 - 1	
-10 - -5	
-25 - -10	
-50 - -25	
-100 - -50	
-500 - -100	
<-500	

Kidney

	HLI 90 6hrs	HLI 90 24hrs
Adm		
Atf3		
Bcl2	0.01	
Bcl3	0.07	
Bid	0.01	
Casp8		
Ccl2	0.009	
Ccl20		
Cxcl1		
Cxcl2		0.05
Fos		
Gapdh		
Hif1a		
Hspa1b		
Hspd1		
Il1b		
Il6		
Jun	0.02	
Lcn2		
Maff		
Myc		
Nfkbiz		
Nos2		0.03
Nr4a1		
S100a8		
Serpine1		0.04
Socs3		
Sod2		

	HLI 150 6hrs	HLI 150 24hrs
Adm		
Atf3		
Bcl2		
Bcl3		
Bid		
Casp8		0.006
Ccl2		
Ccl20		
Cxcl1		
Cxcl2		
Fos		
Gapdh		
Hif1a		
Hspa1b		0.02
Hspd1		
Il1b		
Il6		
Jun		
Lcn2		
Maff		
Myc	0.009	0.05
Nfkbiz	0.03	
Nos2		
Nr4a1	0.04	0.02
S100a8		
Serpine1		0.02
Socs3		
Sod2		

Fold change of blast minus non blast group	
500+	
100 - 500	
50 - 100	
25 - 50	
10-25	
5 - 10	
1 - 5	
-1 - 1	
-10 - -5	
-25 - -10	
-50 - -25	
-100 - -50	
-500 - -100	
<-500	

Muscle

	HLI 90 6hrs	HLI 90 24hrs
Adm		0.009
Atf3		0.05
Bcl2		0.003
Bcl3		0.01
Bid		
Casp8		0.004
Ccl2		
Ccl20		
Cxcl1		0.03
Cxcl2		
Fos		
Gapdh		
Hif1a		
Hspa1b		
Hspd1		
Il1b		0.005
Il6		0.04
Jun		
Lcn2		0.04
Maff		
Myc		
Nfkbiz		0.02
Nos2		
Nr4a1		
S100a8		
Serpine1		0.003
Socs3		
Sod2		

	HLI 150 6hrs	HLI 150 24hrs
Adm		
Atf3		
Bcl2	0.02	
Bcl3	0.03	
Bid	0.04	
Casp8		
Ccl2		
Ccl20		
Cxcl1		
Cxcl2	0.03	
Fos		
Gapdh	0.007	
Hif1a	0.01	
Hspa1b		
Hspd1		
Il1b		0.04
Il6	0.03	
Jun	0.008	
Lcn2	0.05	
Maff		
Myc	0.004	
Nfkbiz	0.004	
Nos2		
Nr4a1	0.02	
S100a8	0.04	
Serpine1	0.007	
Socs3	0.03	
Sod2	0.04	

Fold change of blast minus non blast group	
500+	
100 - 500	
50 - 100	
25 - 50	
10-25	
5 - 10	
1 - 5	
-1 - 1	
-10 - -5	
-25 - -10	
-50 - -25	
-100 - -50	
-500 - -100	
<-500	

Ischemia reperfusion injury gene expression data displayed as a ratio of fold change between complex HLI 150 and complex control groups. P-values of significance shown between groups using unpaired t-test

Fold Change between Complex HLI 150 and Complex Control groups	
50+	
25-50	
10-25	
5-10	
2-5	
1.5-2	
1.25-1.5	
1-1.25	
0.75-1	
0.5-0.75	
0.2-0.5	
0.1-0.2	
<0.1	

Kidney		6hrs	24hrs	Day 3	Day 7		Fold Change between Complex HLI 150 and Complex Control groups
	Adm	0.02	0.04				50+
	Atf3						25-50
	Bcl2						10-25
	Bcl3						5-10
	Bid						2-5
	Casp8						1.5-2
	Ccl2		0.05	0.008			1.25-1.5
	Cd20						1-1.25
	Cxcl1						0.75-1
	Cxcl2						0.5-0.75
	Fos						0.2-0.5
	Gapdh						0.1-0.2
	Hif1a				<0.001		<0.1
	Hspa1b				0.05		
	Hspd1						
	Il1b						
	Il6						
	Jun				0.001		
	Lcn2						
	Maff						
	Myc	0.03	0.04				
	Nfkbiz						
	Nos2						
	Nr4a1						
	S100a8						
	Serpine1		0.03				
	Socs3						
	Sod2				0.005		

Muscle		6hrs	24hrs	Day 3	Day 7		Fold Change between Complex HLI 150 and Complex Control groups
	Adm	0.001					50+
	Atf3						25-50
	Bcl2						10-25
	Bcl3						5-10
	Bid						2-5
	Casp8						1.5-2
	Ccl2						1.25-1.5
	Cd20						1-1.25
	Cxcl1						0.75-1
	Cxcl2						0.5-0.75
	Fos						0.2-0.5
	Gapdh		0.04				0.1-0.2
	Hif1a						<0.1
	Hspa1b	0.04					
	Hspd1						
	Il1b						
	Il6						
	Jun	0.03					
	Lcn2						
	Maff						
	Myc						
	Nfkbiz						
	Nos2						
	Nr4a1						
	S100a8						
	Serpine1						
	Socs3						
	Sod2						

Table 11. Fold difference between complex HLI 150 and complex control groups expressed as absolute fold difference by subtraction of groups

Lung		6hrs	24hrs	Day 3	Day 7	Fold change of complex HLI 150 minus non complex control
Adm						500+
Atf3				0.02	0.002	100 - 500
Bcl2				0.04		50 - 100
Bcl3		0.02			0.03	25 - 50
Bid					0.01	10-25
Casp8					0.004	5 - 10
Ccl2						1 - 5
Ccl20						-1 - 1
Cxcl1						-1 - -5
Cxcl2						-10 - -5
Fos						-25 - -10
Gapdh						-50 - -25
Hif1a					0.005	-100 - -50
Hspa1b						-500 - -100
Hspd1					0.04	<-500
Il1b						
Il6					0.03	
Jun					0.02	
Lcn2		0.02		0.04		
Maff						
Myc						
Nfkbiz		0.04	0.006	0.04	0.03	
Nos2						
Nr4a1						
S100a8						
Serpine1						
Socs3			0.01		0.02	
Sod2						

Liver		6hrs	24hrs	Day 3	Day 7	Fold change of complex HLI 150 minus non complex control
Adm						500+
Atf3						100 - 500
Bcl2				0.001		50 - 100
Bcl3						25 - 50
Bid						10-25
Casp8						5 - 10
Ccl2				0.003		1 - 5
Ccl20				0.009		-1 - 1
Cxcl1		0.03				-1 - -5
Cxcl2				0.03		-10 - -5
Fos						-25 - -10
Gapdh						-50 - -25
Hif1a						-100 - -50
Hspa1b						-500 - -100
Hspd1						<-500
Il1b						
Il6			0.02	0.009	0.03	
Jun		0.004				
Lcn2						
Maff		0.03	0.05			
Myc						
Nfkbiz						
Nos2				0.02		
Nr4a1						
S100a8						
Serpine1		0.02				
Socs3						
Sod2						

Kidney

	6hrs	24hrs	Day 3	Day 7
Adm	0.02	0.04		
Atf3				
Bcl2				
Bcl3				
Bid				
Casp8				
Ccl2		0.05	0.008	
Ccl20				
Cxcl1				
Cxcl2				
Fos				
Gapdh				
Hif1a				<0.001
Hspa1b				0.05
Hspd1				
Il1b				
Il6				
Jun				0.001
Lcn2				
Maff				
Myc	0.03	0.04		
Nfkbiz				
Nos2				
Nr4a1				
S100a8				
Serpine1		0.03		
Socs3				
Sod2				0.005

Fold change of complex HLI 150 minus non complex control	
500+	
100 - 500	
50 - 100	
25 - 50	
10-25	
5 - 10	
1 - 5	
-1 - 1	
-1 - -5	
-10 - -5	
-25 - -10	
-50 - -25	
-100 - -50	
-500 - -100	
<-500	

Muscle

	6hrs	24hrs	Day 3	Day 7
Adm	0.001			
Atf3				
Bcl2				
Bcl3				
Bid				
Casp8				
Ccl2				
Ccl20				
Cxcl1				
Cxcl2				
Fos				
Gapdh		0.04		
Hif1a				
Hspa1b	0.04			
Hspd1				
Il1b				
Il6				
Jun	0.03			
Lcn2				
Maff				
Myc				
Nfkbiz				
Nos2				
Nr4a1				
S100a8				
Serpine1				
Socs3				
Sod2				

Fold change of complex HLI 150 minus non complex control	
500+	
100 - 500	
50 - 100	
25 - 50	
10-25	
5 - 10	
1 - 5	
-1 - 1	
-1 - -5	
-10 - -5	
-25 - -10	
-50 - -25	
-100 - -50	
-500 - -100	
<-500	

Table 12. Relative fold difference between complex HLI 150, complex HLI 90 and complex control group for HO associated gene expression
Heterotopic ossification associated gene expression data displayed as a ratio of fold change between complex HLI 150 and complex control groups. P-values of significance shown between groups using unpaired t-test

Fold change between Complex HLI 150 and Complex Control					
	6hrs	24hrs	D3	D7	D10
Acan					
Adipor1				0.002	
Alpl		0.007		0.001	
Angpt2				<0.001	
Bglap					
Bmp2	0.02			0.001	
Bmp4					
Bmp6		0.009			
Ccl2					
Ccl3				0.02	
Cd44					
Cebpa					
Col1a1				<0.001	
Col1a1				0.005	
Col1a1				0.003	
Comp				0.02	
Cx3				0.008	
Cxcl1					0.03
Cxcl10		0.01			
Cxcl12				<0.001	
Cxcl5					
Eng		0.03		0.02	
Fabp4				<0.001	
Fgf1					
Fgf10				<0.001	
Fgf2					
Fhl1		0.02		0.003	
Gli2		0.01		0.002	
Gusb		0.05		0.002	
Has1				0.001	
Has2				0.002	
Hat1			0.01	<0.001	
Hdac1				0.001	
Hif1a				0.004	
Hif1a				0.04	
Ibsp					
Igf2		0.02		0.004	
Il10				0.022	
Il1b				0.008	0.02
Il6				0.01	0.04
Itga1		0.009		0.002	
Itga2				0.002	
Itgam				0.001	
Itgav		0.03		0.001	
Itgav				0.001	
Jag1				0.001	
Lrp5		0.02		0.002	
Mmp9				0.006	
Myd1					
Notch1				0.002	
Omd					
Pdgfa	0.04	0.05	0.008	<0.001	0.04
Phex				0.04	
Prarg				0.02	
Pich1				<0.001	
Pik2				0.007	
Rhoa		0.04		0.002	
Runx2				0.005	
Scarb1		0.02		0.001	
Smad2				<0.001	
Smc		0.02		0.001	
Smurf1		0.03		0.001	
Sux9				<0.001	
Sp1				<0.001	
Sparc				0.003	
Spp1				0.01	
Ter1					
Tgfb1				0.001	
Tgfb3				0.004	
Tnf				0.01	
Twist1				0.02	
Vegfa		0.05		0.02	
Wnt5a				0.001	

Fold change between Complex HLI 150 and Complex HLI 90				
	6hrs	24hrs		D7
Acan				
Adipor1		0.01		
Alpl				
Angpt2				0.04
Bglap				
Bmp2				0.05
Bmp4				
Bmp6				
Ccl2				
Ccl3				0.05
Cd44				
Cebpa				
Col1a1				
Col1a1		0.03		
Col1a1				
Comp				
Cx3				
Cxcl1				
Cxcl10				
Cxcl12				
Cxcl5				
Eng		0.03		
Fabp4				0.005
Fgf1				0.03
Fgf10				0.003
Fgf2	0.03			
Fhl1		0.03		
Gli2				
Gusb				0.05
Has1				
Has2				
Hat1				0.02
Hdac1				
Hif1a				
Hif1a				
Ibsp				
Igf2				
Il10				0.04
Il1b				0.04
Il6				
Itga1				0.003
Itga2				0.006
Itgam				0.03
Itgav				0.01
Itgav				0.02
Jag1		0.04		
Lrp5				
Mmp9				
Myd1				
Notch1		0.007		
Omd				0.01
Pdgfa				0.03
Phex				0.03
Prarg				0.03
Pich1				
Pik2				
Rhoa		0.03		
Runx2				
Scarb1				0.03
Smad2		0.02		
Smc		0.01		
Smurf1		0.009		
Sux9				
Sp1		0.02		0.02
Sparc		0.01		0.05
Spp1				
Ter1				
Tgfb1		0.01		0.05
Tgfb3		0.02		
Tnf				
Twist1				
Vegfa				
Wnt5a				

Fold change between Complex HLI 90 and Complex Control				
	6hrs	24hrs		D7
Acan				
Adipor1				0.003
Alpl				0.005
Angpt2				0.004
Bglap				
Bmp2				
Bmp4				0.02
Bmp6				0.05
Ccl2				
Ccl3				
Cd44				0.04
Cebpa				0.01
Col1a1		0.05		
Col1a1				0.05
Col1a1				
Comp				0.03
Cx3				0.01
Cxcl1				
Cxcl10				0.04
Cxcl12				0.002
Cxcl5				
Eng				
Fabp4				0.004
Fgf1				0.04
Fgf10				0.05
Fgf2		0.02		0.03
Fhl1				0.003
Gli2				
Gusb				0.05
Has1		0.05		0.02
Has2				0.04
Hat1				<0.001
Hdac1				0.01
Hif1a				0.02
Hif1a				0.02
Ibsp				
Igf2				0.001
Il10				0.04
Il1b		0.04		
Il6				
Itga1				0.003
Itga2				0.006
Itgam				0.005
Itgav				0.01
Itgav				0.03
Jag1				0.003
Lrp5				0.004
Mmp9				
Myd1		0.05		0.04
Notch1				0.005
Omd				
Pdgfa				0.03
Phex				
Prarg				
Pich1				0.02
Pik2		0.03		0.05
Rhoa				0.02
Runx2				0.02
Scarb1		0.02		
Smad2				
Smc				0.04
Smurf1				0.002
Sux9				0.02
Sp1				0.001
Sparc				0.01
Spp1				
Ter1				
Tgfb1				0.007
Tgfb3				0.006
Tnf				0.04
Twist1				
Vegfa				0.02
Wnt5a				0.01

Fold Change between Complex Injured Groups	
50+	
25-50	
10-25	
5-10	
2-5	
1.5-2	
1.25-1.5	
1-1.25	
0.75-1	
0.5-0.75	
0.2-0.5	
0.1-0.2	
<0.1	

Table 13. Fold difference between complex HLI 150, complex HLI 90 and complex control group HO associated genes expressed as absolute fold difference by subtraction of groups

Fold change between Complex HLI 150 and Complex Control						Fold change between Complex HLI 150 and Complex HLI 90					Fold change between Complex HLI 90 and Complex Control					Difference in fold change of complex injured groups by subtraction
	Complex HLI 150 minus Complex Control 6hrs	Complex HLI 150 minus Complex Control 24hrs	Complex HLI 150 minus Complex Control D3	Complex HLI 150 minus Complex Control D7	Complex HLI 150 minus Complex Control D10		Complex HLI 150 minus Complex HLI 90 6hrs	Complex HLI 150 minus Complex HLI 90 24hrs		Complex HLI 150 minus Complex HLI 90 D7		Complex HLI 90 minus Complex Control 6hrs	Complex HLI 90 minus Complex Control 24hrs		Complex HLI 90 minus Complex Control D7	
Acan						Acan					Acan					500+
Adipor1				0.002		Adipor1		0.01			Adipor1				0.003	100 - 500
Alpl		0.007		0.001		Alpl					Alpl				0.005	50 - 100
Angpt2				<0.001		Angpt2				0.04	Angpt2				0.004	25 - 50
Bglap						Bglap					Bglap					10-25
Bmp2	0.02			0.001		Bmp2				0.05	Bmp2					5 - 10
Bmp4						Bmp4					Bmp4				0.02	1 - 5
Bmp6		0.009				Bmp6					Bmp6				0.05	-1 - 1
Ccl2						Ccl2					Ccl2					-1 - -5
Ccl3					0.02	Ccl3				0.06	Ccl3					-10 - -5
Ccl44						Ccl44					Ccl44				0.04	-25 - -10
Cebpa				<0.001		Cebpa					Cebpa				0.03	-50 - -25
Col1a1						Col1a1		0.03			Col1a1	0.05				-100 - -50
Col1a1				0.005		Col1a1					Col1a1				0.05	-500 - -100
Col1a1				0.003		Col1a1					Col1a1					<-500
Comp				0.02		Comp					Comp				0.03	
Cxcl2				0.006		Cxcl2					Cxcl2				0.01	
Cxcl3					0.03	Cxcl3					Cxcl3					
Cxcl10		0.01		0.02		Cxcl10					Cxcl10				0.04	
Cxcl12				<0.001		Cxcl12					Cxcl12				0.002	
Cxcl5						Cxcl5					Cxcl5					
Eng		0.03		0.02		Eng		0.03			Eng					
Fabp4				<0.001		Fabp4				0.005	Fabp4				0.004	
Egfr						Egfr		0.03			Egfr				0.04	
Egfr						Egfr				0.003	Egfr				0.05	
Egfr				<0.001		Egfr	0.03				Egfr	0.02			0.03	
Flt1		0.02		0.003		Flt1		0.03			Flt1				0.003	
Gli2		0.01		0.002		Gli2					Gli2					
Gusb		0.05		0.002		Gusb				0.05	Gusb				0.03	
Hes1				<0.001		Hes1					Hes1	0.06			0.02	
Hes2				0.002		Hes2					Hes2				0.04	
Hes1			0.01	<0.001		Hes1				0.02	Hes1				0.000	
Hdac1				0.001		Hdac1					Hdac1				0.01	
Hif1a				0.004		Hif1a					Hif1a				0.02	
Hif1a				0.04		Hif1a					Hif1a				0.02	
Ibsp						Ibsp					Ibsp					
Igf2		0.02		0.004		Igf2					Igf2				0.001	
Il1b				0.02		Il1b					Il1b				0.04	
Il1b				0.006	0.02	Il1b				0.04	Il1b		0.04			
Il6				0.01	0.04	Il6				0.03	Il6					
Irga1		0.009		0.002		Irga1					Irga1				0.005	
Irga2				0.002		Irga2					Irga2				0.006	
Irgam				0.001		Irgam				0.03	Irgam				0.005	
Irgav		0.03		<0.001		Irgav					Irgav				0.01	
Irgav				0.001		Irgav				0.02	Irgav				0.03	
Jag1				0.001		Jag1		0.04			Jag1				0.005	
Lrp5		0.02		0.002		Lrp5					Lrp5				0.004	
Mmp9				0.006		Mmp9					Mmp9					
Myod1				0.05		Myod1					Myod1		0.05		0.04	
Notch1				0.002		Notch1		0.007			Notch1				0.005	
Omd						Omd					Omd					
Pdgfra	0.04	0.05	0.006	<0.001	0.04	Pdgfra				0.01	Pdgfra				0.02	
Pdx				0.04		Pdx					Pdx					
Pparg				0.02		Pparg				0.03	Pparg					
Pich1				<0.001		Pich1				0.03	Pich1				0.02	
Pik2				0.007		Pik2					Pik2	0.03			0.05	
Rhoa		0.04		0.002		Rhoa		0.03			Rhoa				0.02	
Runx2				0.005		Runx2					Runx2				0.02	
Scarb1		0.02		0.001		Scarb1				0.03	Scarb1		0.02			
Smad3				0.005		Smad3		0.02			Smad3					
Smo		0.02		0.001		Smo		0.01			Smo				0.04	
Smurf1		0.03		0.001		Smurf1		0.009			Smurf1				0.002	
Sox9				<0.001		Sox9					Sox9				0.02	
Sp1				<0.001		Sp1		0.02		0.02	Sp1				0.001	
Spare				0.003		Spare		0.01			Spare				0.01	
Spp1						Spp1				0.06	Spp1					
Tert						Tert					Tert					
Tgfb1				0.001		Tgfb1		0.01		0.05	Tgfb1				0.007	
Tgfb3				0.004		Tgfb3		0.02			Tgfb3				0.006	
Tnf				0.01		Tnf					Tnf				0.04	
Twist1				0.02		Twist1					Twist1					
Vegfa		0.05		0.02		Vegfa					Vegfa				0.02	
Wnt5a				0.003		Wnt5a					Wnt5a				0.01	

APPENDIX 2

Table 14. Gene design information for the ischemia reperfusion injury qRT-PCR assay.

Gene Name	Entrez Gene ID	Sequence	Assay Design
Adm	25026	ATATTGTCACCTTCCCCTATTTTAAGAAGTGAATGTCTCAGCAAGGT GTAAGGTTGTTTTGGTTCCGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTAAGGTGGAGAGCGCCTGATTACCGCCTGTGGATG	Exonic
Aft3	25389	GTGACTGACATCTCCAGAGGCTCTGTTGTTGATGGTGACTGACTCCAG CGCAGAGGACATCCGATGGCAAAGGTGCCTTGTTCTGGATGGCGAATC TCAGCTCTTCCTTGACAAAAG	Exonic
Bcl2	24224	CTGTGACAGCTTATAATGGATGTACTTCATCACGATCTCCCGGTTATC ATACCCTGTTCTCCCGGCTTGCGCCATCCTTCCGGGGAAAGAAGCTG CAGGTACCAATAGCACTTCGCGTCCCGGCTCCCGCCCC	Exonic
Bcl3	680611	CTGGCACCAGCTGTCACCAGGAGCCGGACTATATCTGGTAATGTGGT GATCACAGCCAGGTGGAGCGGGTCTGTGCGAGGTTGTTGTGGACAT CGACTTCCCTGTTCCCAAGCTTGAAAAGGCTGAGAATTCGGTAGACG GCCGTCTTG	Intron
Bid	64625	CACCTGCTTGGGCGAGATGCCTGGCAATGTTGTGGATGACTTCATCC TGACTTTCAGAATCTGGCTCTATTCTTCCATGATAGAAGGAGCGGT AGCCCGGCTG	Intron
Casp8	64044	CAACGAAGAAGTGGCTGCCCTCAAGTTCCTGTGCTTGGACCACATCC CGCAGAAGAACGAGGAGTCCAATGATGTCTTGGTGCTATTT	Exonic
Ccl2	24770	TAAATCTGAAGCTAATGCATCCACTCTCTTTCCACAACCACCTCAA GCACTTCTGTAGAAGTGACCAAGTATGACAGAGAAGTGTGTGATTG GAATGTGATGCCTTAAGTAATGTTAAACTTATTTAACT	Exonic
Ccl20	29538	CACCTGCACATCACTGCACAGACCTGATTTGTGTCCAGTGGTCTTGT CCAATGGATGAAGTTGATTCAATTGCATCATAGTGTGTCATATTTA AGCTCATATTAGAG	Exonic
Cxcl1	81503	CAAAACATACAAACATAGCCTCTCACACATTTCCTCACCCCTAACACA AAACAGATCCCGAGACTCTCATCTCTCCGCCCTTCTTCCCGCTCAACA CCTTCTAGCAGTGGTTG	Exonic
Cxcl2	114105	CAACTACATAAGTAAAAATAACGCTGACTGAACACATTGAACATTATT ACAATAAACTTCAACATATTTAAATGACCTCTTAAAGATACTACAGTG AGCTGGCCAATGCATATCTTTAAATATCATAGTAAACG	Exonic
Fos	314322	ACCAGCTCTCAGGTGTCACTACAAACAATACACTCCATGCGGTGCT TTTGATTTTTTGTTTTGTTTTTTTTTTGTTTTGTTTTTTTTTGCT ACATCTCCGGAAGAGGTGAGGACTGGAGTCCAGATGTGGAT	Exonic
Gapdh	24383	TGATGGCAACAATGTCCACTTTGTCACAAGAGAAGGCAGCCCTGGTA ACCAGCGTCCGATACGGCCAAATCCGTTACACCCGACCTTCACCAT CTTGCTCTATGAGACGAGGCTGGCACTGCACAAGAAGATGCGGCTGTC TCTA	Intron
Hif1a	29560	CCCAGATTCAAGATCAGCCAGCAAGTCCTTCTGATGGAAGCACTAGA CAAAGCTCACCTGAGCCTAACAGTCCCACTGAGTACTGCTTTGATGT GGACAGCGATATGGTCAATGTATTCAAGTTGG	Intron
Hprt1	24465	TTCATGCAAAAGCTTTACTAAGTAGATGGCCACAGGACTAGAACGTC TGCTAGTCTTTACTGGCCACATCAACAGGACTCTTGTAGATTCAACT TGCCGCTGTCTTTT	Exonic
Hspa1b	294254	GCCCTTGTCGTTGGTGATGGTGATCTTGTTGGCCTTGCCGGTGCTCTT GTCAGTGGCCGTGACGTTCAAGATGCCGTTGGCGTCGATGTCGAAGG TCACCTCG	Exonic
Hspd1	63868	CCAGTAACTGATGGTTACAGTGATCAGCCAGCCTTCTCTTCAGTCATT TTCTTCAGGTGACTTCTCTGAAGTTATTGGTGAGGAACCTGCCTTGAG CTTCTCGCCACAGTTCATTGAT	Exonic
Ii1b	24494	TGAAAGCTCTCCACCTCAATGGACAGAACATAAGCCAACAAGTGGT ATTCTCCATGAGCTTTGTACAAGGAGAGACAAGCAACGACAAAATC	Intron

		CCTGTGGCCTTGGGCCTCAAGGGGAAGAATCTATACCTGTCCTGTGT GATGAAAGACG	
Il6	24498	CTTCACAGAGGATACACCCACAACAGACCAGTATATACCACTTCAC AAGTCGGAGGCTTAATTACATATGTTCTCAGGGAGATCTTGAAATG AGAAAAGAGTTGTGCAATGGCAATTCTGATTGTATGAACAGCGATG ATGCACTGTC	Intron
Jun	24516	GGAGGTGACACTGGGCAGCGTATTCTGGCTATGCAGTTACAGTAGGG CGCGCACGAAGCCTTCGGCGAAGCCCTCCTGCTCGTCGGTCACGTTT TTGGGGCACAAGAAGTGAAGTGGGGTGGTGTAGTGGTGATGTGCC CATTGCTGG	Exonic
Lcn2	170496	GATCCAGTAGCGACAGCCCTGGCCCCTGACGAGGATGGAAGTGACG TTGTAGCTATTGTCTTCCTGTAGCTCATAGATGGTGCTGTACATGGTA AAGC	Exonic
Maff	366960	AGATGATGACCACTGTGGCAGAGACAGACACTACAGTCCTGATGCG GGAAAGGGGTGGGGGTGGGGTGAAGTTGAATTAAGGCTTCCGCTTT GGTCCCGAGTATCTGTGGTGGTTCTGGGGGGCT	Exonic
Myc	24577	GAGCCCCTAGTGCTGCATGAAGAGACACCGCCACCACCAGCAGCG ACTCTGAAGAAGAACAAGATGATGAGGAAGAAATTGATGTGGTGTC TGTGAAAAGAGGCAACCCCTGCCA	Intron
Nfkbiz	304005	AAGAATAAGAGCTTGGTTGACACCATTAAAGTGTCTGATTCAGATGGG AGCGGCCGTGGAGGCGAAGGATCGTAAAAGTGGCCGCACGGCCCTG CATTGGCAGCGGAGGAAGCAAATCTGGAACCTATCGCCTCTTTTT GGAGTT	Intron
Nos2	24599	GTGAGGAACTGGGGGAAACCATTTTGTATGCTTGTGACTCTTAGGGTC ATCCTGTGTTGTTGGGCTGGGAATAGCACCTGGGGTTTTCTCCACGTT GTTGTTAATGTCCTTTTCTCTTTCAGGTCACCTGGTAGGATT	Intron
Nr4a1	79240	AGCTGGGCAAGCCTCTCGAGCCTCCAACCTGAGATCTCTCCGCTCT CCTTCCATGTGTACATAGCTGTCACTCAAGAAGGTGATTGACAGATT CTGATTT	Exonic
S100a8	116547	AATTTTATCGACAATGGCAACTGAACTGGAGAAGGCCTTGAGCAAC GTCATTGAAGTCTACCACAATTATCTGGTATAAAAAGGGAATCACC	Exonic
Serpine1	24617	GTGGACTTCTCAGAGATGGAAAGAGCCAGATTCATCATCAACGACTG GGTGGAGAGGCACACCAAAGGTATGATCAGTGACTTACTGGCCAAG GGGGCTGTAAATGAGCTGACACGCCTGGTGCT	Intron
Socs3	89829	GTCCAGGTGACCGTTGACAGTCTTCCGACAAAGATGCTGGAGGGTAG CCACGTTGGAGGAGAGAGGTGCGCTCAGTACCAGCGGGATCTTCTCG CCCCAGAATAGATGTAGTAAGCTCTCTTGGGGGTACCCCGGGGA GAAGTACCACGAGGCGCTGGCCAAGGAGATGTTACAACCTCAGGTT GCTCTTCAGCCTGCACTGAAGTTC	Exonic
Sod2	24787	GCAAGCACCACGCGACCTACGTGAACAATCTGAACGTCAACGAGGA GAAGTACCACGAGGCGCTGGCCAAGGAGATGTTACAACCTCAGGTT GCTCTTCAGCCTGCACTGAAGTTC	Intron

Table 15. Gene design information for the heterotopic ossification qRT-PCR assay.

Gene Name	Entrez Gene ID	Sequence	Assay Design
Acan	58968	CCCCTACCCTTGCTTCTCCAGAAGGGTCAGGAGAAACAGAGTCAACC GCTGCAGACATGTATGAGTGCCTCTCAAGCCCTTGTCTGAATGGAGC CACCTGCGTGGATGCCTTGGACACTTTCACATGCTTATGCCTTCC	Intron
Actb	81822	CGTCCACCCGCGAGTACAACCTTCTTGCAGCTCCTCCGTCGCCGGTC CACACCCGCCACCAAGTTCGCCATGGATGACGATATCGCTGCGCTCGT CGTCGACAAC	Intron
Adipoq	246253	TTCATGACTGGGCAGGATTAAGAGGAACAGGAGCGCTTGCAACAGT AGCATCCTGAGTCTTGAACTTTTGGTGTAATCAGATCCACTGGCAC TCGTACAGACAGGAATGAAG	Intron
Adipor1	289036	GCAAGCCCACCATGCCATGGAGAAGATGGAGGAGTTCGTATATAAG GTCTGGGAGGGGCGTTGGAGGGTCATCCCATACGATGTGCTTCCTGA CTGGCTGAAAGACAATGACTACCTGCTACATGGCCACAGACCA	Intron

Alpl	25586	AGGTGCCGATGGCCAGTACTAAAAATGGCAAGATCATGGTGTACCC CGAGATCCGTTCCTCGCTGGAGCCCAGATGGGTGGGAAGAGGTGGG GGGCAGTGTCTAGCCGTTAATTGACGTTCCGA	Exonic
Angpt2	89805	GCACACTCTCTGTATGAGCACTTCTACCTGTCCGGCGAGGAGTCCAA CTACAGGATTACCTTACAGGACTCACAGGCACGGCGGGCAAAATC AGTAGCATCAGCCAACCAGGAAGTGATTTTAGCACAAA	Intron
B2m	24223	GAAAACTCCCCAAATTCAAGTGTACTCTCGCCATCCACCGGAGAATG GGAAGCCCAACTTCCTCAACTGCTACGTGTCTCAGTTCACCCACCT CAAATAGAAATTGAGCTACTGAAGAATGGAAAGAAGATACCA	Exonic
Bglap	25295	AACAGGGAGGGTTCGAGTCTGGAGAGTAGCCAAAGCTGAAGCTGCC GTTGGGCTCCAGGGCAACACATGCCCTAAACGGTGGTGCCATAGATG CGCTTGTAGGCGTCTTGGAAAGCCAATGTGGTCCGCTAGC	Exonic
Bmp2	29373	GCCCCCTAGTGCTTCTTAGACGGACTGCGGTCTCCTAAAGGTGACC ATGGTGGCCGGGACCCGCTGTCTTCTAGTGTGCTGCTTCCCCAGGTC CTCCTGGGCGGCGC	Intron
Bmp4	25296	GATAATTTTTCAACACCACCTTGTCTGTAAGTCTCGTCCAGATACAACATG GAAATGGCGCTCAGTTCGGTGGGGACACAGCAGGCCTTAGGGATGC TAGAATTAACGGAGTTGACCAGGGTCTGCACAATGGCATGATTGGTT GAGTTGAGGT	Exonic
Bmp6	25644	ACTCAATGACATCCACAAGCTCTCACAACCATGTTCTGTATTTTTTC AAGATGACATTGGAGTTGTCTGTCGAAGTAAAGAACCGAGATGGCAT TCAGTTTGTT	Exonic
Ccl2	24770	TAAATCTGAAGCTAATGCATCCACTCTCTTTTCCACAACCACCTCAA GCACTTCTGTAGAAGTGACCAGTATGACAGAGAACTAGTGTGATTG GAATGTGATGCCTTAAGTAATGTTAAACTTATTAACT	Exonic
Ccl3	25542	GGAGATTGTCAGGTGGCAGGAATGTTCTGGGGCTCAAGCCCCTGCTC TACACGGGGCCACGGAGGTTTGGGGGTTCTTGCTGCCTCTAATCT CAGGCATTTAGTTCAGCTCAGTGATGTATTCTTGGACCCAGGTCTCT	Exonic
Cd44	25406	ATGTAGCCTTCTGGGGTGCTCTTCTCAATGGTGGATCCACTGCTGAC ATCCTCATCTATAATGTTTAGGCATCGATGTCTTCTTGGTGTGTTCT ATACTCGCCCTTCTTGCTGTA	Exonic
Cebpa	24252	GCCGGCCCCCTGGCTGGGGACCCGGAGGATGGTTTAGGGTCGCTGG ATCTCTAGGCTGCCCGGACCGTGCAAGCCAGGACTAGGAGATTCTGG TGTCGCCTGAAAGCC	Exonic
Col1a1	29393	GGAGAGAGCATGACCGATGGATTCCAGTTCGAGTATGGAAGCGAAG GTTCCGATCCTGCCGATGTGCTATCCAGCTGACCTTCCTGCGCCTGA TGTCCACCGAGGCCTCCCAGAACATCACCTATCACTGCAAGAACAG	Exonic
Col2a1	25412	CAAGGCCTTCTTGAGGTTGCCGGCTGCTTCGTCCAGGTAGGCAATGC TGTTCTTACAGTGGTAGGTGATGTTCTGGGAGCCCTCAGTGGACAGT AGACGGAGG	Exonic
Col4a3	363265	GAGATAAGGGGGAGCCAGGTTATTCAGAAGGTGCAAGCCCAGGACC ACCAGGACCGAAGGGTGATCCAGGATTGCCAGGTGACAAAGGGAAG AAAGGAGAGAGAGGACTACCTGGGCCACCTGGACATTCCG	Intron
Col10a1	25681	CGTTTTATGCTGAGCGGTACCAAACTCCCACAGGTGTGAAGAGCGCA CTTGCCAGCCCCAGGACACAATACTTCATCCCATAACCTATAAAGGG TAAAGAGATTTCAGTAAGAGGAGAACAAGGCATTCTGGTCCACCA GGCCCAGCTG	Intron
Col11a1	25654	GATGGCTTACCCGGTCTCCAGGAACCATGTTAATGTTACCATTCGG CTATGGGGGTGACGGCTCCAAAGGACCAACCATCTCTGCGCAGGAA GCCAGGCTCAGGCGATTCTTCAGCAGGCTCGGATTGCTCTGA	Intron
Comp	25304	CGGGGACAGTTGTCAGCTACATTTCCGATTTCGATCGCCATCTATGTC GTCGTGCGAGGCATCGCCCTGGCCATCCCGGTCTGTATCTTTCTGGTC ATCATTCTTCTGGGACCGGCAGTTGTGCGCAGGCATCTCCC	Intron
Csf3	25610	GCCCTACAGCTGCTGTGTGGCAGTGCATATGGTCAGGACAAGA GGCCATTCCCCTGCTCAGCTCAGCTCTGTCACCATCCCTGCCTTT GCCCCGAAGCTTTCTGCTTAAAGTCCTTGAGCAAGTGAGGAAGATT AGGCC	Exonic
Cxcl1	81503	CAAAACATACAAACATAGCCTCTCACACATTTCTCACCCTAACACA AAACACGATCCCAGACTCTCATCTCTCCGCCCTTCTTCCCGCTCAACA CCTTCTAGCACAGTGGTTG	Exonic

Cxcl5	60665	AATATTTTCTGAATGACTTTCTTTATCAACGGAGCTTCTGGGTCAAGA CAAACATTATCCTTCTGGTCTTCAACTTAGCTATGACTTCCACCTTG GGGCAA	Exonic
Cxcl10	245920	AGGCTTCCCAATTCTCTAAGAGCTGGTCCGAATCTTCCCTCAGGCAG CTATGACGGCTCTCCTAGCTCTGTTCTGTAAGCTATGTGCAGGTACTA ATCTCTTCAGCATGTGCCATGCCCCAGCCTGCTC	Exonic
Cxcl20	24772	TGCCCCTGCCGATTCTTTGAGAGCCATGTGCCAGAGCCAACGTCAA ACATCTGAAAAATCCTCAACACTCCAAACTGTGCCCTTCAGATTGTTG CAAGGCTGAAAAAG	Exonic
Eng		AAGTCTACAAGACTGTCTCCATGCGCCTGAACATCGTCAGTCCTGAC CTGTCTGGCAAAGGCCCTGCTCTGCCCTCTGTACTGGGCATCACCTTT GGTGCCTTCTCATTGGGGCCCTGCTCACAGCTGCACTCTGGTACATC TATTCTCACACACGTGAGTATCCCAAGCCTCTCCACCCCTTCTCTACA GTGAGTGCTCAGGGCCGGTCCACACCACCCAGGGCA	Intron
Fabp4	79451	AATGTGCGACGCCCTTGTGTTGGGACCTGGAAACTCGTCTCCAGTGAGA ACTTCGATGATTACATGAAAGAAGTGGGAGTTGGCTTCGCCACCAGG AAAGTGGCCGGTATGGCCAAGCCCAACTTGATCATCAGCGTAGAAG GGGACT	Intron
Fgf1	25317	CTATTCCTGGAAGGCTAGAAGAAAACCATTATAACACTTACACATC CAAGAAGCACGCGGAGAAGAACTGGTTTGTGGGCCCTCAAGAAGAAC GGGAGTTGTAAGCGCGGTCTCGG	Exonic
Fgf2	54250	CACCTCAAGGATCCCAAGCGGCTCTACTGCAAGAACGGCGGCTTCTT CCTGCGCATCCATCCAGACGGCCGCGTGGACGGCGTCCGGGAGAAG AGCGACCCACACGTCAAACCTACAGCTCCAAGCAGAAGAGAGAGGAG TTGTGTCCAT	Intron
Fgf10	25443	TCGGAGTTGTTGCCGTCAAAGCCATTAACAGCAACTATTACTTAGCC ATGAACAAGAAGGGGAAACTCTATGGCTCAAAAAGAAATTTAACAATG ACTGTAAACTGAAAGAGAGGATAGAGGAAAATGGATACAACACGT	Intron
Gapdh	24383	TGATGGCAACAATGTCCACTTTGTCAACAAGAGAAGGCAGCCCTGGTA ACCAGGCGTCCGATACGGCCAAATCCGTTACACCCGACCTTCACCAT CTTGTCTATGAGACGAGGCTGGCACTGCACAAGAAGATGCGGCTGTC TCTA	Intron
Gli2	304729	CCTCAGCCTCAGTCTTGACCTTGCTCCGCTTATGGATGGTGATGGGGT TCACGGTGCTGCTCACGGCCGACTCGCTGTTCTGCTTATTCTGGTTGG CATCACTTATACAGTTGCTGCTATTGCTGCCTAGCTGG	Intron
Gusb	24434	ACCCTGCGGTCGTGATGTGGTCTGTGGCCAATGAGCCTGTCTCTTCTC TGAAACCTGCCGGATATTACTTCAAGACGCTGATCGCCACACCAAAA GCCCTGGACCCCAACCCGCTCTGTGACCTTTGTGAGCAATACCAGATA TGACGCAG	Intron
Has1	282821	TGGCTCAGCCAACGAAGGAAAGAGGAGGGCGTCTCTGAATAGCATC TGGAGCGCGAGGTATACTTGGTAGCGTAGCCCATGCTAAGCATGCGG TTGGTGAG	Intron
Has2	25694	ACACTCCAAACAGTGTGGTTCCGATTATTCTCAGGACACATAGAAAC CTCTACAATGCATCTTGTTTCAGCTCCTGCTCATAGACTCGTCTCCG CCTGTCTGTGTGGTCCCGAG	Intron
Hat1	296501	TTGGTTACAAGGGCTGAAGATCTTGTGTACTATATTGCTGGTAGC CTGTCAACTATGTTCCGTGTTGAATATGCATCTAAAGTTGATGAGAA CTTTGATTGTGTGGAGGCAGATGACGTTGAAGGCAAAATCAGACAA ATCATTTCC	Intron
Hdac1	297893	GAAAAGCTTAACAAGGCCATCCTAGCAGAGCTCCTAAGCAGGCACT TGCAATTACAGGATTTAGTCTTCCGGTTGTTTTCTTTTACCTGGACCC TACGTGGTTCAAAACTCAGACCTAGGGCAGGACGGACAAGGGAGCT CTGGGAAG	Exonic
Hif1a	29560	CCCAGATTCAAGATCAGCCAGCAAGTCCTTCTGATGGAAGCACTAGA CAAAGCTCACCTGAGCCTAACAGTCCCAGTGAGTACTGCTTTGATGT GGACAGCGATATGGTCAATGTATTCAAGTTGG	Intron
Hnf1a	24817	CCTCTGGCTGCAGGGCCCTACAACCTTCTTCTGGTGTGAGAGACGT GTAGAAGTCGGAAGACAAGGCCTGAGCAATGTCTAGTGAGAGAGT GCTGAGA	Exonic

Hprt1	24465	TTCATGCAAAAGCTTTACTAAGTAGATGGCCACAGGACTAGAACGTC TGCTAGTTCCTTTACTGGCCACATCAACAGGACTCTTGATAGATTCAACT TGCCGCTGTCTTTT	Exonic
Ibsp	24477	TCCCCGCGAGGCTCCCCGTTGTTCTCGTCATAGGTTTCATACGCAGTG TTGTACTCGTTGCCTATTGTTCGTATTCTTCCCCATACTCAACCGTG CTGCTCTTTCTGGCTGG	Exonic
Igf2	24483	TCACGATGGTTGCTGGACATCTCCGAAGAGGCTCCCCCGTGGGCGG GGTCTTTGGGTGGTAACACGATCAGGGGACGGTGGCGCTTGGCCTCT CTGAACGCTTCGAGCTCTTTGGCAAGCATGCGACCCCGGCGGGCA	Exonic
Il1b	24494	TGAAAGCTCTCCACCTCAATGGACAGAACATAAGCCAACAAGTGGT ATTCTCCATGAGCTTTGTACAAGGAGAGACAAGCAACGACAAAATC CCTGTGGCCTTGGGCTCAAGGGGAAGAATCTATACCTGTCTGTGT GATGAAAGACG	Intron
Il6	24498	CTTACAGAGGATACCACCCACAACAGACCAGTATATACCACTTCAC AAGTCGGAGGCTTAATTACATATGTTCTCAGGGAGATCTTGAAATG AGAAAAGAGTTGTGCAATGGCAATTCTGATTGTATGAACAGCGATG ATGCACTGTC	Intron
Il10	25325	CTGCCTTCAGTCAAGTGAAGACTTTCTTTCAAAGAAGGACCAGCTG GACAACATACTGCTGACAGATTCTTACTGCAGGACTTTAAGGGTTA CTTGGGTGCCAAGCC	Intron
Itga1	25118	GTATAAGAATCTTTGTGCGATGTCAATTGTTGTTAAGACACTACCAAA GTAGGAACCAATCTGCTCTCCGCCGAGTGTCTGCAGAATGTTGATGT TCCCATCTCCATCTTGTAGATGACGACCTGGCCCGTATGATTGTACC GA	Intron
Itga2	170921	GCAGAGGAATGTGGATGGTTACTGATGCCATGCTCACTGGAGCACTT CCCGCTGTTATCTTGAGAGAGAAGATGAATTCAGGACCAATATCTTC AACACTGTTTATGACAG	Intron
Itgam	25021	CAAGGAGTGTGTTTGCCTGTCAAGAAGAAGTAGTCAAAAACAAGGA TGCTGGGGAGGTCAGAGTCTGCCTCCATGTCCGCAAGAACACCAAG GACAGGCTGCGAGAAGGAGACATCCAGAGCACTGTCACTTATGACC TGGCTTTAGACC	Intron
Itgav		CTTTGCTGTACATTCTCCATTACGACATTGATGGGCGGATGAACTGTA CTGCCGACACAGAGATCAACCCTCTGAGAATTAAGACGCCCGAAAA GAATGACACAGCAGCTGAGCAAGGAGGAAGAAATCATCTCATC	Intron
Itgax	499271	GGAAAGAAGGGGACAGGTTGGACTATGGTGATGTCATCCCCATGGC GGAGGCTGCAGGCATCATCCGTTATGCAATTGGGGTAGGACAGGCCT TTTACCAAGCACAGTCCAGGCAAGAATTAAGGACATTGCGTCGTCG	Intron
Jag1	29146	AAGGCTGTCACCAAGCAACAGACCCAAGCCACTGTTAAGACAGAGC TCAGCAGAGGAACCAAGGAAATCTGTTCTGTTTTTCAGAGGGCGCCTC TGAACCTCTGACTTCTGCAACAGCAGCGATAAGTGAGCTGTTCC	Intron
Kdr	25589	GCAACTATGTCTGCTCTGCTCAAGACAAGAAGACCAAGAAAAGACA TTGCCTAGTCAAGCAGCTCGTCATCCTAGAGCGCATGGCACCCATGA TCACTGGAAATCTGGAGAATCAGACAACAACCATTTGGTGAGACCAT CGAAGTTGTT	Intron
Lep	25608	GACCCTGGCAGTCTATCAACAGATCCTCACCAGCTTGCCTTCCAAA ACGTGCTGCAGATAGTTCATGACCTGGAGAACCTGCGAGACCTCCTC CATCTGCTGGC	Exonic
Lrp5	293649	AAGTCAGGGGTGTCCAGAGAGATCCTTCTCAGGTCTGTCTCCGGGC CAGCAGCAGCACTTCCTCAGCCCCTGCCTTACACGTCTTGCCATTGTC CTGCAACTGTAC	Intron
Mmp9	81687	CTGCACCACTAAAGGTCGCTCGGATGGTTATCGCTGGTGCGCCACCA CCGCCAACTATGACCAGGATAAGCTGTATGGCTTCTGTCTACTCGA GCCGAC	Exonic
Myod1	337868	ACGCCATCCGCTACATTGAAGGTCTGCAGGCTCTGCTGCGCGACCAG GACGCCGCTCCCCCTGGCGCCGCTGCCTTCTACGCACCTGGCCCGCT GCCCCAGGCCGTGGCAGTGAGC	Exonic
Notch	25496	AGGGGCTGCCCGCACATTTCGTCATGTTGACATTACACATGCTCCCT GTGTAGCCTGGCTCGCACGCACACTCGTAGCCATCGATCTTGTCCAG	Intron
Omd	83717	AATCCTCACTTGGCTCTTGCTCATAATCTTCATCCCACTGGTAAGCTT CATATTGGCCGTATACTCTAACTCCAAAACAGAAGAAGAGGACATAT	Intron

		ATTGGACTTAAAAAGCCCATTTCTTCTCCTGTTGAGATTGCTTTGGTT TCA	
Pdffa	25266	CCTCCAACCTGAACCCAGACCATCGGGAGGAGGAGACGGATGTGAG GTGAGATGAGCTGGCTGCCCTCTCCCGGGACACGGATGTACGTGGCG TGTGACATTCTGAACATACTATGTATGGTGCTTCATTGCCAATGTGC GTGCAGT	Intron
Phex	25512	AGGCATCCAATGAGCATATCTTGAAGTTGGACCAAGCAACACTCTCC CTGGCTGTGAGGGAAGACTTTCTGGATAACACTACCGAAGCCAAATC TTATCGTGATGCCCTTTACAAATTCAT	Intron
Pou5f1	294562	GCAGCTTGGCAAACCTGCTCTAGCTCCTTCTGCAGGGCTTTCATATCCT GGGACTCCTCGGGACTAGGTTCCACCTTCTCCAACTTCACGGC	Intron
Pparg	25664	CCCTTTGGTGACTTTATGGAGCCTAAGTTTGTAGTTTGTGTGAAGTTC AATGCACTGGAATTAGATGACAGTGACTTGGCCATATTTATAGCTGT CATTATTCTCAGTGGAGACCGCCAGGCTTGCTGAACGTGAAGCCCA TCGAGGACATCCAAGACAACC	Intron
Ptch1	89830	TAGGGCCTCTGTGGTCAGGACATTAGCGCCTTCTTCTTTTGGAGTCTG AATCATGAGTTGAGGATTAAACATAGCCTCTTCTCCTATCTTCTGGCG GGTATAATTTAATTCTCGACTCACTCGTCCACCAACTTCCAC	Exonic
Ptk2	25614	ATAAGATCAAAGATGCTAGATCCAAGCTGTATTTCTTACTTGCAAA AATGACCTCAGCTCTCCAAGTGTGCACAACCTCATGATTATCCAAAC TGGGTCTCTGTAAATGACTCCAATCAGCTTCACGATGTGAGGATGGT CAAAC	Intron
Rhoa	117273	AGGAAGATTATGACCGTCTGAGGCCTCTCTCCTACCCAGACACTGAT GTTATACTGATGTGTTTTTCCATCGACAGCCCTGATAGTTTAGAAAAC ATCCAGAAAAATG	Exonic
Rplp0	64205	TCTTTCTCAAATTAAGCAGGCTGACTTGGTGTGAGGGGCTTAGTCGA AGAGACCGAATCCCATGTCTCATCGGATTCTCCGACTCTTCCTTTG CTTCGACCTTGGCTGGGGCAGCAGCTGCA	Exonic
Runx2	367218	CACACGTGTAGTAAAGGCTCAACCATGACACGTACAGCCTAGGAAC ACAGATGCATTAGGAGTCTGCACCCCCCCCCCAGCCTTACCACACAG CCATTACAGGGG	Exonic
Scarb1	25073	GTAGGAGCTGGCGCCGCCGGGGCTCCTGACTTTCTCCGCTTTTCTCCA GCAGGCTGAAAGCCTTAGTTCCACAGAGGACACAGATCTGTCACTC CTAGGGGCTGGGCAAACATCGGCCTCTTGCGTCACGGCTGGACAAGC AGTTCCAGATCCTGACCAGTCTGCACACACACTCTGGCGCTGTATCT GGCCTTCTCCTACA	Exonic
Smad3	25631	CAGCAGCTCATAGCTCATTTTAAACTCGGAATTCAGAATGGCATCAC AGGAGCTACTTGCTCTCTGCCATGGCTGAGAAGAGAGGACCATGAA TACT	Intron
Smo	25273	TGGCTCAGGTAGATGGGGACTCCGTGAGTGGCATCTGCTTTGTAGGC TACAAGAACTATCGGTACCGTGCTGGCTTTGTACTTGCCCCAATTGG CCTGGTGCTTATTGTGGGAGGCTAC	Exonic
Smurf1	690516	CCATCCAGCTGTGCGATCTGGAGTCAGTGGACCCAGAACTTCACAAG AGCTTGGTGTGGATTCTAGAGAATGACATCACGCCAGTGCTGGATCA CACCTTCTGC	Intron
Smurf2	303614	ATGTGAAACCACCATCAATATAATGTCCATGGAATACAGCCATTCCC ATTATTCTGTCCAACAAAGTGGAATAGGACAAATGCTCCGGATTAAAC TGCAGAATCGGGATTGATCTGTAACGTGTAGATATCAT	Intron
Sox2	499593	GCAGTACAACCTCCATGACCAGCTCGCAGACCTACATGAACGGCTCGC CCACCTACAGCATGTCTACTCGCAGCAGGGCACCCCCGGTATGGCG CTGGGCTCCATGGGCTCTGTGGTCAAGTCCGAGGCCAGTTCC	Exonic
Sox9	140586	ACTCCCCGCAACAGATCTCTACAGCCCCCTTCAACCTTCCGCACTAC AACCCCTCCTACCCAACCATCACGCGCTCGCAGTATGACTACACCGA CCACCAGAACTCCGGCTCCTACTACAG	Exonic
Sp1	24790	CTCCAGACCATTAACTCAGTGCAATTGGGTACTTCAGGGATCCAGGT GCACCAGCTTCCAGGCCTGCCCTTTGGCTATAGCAAACACCCAGGTG ATCATGGAGCTCAACTTGGCCTTCATGGACCTGGCGGTGATGGA	Intron
Sp7	300260	ATCTTGGGGTAGGACATGCTGGGGACCTGGTTGCAAGAGGTGGGGT GCTGGATAGGGAGCTGGGTTAAGGGGGGCAAAGTCAGACGGGTAAAG TAGGCAGCT	Exonic

Sparc	24791	GTTGCCCTCATCTCTCTCGTACAAGGTGACCAGGACGTTTTTGAGCC AGTCACGCATGCGCAGAGGGAATTCGGTCAGCTCAGAATCCAGGCA GGGGGCAATGTATTTC	Exonic
Spp1	25353	TGTGTGCTGGCAGTGAAGGACTCATCAGATTCATCGGAATGGTGAGA TTCGTCAGATTCATCCGAGTTCACAGAATCCTCGCTCTCTGCATGGT	Exonic
Tac1	24806	CCAACGATGATCTAAATTATTGGTCCGACTGGTCCGACAGTGACCAA ATCAAGGAGGCAATGCCGGAGCCCTTTGAGCATCTTCTTCAGAGAAT CGCCCGAAGACCCAAGCCTCAGCAGTCTTTGGATTAATGGGCAAAC	Intron
Tbx5	304514	AAATTTACCACTGTACCAAGAGGAAAGATGAAGAATGTTCCAGCAC CGAGCACCCCTATAAGAAGCCGTACATGGAGACCTCCCCCAGCGAG GAGGACACCTT	Intron
Tert	301965	ACCTCCTCTTGTGACAGTTCCTGCTAGCTGAACTCTCTCAAGCTGTTGC CTGATTCCAATGCTCTGCAGCTTGCTCCACACACTCTTACGGTAGAA GAAAAGGCGGTTCTTCTGGAACGTGGTCTCTG	Intron
Tgfb1	59086	TTTTGACGTCACTGGAGTTGTCCGGCAGTGGCTGAACCAAGGAGACG GAATACAGGGCTTTTCGCTTCAGTGCTCACTGCTCTTGTGACAGCAAA GATAATGTACTCCACGTGGAATCAATGGGATCAGTCCCAAACGT	Intron
Tgfb3	25717	CCACCTCTGCCTGCACCACAGAAATCCATGTTCTCACCAGTTTGCCTT CCCCTAACCAACCCACACTTCTTTACCACAGTGATCTCAGAGCTA GCAGAAAGAAATGTTCCAGAAGGAAGCCTCCCTCTC	Exonic
Tnf	24835	CTACTTTGGAGTCATTGCTCTGTGAGGCGACTGGCGTGTTTCATCCGTT CTCTACCCAGCCCCGTGCCCCGACTCTGACCCCCATTACTCTGACCCC TTTATCGTCTACTCCTCAGAGCCCCCAATCTGTGTCCTTCTAACTT	Exonic
Twist1	85489	TAGAGACTCTGGAGCTGGATAACTAAAAATAAATCTATATGACAAA GATTTTCTTGGAATTAGAAGAGCAGAGACCAAATTTACAAGAATC AGGGCGTGGGGCACACTTTTAAAAGAGAAAGCGA	Exonic
Vegfa	83785	TCCCACAGGGGTCTTGGCAAAGAGAAGACACAGTGGTGGAAGAAGA GGCCTGGTAATGGCTCCTCCTCCTCCTCCTGGGAACCCCTCGTCCTCT CCCTACCCCACTTCTTGGGTATAGCTCAGGAGGACCTTGTGTGATCA GACCATGA	Exonic
Wnt5a	64566	CATCCTCATGAACTTGCAACAATGAAGCAGGTGCGAGGACAGTAT ACAACCTGGCAGATGTAGCCTGTAAGTGCCATGGAGTGTCTGGCTCC TGTAGCCT	Intron

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