Validation of Acute Traumatic Coagulopathy in an ovine model of trauma and haemorrhage

Natasha van Zyl
BVSc (Hons) MBBS MANZCVS(Equine Surgery)

A thesis submitted for the degree of Master of Philosophy at The University of Queensland in 2016
School of Medicine.
Abstract

Perturbations in coagulation function are common following trauma and are independently associated with poor clinical outcomes. Trauma induced coagulopathy (TIC) was traditionally considered an iatrogenic process, attributed to the loss, dilution or dysfunction of coagulation proteases. It is now recognised that an acute endogenous coagulation dysfunction develops prior to medical intervention in response to a combination of tissue injury and hypoperfusion. This acute traumatic coagulopathy (ATC) is still defined clinically using traditional assays of coagulation function as a 20% increase in international normalised ratio (INR). Efforts have been made to characterise ATC using viscoelastic point of care testing; however there is no current universally accepted viscoelastic definition.

The pathogenesis of ATC remains poorly understood. Activation of the protein C pathway, fibrinolysis, platelet dysfunction and endothelial glycocalyx shedding are all hypothesised to play a role in development. However the exact contributions are still unknown and this current knowledge gap is impeding the development of effective and tailored resuscitation strategies for this subset of patients.

Pre-clinical animal research is a necessary adjunct for improving the understanding and management of ATC. Despite considerable interest in developing animal models of ATC there are few clinically relevant models that reflect the contemporary understanding of the condition. The development of a well-designed animal model of trauma may improve mechanistic understanding of ATC and facilitate the development of targeted treatment strategies.

This thesis describes an ovine model of complex trauma and haemorrhage that demonstrates coagulation changes using both traditional plasma based assays and point of care viscoelastic assays that are consistent with current definitions of ATC. The degree of coagulopathy was correlated with the degree of shock as quantified by arterial lactate. Coagulopathy was also associated with activation of the protein C pathway and shedding of the endothelial glyocalyx. Fibrinolysis did not make a significant contribution to the coagulopathy observed and there was no evidence of altered platelet function in this model.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature


Publications included in this thesis

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<td>Reviewed studies for inclusion (90%)</td>
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<td>Michael C Reade</td>
<td>Designed literature search (5%)</td>
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<td>Designed literature search (5%)</td>
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| Elissa M Milford             | Laboratory testing (70%)  
Analysis and interpretation of data (75%)  
Writing and editing paper (70%)  |
| Sara Diab                    | Conception and Design (10%)  
Ethics application (30%)  
Animal experiments (2.5%)  
Analysis and interpretation of data (15%)  
Writing and editing paper (10%)  |
| Kimble Dunster               | Conception and design (2.5%)  
Animal experiments (10%)  |
| Peter McGiffin               | Laboratory testing (30%)  |
| Stephen G Rayner             | Animal experiments (7.5%)  
Writing and editing paper (2.5%)  |
| Andrew Staib                 | Application for funding (80%)  |
| Michael C Reade              | Conception and design (5%)  
Ethics application (2.5%)  
Analysis and interpretation of data (5%)  
Writing and editing paper (7.5%)  |
| John F Fraser                | Application for funding (10%)  
Conception and design (5%)  
Ethics application (2.5%)  
Analysis and interpretation of data (5%)  
Writing and editing paper (7.5%)  |
Contributions by others to the thesis

The concept and design of the model utilised in this study was a joint collaboration between myself, John Fraser, Michael Reade, Elissa Milford, Sara Diab and Kimble Dunster. The specially designed instruments used to create crush injuries, contusions and fractures were constructed and calibrated by Kimble Dunster. Assistance with instrumentation of the animals during the experimental period was received from Sara Diab, Stephen Rayner and Kimble Dunster. All point of care viscoelastic testing was performed during the experimental period by Peter McGiffin. Chiara Palmeri assisted in the processing and evaluation of tissue histology. The ELISA assays for syndecan-1, activated protein C and thrombomodulin were performed with the assistance of John Cardinal. The hyaluronan ELISA assay was performed by Elissa Milford. The remaining serum and plasma assays were performed by Queensland Pathology. Data organisation was assisted by Elissa Milford, and statistical analysis was assisted by Marcella Kwan. Michael Reade and John Fraser reviewed drafts of this thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

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**Keywords**
Trauma, acute traumatic coagulopathy, coagulation, haemorrhage, protein C, sheep, pre-clinical research

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ANZSRC code: 110305 Emergency medicine 40%
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FoR code: 1102 Cardiorespiratory Medicine and Haematology 35%
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List of abbreviations

ADP  adenosine diphosphate
AIS  abbreviated injury score
ALI  acute lung injury
aPC  activated protein C
aPTT activated partial thromboplastin time
ATC  acute traumatic coagulopathy
ARDS acute respiratory distress syndrome
Ca^{2+} ionised calcium
CFT  clot formation time
CL  clot lysis
CT  clotting time
DAMPS damage associated molecular patterns
DIC disseminated intravascular coagulation
eTM endothelial bound thrombomodulin
FFP fresh frozen plasma
FV factor V
FVIII factor VIII
HMK high molecular weight kininogen
IHC immunohistochemistry
IL-1 interleukin-1
INR international normalised ratio
ISS injury severity score
ISTH International Society on Thrombosis and Haemostasis
JAAM Japanese Association for Acute Medicine
LY lysis
MA maximum amplitude
MCF mean clot firmness
PAI-1 plasminogen activator inhibitor-1
PK prekallikrein
PL phospholipid
POC point of care
PMP platelet derived micro-particle
PT prothrombin time
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>R</td>
<td>reaction time</td>
</tr>
<tr>
<td>ROTEM</td>
<td>rotational thromboelastometry</td>
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<tr>
<td>sTM</td>
<td>soluble thrombomodulin</td>
</tr>
<tr>
<td>SBL</td>
<td>Samm Border Leicester</td>
</tr>
<tr>
<td>TEG</td>
<td>thromboelastography</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
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<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
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<td>TIC</td>
<td>trauma induced coagulopathy</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>TM</td>
<td>thrombomodulin</td>
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<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
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<tr>
<td>TXA</td>
<td>tranexamic acid</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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CHAPTER 1: INTRODUCTION

Trauma remains a leading cause of death and disability worldwide, with approximately 10% of all deaths occurring due to traumatic injury (1). It predominately affects individuals younger than 44 years, with an average of 36 life years lost per trauma death (2, 3). However trauma has a wider impact than mortality statistics alone can illustrate. For every trauma fatality are two survivors who sustain serious or permanent disability, with over 1.8 billion disability adjusted life years lost annually as a result of traumatic injury (4). Trauma therefore has a significant financial impact on society as a consequence of healthcare costs and lost productivity, as well as having far reaching personal and emotional effects on the individual.

The causative mechanisms of trauma can be divided into blunt and penetrating injuries (5). Regardless of the initiating mechanism the temporal distribution and cause of mortality in trauma patients remains similar. Between one third and two thirds of trauma deaths occur prior to hospital admission and generally result from catastrophic head injury or major vessel disruption that is unresponsive to medical intervention and difficult to prevent (5-7). Of those trauma patients that survive until arrival to hospital the vast majority (up to 80%) will die, primarily during the first 48 hours of admission (3, 8). Despite advances in resuscitation, surgical management and critical care, uncontrollable haemorrhage remains the leading cause of preventable death during this period (3).

Haemostasis is precipitated by damage to the vascular endothelium, which triggers reflex local vasoconstriction and platelet activation in an attempt to minimise haemorrhage volume (9). Platelets adhere to the exposed sub-endothelial matrix via a collagen receptor and glycoprotein Ib which facilitates binding of von Willebrand factor (10). Adhesion triggers platelet activation resulting in changes in the cytoskeleton shape and the secretion of agonists such as adenosine diphosphate (ADP), thromboxane A2, adrenaline and serotonin to recruit additional platelets (10, 11). The rapid development of this platelet plug represents primary haemostasis and is effective in sealing small endothelial lesions in isolation. However platelet activation also triggers a cascade of pro-coagulant enzymes that results in the formation of a fibrin mesh to stabilise and support the platelet plug, a process known as secondary haemostasis.
The cascade model of coagulation depicts secondary haemostasis as two separate pathways triggered by different stimuli that ultimately result in the formation of fibrin: the contact activation (or intrinsic) pathway and the tissue factor (or extrinsic) pathway (figure 1) (12, 13). The cascade model accurately reflects the identity, function and interactions of the individual proteases involved in secondary haemostasis (12, 13). However it fails to take into account the contribution of the endothelium and other circulating cells to the coagulation process, and is therefore unable to fully explain many clinical bleeding syndromes. This has led to the proposal of the cell based model of haemostasis to better reflect the process of coagulation in vivo (14). The cell based model consists of 3 phases: initiation, amplification and propagation (figure 2). These phases are isolated to specific cell surfaces and suggest that the intrinsic and extrinsic pathways of coagulation are not redundant systems, but rather operate in parallel using different cell surfaces (14).

**Intrinsic Pathway**

- Factor XII
- HMK
- PK
- Factor XI → Factor Xa
- Factor IX → Factor IXa

**Extrinsic Pathway**

- Factor VII
- Tissue factor
- PL, Ca^{2+}
- Factor IXa → Factor IXa
- Factor VIIIa

**Common Pathway**

- Factor X
- Factor Va
- PL, Ca^{2+}
- Prothrombin → Thrombin
- Fibrinogen → Fibrin

*Figure 1. The cascade model of coagulation. Adapted from Hoffman and Monroe 2001 (14)*
Normal pro-coagulant function is balanced by a number of endogenous anti-coagulant mechanisms that aim to restrict thrombin formation to the site of injury, avoid uncontrolled clotting of the entire vascular tree and inhibit local vessel thrombosis to maintain blood flow during periods of reduced perfusion (15-17). Intact endothelium prevents platelet adhesion to the thrombogenic subendothelial extracellular matrix and expresses heparin like molecules that activate anti-thrombins, inhibiting thrombin and several serine proteases including factors IXa, Xa, Xia and XIIa (15). The endothelium also expresses thrombomodulin (TM) which activates protein C and S to proteolytically inactivate factors Va and VIIIa (16), and produces tissue factor pathway inhibitor (TFPI) which prevents the ongoing tissue factor-factor VII interactions that initiate the extrinsic pathway (18).

Patients experiencing severe trauma often demonstrate impaired coagulation function, which complicates efforts to achieve effective haemostasis, increases transfusion requirements and contributes to higher mortality rates (19, 20). This trauma induced coagulopathy (TIC) has traditionally been attributed to the loss, dilution and dysfunction of coagulation proteases in response to fluid resuscitation, hypothermia and acidosis (20, 21). However it is now recognised that a mechanistically distinct acute traumatic coagulopathy (ATC) develops in the hyper-acute post trauma period independent of these factors (22, 23). The existence of this early coagulopathy has since been confirmed in a number of studies performed by independent research groups (22-26) with a strong association between coagulopathy and mortality reported in all studies (table 1).
The presence of an early endogenous coagulopathy has been confirmed by a number of independent research groups. In all studies the presence of coagulopathy was associated with a higher mortality rate.

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The identification of ATC was originally based upon the traditional transfusion triggers recommended by massive transfusion guidelines (27, 28), namely a 50% prolongation of prothrombin time (PT), activated partial thromboplastin time (aPTT) or international normalised ratio (INR) (22, 23). However subsequent epidemiological work has shown a 20% increase in INR to be the clinically significant threshold for mortality and blood product requirements in trauma (29). More recently efforts have been made to characterise ATC using both thromboelastometery (ROTEM) or thromboelastography (TEG), reflecting the increasing use of viscoelastic point of care assays in the trauma setting (30-35). However there remains no universally accepted viscoelastic definition of ATC (36).

ATC appears to develop in response to a combination of tissue injury and systemic hypoperfusion (29, 37) and may simply represent the over-zealous activation of normal anti-coagulant processes. However the exact pathophysiological mechanisms underlying its development remain unclear. It has been suggested that activation of the protein C pathway may play a central role, as clinical studies demonstrate an association between protein C reduction, coagulopathy and mortality (37-39). The primary theory is that pathological activation of protein C results in systemic anticoagulation and hyperfibrinolysis via the inactivation of factors Va, VIIIa and plasminogen activator inhibitor-1 (PAI-1) (37, 38, 40). However the circulating concentration of PAI-1 is roughly ten times that of protein C, raising doubts about the ability of activated protein C (aPC) to deplete PAI-1 levels to the extent required to accelerate fibrinolysis (41). This has led to suggestions that ATC is a fibrinolytic form of disseminated intravascular coagulation (DIC) driven by increased tissue
plasminogen activator (tPA) release from the site of injury (21, 42-44). There is also emerging evidence to implicate platelet dysfunction and endothelial glycocalyx degradation in the development of ATC (45-48), although the exact contributions of these factors remains unclear.

Coagulation is also an integral component of the innate immune system, with coagulation dysfunction triggering a systemic inflammatory response that is further compounded by immunologic responses to blood product transfusion and fluid resuscitation (49, 50). Haemostatic resuscitation using combinations of red blood cells, plasma, platelets, cryoprecipitate and fibrinogen remains the core therapeutic approach to severe trauma (51, 52), and has been combined with adjuncts such as prothrombin complex, recombinant activated factor VII and tranexamic acid (TXA) to further improve haemorrhage control (53). This may improve short term mortality rates (52); however the effect on the subsequent pro-coagulant state still needs to be quantified. TXA has also been shown to impede neutrophil adhesion pathways via the inhibition of plasmin (54, 55). This may further alter the immune response in a patient population susceptible to sepsis and influence the medium-long term complication rate. Targeted treatment strategies are required to further improve outcomes for patients with ATC; however the development of these is currently restricted by the limited mechanistic understanding of the condition.

Pre-clinical animal research has become an attractive option for investigating the pathogenesis and management of ATC. Human research in the trauma setting can be problematic, as the dynamic nature of traumatic injury creates difficulties in the recruitment of patients to randomized controlled trials and increases the risk of bias and confounders in both prospective and retrospective studies (56, 57). Animal models provide an alternative option as they facilitate the controlled and systematic evaluation of isolated insults in vivo, which may enhance mechanistic understanding and identify novel therapeutic targets. However animals are genetically distinct from humans, and demonstrate differences in coagulation assay parameters, coagulation factor concentrations and platelet function that may alter the susceptibility to ATC (58-62). For an animal model to be clinically relevant there needs to be more similarities than differences between the human coagulation system and that of the chosen animal species.

There are a number of published animal models that have attempted to characterise changes in coagulation function in response to trauma and haemorrhage (63, 64).
However there remains no consensus as to the species or type of traumatic insult that most closely replicates the human condition. Rodents have consistently demonstrated the ability to achieve clinical definitions of ATC in response to trauma and haemorrhage (29, 40, 65), however they are limited by animal size and recognized differences in coagulation function (58). Pigs are the only large animal species that has been used for evaluating ATC, which would appear appropriate given the established physiological similarities between pigs and humans (66). However comparative studies of coagulation function have shown pigs to be relatively hypercoagulable compared to humans, with protein C levels only 36% of human values (58, 60). Given the hypothesized role of protein C in the development of ATC it is possible that this fundamental difference may impede the ability to successfully model ATC in pigs. The only published porcine model to develop coagulation changes consistent with clinical definitions of ATC reports a 25% mortality rate in the trauma group (67). This may reflect the severity of injury required to produce ATC in this species and raises concerns over the ethical acceptability of this model. The predisposition for porcine models may therefore be limiting information about ATC that could be obtained from alternative large animal species.

Sheep have been widely used in biomedical research to model a number of human pathologies including asthma (68, 69), osteoporosis (70), sepsis (71) and acute lung injury (ALI) (72-74), as they also share many physiological similarities with humans (75-77). Comparative studies of human, porcine, rodent, canine and ovine coagulation function using routine coagulation tests, rotational thromboelastometry (ROTEM) and clotting factor assays also suggest that the human coagulation system demonstrates the greatest similarity with that of sheep (58, 59). However there are no current published ovine models of ATC. The development of an ovine model of ATC may therefore improve understanding of pathophysiology and better inform subsequent human studies.

The primary aims of this thesis were three fold. The first was to undertake a systematic review of the literature to identify and evaluate existing animal models of ATC, to better inform the design of an alternative large animal model. The second was to develop an ovine model of trauma and haemorrhage that demonstrated coagulation changes consistent with ATC as defined by INR, aPTT and ROTEM. The third was to evaluate the relationships between coagulopathy, the protein C pathway, endothelial glycocalyx, platelet function and fibrinolysis within the ovine model to better inform future mechanistic studies.
2.1 Background of Acute Traumatic Coagulopathy

Coagulopathy in trauma has classically been considered an iatrogenic process developing late after injury in response to haemodilution, acidosis and hypothermia (20). In 2003 two separate retrospective clinical studies described an endogenous coagulopathy present at hospital admission that had developed in the absence of these physiological derangements (22, 23). The existence of this acute traumatic coagulopathy (ATC) has since been confirmed in multiple publications from independent research groups and has changed our comprehension of coagulopathy in trauma (24-26, 29, 30, 32, 39). It is now understood that haemostatic equilibrium is disrupted early post trauma by an endogenous dysfunction which is further aggravated by medical interventions promoting the development of haemodilution, acidosis and hypothermia (figure 3).

![Figure 3](image)

**Figure 3.** ATC is an endogenous coagulation dysfunction developing in response to tissue injury and haemorrhagic shock. Acidosis, hypothermia and haemodilution simply exacerbate this underlying dysfunction, signalling progression to TIC.

The reported incidence of ATC at hospital admission varies from 24-41% depending on the diagnostic criteria that is used (table 2) (22-25, 29, 32, 39). However in all studies it is associated with a negative impact on patient outcomes. ATC has been independently associated with higher transfusion requirements, increased incidence of post injury multi-organ failure, longer critical care unit stays and a four-fold increase in the risk of mortality (22-25, 29). The inability to control bleeding is the biggest factor contributing to mortality.
and morbidity in patients with ATC (78). However the presence of ATC is also a strong predictor of venous thromboembolism which can further complicate the clinical course (79) and knowing when to instigate thromboprophylaxis is difficult in patients presenting with coagulopathy.

Table 2. Various definitions of ATC have been proposed in the literature. ATC is associated with a negative impact on patient outcomes regardless of the definition used.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Number</th>
<th>Definition</th>
<th>% with ATC</th>
<th>Mortality with ATC</th>
<th>Mortality without ATC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brohi et al (22)</td>
<td>1,088</td>
<td>PT&gt;18s or aPTT&gt;60s or INR&gt;1.5</td>
<td>24.4%</td>
<td>46.0%</td>
<td>10.9%</td>
</tr>
<tr>
<td>MacLeod et al (23)</td>
<td>7,638</td>
<td>PT&gt;14s or aPTT&gt;34s</td>
<td>28.0%</td>
<td>19.3%</td>
<td>6.3%</td>
</tr>
<tr>
<td>Maegele et al (24)</td>
<td>8724</td>
<td>PT &lt;70% or platelets &lt;100x10⁹</td>
<td>34.2%</td>
<td>28.0%</td>
<td>8.4%</td>
</tr>
<tr>
<td>Niles et al (25)</td>
<td>347</td>
<td>INR&gt;1.5</td>
<td>38.0%</td>
<td>24.0%</td>
<td>7.0%</td>
</tr>
<tr>
<td>Frith et al (29)</td>
<td>3,646</td>
<td>INR&gt;1.2</td>
<td>36.2%</td>
<td>22.7%</td>
<td>7.0%</td>
</tr>
<tr>
<td>Davenport et al (32)</td>
<td>300</td>
<td>ROTEM A5≤35mm</td>
<td>18.0%</td>
<td>23.4%</td>
<td>6.2%</td>
</tr>
<tr>
<td>Cohen et al (39)</td>
<td>1,245</td>
<td>INR&gt;1.3 or aPTT&gt;35s</td>
<td>41.6% by INR</td>
<td>32.6%</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

PT = prothrombin time, aPTT = activated partial thromboplastin time, INR = international normalised ratio, ROTEM A5 = clot amplitude at 5 minutes using rotational thromboelastometry.

The recognition of ATC has driven changes in the clinical management of traumatic injury. Resuscitation practices in trauma have shifted away from crystalloid based regimens towards the use of whole blood, or red cell concentrates in combination with plasma, fibrinogen, cryoprecipitate and platelets (51, 52, 80, 81). Improvements in survival rates have been associated with these ‘damage control’ or ‘haemostatic’ resuscitation regimens that have been retrospectively attributed to improvements in haemostatic function (51, 52, 82). However a recent study by Kahn and colleagues suggests this is not the case, with ongoing deterioration of coagulation function evident in the face of haemostatic resuscitation (80). Rather than directly addressing coagulopathy it is possible that factors such as the prevention of haemodilution, type of injury, rate of haemorrhage, survivor bias or other confounders may have contributed to the survival benefits associated with
haemostatic resuscitation regimens (83). The optimum composition of blood products for resuscitation is also a point of contention, with the ratios and types of products used varying between countries and institutions (84-86). This divergence in clinical practice reflects a lack of knowledge regarding the benefits and risks of individual blood components in the management of ATC.

2.2 The pathophysiology of Acute Traumatic Coagulopathy

A combination of tissue injury and tissue hypoperfusion has been shown to be a necessary pre-requisite for the development of ATC in clinical studies (25, 29, 37, 39, 87-90), in vitro experiments (91) and animal models (29, 40, 65). A normal base deficit is not associated with coagulopathy in trauma regardless of the injury severity (29, 37, 92, 93). However systemic hypoperfusion has a dose-dependent effect on coagulation function in the face of tissue injury (39). ATC is most likely to occur when a base deficit of more than 6mmol/L is combined with an injury severity score (ISS) greater than 15, and the incidence and severity of coagulopathy in patients with haemorrhagic shock increases significantly when the ISS is 25 or greater (29).

The presence of ATC is characterised by systemic hypocoagulability, dysfibrinogenaemia and hyperfibrinolysis (89, 94). However the underlying mechanisms triggering the development of ATC remain unclear and this current knowledge gap remains a key barrier to the development of targeted treatment strategies for this subset of patients. Protein C activation, tissue factor release, endothelial glycocalyx shedding, platelet dysfunction and fibrinolysis have all been hypothesised to play a role in the development. However the evidence for many of these is limited and their role in the haemostatic response to tissue injury and shock requires further clarification.

2.2.1 Activation of the protein C pathway.

Activation of the protein C pathway is considered by many to be instrumental to the development of ATC, with the accompanying protein C depletion postulated to contribute to the increased risk of post injury multi-organ failure and mortality (37, 38). Clinical studies have demonstrated a correlation between protein C reduction or activated protein C (aPC) increase, prolonged PT/aPTT and reduced clot strength (37-39, 95). Mechanistic
Evaluation in a murine model of ATC has added further weight to the theory, with coagulopathy prevented by the administration of aPC antibodies (40).

Protein C is a vitamin K dependent glycoprotein that is activated on the surface of endothelial cells by thrombin bound concurrently to the endothelial protein C receptor (EPCR) and the transmembrane glycoprotein thrombomodulin (TM) (16, 37). It is postulated that upregulation of TM expression occurs in response to tissue hypoperfusion (37, 39). When this is combined with increased thrombin generation from tissue trauma an increase in thrombin-TM complex formation occurs, resulting in pathological protein C activation (figure 4)(16). aPC then inhibits the coagulation cascade via the inactivation of factors Va and VIIIa and promotes fibrinolysis through the consumption of PAI-1 (figure 5)(16, 38, 96, 97). The protein C hypothesis therefore provides an explanation for both systemic anticoagulation and hyperfibrinolysis, although the magnitude of these effects in the development of ATC has not been quantified.

Figure 4. Thrombomodulin is released from activated endothelial cells and combines with thrombin to activate protein C. Activated protein C then inhibits coagulation by inactivating factors Va and VIIIa. Adapted from Brohi et al 2007 (98)
The significance of the protein C pathway in ATC has recently been questioned by Campbell and colleagues (44) who have shown that platelet and plasma factor Va pools are resistant to cleavage by aPC in vitro, both at concentrations observed in trauma patients and following the therapeutic doses of recombinant human aPC used in sepsis. In addition they found no evidence of fibrinolysis, arguing that the high circulating levels of PAI-1 are unlikely to be inactivated to the extent required to drive the fibrinolytic picture seen with ATC. The application and interpretation of these findings from a static system is limited given the complex, dynamic process of coagulation in vivo. Further evaluation of the interaction between the protein C pathway, platelet function and fibrinolysis is warranted to better understand their contribution to ATC.

2.2.2 The importance of fibrinogen and fibrinolysis

Haemostasis relies upon fibrinogen for adequate clot formation (99). Hypofibrinogenemia is well documented in clinical and experimental studies of ATC, and has been correlated with both early and late mortality (100-103). Early fibrinogen replacement has been shown to improve outcomes and correct coagulopathy (87, 100, 104, 105). However there is controversy regarding the critical level for replacement. Levels below 2.29g/L were associated with increased morbidity and mortality rates in a large multicentre observational study (94), which is well above the threshold for replacement of 1.0 g/dL recommended by current guidelines (106). This incongruity reflects a lack of understanding of the underlying benefits and risks of fibrinogen replacement in trauma. A randomised controlled trial to better evaluate the role of fibrinogen replacement in trauma is in the preliminary stages.
In the interim a controlled animal model of ATC may facilitate mechanistic evaluation of fibrinogen replacement and improve understanding of the role of fibrinogen replacement in trauma.

The cause of hypofibrinogenaemia in ATC is unclear. Acidosis, hypothermia and hemodilution have all been shown to lower fibrinogen levels (108), however these are not significant contributors to ATC. The formation of weak clots that are susceptible to increased fibrinolysis or fibrinogenolysis has been proposed as a cause (109). It is also possible that activation of the protein C pathway and subsequent disinhibition of the fibrinolytic pathways may lead to fibrinogen consumption and subsequent hypofibrinogenaemia (110).

Fibrinolysis appears to develop in a subset of patients with ATC (39, 96, 111, 112) and is strongly correlated with poor clinical outcomes (96, 111-115). The CRASH-2 study provides level I evidence of the importance of fibrinolysis, demonstrating a significant survival benefit following administration of the anti-fibrinolytic tranexamic acid (TXA)(116, 117). However a cautious approach to the empirical use of TXA is required. As ATC progresses a pro-thrombotic state begins to predominate (118), with a high incidence of thromboembolic complications evident following major trauma (79, 119). The development of this pro-coagulant tendency has been attributed to inflammation, circulating microparticles and dysregulation of tissue factor and thrombin, which act to increase PAI-1 production and shutdown fibrinolysis (120, 121). The survival benefits from TXA in the CRASH-2 study were time limited, with increased mortality rates observed if administered more than 3 hours following injury (116). Administration of TXA after the benefit of reduced fibrinolysis has passed may potentiate the prothrombotic state, increasing the risk of thromboembolic complications. TXA has also been shown to impede the adherence and transmigration of neutrophils which may have an indirect effect on outcomes (54). Impeding leucocyte-endothelial interactions has been shown to dampen the systemic inflammatory response and improve outcomes in animal models of sepsis (122, 123). Therefore TXA administration could theoretically play a role in decreasing the risk of sepsis in trauma, although stage 2 and 3 human trials have shown minimal benefit from these anti-adhesion therapies (123, 124). Conversely the late administration of TXA could theoretically contribute to an increased risk of sepsis and mortality by inhibiting the innate immune response, predisposing the patient to systemic bacterial infection (122).
The mechanism behind fibrinolysis in ATC is unclear. Increased levels of tissue plasminogen activator (tPA) are evident in trauma, and PAI-1 is the primary inhibitor of tPA (96). As previously mentioned it has been suggested that PAI-1 consumption by aPC might disinhibit fibrinolysis, contributing to the fibrinolytic picture observed (96, 97). Others argue the increased tPA levels are due to massive release following injury, which when augmented by increased thrombin production, catecholamine and vasopressin release triggers fibrinolysis (44, 125). Recent work in a rodent model has demonstrated that TEG detectable fibrinolysis is inhibited by tissue injury and promoted by haemorrhagic shock, although the hypofibrinolytic phenotype of rats makes the significance of this finding unclear (126). At face value it may also appear that ATC is actually DIC with a fibrinolytic phenotype, as the initial changes of ATC are positive on both International Society on Thrombosis and Hemostasis (ISTH) and Japanese Association for Acute Medicine (JAAM) scoring systems (125, 127, 128). However there is no histopathological evidence of inappropriate disseminated clot formation in trauma patients to suggest ATC and DIC are one and the same (129).

2.2.3 Endothelial injury
The endothelial glycocalyx is a negatively charged anti-adhesive and anti-coagulant surface layer that protects the endothelial cells and maintains vascular barrier function (130). Traumatic injury and shock result in tissue ischaemia, activation of the inflammatory system and stimulation of the neuro-humoral axis with a subsequent catecholamine surge (45, 131-134). This can lead to endothelial cell activation, glycocalyx degradation and luminal expression of anticoagulant/profibrinolytic proteins (45, 135) with emerging evidence suggesting these factors may play a role in ATC (45, 46, 132). Trauma patients have demonstrated an increase in syndecan-1, a soluble marker of glycocalyx shedding, which correlates with the severity of shock, levels of circulating catecholamines, tissue injury and markers of fibrinolysis (45). Elevated syndecan-1 is also associated with a higher incidence of ATC and mortality regardless of ISS, suggesting the downstream effects of trauma modulate the response rather than the injury itself (45).

Endothelial glycocalyx shedding appears to trigger thrombin generation and fibrinolysis, which in the presence of increased soluble thrombomodulin (sTM) from damaged endothelial cells may enhance protein C activation (45, 135). A volume of plasma containing heparin like substances is also held within the glycocalyx (136), and release of this following degradation may lead to direct anticoagulant effects from endogenous
heparinisation (46). Electron microscopy data from experimental models of haemorrhagic shock suggests that the endothelium may be a potential therapeutic target in ATC (137). However further work to better evaluate the microvasculature and its role in ATC is still required.

2.2.4 Role of platelet function and microparticles

The cell based model of haemostasis recognizes the fundamental role of platelets in the balanced assembly of a fibrin clot (14), with a recent study demonstrating that platelets contribute to two thirds of overall clot strength (99). Minor reductions in the admission platelet count of trauma patients are predictive of mortality, even if they remain within the normal range (138, 139). Decreased responsiveness to collagen, ADP and arachidonic acid agonists has also been demonstrated in trauma using TEG and whole blood aggregometry (Multiplate), and is strongly associated with mortality (47, 48, 140). These observations may explain the improved outcomes associated with early platelet transfusion in trauma, with the degree of improvement further impacted by the quality of transfused platelets (85, 141, 142).

The role of platelet dysfunction in ATC remains obscure. It has been suggested that initial platelet hyperactivation in trauma may render platelets unresponsive to subsequent stimulation, resulting in reduced aggregation parameters and decreased clot strength (143). A similar phenomenon has been observed in conditions such as transplant rejection and thrombotic thrombocytopenic purpura, in which acquired defects in platelet function develop following prolonged activation in vivo (144). Platelet dysfunction may also play a role in hyperfibrinolysis. ADP release into the circulation has been shown to increase sensitivity to tPA, resulting in increased fibrinolytic activity (145). However mechanistic studies evaluating platelet function in trauma are limited and further work is required to better characterise the contribution to ATC.

Recent work has also focused on the contribution of microparticles to coagulopathy. These small vesicles are derived from blood and endothelial cells and contribute to normal haemostatic function (146). Platelet derived microparticles (PMPs) are the most abundant and are highly pro-coagulant (147, 148). The PROMMT study demonstrated an association between decreased levels of PMPs and poor clot strength, increased blood product requirements and mortality in coagulopathic trauma patients (149). An alternate study demonstrated no significant difference in microparticle concentration with time in
trauma patients (150), while a third study reported elevated PMPs in trauma patients at hospital admission that were implicated in the development of post injury multi-organ failure (151). Alterations in PMP levels may be a separate component of platelet dysfunction developing independently to impaired aggregation capacity, and may play an important part in the haemostatic and inflammatory responses to trauma. However further work is required to determine the precise contribution to ATC.

2.3 Current definitions of Acute Traumatic Coagulopathy

Laboratory diagnosis is required for identification of ATC; however there remains no consensus regarding the laboratory definition that should be used. ATC was originally characterised by Brohi et al as PT > 18s, aPTT > 60s or INR >1.5 and by MacLeod et al as a PT >14s and aPTT > 34s (table 2)(22, 23). Subsequent epidemiological work has found a 20% increase in INR to be the clinically significant threshold for blood product requirements and mortality, with the previously cited thresholds failing to identify 16% of trauma patients experiencing poorer outcomes (29). These traditional assays of coagulation function remain the basis for identification of ATC as they are readily available and have demonstrated an ability to predict mortality and need for transfusion in the trauma patient (25).

In recent times the value of these traditional assays in the contemporary management of ATC has been questioned. While prolongation of these assays has been associated with poorer patient outcomes it is conceivable that they may simply be a marker of injury severity, rather than an accurate reflection of coagulation function. These tests were initially developed to identify specific coagulation factor deficiencies and have been shown to be poor predictors of haemorrhage in the face of multiple acquired deficiencies (152, 153). The assays are performed on platelet poor plasma and reflect only the initial 20-60 seconds of clot formation, failing to take into account the contribution of platelets to haemostasis, the role of fibrinolysis or thrombin generation, or the global interaction between coagulation enzymes (32). Furthermore the turn-around times of 30-60 minutes often negates the value of results in the rapidly evolving setting of acute trauma (32). This prolonged turnaround time could be addressed through the use of point of care (POC) INR devices, which have shown to correlate well with formal laboratory testing in the trauma setting (154-157). However the acknowledged limitations of these traditional assays
remain, which has precluded widespread uptake of POC INR testing in the trauma setting (158).

Viscoelastic coagulation testing is widely used in cardiac and transplant surgery to guide resuscitation, and has been associated with decreased blood product use and improved outcomes (159-163). These tests are performed on whole blood and produce a trace that is representative of [1] platelet activation, [2] thrombin burst, [3] function of plasma proteins including fibrinogen and [4] the fibrinolytic system (164) (figure 6). Unlike the traditional assays of coagulation function viscoelastic tests provide an early and more comprehensive assessment of secondary haemostasis (164, 165). The need for an accurate and rapidly available diagnostic test of ATC has prompted the increased utilisation of viscoelastic testing in the trauma setting.

![Viscoelastic Trace Diagram](image)

**Figure 6.** Schematic viscoelastic trace demonstrating the commonly reported variables of TEG (upper part) and ROTEM (lower part). Reaction time (R)/clotting time (CT) is partially representative of platelet function (1), alpha angle (α) reflects the thrombin burst (2), maximum amplitude (MA)/maximum clot firmness (MCF) reflects the function of plasma proteins (3) and clot lysis (CL/LY) represents fibrinolysis (4).

Attempts to define ATC using both rotational thromboelastometry (ROTEM) and thromboelastography (TEG) have been made (30-33, 166-170). ATC has a viscoelastic profile characterised by slow clot formation time and reduced clot amplitude (32, 33, 167-169). ROTEM EXTEM A5 values ≤35mm have been associated with increased transfusion
requirements and mortality compared to an INR > 1.2 (32, 34). Others suggest an EXTEM A10 ≤40mm correlates best with platelet count, fibrinogen level and blood product requirements (30, 170). Similar findings are evident with TEG, with maximum amplitude (MA) <55mm, K-time >2.5s and R value >1.1s associated with poor outcomes and increased need for transfusion (33, 167, 168). However there remains no universally accepted viscoelastic definition of ATC (36, 93).

The ability of viscoelastic testing to distinguish between different haemostatic abnormalities suggests that it may provide a means of individualizing haemostatic resuscitation in the trauma patient (33, 113). However there are no viscoelastic based resuscitation algorithms for trauma that have been validated by randomised trials. Viscoelastic tests also fail to take into account the contribution of the endothelium to coagulation, which may play an important role in platelet and fibrinogen function (95). These assays are also relatively insensitive to the detection of fibrinolysis. Over 80% of trauma patients with an ISS > 15 demonstrate fibrinolysis using plasmin-antiplasmin assays (112), however only 5-10% of these patients are detected using viscoelastic testing (96, 111, 112). Further work is required to identify the viscoelastic triggers that will improve outcomes for patients with ATC. The development of additional tests to fully evaluate coagulation function may be required to facilitate individualised treatment strategies for the trauma population.
3.1 An introduction to this systematic review

Preclinical animal research is an attractive option for evaluating the pathophysiology and management of ATC. The variable and dynamic nature of trauma and its management means that human trauma research can be limited by confounders, difficulties with patient recruitment and bias \( (56, 57) \). Animal models offer an alternative as they allow the systematic evaluation of isolated insults \textit{in vivo}. This may reduce the impact of confounders whilst simultaneously improving mechanistic understanding. Based upon the perception of an iatrogenic process a number of animal models have investigated the effects of hypothermia, acidosis and haemodilution on systemic coagulation function \( (63, 64) \). However to study ATC effectively animal models need to reflect our current understanding of the condition as an endogenous response to tissue injury and hypoperfusion.

The use of animals for medical research has significant ethical considerations. All proposed animal experimentation is now appropriately scrutinised by institutional animal ethics committees and must encompass methods to protect and promote the welfare of animals \( (171) \). These methods include means to replace animals with alternatives where possible, reduce the number of animals used to the minimum required for statistical validity and refine experimental techniques to reduce the overall impact on the animals \( (171) \). Consideration of these ‘3R’ principles is therefore essential in the development of any animal study in order to maximise benefits and improve ethical acceptability.

This paper addresses the first aim of this thesis: to perform a systematic review of the literature in order to better inform the design of the animal model utilised in this thesis. Existing animal models of traumatic coagulopathy published following the recognition of ATC in 2003 were identified. The initiating mechanism of coagulopathy, mechanism of
injury, type of haemorrhage and animal species used were then assessed for clinical relevance, variability of response and survivability of injury. Collating this information identified experimental techniques that could be refined and combined in different ways in future animal models. It also identified data upon which to base sample size and power calculations, facilitating the development of a novel ovine model in an ethically acceptable manner.

3.2 Reprint of accepted manuscript.

Experimental animal models of traumatic coagulopathy: a systematic review.
Natasha van Zyl 1,2, Michael C Reade 3,4, John F Fraser 2
1. The University of Queensland, School of Medicine, Herston, QLD, Australia
2. Critical Care Research Group, The Prince Charles Hospital, Brisbane, QLD, Australia
3. Burns, Trauma and Critical Care Research Centre, The University of Queensland, Brisbane, QLD, Australia
4. Joint Health Command, Australian Defence Force, Canberra, Australia

Abstract
Introduction
Perturbations in coagulation function are common following trauma and are associated with poor clinical outcomes. Traditionally considered an iatrogenic process, it is now recognized that an acute coagulation dysfunction develops prior to medical intervention. The mechanisms underlying the development of this acute traumatic coagulopathy (ATC) remain poorly understood. Pre-clinical animal research is a necessary adjunct to improve mechanistic understanding and management of this condition. This review aims to identify and evaluate existing animal models of traumatic coagulopathy for clinical relevance.

Methods
A structured search of MEDLINE/Pubmed was performed in September 2014 in accordance with the PRISMA guidelines.

Results
A total of 62 relevant publications describing 27 distinct models of traumatic coagulopathy were identified. Porcine models predominated and hemodilution in isolation or in combination with hypothermia and/or acidosis was the principal mechanism for inducing
coagulopathy. Acute coagulation changes in response to tissue injury and hemorrhage were evident in 5 publications, and pathophysiological evaluation of postulated mechanisms was performed in 3 studies.

**Conclusions**
There are few clinically relevant animal models that reflect the contemporary understanding of traumatic coagulopathy. This relative deficiency highlights the need for further development of valid and reproducible animal models of trauma. Well-designed models will facilitate improved mechanistic understanding and development of targeted treatment strategies for traumatic coagulopathy.

**Keywords:** trauma, coagulation, hemorrhage, clotting function, pre-clinical research

**Introduction**
Trauma remains the principal cause of death and disability in people aged between 1-44 years (1). Severe hemorrhage is the leading cause of preventable death in the trauma population, responsible for up to 40% of all deaths occurring within 24 hours of trauma (78, 172).

Coagulation dysfunction following trauma is well recognized and influences the ability to achieve effective hemostasis. Trauma induced coagulopathy (TIC) was traditionally attributed to the loss, dilution and dysfunction of coagulation proteases in response to fluid resuscitation, hypothermia and acidosis (20). However, over the past decade derangements in coagulation function developing independent of these factors have been identified in the hyper-acute post-trauma period (22, 23). Identification of this acute traumatic coagulopathy (ATC) has altered the perceived natural history of TIC. The physiologic derangements that characterize TIC are now recognized to develop in a hemostatic system that is already unbalanced by an endogenous coagulation dysfunction.

The reported incidence of ATC in trauma patients at hospital admission varies between 24-41%, reflective of the multiple definitions of ATC that have been proposed (22, 23, 29, 39). ATC was originally characterized as a 50% prolongation in prothrombin time (PT), activated partial thromboplastin time (APTT) or international normalized ratio (INR) (22, 23). More recently an INR >1.2 has been shown to be the clinically significant threshold for increased mortality and blood product requirements (29). The identification of ATC at
hospital admission continues to be based upon these traditional assays of coagulation function. However these assays have limitations in the contemporary management of traumatic coagulopathy as they are performed on platelet poor plasma, reflect only the initial 20-60 seconds of clot formation and usually require 30-60 minutes for processing (32). Efforts have been made to define ATC with point-of-care viscoelastic coagulation measurements using both thromboelastometry (ROTEM) or thromboelastrography (TEG), however there is no current universally accepted viscoelastic definition, assay or validated algorithm for ATC (30-32, 95).

The pathophysiological mechanisms underlying the development of ATC remain poorly understood. A combination of tissue injury and shock resulting in tissue hypoperfusion appears to be a necessary requirement, as neither of these insults in isolation is associated with deranged coagulation function (29, 37, 39). Activation of protein C (aPC) may play a central role by diverting hemostasis towards hypocoagulability and hyperfibrinolysis through the inactivation of factors Va, VIIIa and plasminogen activator inhibitor-1 (PAI-1) (37, 38, 40, 173). Dissenters of this theory suggest that the concentrations of aPC associated with ATC are insufficient to produce these effects (44). It has been postulated that ATC is disseminated intravascular coagulation (DIC) with a fibrinolytic phenotype induced by tissue factor release from the site of injury, although histologic evidence of DIC is absent (43, 129). There is also emerging evidence to suggest that platelet dysfunction and endothelial glycocalyx degradation make a significant contribution to ATC, with catecholamine induced endothelial damage proposed as an alternative initiating mechanism for coagulopathy (44-47, 132).

The adverse outcomes related to ATC are not limited simply to the effects of acute blood loss. Coagulation dysfunction can trigger a de novo systemic inflammatory response which is compounded by immunologic responses to blood product transfusion and fluid resuscitation (49, 50). This results in an increased risk of post injury multi-organ failure, sepsis and thromboembolic complications in the long term (98). The current core therapeutic approach to patients with traumatic injury is the use of hemostatic resuscitation to restore circulatory homeostasis and control hemorrhage (51, 81). Whilst this improves mortality rates it may influence the development of a subsequent pro-coagulant state (121). Further improvements in patient outcomes require the development of targeted treatment strategies for this population, along with an understanding of when to switch from pro-coagulant to anti-coagulant therapies. This is currently restricted by our limited
knowledge of the pathogenesis of ATC and underlines the need for improved mechanistic understanding.

The pathogenesis and management of ATC has become a prime target for pre-clinical research, as the nature of traumatic injury creates difficulties for human research in the emergency setting. Recruitment to randomized controlled trials is difficult in a population where treatment begins in the field and consent can be difficult to obtain, reducing study power and increasing the risk of bias (56). Retrospective human studies can have multiple confounders, making it difficult to separate association and causation in the acutely ill trauma patient. They are also reliant on the quality of available data and are associated with survivor bias (174).

Animal models offer the opportunity to investigate isolated insults in vivo in a controlled and systematic fashion. This can improve mechanistic understanding of pathophysiology, facilitate the development of novel therapeutic strategies and provide a platform for the evaluation of existing and developing therapeutic interventions. While there has been considerable interest in developing animal models of ATC, coagulation mechanisms vary between species (58-60). For these models to be clinically relevant there needs to be more similarities than differences between the human coagulation system and that of the model investigated. As yet there appears no consensus as to the species, traumatic insult and resuscitation strategies that most closely replicate the human condition.

The aim of this review was to identify the experimental animal models that have been used to investigate traumatic coagulopathy following the identification of ATC in 2003. The models were evaluated for clinical relevance, ability to characterize underlying pathophysiologic mechanisms and ability to evaluate the effectiveness of hemostatic interventions. A systematic review of the literature was performed.

**Methods**

**Search Strategy**

The indexed online database MEDLINE/Pubmed was searched in September 2014 using the terms ("animals"[MeSH Terms] OR animal[All Fields] OR preclinical[All Fields] OR model[All Fields] AND ("injuries"[Subheading] OR "injuries"[All Fields] OR "trauma"[All Fields] OR "wounds and injuries"[MeSH Terms] OR ("wounds"[All Fields] AND "injuries"[All Fields]) OR "wounds and injuries"[All Fields]) AND ("haemorrhage"[All Fields] OR
Selection criteria
Abstracts and citations identified by the search were screened for relevance. Full publications of studies considered relevant were retrieved and reviewed. Additional relevant publications cited within the retrieved articles were also reviewed. Publications were included if they described an animal model of trauma or hemorrhage and reported systemic measures of coagulation function. Animal models of burn injury and traumatic brain injury were excluded due to acknowledged differences in the processes leading to coagulopathy in these conditions (175, 176).

Results
The search process produced 448 abstracts, with an additional 7 relevant publications identified from other sources. A total of 71 publications were considered relevant and their full text reviewed. Following exclusions a total of 62 publications were available for review (see Figure 1.) Of these 62 publications 26 were conducted to characterize the time course of coagulopathy or investigate pathophysiological mechanisms associated with the development of coagulopathy. The remaining 36 studies aimed to investigate a therapeutic intervention. Many of the studies were published by the same authors or research groups and described previously published models with only minor variations in study protocol. The studies that were considered to represent an original and distinct model of traumatic coagulopathy are summarized in table 1.

Study Characteristics
Six different animal species were featured in the studies evaluated (see table 1). Porcine models predominated (n=45), with the remainder comprised of rat (n=10), rabbit (n=4), sheep (n=1), mouse (n=1) and hamster (n=1) models. All studies utilized general anesthesia administered via the intravenous, intraperitoneal and/or inhalational routes.

Coagulation function was assessed by traditional plasma based assays (PT, APTT, INR, ACT) and/or viscoelastic assays (TEG or ROTEM). In most studies tests of coagulation
function were used to establish the presence/absence of coagulopathy following the initiating mechanism, with repeat assessment to evaluate the coagulation response to time or therapeutic intervention. Most studies also reported changes in platelet count and fibrinogen concentration. Only 3 studies assessed components of the hypothesized mechanisms of ATC development by evaluating changes in platelet function (177), response to activated protein C (aPC) blockade (40) and endothelial glycocalyx thickness and plasma syndecan-1 (178).

Methods used to induce coagulopathy.
Iatrogenic hemodilution, hypothermia and/or acidosis were the most common mechanisms utilized to induce deranged coagulation function. Hemodilution prolonged routine coagulation assays and exacerbated hemorrhage from standardized visceral injury (179-185). Induced hypothermia to 33°C in combination with hemodilution was correlated with a further increase in organ bleeding time (186, 187). Isolated hypothermia was only associated with prolonged coagulation assays when the tests were performed at the core body temperature of the animal, with no abnormalities present when performed at the regulation 37°C (188-190). Isolated acidosis was induced via intravenous administration of hydrochloric acid or cross clamping of the aorta following fixed volume hemorrhage to produce ischemia-reperfusion (191-194). It was associated with coagulation dysfunction that failed to improve following reversal with bicarbonate and the effects were compounded by concurrent hypothermia (193-195).

Isolated hemorrhagic shock failed to significantly prolong routine coagulation assays in all but one study in which a 10 fold increase in APTT was observed after 60 minutes of shock (29, 196-198). Isolated tissue trauma was not associated with prolongation of coagulation assays in any study (29, 40). One study utilized an intravenous infusion of tissue factor to simulate the effects of tissue factor release from the site of trauma (199). This produced hyperfibrinolysis, increased D dimer levels and significant prolongation of coagulation assays, although the absence of physical injury limits the clinical applicability of these findings.

The combined effect of trauma and hemorrhagic shock was evaluated in 3 rodent and 7 porcine models with varying effects on coagulation function observed (29, 40, 51, 65, 67, 177, 200-203). All rodent models demonstrated a minimum 20% prolongation in PT, aPTT or PT ratio (PTr) (29, 40, 65) which was attenuated by monoclonal antibodies to aPC in
One study (40). One porcine model demonstrated a brief hypercoaguable response that was followed by significantly reduced viscoelastic function and a 20% prolongation of INR, although a 25% mortality rate was evident within the experimental group (67). A second porcine model demonstrated altered platelet function and prolonged PT that was exacerbated by fluid resuscitation but unaltered by induced hypothermia, although the initial prolongation in PT failed to attain the current clinical definition of ATC (177). Non-significant changes in PT or INR were evident in the remaining 5 porcine models (51, 200-203), although a significant reduction in ROTEM mean clot firmness was evident in one study (203) and significant changes developed following fluid resuscitation and/or the induction of hypothermia and acidosis in the remaining three studies (51, 201, 202).

Discussion

This review identifies a large diversity of animal models utilized to investigate coagulation changes following trauma. This may reflect both the clinical importance of such an area as well as the fact that the pathophysiology of ATC is poorly understood and may be difficult to model accurately. Despite our current understanding of the natural history of traumatic coagulopathy only 10 models attempted to reflect it as an endogenous dysfunction induced by initiating injury and hemorrhage which may be exacerbated by hypothermia, acidosis and hemodilution. Consequently there remains an overall lack of reproducible, clinically relevant models for investigating pathophysiology and evaluating therapeutic interventions.

There are many animal models which have provided information on the effects of hypothermia, acidosis and hemodilution on systemic coagulation function (179-195). Restoration of plasma volume with crystalloid and/or synthetic colloid solutions produces a diffuse microvascular bleeding tendency due to dilution of clotting factors, accelerated endothelial glycocalyx degradation and direct interactions with fibrin polymerization (178, 183, 204). The induction of acidosis to a pH of less than 7.2 via intravenous administration of hydrochloric acid or ischemia-reperfusion injury has been shown to reduce the enzymatic activity of coagulation proteases, producing a coagulopathy that cannot be readily reversed with simple correction of the acidosis (192-194). Induced hypothermia to less than 33°C has similar effects on coagulation protease and platelet function, contributing to coagulation dysfunction and exacerbating existing coagulopathy induced by hemodilution and/or acidosis (188-190, 195). However in the majority of models investigating these insults the hemodilution, hypothermia and/or acidosis precede tissue
injury and hemorrhage. Given our current understanding of the natural history of traumatic coagulopathy the clinical value and relevance of models without initiating tissue trauma and shock is questionable.

Hemodilutional coagulopathy in isolation or in combination with hypothermia was the most common model used to evaluate the therapeutic efficacy of specific hemostatic interventions in trauma. However in the vast majority of these models the resuscitation fluids used to produce hemodilution were not reflective of contemporary resuscitation practices (82, 205). The absence of initiating tissue injury and divergence from current clinical practice may limit translatability of therapeutic information obtained from these models. The effects of spontaneous hypothermia and acidosis are also essentially uncharacterized by current animal models. Changes associated with central cooling induced hypothermia may differ from the spontaneous hypothermia observed in human trauma patients given that they rarely present with a body temperature as low as 33°C (206). The applicability of acidosis induced by hydrochloric acid infusion or ischemia-reperfusion in the absence of tissue trauma and shock is also dubious. The deleterious outcomes associated with acidosis developing in response to tissue hypoxia and trauma may simply reflect the severity of the insult responsible for the acidosis rather than being a direct effect of the acidosis itself.

The mechanism underlying the development of ATC is still incompletely understood. Clinical studies suggest a combination of tissue injury and hypoperfusion as a prerequisite (29, 37). Animal models investigating the effects of isolated trauma and hemorrhagic shock support this, with altered coagulation function in response to isolated insults only evident in one study (196). In contrast animal models of combined trauma and hemorrhage have shown varying degrees of coagulation dysfunction, which may reflect both the severity of the insult used and the intrinsic coagulation function of the animal. The majority of these models have provided information on the presence and time course of coagulopathy in response to trauma and hemorrhage (29, 65, 67, 177, 200, 201). However only 3 models have evaluated aspects of the proposed pathophysiologic mechanisms, and as a result animal models are yet to significantly contribute to the improved mechanistic understanding of ATC (40, 177, 178).

Animal models of trauma enable particular clinical scenarios to be evaluated under controlled conditions and provide opportunities for invasive monitoring and diagnostic
methods that cannot be performed in human trauma patients. Incorporating techniques such as electron microscopy to assess the endothelial glycocalyx, organ microdialysis and sidestream dark field (SDF) camera application to evaluate the microcirculation and immunohistochemistry (IHC) to investigate tissue expression of coagulation proteases in conjunction with a structured coagulation component analysis will facilitate a more sophisticated contribution to the mechanistic understanding of traumatic coagulopathy in future animal models of trauma (137, 207-209). However the use of animals for trauma research also has limitations. The ethical necessities of sedation and anesthesia alter the compensatory physiologic responses to injury and have an unknown effect on the overall response observed (210, 211). Animals are also genetically distinct from humans and demonstrate differences in coagulation assay parameters, coagulation factor concentrations and platelet function that may alter susceptibility to ATC (58-60).

Rodent models are inexpensive and transgenic techniques such as protein C gene mutations provide opportunities to manipulate and evaluate isolated pathways within the coagulation system (212). Rodents have also consistently demonstrated the ability to achieve the clinical definition of ATC in response to trauma and hemorrhage (29, 40, 65). However rodent models are limited by animal size which restricts sample volumes and subsequent assays, hampering the ability to further investigate mechanisms associated with coagulopathy. The genomic response of mice to trauma has also been shown to correlate poorly with humans, bringing the clinical relevance of murine trauma models into question (213).

Pigs are the predominant large animal species that have been used for the investigation of traumatic coagulopathy, which appears appropriate given the established similarities in cardiovascular physiology between pigs and humans (66). However pigs have demonstrated significant variability in coagulation function response to trauma and hemorrhage (51, 67, 177, 200-203). Both viscoelastic and plasma based assays of coagulation function have shown pigs to be relatively hypercoaguable compared to humans and to have protein C levels that are only 36% of human values (58, 60). Given that activation of protein C is hypothesized to be a central driver of ATC, this fundamental difference may impede the ability to model ATC successfully in this species. The only porcine model to achieve the clinical definition of ATC in response to trauma and hemorrhage was associated with a 25% mortality rate (67). This may reflect the severity of the injury required to produce ATC in this species and brings into question the
reproducibility and ethical acceptability of this model. The abundance of porcine models may be limiting information about traumatic coagulopathy that could be gained from other large animal species. Evaluating pathophysiological mechanisms and therapeutic efficacy in a variety of animal species subjected to varying insults and intercurrent treatments would improve the translatability of results and better inform subsequent human trials (214).

Traumatic insults resulting in tissue damage and uncontrolled hemorrhage occur simultaneously in the human trauma patient. To control the degree of tissue trauma and shock the majority of animal models apply standardized insults in a staggered fashion and utilize either fixed pressure or fixed volume bleeds via an indwelling vascular catheter. The influence of this non-pathophysiologic sequence of insults on overall response is unknown. Fixed pressure hemorrhage controls the degree of hypotension via repeated blood withdrawal and volume replenishment; however it does not mimic the clinical situation and introduces the potential confounders of iatrogenic hemodilution and anti-coagulation (29, 67). Fixed volume hemorrhage allows assessment of the hemodynamic responses to hypotension, although the degree of hypotension is not well defined and variability in the physiologic response is evident (215). Uncontrolled hemorrhage most closely resembles the clinical situation; however it introduces significant physiological variability and risk of animal mortality (189, 216-218). This influences the repeatability of the model and compromises the ethical acceptability by increasing the number of animals required for statistically significant results to be achieved (219, 220).

Existing animal models of trauma and hemorrhage demonstrate considerable variation in the degree of injury severity and target pressure or volume of hemorrhage (29, 40, 51, 65, 67, 177, 200-203). This contributes to inter-model variations in coagulation function response and limits the reproducibility and comparability of these existing models. To improve future models of traumatic coagulopathy the degree of trauma and hemorrhage needs to be titrated to variables that have been shown to correlate with the onset of ATC. Evidence suggests that an injury severity score (ISS) of 25 combined with a base deficit of 6mmol/L is necessary for ATC to develop (29). While the translatability of ISS across species is uncharacterized, future models should aim to produce a reproducible and survivable tissue injury that correlates with an ISS of 25. The degree of hemorrhage should be titrated to a base deficit of 6mmol/L to ensure the necessary degree of tissue hypoxia is achieved consistently, removing the variability that may accompany a fixed
pressure or volume bleed due to differences in pre-experiment fluid balance and physiological response (215). This should improve the clinical relevance and reproducibility of future animal models, facilitating more efficient inter-species comparison and improving the translatability of pre-clinical research.

The presence of ATC was originally characterized as a 50% prolongation of PT, aPTT or INR (22, 23). Subsequent epidemiological work described an INR >1.2 as the clinically significant threshold associated with an increased risk of adverse outcomes (29). However many of these patients show no clinical evidence of prolonged hemorrhage, with abnormalities in coagulation function only evident in laboratory assays of coagulation function (29). It is possible that in these patients abnormal coagulation function develops as a marker of injury severity, which may explain why ATC as defined can be associated with an increased risk of adverse outcomes in the absence of overt clinical coagulopathic hemorrhage.

The use of viscoelastic assays to assess coagulation function in animal models of trauma is increasing, which parallels the emerging role of viscoelastic assays in the acute trauma setting. The use of traditional plasma based assays of coagulation function in animal models of trauma is appropriate given that ATC continues to be defined clinically in terms of these assays. However the value of continuing to define ATC in terms of traditional assays has been questioned due to their inability to characterize global coagulation function and delayed availability of results (98). Viscoelastic assays provide a more complete assessment of coagulation function via the evaluation of clot formation, clot strength and fibrinolytic activity and have been validated in a number of animal species (221, 222). However they neglect the contribution of the endothelium, which may continue to be a limitation given the postulated role of the endothelium in the development of ATC. Attempts to define ATC using viscoelastic parameters have been made, as trauma patients with an INR >1.2 show changes in clot formation time, clot strength and fibrinolytic activity (30, 32). Various thresholds have been proposed, however there is no consensus on the viscoelastic definition that should be used (30, 32, 33, 95). Further work is required to ascertain the viscoelastic triggers that identify ATC, and relevant animal models will play an important role in this process.
Conclusion

Traditional animal models of traumatic coagulopathy were inspired by a perceived iatrogenic process and investigated hemodilution, hypothermia and acidosis as the pathophysiological initiators of coagulation dysfunction. The recognition of ATC as an endogenous response to tissue injury and hypoperfusion has altered the perceived etiology and chronology of traumatic coagulopathy. In order to be clinically relevant new animal models should reflect the current natural history of traumatic coagulopathy and utilize a variety of animal species to account for inter-species variation in coagulation function.

Creating a clinically relevant model of trauma and hemorrhage that allows translation of results to humans is difficult given the complexity of the condition and known limitations of animal research. It is likely that no single animal model will answer all questions and utilizing the appropriate model for the question at hand will continue to be a challenge. However preclinical animal studies are necessary to continue improving the management of trauma patients given the limitations of human research in the emergency setting. The development of a valid, reproducible and clinically relevant animal model of trauma is needed to contribute to improved mechanistic understanding of the pathophysiology of ATC and allow future evaluation of novel therapeutic agents in whole biological systems.
Figure 1. PRISMA flow diagram for experimental animal models of traumatic coagulopathy


For more information, visit www.prisma-statement.org.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year of Publication</th>
<th>Animal species</th>
<th>Number of animals per study arm</th>
<th>Method used to induce coagulopathy</th>
<th>Principal coagulation findings</th>
<th>Motivation behind study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martini WZ et al (195)</td>
<td>2005</td>
<td>Pig</td>
<td>6</td>
<td>Combined acidosis and hypothermia</td>
<td>Increased splenic bleeding time Reduced platelet count Increased PT and aPTT Increased R value on TEG.</td>
<td>Pathophysiology of combined hypothermia and acidosis.</td>
</tr>
<tr>
<td>Klemcke HG et al (187)</td>
<td>2005</td>
<td>Pig</td>
<td>18</td>
<td>Combined hypothermia and hemodilution</td>
<td>Increased PT and aPTT Decreased TEG α angle and maximum amplitude Decreased fibrinogen.</td>
<td>Assess effect of recombinant FVIIa</td>
</tr>
<tr>
<td>Fries D et al (180)</td>
<td>2005</td>
<td>Pig</td>
<td>14</td>
<td>Hemodilution followed by liver injury</td>
<td>Decreased fibrinogen and platelet count Increased PT and aPTT</td>
<td>Assess effects of fibrinogen concentrate</td>
</tr>
<tr>
<td>Martini WZ et al (179)</td>
<td>2006</td>
<td>Pig</td>
<td>6</td>
<td>Hemodilution</td>
<td>Decreased fibrinogen concentration Reduced clotting time and clot strength on TEG.</td>
<td>Pathophysiology of hemodilution</td>
</tr>
<tr>
<td>Kiraly LN et al (216)</td>
<td>2006</td>
<td>Pig</td>
<td>15</td>
<td>Grade V liver injury and fluid resuscitation</td>
<td>Increased PT and aPTT Increased alpha angle and clotting index on TEG</td>
<td>Efficacy of Hartman’s vs 0.9% saline resuscitation</td>
</tr>
<tr>
<td>Martini WZ et al (193)</td>
<td>2006</td>
<td>Pig</td>
<td>7</td>
<td>Isolated acidosis induced by hydrochloric acid infusion</td>
<td>Reduced fibrinogen and platelet count Increased PT, aPTT and ACT Decreased TEG alpha angle and maximum amplitude</td>
<td>Pathophysiology of isolated acidosis</td>
</tr>
<tr>
<td>Martini WZ et al (188)</td>
<td>2007</td>
<td>Pig</td>
<td>6</td>
<td>Isolated hypothermia (&lt;33°C)</td>
<td>Increased TEG clotting time and α angle Decreased fibrinogen concentration</td>
<td>Pathophysiology of isolated hypothermia</td>
</tr>
<tr>
<td>Kheirabadi B et al (186)</td>
<td>2007</td>
<td>Rabbit</td>
<td>8</td>
<td>Combined hypothermia and hemodilution</td>
<td>Increased PT and aPTT Decreased fibrinogen concentration Decreased TEG alpha angle and amplitude</td>
<td>Evaluate ability of blood tests to detect coagulopathy</td>
</tr>
<tr>
<td>Chesebro BB et al (40)</td>
<td>2009</td>
<td>Mice</td>
<td>10</td>
<td>Combined laparotomy and fixed pressure hemorrhage</td>
<td>Increase in aPTT, attenuated by monoclonal antibodies to activated protein C</td>
<td>Contribution of protein C to coagulopathy</td>
</tr>
<tr>
<td>Cho SD et al (201)</td>
<td>2009</td>
<td>Pig</td>
<td>37</td>
<td>Combined femoral fracture and fixed volume haemorrhage, hemodilution, induced hypothermia and induced acidosis</td>
<td>Increased liver bleeding time Prolonged PT/aPTT following hemodilution Reduction in maximum amplitude of clot strength on TEG</td>
<td>Reproducibility of a multi combat model between institutions</td>
</tr>
<tr>
<td>Pragst I et al (181)</td>
<td>2010</td>
<td>Rabbit</td>
<td>17</td>
<td>Hemodilution</td>
<td>Increased kidney bleeding time Increased PT time</td>
<td>Efficacy of prothrombin complex concentrate</td>
</tr>
<tr>
<td>Frith D et al (29)</td>
<td>2010</td>
<td>Rat</td>
<td>10</td>
<td>Combined laparotomy, femoral fractures and fixed pressure haemorrhage</td>
<td>Increased PT and aPTT</td>
<td>Evaluate pathophysiology and definition of ATC</td>
</tr>
<tr>
<td>Study Authors and Year</td>
<td>Species</td>
<td>Animals</td>
<td>Intervention</td>
<td>Outcomes</td>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
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</tr>
<tr>
<td>Iwamoto S et al (190)</td>
<td>2010</td>
<td>Rat</td>
<td>12</td>
<td>Hypothermia and hemodilution</td>
<td>Increased ACT</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>White NJ et al (200)</td>
<td>2010</td>
<td>Pig</td>
<td>18</td>
<td>Combined unilateral femoral fracture and fixed pressure hemorrhage</td>
<td>Decreased fibrinogen No significant change in PT or aPTT</td>
<td>Pathophysiology of combined trauma and hemorrhage</td>
</tr>
<tr>
<td>Letson HL et al (196)</td>
<td>2012</td>
<td>Rat</td>
<td>10</td>
<td>Isolated fixed pressure hemorrhage</td>
<td>Increased aPTT</td>
<td>Efficacy of 7.5% Na Adenocaine and Mg^{2+}</td>
</tr>
<tr>
<td>Mulier KE et al (202)</td>
<td>2012</td>
<td>Pig</td>
<td>8</td>
<td>Combined lung contusion, liver fractures and fixed pressure haemorrhage</td>
<td>Reduced platelet count Increased rate of clot formation and reduced clot stiffness on TEG</td>
<td>Pathophysiology of multi-trauma and haemorrhage</td>
</tr>
<tr>
<td>Doran CM et al (51)</td>
<td>2012</td>
<td>Pig</td>
<td>6</td>
<td>Combined blast injury, fixed volume haemorrhage and hemodilution</td>
<td>Increased PT</td>
<td>Evaluate the effect of targeted resuscitation</td>
</tr>
<tr>
<td>Lesperance RN et al (192)</td>
<td>2012</td>
<td>Pig</td>
<td>28</td>
<td>Fixed volume haemorrhage and ischemia-reperfusion injury</td>
<td>Increased INR Increased CT, CFT, and reduced α angle and MCF on ROTEM</td>
<td>Assess effects of lactic acidosis on coagulopathy.</td>
</tr>
<tr>
<td>Fung YL et al (197)</td>
<td>2013</td>
<td>Sheep</td>
<td>5</td>
<td>Isolated fixed volume haemorrhage</td>
<td>No significant changes in PT or aPTT or any ROTEM parameters</td>
<td>Evaluate the effects of stored blood transfusion</td>
</tr>
<tr>
<td>Park KH et al (189)</td>
<td>2013</td>
<td>Rat</td>
<td>8</td>
<td>Hypothermia and uncontrolled hemorrhage from splenic laceration</td>
<td>Increased CT, CFT and reduced α angle and MCF on ROTEM</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Darlington DN et al (65)</td>
<td>2013</td>
<td>Rat</td>
<td>7</td>
<td>Combined femoral fracture, soft tissue trauma and fixed pressure hemorrhage.</td>
<td>Increased PT and aPTT Decreased MCF on ROTEM</td>
<td>Pathophysiology.</td>
</tr>
<tr>
<td>Nishi K et al (218)</td>
<td>2013</td>
<td>Rat</td>
<td>6</td>
<td>Uncontrolled hemorrhage following tail amputation</td>
<td>Prolonged ACT and clot rate following fluid resuscitation</td>
<td>Effects of fluid resuscitation.</td>
</tr>
<tr>
<td>Mohr J et al (177)</td>
<td>2013</td>
<td>Pig</td>
<td>10</td>
<td>Blunt chest trauma, liver laceration, fixed pressure hemorrhage and hypothermia</td>
<td>Prolonged PT and decreased Fibrinogen Increased CT, CFT and reduced MCF on ROTEM</td>
<td>Evaluate coagulation function in a multi-trauma model.</td>
</tr>
<tr>
<td>Hayakawa M et al (199)</td>
<td>2013</td>
<td>Rat</td>
<td>6</td>
<td>Administration of tissue factor</td>
<td>Reduced platelet count and fibrinogen Increased PT and D-dimer Reduced anti-thrombin levels.</td>
<td>Effects of tissue factor on coagulation and fibrinolysis.</td>
</tr>
<tr>
<td>Hagemo JS et al (203)</td>
<td>2013</td>
<td>Pig</td>
<td>8, 4, 2</td>
<td>Bilateral femoral fractures and soft tissue injury, fixed volume hemorrhage</td>
<td>Decreased fibrinogen and INR Increased CT on ROTEM</td>
<td>Characterize fibrinogen changes with trauma and hemorrhage.</td>
</tr>
<tr>
<td>Martini J et al (183)</td>
<td>2013</td>
<td>Hamster</td>
<td>5</td>
<td>Hemodilution</td>
<td>Decreased MCF and α angle, increased CFT on ROTEM</td>
<td>Evaluate effects of fibrinogen on clotting.</td>
</tr>
<tr>
<td>Duan K et al (67)</td>
<td>2014</td>
<td>Pig</td>
<td>16</td>
<td>Right femoral fracture, small intestinal crush and grade III liver injury, fixed pressure haemorrhage</td>
<td>Increased PT and INR Increased R value on TEG.</td>
<td>Characterize changes in clotting function following trauma.</td>
</tr>
</tbody>
</table>

PT=prothrombin time, aPTT=activated partial thromboplastin time, INR=International normalized ratio, TEG=Thromboelastography, ROTEM=thromboelastometry, PTr=prothrombin ratio, aPTr=activated partial thromboplastin ratio, CT=clotting time, MCF=mean clot firmness, CFT=clot formation time, ACT=activated clotting time
3.3 Discussion of this review article

This systematic review identified 27 distinct animal models developed after 2003 to investigate coagulation dysfunction in trauma. Of these 27 models, 17 still utilised haemodilution, acidosis or hypothermia either alone or in combination as the initiating mechanism for coagulopathy, prior to the creation of tissue injury. This has provided information about the haemostatic effects of these separate insults in complex biological systems. However given our current understanding of the natural history of ATC the ongoing value of models devoid of initiating tissue injury and haemorrhagic shock is limited.

Ten distinct animal models that investigated the combined effect of tissue injury and haemorrhagic shock on coagulation function were identified by this review. Three of these were rodent models, all of which achieved current clinical definitions of ATC using traditional assays of coagulation function (29, 40, 65). Elements of pathophysiology were examined in one of these rodent models, with coagulopathy prevented by the administration of aPC antibodies (40). However further evaluation of alternative pathophysiological mechanisms in these models was restricted by animal size, which limited sample collection and the number of assays that could be performed. The remaining 7 models were porcine models, with considerable inter-model variability in the coagulation function response evident (51, 67, 103, 177, 200-202). No significant change in coagulation function was observed in 6 of these models (51, 103, 177, 200-202). A statistically insignificant reduction in mean clot firmness (MCF), increase in PT and alteration in platelet function was observed in 2 models; however clinical definitions of ATC were not met until after fluid resuscitation had commenced (103, 177). The remaining model is the only porcine model to have achieved clinical definitions of ATC in response to trauma and haemorrhage alone, demonstrating a 20% increase in INR/PT (67). However this model was associated with a 25% mortality rate in the trauma group, creating doubt about the repeatability and ethical acceptability of this model.

This review also highlighted a lack of consensus as to the animal species that may best simulate the human condition. Animals are genetically distinct from humans and demonstrate fundamental differences in coagulation assay parameters, coagulation factor levels and platelet function (58-62). These differences may alter the susceptibility to ATC and influence the translatability of the chosen experimental model. Refinement of future
animal models of ATC requires careful consideration of the chosen animal species. In order to be clinically relevant the chosen animal species must demonstrate more similarities than differences with human cardiovascular physiology and coagulation function.

The reporting of coagulation function testing was mandated as an inclusion criterion for this systematic review, as the overarching aim was to inform the development of an alternative model of traumatic coagulopathy. However the concept of traumatic shock is not limited to coagulation dysfunction, as only a proportion of trauma patients demonstrate impaired coagulation function in response to trauma (22, 29). More broadly traumatic shock encompasses the local and systemic alterations that occur in response to tissue injury, haemorrhage, organ ischaemia, tissue reperfusion and resuscitation (223). It is associated with a post traumatic immune response characterised by an increase in inflammatory mediators and influx of inflammatory cells that predisposes to coagulopathy, sepsis and post-injury multi-organ failure (215, 223). The exclusion of animal models of trauma that failed to describe coagulation function testing is a limitation of this review, as it restricted the ability to fully evaluate additional information regarding traumatic shock that could be obtained from these other models.

3.3.1 The need for an alternative large animal model
Large animal models of coagulopathy are preferable as they facilitate sample collection and allow the application of standard human monitoring techniques (224). Rodent models are more affordable; however the ability to fully investigate pathophysiological mechanisms of coagulopathy in vivo is hampered by animal size. Rodents also demonstrate significant differences in coagulation function and the genomic response to trauma when compared to humans, further limiting the clinical relevance of rodent trauma models (58, 213).

As noted in this review, pigs are the only large animal species to have been used to investigate the coagulation function response to trauma and haemorrhage. This may appear appropriate given the number of physiological similarities pigs share with humans (66). However pigs demonstrate significant differences in coagulation function, which may impact the ability to successfully model ATC in this species. Pigs are hypercoaguable compared to humans on both traditional plasma based assays and viscoelastic assays of coagulation function, and have significantly lower levels of protein C (58, 60). This may
explain why pig models fail to demonstrate the degree of ATC seen in rodents despite the use of similar protocols. The use of an alternative large animal species may therefore improve understanding of coagulopathy in trauma and better inform subsequent human studies.

Sheep may represent the ideal candidate animal, as they share many similarities in haemodynamic, microcirculatory and immunologic function with humans (75-77). Sheep have been widely used in biomedical research to model other human pathologies including asthma (68, 69), myocardial reperfusion (73), burn injury (225), haemophilia (226), osteoporosis (70), sepsis (71, 74, 227) and various presentations of acute lung injury (ALI) (72, 77, 228). Comparative studies of human, porcine, rodent, canine and ovine coagulation function using routine coagulation tests, ROTEM and clotting factor assays also indicate that the human coagulation system demonstrates the greatest similarity with that of sheep (58, 59).

3.3.2 Animal model design
Considerable variability in the type of injury and method of haemorrhage was evident in the models of trauma and haemorrhage identified by this review. Hind-limb fractures formed the basis of tissue injury in all models, combined with varying combinations of soft tissue contusions (103, 200) blast injuries (51), intestinal crush injuries (65, 67), laparotomy incisions (29, 40) and liver injuries (67, 177, 202) to increase the degree of tissue trauma. Fixed pressure haemorrhage was utilised in the majority of models (29, 40, 65, 67, 177, 200, 202), although there was no apparent agreement with regards to the desired target pressure. This lack of consensus in the type of injury and degree of haemorrhage has contributed to inter-model variations in haemodynamic and coagulation function response, limiting the reproducibility and clinical relevance of these existing models. Targeting the severity of injury and type of haemorrhage to variables that correlate with the development of ATC may facilitate the development of a more clinically relevant, reproducible and ethically acceptable model.

3.3.2.1 Severity of injury
Retrospective human studies indicate that an injury severity score (ISS) of more than 15 is a necessary condition for the development ATC, with the majority of cases associated with an ISS > 25.(29, 39, 92, 229). The ISS was first introduced in 1974 and continues to be one of the most widely used measures of injury severity (230). It is based upon an
abbreviated injury score (AIS) which is an anatomic scoring system used to describe the site of injury, force applied and extent of damage (231). The ISS was introduced to take into account the contribution of second and subsequent injuries to morbidity and mortality and is calculated by assigning injuries to one of six body regions (230). The severity of each injury is then ranked on a scale of 1 (minor) to 5 (critical) (231). The three highest scores are then squared and added together to produce the ISS (table 3).

The translatability of the ISS across species is yet to be characterised. However creating a survivable and reproducible tissue injury that correlates with an ISS of 25 may improve the clinical relevance of future models. Hind limb fractures would appear to be an appropriate basis for the tissue injury, however are insufficient in isolation as they only result in an ISS of 9 (table 3). Additional injuries are required to meet the proposed ISS, and the chosen injury needs to minimise potential confounders of uncontrolled haemorrhage, endotoxaemia and tissue ischaemia. Pulmonary contusions have been utilised in an ovine model investigating the effects of fat emboli and acute respiratory distress syndrome (ARDS) following femoral fracture, and have been associated with a low mortality rate (232). As pulmonary contusions make a significant contribution to ISS, the use of pulmonary contusions in conjunction with hind limb fractures may facilitate the creation of a reproducible and survivable tissue injury that has an ISS of 25 (table 3).

### Table 3. Example calculation of an ISS

<table>
<thead>
<tr>
<th>Region</th>
<th>Injury Description</th>
<th>AIS</th>
<th>Highest AIS²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and Neck</td>
<td>No injury</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Face</td>
<td>No injury</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chest</td>
<td>Multiple lung contusions</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Chest wall laceration</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Abdomen</td>
<td>No injury</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Extremity</td>
<td>Displaced compound tibial fracture (upper third)</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>External</td>
<td>No injury</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injury Severity Score</td>
<td></td>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>
3.3.2.2 Type of haemorrhage

Retrospective human studies indicate that in the presence of severe tissue injury a base deficit of 6mmol/L is associated with the onset of ATC (29). This is thought to reflect systemic hypoperfusion developing secondary to haemorrhagic shock. In the human trauma patient haemorrhagic shock develops in response to uncontrolled haemorrhage, thus the use of uncontrolled haemorrhage in experimental models would most closely replicate the clinical situation. However uncontrolled haemorrhage introduces significant physiological variability, compromising the reproducibility of the model (189, 218). It also increases the risk of intra-experimental animal mortality, which would in turn impact on the ethical acceptability of the model.

Two options for the creation of controlled haemorrhagic shock were identified by this review: fixed volume or fixed pressure haemorrhage. Fixed pressure haemorrhage is more commonly utilised by existing trauma and haemorrhage models, as it allows the degree and duration of hypotension to be controlled (224). However in order to maintain the mean arterial pressure at the target level, repeated blood withdrawal and volume replacement with crystalloid solutions or anticoagulated blood is required (29, 65, 67, 200). This increases the likelihood of iatrogenic haemodilution or anti-coagulation, and is not reflective of the clinical situation in the human trauma patient. Fixed volume haemorrhage offers an alternative, as it allows the degree of haemorrhagic shock to be controlled whilst facilitating assessment of the haemodynamic responses to hypovolaemia (224). This is more reflective of the clinical situation seen with uncontrolled haemorrhage; however it does increase the potential for physiological variability.

3.3.3 Summary

An alternative large animal model of coagulopathy is desirable. Pigs are the only large animal species that have been used to evaluate the haemostatic response to trauma and haemorrhage. However recognised differences in coagulation function compared to humans appear to be limiting the ability to successfully model ATC in this species. Sheep demonstrate similarities in haemostatic, haemodynamic and microcirculatory function with humans, suggesting they may be the ideal candidate animal in which to develop an alternative large animal model of ATC. Combining fixed pressure haemorrhage with a reproducible tissue injury that equates to an ISS of 25 may facilitate the development of a more clinically relevant animal model of ATC, which may improve understanding of the condition and facilitate future evaluation of alternative therapeutic strategies.
CHAPTER 4: ACTIVATION OF THE PROTEIN C PATHWAY AND ENDOTHELIAL GLYCOCALYX SHEDDING IS ASSOCIATED WITH COAGULOPATHY IN AN OVINE MODEL OF TRAUMA AND HAEMORRHAGE.


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4.1 An introduction to this peer reviewed publication

This manuscript addresses the second and third aims of this thesis: to develop an ovine model of trauma and haemorrhage that demonstrates coagulation changes consistent with current definitions of ATC, and to observe the relationship between coagulopathy and the protein C pathway, endothelial glycocalyx, platelet function and fibrinolysis within this model.

The information obtained from the systematic review discussed in Chapter 3 facilitated the design of the large animal model utilised in this manuscript. A need for an alternative large animal model was identified, with sheep chosen for their similarities with humans with regards to size, cardiovascular physiology and coagulation function, and the experience of the research group with ovine models of critical illness. Controlled haemorrhagic shock was created using fixed volume haemorrhage to enable assessment of the physiologic responses to hypovolaemia. Bilateral tibial fractures were combined with pulmonary contusions to produce a repeatable tissue injury that equated to an ISS of 25. A graded degree of injury severity was also assessed in this thesis through the inclusion of two different injury groups. The moderate injury group underwent bilateral tibial fractures, single left upper and left lower lung lobe contusions and haemorrhage of 20% blood volume. The severe injury group underwent bilateral tibial fractures, bilateral hamstring crush injuries, two left upper and left lower lung lobe contusions and haemorrhage of 30% blood volume. Grading the degree of trauma in this way allowed the effects on base deficit
and coagulation function to be assessed in a step wise manner in the hope that a more reproducible, clinically relevant and ethically acceptable animal model would result.

The development of coagulopathy in this model was assessed using both traditional and viscoelastic assays of coagulation function, and the coagulation changes displayed by the animals are outlined in this manuscript. It was considered necessary to demonstrate coagulopathy consistent with INR (29), aPTT (23) and ROTEM (30) based definitions in order to validate this model as clinically relevant. This was achieved, with the severe trauma group of animals developing changes in INR, aPTT and EXTEM A10 that were comparable with current clinical definitions of ATC.

The proposed pathophysiological contributors to ATC were then evaluated in this model. Changes in the protein C pathway were assessed by evaluating changes in sTM, protein C, aPC, factor V, factor VIII and PAI-1. A significant association between the protein C pathway and coagulopathy was evident, with coagulopathy correlated with an increase in sTM and aPC and a reduction in protein C, factor VIII and PAI-1. The contribution of the endothelial glycocalyx to ATC was assessed by evaluating changes in syndecan-1 and hyaluronan, which have been utilised as markers of endothelial glycocalyx damage in human studies. Coagulopathy was correlated with an increase in both syndecan-1 and hyaluronan in this model, further supporting the hypothesised contribution of endothelial glycocalyx shedding to ATC. Fibrinolysis was assessed in this model by evaluating changes in D-dimer levels, FIBTEM parameters. Platelet function was evaluated using impedance aggregometry (Multiplate analyser) with collagenase and ADP agonists. There was no evidence of significant fibrinolysis or altered platelet function to suggest a contribution to coagulopathy in this model.
Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and hemorrhage.

Natasha van Zyl BVSc (Hons) MBBS  1,2, Elissa M Milford MBBS  2,3, Sara Diab BN  1, Kimble Dunster BSc (Hons)  1, Peter McGiffin BSc  1, Stephen G Rayner BVSc (Hons)  4, Andrew Staib MBBS FACEM  2,5, Michael C Reade DPhil FCICM  2,3,6, John F Fraser PhD FCICM  1,2

1. Critical Care Research Group, The Prince Charles Hospital, Brisbane QLD Australia
2. The University of Queensland, School of Medicine, Herston QLD Australia
3. Australian Defence Force, Canberra, Australia
4. Darling Downs Vets, Westbrook QLD Australia
5. The Princess Alexandra Hospital, Woolloongabba QLD Australia
6. Burns, Trauma and Critical Care Research Centre, The University of Queensland, Brisbane QLD Australia

Abstract

Introduction: Acute traumatic coagulopathy (ATC) is an endogenous coagulopathy that develops following tissue injury and shock. The pathogenesis of ATC remains poorly understood, with platelet dysfunction, activation of the protein C pathway and endothelial glycocalyx shedding all hypothesized to contribute to onset. The primary aim of this study was to develop an ovine model of traumatic coagulopathy, with a secondary aim of assessing proposed pathophysiological mechanisms within this model.

Methods: Twelve adult Samm-Border Leicester cross ewes were anesthetized, instrumented and divided into three groups. The moderate trauma group (n=4) underwent 20% blood volume hemorrhage, bilateral tibial fractures and pulmonary contusions. The severe trauma group (n=4) underwent the same injuries, an additional hamstring crush injury and 30% blood volume hemorrhage. The remaining animals (n=4) were uninjured controls. Blood samples were collected at baseline and regularly post injury for evaluation of routine hematology, arterial blood gases, coagulation and platelet function, factor V, factor VIII, plasminogen activator inhibitor-1, syndecan-1 and hyaluraran levels.

Results: At 4 hours post injury a mean increase in international normalised ratio (INR) of 20.50±12.16% was evident in the severe trauma group and 22.50±1.00% in the moderate trauma group. An increase in activated partial thromboplastin time (aPTT) was evident in
both groups, with a mean of 34.25±1.71s evident at 2 hours in the severe trauma animals and 34.75±2.50s evident at 4 hours in the moderate trauma animals. This was accompanied by a reduction in ROTEM EXTEM A10 in the severe trauma group to 40.75±8.42mm at 3 hours post injury. Arterial lactate and indices of coagulation function were significantly correlated (R =-0.86, p<0.0001). Coagulopathy was also correlated with activation of the protein C pathway and endothelial glycocalyx shedding. Whilst a significant reduction in platelet count was evident in the severe trauma group at 30 minutes post injury (p=0.018) there was no evidence of altered platelet function on induced aggregation testing. Significant fibrinolysis was not evident.

**Conclusions:** Animals in the severe trauma group developed coagulation changes consistent with current definitions of ATC. The degree of coagulopathy was correlated with the degree of shock, quantified by arterial lactate. Activation of the protein C pathway and endothelial glycocalyx shedding were correlated with the development of coagulopathy; however altered platelet function was not evident in this model.

**Keywords:** Acute traumatic coagulopathy, ovine, protein C

**Background**

Severe hemorrhage remains the leading cause of preventable death in trauma, responsible for up to 40% of trauma related mortality (3). Efforts to achieve hemostasis are complicated by a trauma induced coagulopathy (TIC), which was traditionally attributed to the loss, dilution and dysfunction of coagulation proteases secondary to hemodilution, hypothermia and acidosis (20). However it is now recognized that a mechanistically distinct acute traumatic coagulopathy (ATC) develops independent of these factors and is present in 24-41% of trauma patients at hospital admission (22, 23).

Multiple definitions of ATC have been proposed; however there remains no consensus regarding the laboratory definition that should be used. ATC was originally characterized as a 50% prolongation in international normalised ratio (INR) or prothrombin time (PT), or an activated partial thromboplastin time (aPTT) > 34s (22, 23). Subsequent epidemiological work has found a 20% prolongation of INR to be clinically significant for mortality and blood product transfusion (29). More recently attempts have been made to define ATC using both thromboelastometry (ROTEM) or thromboelastography (TEG). A clot amplitude < 35mm at 5 minutes (A5), <40mm at 10 minutes (A10), reaction time (R value) >1.1 seconds and K time >2.5 seconds have all been associated with increased
transfusion requirements and mortality in trauma (30-34). However there remains no universally accepted viscoelastic definition of ATC.

A combination of tissue injury and tissue hypoperfusion appears necessary for ATC development (29, 37, 39). However the exact causative mechanisms remain poorly understood. Protein C activation may play a central role, with clinical studies demonstrating an association between protein C reduction, coagulopathy and mortality (37-39). One theory is that pathological activation of protein C results in systemic anticoagulation and hyperfibrinolysis through the inactivation of factors Va, VIIIa and plasminogen activator inhibitor-1 (PAI-1) (37, 38, 40). However the validity of this hypothesis has been questioned by recent in vitro work showing that factor Va pools are resistant to cleavage by activated protein C (aPC) at the concentrations observed in trauma patients (44). It has also been demonstrated that PAI-1 circulates at roughly ten times the concentration of aPC, raising doubts about the ability of aPC to deplete PAI-1 levels to the extent required to accelerate fibrinolysis (41). Consequently it has been suggested that increased tissue plasminogen activator release may drive fibrinolysis, and that ATC should instead be classified as a form of disseminated intravascular coagulation (DIC) with a fibrinolytic phenotype (43, 44). There is also emerging evidence to suggest that platelet dysfunction and shedding of the endothelial glycocalyx may contribute to ATC, although the exact contributions of these factors remain unclear (45-48).

There are several published porcine models that have attempted to characterize changes in coagulation function secondary to trauma and hemorrhage (63). However few have achieved proposed definitions of ATC, and evaluation of pathophysiological mechanisms was limited in most cases (63). Comparative studies of coagulation function have shown pigs to be relatively hypercoaguable compared to humans, which may impede the ability to model ATC in this species (58, 60). The effect of non-traumatic hemorrhage of 30% blood volume on coagulation function has been evaluated in sheep (197); however there are no published ovine models evaluating the combined effects of trauma and hemorrhage. Sheep share a number of similarities in cardiorespiratory and hemostatic function with humans, demonstrating equivalent values for fibrinogen, aPTT, and ROTEM parameters (58, 59, 76). These similarities suggest that they may be a suitable species in which to develop an alternative large animal model of ATC.
This study describes a model development project, with the primary aim of creating an ovine model of trauma and hemorrhage that developed coagulation changes consistent with ATC as defined by aPTT, INR and ROTEM. The secondary aim was to observe associations between coagulopathy, the protein C pathway, fibrinolytic system, endothelial glycocalyx and platelet function within this model.

**Materials and Methods**

This study was approved by the University Animal Research Ethics Committee of both the Queensland University of Technology and University of Queensland. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific purposes. Animals were housed in a dedicated facility with ad libidum access to food and water. Twelve female 2 year old Samm Border-Leicester cross sheep (weight 43.9 ± 1.2kg) were used in the study.

**Instrumentation and Anesthesia**

Prior to surgical intervention all animals underwent a routine health check and were fasted for 12 hours. A triple lumen central line (Arrow, PA, USA) was placed in the right internal jugular vein for drug delivery. Two 8.5 Fr percutaneous sheath introducers (CCombo, Edwards Lifesciences, Singapore) were placed in the left jugular vein for hemorrhage and pulmonary artery catheter placement. Anesthesia was induced with intravenous (IV) buprenorphine (0.01mg/kg), midazolam (0.5mg/kg) and alfaxalone (3mg/kg) and maintained with continuous rate infusions of alfaxalone (6mg/kg/hr), midazolam (0.25mg/kg/hr), fentanyl (15µg/kg/hr) and ketamine (10mg/kg/hr) with rates titrated to maintain surgical anesthesia. Animals were intubated and ventilated with a Galileo ventilator set to 12 breaths/minute, a tidal volume of 10ml/kg and 21% FiO$_2$ (233). Electrocardiogram and pulse oximetry monitoring was commenced. A 20G cannula was placed in the left facial artery (Leadercath, Vygon, UK) for arterial blood pressure monitoring and sampling. A pulmonary artery catheter was placed via the proximal left jugular sheath for monitoring of continuous cardiac output (CCO), mixed venous oxygen saturation (SvO$_2$) and temperature via an Edwards Vigilance Monitor (Edwards Lifesciences, CA, USA), with normothermia maintained throughout. All animals received a 500ml bolus of lactated Ringer’s solution followed by a 3ml/kg/hr infusion during the instrumentation period to replace estimated third space and salivary losses (234). Splenic ligation was performed to attenuate physiological differences in the red cell storage capacity of the ovine spleen. Splenic contraction in humans has minimal effect on
circulating red cell mass; however splenic contraction in response to physiological stress in sheep can increase circulating red cell mass by up to 26% (235, 236). Ligation was performed via a paracostal approach with intrasplenic administration of 0.2ml of 1:10,000 epinephrine prior to ligature placement to promote splenic contraction (237), maximizing the circulating red cell mass prior to hemorrhage. Bilateral femoral nerve blocks were performed using 20ml of 0.25% bupivacaine for regional anesthesia of the hind limbs.

**Experimental interventions.**

At the completion of instrumentation sheep were allocated to one of three groups: uninjured controls (n=4), moderate trauma (n=4) or severe trauma (n=4). Sample size was determined using a longitudinal power calculation, giving 80% power to detect a difference in EXTEM A10 of ≥5mm per hour between the groups. This calculation was based upon EXTEM A10 as ovine reference ranges and variability have been established, and it has been shown to be a sensitive predictor of coagulopathy in trauma (30, 59). Crystalloid administration was reduced to 10ml/hr and the animals underwent an injury phase. Standardized 2cm x 2cm left lung lobe contusions were created through a left thoracotomy using a custom made pneumatic device calibrated to 15 psi (232). The moderate trauma sheep received single upper and lower lobe contusions, whilst the severe trauma sheep received two upper and lower lobe contusions. A 20 Fr Argyle chest drain was inserted into the pleural space and the thoracotomy closed. A custom built guillotine device weighted to 8.2kg and released from a height of 1.2 meters was used to create bilateral tibial fractures (238). A standardized soft tissue injury was created in the hamstring region of the severe trauma sheep using a custom made pneumatic vice calibrated to 120psi. Controlled hemorrhage was performed over a 10 minute period via gravity assisted drainage through the distal sheath in the left jugular vein to a target of 20% blood volume (moderate trauma sheep) or 30% blood volume (severe trauma sheep), with blood volume calculated as 65ml/kg (239). Following completion of the injury phase all groups were monitored for 6 hours with no further intervention and then euthanized with 10ml pentobarbitone sodium (325mg/ml).

**Sample collection and storage**

Blood samples were collected into EDTA, 3.2% sodium citrate, lithium heparin and hirudin blood tubes at baseline, 30 minutes post injury, then hourly post injury until completion of the 6 hour monitoring period. Samples were separated and stored at -80°C pending analysis. ROTEM, platelet function and hematology were performed on whole blood within
30 minutes of collection. Post mortem tissue samples were collected from the lungs, myocardium, left kidney and liver and fixed in 10% formalin. All assays were performed according to manufacturer’s instructions.

**Coagulation function and hematology analysis**

Citrated plasma was analyzed for PT, aPTT, D-dimer levels and fibrinogen (Clauss) on the ACL TOP analyzer (Werfen, Sydney, Australia). Platelet function was assessed on the Multiplate analyzer (Haemoview Diagnostics, Brisbane, Australia) using collagen and adenosine diphosphate (ADP) agonists, as trauma patients demonstrate changes in platelet aggregometry with these agonists, ovine reference ranges for these agonists exist, and ovine platelets have proven unresponsive to TRAP-6, arachidonic acid and risocetin agonists (48, 59). Thromboelastometry (EXTEM, INTEM and FIBTEM assays) was performed on the ROTEM (Haemoview Diagnostics). Full blood evaluation was performed using the veterinary mode of the Act Diff hematology analyzer (Beckman Coulter Australia Pty Ltd).

**Blood component analysis**

Quantitative measurements of factor V (FV), factor VIII (FVIII), protein C and PAI-1 were performed on citrated plasma using the ACL TOP analyzer; with protein C values quantified using a chromogenic assay. Soluble thrombomodulin (sTM) and aPC levels were measured in duplicate on EDTA plasma using commercially available immunoassays (Sheep soluble thrombomodulin ELISA kit, Mybiosource, San Diego USA; Sheep activated protein C ELISA kit, BlueGene lifesciences, Shanghai, China).

**Metabolic analysis**

Arterial blood samples were analyzed for pH, lactate (mmol/L), base excess/deficit (mmol/L) and bicarbonate (mmol/L) using an automated blood gas analyzer (ABL System 625, Radiometer, Denmark). Plasma catecholamine levels were measured using a Waters 2695 Separation Module (Waters Corporation, NSW, Australia).

**Endothelial glycocalyx evaluation**

Endothelial glycocalyx breakdown products were measured in duplicate using commercially available immunoassays in serum (Sheep syndecan-1/CD138 ELISA kit, Mybiosource, San Diego USA; Hyaluronan ELISA, Echelon Biosciences, Salt Lake City, USA)
**Histology**

Formalin fixed tissue samples underwent routine processing and paraffin embedding. 4µm sections were cut and stained with hematoxylin and eosin. Slides were analyzed for fat emboli and histological evidence of DIC (240).

**Statistical analysis**

Normally-distributed continuous data measurements were described using the mean and standard deviation. The mean values were grouped by time points and compared for significant differences over time within the experimental groups using a two-way repeated measures analysis of variance (ANOVA). Tukey Kramer significance adjustment was used to compare significant differences between groups. The relationship between data sets was evaluated using a two tailed Pearson correlation. Significant differences were defined as a two tailed p value <0.05. All statistical analyses were performed using GraphPad PRISM 6 for Windows (GraphPad Software, San Diego, USA).

**Results**

**Hemodynamic changes**

Changes in hemodynamic parameters are depicted in figure 1. At baseline all variables were comparable between the three groups. Immediately following trauma and hemorrhage MAP decreased significantly from baseline in both the moderate trauma (116.5±4.04 to 41.25±8.60mmHg, p<0.001) and severe trauma (113±7.20 to 30.50±4.04mmHg, p<0.001) groups, remaining significantly lower than the control group mean throughout the monitoring period. The mean CCO decreased from baseline immediately following injury in both the moderate trauma (4.5±0.58 to 3.5±0.48L/min, p<0.01) and severe trauma (4.25±0.96 to 3.25±0.34L/min, p<0.001) groups, returning to baseline levels by 4 hours. A significant rise in heart rate was evident from 1.5 hours post injury in both the moderate trauma (82.5±7.6 to 113.5±13.0 bpm, p=0.035) and severe trauma (79.0±2.6 to 116.8±19.4 bpm, p=0.033) groups.

**Hematology and metabolic function**

Hematology and metabolic parameters are summarized in table 1. All groups demonstrated an initial rise in mean hemoglobin secondary to induced splenic contraction. Following hemorrhage a significant reduction in mean hemoglobin was evident in both trauma groups compared to the control group mean (p<0.001). The platelet count in the
severe trauma group was lower than control from 30 minutes post injury (\( p=0.018 \)), however there were no significant differences in ADP or collagen-induced platelet aggregation between the groups. Lactate rose significantly at 1 hour post injury in the severe trauma group (\( p=0.017 \)) and at 4 hours post injury in the moderate trauma group (\( p=0.011 \)). This was accompanied by a significant reduction in base excess in the severe trauma animals from 1-3 hours post injury, however was not associated with the development of a base deficit or acidosis. The high bicarbonate levels in all animals suggest this may have been due to the development of a co-existing metabolic alkalosis of unknown cause.

**Coagulation function.**
Changes in coagulation function are summarized in table 2. Significant prolongation of aPTT was evident from 30 minutes post injury in the severe trauma group (\( p=0.002 \)) and 1 hour post injury in the moderate trauma group (\( p=0.011 \)). A significant elevation in INR was evident in both trauma groups at 5 hours post injury, with a mean increase from baseline of 20.50±12.16% in the severe trauma group and 22.50±1.00% in the moderate trauma group at 4 hours post injury (figure 2). Fibrinogen decreased significantly in the severe trauma group compared to controls at 4 hours post injury (\( p=0.037 \)). EXTEM A10 was significantly reduced in the severe trauma group from 3 hours post injury (figure 2). The severe trauma group also demonstrated a significant reduction in INTEM A10 (\( p=0.01 \)) and INTEM \( \alpha \) angle (\( p=0.015 \)) compared to controls at 5 hours post injury. FIBTEM measurements were not significantly different between groups.

**Coagulation component and endothelial glycocalyx analysis**
Changes in coagulation proteases and endothelial glycocalyx markers are summarized in table 3. FVIII levels were reduced in the severe trauma group compared to controls from 30 minutes post injury (\( p=0.002 \)). The severe trauma group also demonstrated a significant reduction in FV levels from baseline over time (\( p<0.0001 \)), however when compared to the control group a statistically significant difference was not evident. Protein C levels decreased significantly in the severe trauma group compared to control from 1 hour post injury (\( p=0.06 \)), with a significant elevation in aPC levels evident from 3 hours. sTM levels were increased in the severe trauma group from 1 hour post injury (\( p<0.0001 \)) and PAI-1 levels were reduced from 30 minutes post injury (\( p=0.006 \)). No significant change in D-dimer levels was evident. Syndecan-1 levels were significantly elevated in the severe trauma group compared to the control group from 1 hour post injury (\( p=0.018 \)),
and hyaluronan significantly higher from 3 hours post injury (p=0.018). There was no significant change in plasma catecholamine levels evident.

**Histology**
There was no evidence of fat emboli and no evidence of perivascular hemorrhage, microthrombi, microinfarction, fibrin exudation or hyaline membrane formation in any tissue to suggest DIC.

**Correlations between coagulation function, metabolic function, coagulation protease and endothelial glycocalyx parameters in the severe trauma group.**
Pearson correlation coefficients are outlined in table 4. Coagulopathy as defined by EXTEM A10 and aPTT was strongly correlated with arterial lactate levels (p<0.0001). Correlation between EXTEM A10, fibrinogen concentration and platelet count was evident, with the reduction in fibrinogen and platelet count also correlated with the decrease in INTEM alpha angle. Changes in the protein C pathway were correlated with coagulopathy, with the reduction in protein C, FVIII and FV correlated with both aPTT and EXTEM A10. The rise in sTM and reduction in PAI-1 was also correlated with EXTEM A10, with the rise in aPC correlated with aPTT. Both protein C and aPC were correlated with changes in INTEM alpha angle, FVIII, FV, PAI-1 and sTM. EXTEM A10 was also strongly correlated with the increase in syndecan-1 and hyaluranon. Syndecan-1, hyaluronan and sTM levels were correlated with the rise in arterial lactate concentration.

**Discussion**
This study presents the first ovine model of coagulopathy in response to trauma and hemorrhage, with coagulation changes in the severe trauma group consistent with current definitions of ATC. An increase in aPTT to 34.25 ± 0.85 s was evident at 2 hours post injury, EXTEM A10 had decreased to 40.75 ± 4.21mm at 3 hours post injury and INR had increased by 20.50 ± 6.08% at 4 hours post injury (23, 29, 30). The model was designed to simulate an injury severity score (ISS) of 25 based upon evidence suggesting an ISS of 25 is necessary for ATC to develop, and was combined with fixed volume hemorrhage to allow assessment of the hemodynamic responses to hypovolaemia (29). A graded severity of hemorrhage was used to facilitate development of a survivable insult that achieved the desired end points, with a maximum volume of 30% based upon a previous study of non-traumatic hemorrhage (197). Tissue hypoperfusion was evidenced by the
rise in plasma lactate and was correlated with the onset of coagulopathy, further supporting the importance of tissue hypoperfusion in the development of ATC (29).

Activation of the protein C pathway has been considered instrumental to ATC, with the accompanying protein C depletion postulated to contribute to the increased risk of post injury multi-organ failure and mortality (37, 38). Mechanistic evaluation in a murine model of ATC further supports this theory, with the onset of coagulopathy prevented by the administration of aPC antibodies (40). Protein C is activated by thrombin bound concurrently to thrombomodulin and the endothelial protein C receptor (37). It is hypothesized that hypoperfusion results in increased thrombomodulin release from the endothelium, diverting thrombin away from clot formation towards the activation of protein C (37, 38, 40). aPC then inhibits FV and FVIIIa, with the anti-coagulant effects of aPC mediated primarily through the inactivation of FVIIa (44). In this study the onset of coagulopathy was strongly associated with activation of the protein C pathway. The severe trauma animals showed a significant rise in sTM that was correlated with lactate levels, supporting the role of hypoperfusion in increasing thrombomodulin release. ROTEM alpha angle is reflective of the thrombin burst, with the reduction in INTEM alpha angle in the severe trauma animals consistent with reduced thrombin activity. This was correlated with the rise in sTM and aPC, suggesting diversion of thrombin towards protein C activation. Inhibition of the coagulation cascade was suggested by the strong correlations between changes in aPC, FV and FVIII. The development of global anti-coagulation was supported by the strong correlation between changes in protein C, aPC, FV, FVIII, sTM and coagulopathy as defined by both EXTEM A10 and aPTT. This conflicts with published in vitro findings suggesting that FVa pools are resistant to aPC cleavage at the levels present in trauma patients (44). However correlation is not proof of causation, and given the primary aim of this study was the development of an ovine model of traumatic coagulopathy no mechanistic experiments were undertaken. Future mechanism studies in this model once refined and established are indicated to better evaluate the contribution of the protein C pathway.

A hyperfibrinolytic state has been reported to play an important role in ATC (89, 114). The mechanism behind fibrinolysis is unclear, with PAI-1 inhibition, tissue factor release and t-PA production proposed as triggers (89, 199). In this study inhibition of PAI-1 was suggested by a reduction in PAI-1 levels that was correlated with the increase in aPC. The reduction in PAI-1 was also correlated with the reduction in fibrinogen and EXTEM
A10 values, suggesting loss of fibrinogen may have contributed to coagulopathy in this model. However there was no alteration in D-dimer levels or FIBTEM parameters to suggest the development of overt fibrinolysis, which is consistent with published findings suggesting aPC is unable to inhibit PAI-1 to the extent required to produce clinically relevant fibrinolysis (41). As a gradual decline in fibrinogen levels was also evident in the control animals in this study it is possible that endogenous hemodilution, repeated blood sampling and anesthesia may have contributed to the fibrinogen depletion observed. It has also been proposed that ATC is actually a fibrinolytic form of DIC, as the initial changes are positive on International Society on Thrombosis and Hemostasis (ISTH) DIC scoring systems (129). This appears unlikely given the absence of histological features of DIC in patients with ATC (43, 129), which was further supported by the lack of typical histopathological findings in this study.

The endothelial glycocalyx is a negatively charged anti-adhesive and anti-coagulant surface layer that protects the endothelial cells and maintains vascular barrier function (135). Traumatic injury and shock result in tissue ischemia, activation of the inflammatory system and a catecholamine surge which may lead to endothelial cell activation and glycocalyx degradation (45, 132, 134). Emerging evidence suggests that these factors may play a role in ATC, with endothelial glycocalyx shedding associated with coagulopathy and mortality in trauma patients (45, 46, 132). Degradation of the glycocalyx has been shown to increase local thrombin generation and fibrinolysis, which in the presence of increased sTM from damaged endothelial cells may enhance protein C activation (135). A volume of plasma containing heparin like substances is also held within the glycocalyx, and release of this following degradation may lead to direct anti-coagulant effects from endogenous heparinisation (46). Syndecan-1 and hyaluronan have both been utilized as markers of glycocalyx degradation in human studies (45, 133). In this study both markers were correlated with EXTEM A10, supporting the hypothesized contribution of glycocalyx degradation to coagulopathy. Rodent models of ATC suggest catecholamine excess may underlie glycocalyx degradation, with coagulopathy and glycocalyx shedding prevented by sympathetic blockade (241, 242). However this model failed to demonstrate an association between glycocalyx degradation and catecholamine excess. Given the correlation between lactate, syndecan-1 and hyaluronan in this study it would appear that ischemia secondary to hypoperfusion is the more likely trigger for glycocalyx degradation in trauma.
Dysfunction of platelet activation and adhesion pathways is apparent in severe trauma patients; however the exact contribution to ATC remains unclear (47, 243). The severe trauma animals in this study developed thrombocytopenia post injury, consistent with human findings in ATC (47), which was correlated with reductions in EXTEM A10 and INTEM alpha angle. This suggests thrombocytopenia may have contributed to reduced clot formation and clot strength, although there was no change in induced platelet aggregation parameters to indicate altered function in this model. However it has been demonstrated that the magnitude of ADP and collagen-induced platelet aggregation in sheep is less than half of that in humans, which may have contributed to the lack of significant findings in this model (59).

Any conclusions drawn from this study should be tempered by several inherent limitations. In human casualties tissue damage and hemorrhage occur simultaneously. To facilitate control over the degree of tissue injury and shock this model applied standardized insults in a staggered fashion, and the influence of this non pathophysiologic sequence on overall response is unknown. The ethical necessities of sedation and anesthesia alter the compensatory physiologic responses to injury by reducing sympathetic activation, and have an unknown impact on the overall response (211). Similarities between the ovine and human hemostatic systems have been demonstrated (58, 59); however there are acknowledged differences in primary and secondary hemostasis that may impact the translatability of findings (59). In human patients ATC is evident within 30 minutes of injury (22, 37, 89). In this study the severe trauma animals took 2-4 hours to reach proposed definitions of ATC, and demonstrated variability in metabolic response. Further refinement of the model is indicated to improve reproducibility and clinical relevance. This may be achieved by titrating volume loss to variables shown to correlate with ATC (such as lactate or base deficit) rather than target volume or pressure, ensuring that the required degree of tissue hypoxia is consistently achieved.

**Conclusion**

This study presents an ovine model of coagulopathy in response to poly-trauma and hemorrhage. The model demonstrates changes in INR, aPTT and ROTEM parameters that are consistent with current clinical definitions of ATC. The degree of coagulopathy was closely correlated with the degree of shock as quantified by arterial lactate, supporting the role of hypoperfusion in the development of ATC. The hypothesized role of the aPC pathway and endothelial glycocalyx in ATC was also supported, with coagulopathy
strongly correlated with protein C depletion and markers of endothelial glycocalyx shedding. With further refinement this model may facilitate mechanistic evaluation of hypothesized mechanisms, and enable evaluation of novel therapeutic strategies that may improve outcomes for trauma patients.

Acknowledgements
Funding for this study was provided by the Queensland Emergency Medicine Research Foundation and The Prince Charles Hospital Foundation. Statistical support was provided by Dr Marcella Kwan. John F Fraser was supported by the QHealth research fellowship.

Figure 1. Hemodynamic changes following trauma and hemorrhage. [A] A significant reduction in mean arterial pressure (MAP) from baseline was evident in both trauma groups immediately post injury and maintained throughout the monitoring period. [B] A significant reduction in combined cardiac output (CCO) from baseline was evident in both trauma groups immediately post injury, with a return to baseline levels at 4 hours. [C] A significant elevation in heart rate was evident in both trauma groups from 1.5 hours post injury.
Figure 2. Coagulation changes following trauma and hemorrhage. [A] An increase in INR was evident in the moderate trauma (p=0.002) and severe trauma (p=0.008) groups compared to the control group, with a 20% increase from baseline evident at 4 hours. [B] A reduction in EXTEM A10 was evident in the severe trauma group (p=0.032) from 3 hours post injury.
Table 1. Hematology and metabolic parameters at baseline and time points following trauma and hemorrhage. Values are presented as mean±standard deviation.

<table>
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<th>Variable</th>
<th>Baseline</th>
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<th>30 min</th>
<th>1 hr</th>
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<th>3 hr</th>
<th>4 hr</th>
<th>5 hr</th>
<th>6 hr</th>
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<td>25.8±6.82</td>
<td>25.6±3.70</td>
<td>27.0±1.78</td>
<td>26.4±1.76</td>
<td>28.2±3.84</td>
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</table>

Hb = hemoglobin, Multiplate = induced platelet aggregation, (-) = not assessed, * = significant difference between control and severe trauma groups p<0.05, x = significant difference between control and moderate trauma groups p<0.05
Table 2. Coagulation function at baseline and at time points following trauma and hemorrhage. Values are presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>30 min</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
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<td>32.00±4.97*</td>
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<td>34.75±2.50*</td>
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<td>42.00±8.16*</td>
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<td><strong>A10 (mm)</strong></td>
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</tr>
<tr>
<td>Control</td>
<td>60.75±5.32</td>
<td>54.00±5.56</td>
<td>53.25±6.90</td>
<td>53.75±6.40</td>
<td>53.25±6.02</td>
<td>53.25±6.76</td>
<td>53.50±6.06</td>
<td>53.50±6.06</td>
</tr>
<tr>
<td>Moderate</td>
<td>64.75±3.20</td>
<td>56.00±2.22</td>
<td>56.50±2.38</td>
<td>56.25±1.70</td>
<td>54.00±1.42</td>
<td>54.25±2.62</td>
<td>53.00±0.82</td>
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<tr>
<td>Severe</td>
<td>58.25±5.56</td>
<td>53.00±2.82</td>
<td>50.75±2.50</td>
<td>50.00±4.24</td>
<td>46.75±4.58</td>
<td>44.00±8.52</td>
<td>41.75±10.88$^*$</td>
<td>41.75±10.88$^*$</td>
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<tr>
<td>Control</td>
<td>76.00±1.82</td>
<td>68.25±5.04</td>
<td>71.25±7.22</td>
<td>70.75±2.98</td>
<td>73.25±2.88</td>
<td>71.00±3.74</td>
<td>71.25±3.20</td>
<td>68.00±5.72</td>
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<tr>
<td>Moderate</td>
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<td>71.25±2.98</td>
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<td>Severe</td>
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<td>70.25±2.88</td>
<td>66.25±7.98</td>
<td>62.50±13.53</td>
<td>55.50±19.06$^*$</td>
<td>54.75±19.66$^*$</td>
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<td>$CL_{30}$ (%)</td>
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<td>Moderate</td>
<td>Severe</td>
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<table>
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<tr>
<th>FIBTEM</th>
<th>A10 (mm)</th>
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</thead>
<tbody>
<tr>
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<td>16.50±2.8</td>
<td>13.25±2.76</td>
<td>13.75±2.62</td>
</tr>
<tr>
<td>Moderate</td>
<td>18.00±3.82</td>
<td>13.75±2.22</td>
<td>13.25±1.90</td>
</tr>
<tr>
<td>Severe</td>
<td>21.00±1.42</td>
<td>16.50±2.08</td>
<td>15.50±2.08</td>
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<td>14.50±2.64</td>
<td>14.25±4.28</td>
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<td>12.50±3.70</td>
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<td>11.75±1.26</td>
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<table>
<thead>
<tr>
<th>$\alpha$ angle (°)</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.75±2.06</td>
<td>71.50±6.14</td>
<td>73.25±3.86</td>
</tr>
<tr>
<td>Moderate</td>
<td>78.00±4.54</td>
<td>72.50±4.50</td>
<td>73.00±2.54</td>
</tr>
<tr>
<td>Severe</td>
<td>79.75±0.96</td>
<td>75.75±3.68</td>
<td>74.75±4.72</td>
</tr>
<tr>
<td></td>
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<td>73.00±4.32</td>
<td>69.75±7.64</td>
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<td>69.00±10.24</td>
<td>69.75±7.28</td>
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<td>76.00±1.42</td>
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<td>67.75±6.88</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>65.50±15.02</td>
<td>65.50±15.02</td>
</tr>
</tbody>
</table>

$CL_{30}$ (%)  
Control: 100  
Moderate: 100  
Severe: 100

aPTT = activated partial thromboplastin time, INR = international normalised ratio A10 = clot amplitude at 10 minutes, $CL_{30}$= clot lysis index at 30 minutes, * = significant difference between control and severe trauma groups p<0.05. x = significant difference between control and moderate trauma groups p<0.05
Table 3. Coagulation protease and endothelial glycocalyx markers at baseline and time points following trauma and hemorrhage. Values are presented as mean ± standard deviation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>30 min</th>
<th>1 hr</th>
<th>3 hr</th>
<th>5 hr</th>
</tr>
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<tbody>
<tr>
<td><strong>Factor VIII (U/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.05±1.44</td>
<td>11.2±0.58</td>
<td>10.26±1.22</td>
<td>10.70±2.04</td>
<td>10.17±1.96</td>
</tr>
<tr>
<td>Severe</td>
<td>10.38±0.52</td>
<td>7.92±1.34</td>
<td>7.57±1.24</td>
<td>6.33±0.32</td>
<td>5.46±1.26</td>
</tr>
<tr>
<td><strong>Factor V (U/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.89±0.50</td>
<td>4.56±2.86</td>
<td>3.82±1.34</td>
<td>3.11±0.58</td>
<td>3.34±1.18</td>
</tr>
<tr>
<td>Severe</td>
<td>4.80±2.20</td>
<td>2.37±0.46</td>
<td>2.47±0.58</td>
<td>2.29±0.48</td>
<td>1.79±0.88</td>
</tr>
<tr>
<td><strong>Protein C (mg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.51±0.08</td>
<td>0.48±0.08</td>
<td>0.46±0.06</td>
<td>0.47±0.06</td>
<td>0.47±0.04</td>
</tr>
<tr>
<td>Severe</td>
<td>0.55±0.06</td>
<td>0.36±0.04</td>
<td>0.32±0.06</td>
<td>0.28±0.06</td>
<td>0.22±0.06</td>
</tr>
<tr>
<td><strong>aPC (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>278.3±35.6</td>
<td>(-)</td>
<td>257.3±29.2</td>
<td>317.8±15.8</td>
<td>323.3±37.6</td>
</tr>
<tr>
<td>Severe</td>
<td>283.8±139.8</td>
<td>462.0±158.4</td>
<td>589.3±203.6</td>
<td>706.3±199.4</td>
<td></td>
</tr>
<tr>
<td><strong>PAI-1(U/ml)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>7.32±0.22</td>
<td>6.98±0.30</td>
<td>6.99±0.54</td>
<td>7.04±0.54</td>
<td>7.34±0.64</td>
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<tr>
<td>Severe</td>
<td>6.84±0.70</td>
<td>5.96±0.14</td>
<td>5.68±0.38</td>
<td>4.69±0.38</td>
<td>4.59±0.42</td>
</tr>
<tr>
<td><strong>sTM (ng/ml)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.89±0.12</td>
<td>(-)</td>
<td>0.91±0.14</td>
<td>0.93±0.12</td>
<td>1.00±0.12</td>
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<tr>
<td>Severe</td>
<td>1.04±0.04</td>
<td>1.61±0.12</td>
<td>1.63±0.16</td>
<td>1.88±0.32</td>
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<tr>
<td><strong>D-dimer (ng/ml)</strong></td>
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<tr>
<td>Control</td>
<td>205.25±8.62</td>
<td>204.50±14.24</td>
<td>209.00±9.02</td>
<td>213.50±5.98</td>
<td>208.75±8.54</td>
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<tr>
<td>Severe</td>
<td>205.25±9.28</td>
<td>217.75±6.86</td>
<td>219.75±9.04</td>
<td>212.00±12.14</td>
<td>221.25±3.40</td>
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<td><strong>Syndecan-1(ng/ml)</strong></td>
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<tr>
<td>Control</td>
<td>10.00±0.82</td>
<td>(-)</td>
<td>11.25±0.50</td>
<td>10.75±0.96</td>
<td>12.00±0.82</td>
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<tr>
<td>Severe</td>
<td>10.00±0.96</td>
<td>14.25±0.96</td>
<td>18.25±1.90</td>
<td>20.50±2.64</td>
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<tr>
<td><strong>Hyaluraron (g/ml)</strong></td>
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</tr>
<tr>
<td>Control</td>
<td>0.29±0.16</td>
<td>0.47±0.32</td>
<td>0.55±0.30</td>
<td>0.38±0.34</td>
<td>0.43±0.38</td>
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<tr>
<td>Severe</td>
<td>0.13±0.04</td>
<td>1.07±0.34</td>
<td>1.94±0.44</td>
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<td>2.04±2.54</td>
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<td><strong>Adrenaline (nmol/L)</strong></td>
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<tr>
<td>Control</td>
<td>0.65±0.30</td>
<td>0.35±0.06</td>
<td>0.20±0.12</td>
<td>0.25±0.14</td>
<td>0.23±0.18</td>
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<tr>
<td>Severe</td>
<td>0.88±0.46</td>
<td>0.20±0.08</td>
<td>0.28±0.18</td>
<td>0.35±0.26</td>
<td>0.48±0.28</td>
</tr>
<tr>
<td><strong>Noradrenaline (nmol/L)</strong></td>
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<tr>
<td>Control</td>
<td>6.30±1.46</td>
<td>3.43±3.00</td>
<td>1.63±0.56</td>
<td>1.83±0.54</td>
<td>1.25±0.26</td>
</tr>
<tr>
<td>Severe</td>
<td>3.85±0.14</td>
<td>1.48±0.74</td>
<td>1.15±0.14</td>
<td>1.90±1.08</td>
<td>1.65±1.30</td>
</tr>
</tbody>
</table>

aPC = activated protein C, PAI-1 = plasminogen activator inhibitor-1, sTM = soluble thrombomodulin, (-) = not assessed, * = significant difference between control and severe groups (p<0.05), § = significant difference from baseline.
Table 4. Pearson correlation coefficients for coagulation function parameters, coagulation protease levels, endothelial glycocalyx markers and platelet values in the severe trauma group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lactate</th>
<th>aPC</th>
<th>Protein C</th>
<th>F VIII</th>
<th>F V</th>
<th>PAI-1</th>
<th>sTM</th>
<th>Syndecan-1</th>
<th>Hyaluranon</th>
<th>Platelet count</th>
<th>Fibrinogen</th>
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<tbody>
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<td>EXTEM A10</td>
<td>-0.86***</td>
<td>-0.32</td>
<td>0.72***</td>
<td>0.78****</td>
<td>0.71****</td>
<td>0.49**</td>
<td>-0.64**</td>
<td>-0.89****</td>
<td>-0.93****</td>
<td>0.81****</td>
<td>0.78****</td>
</tr>
<tr>
<td>aPTT</td>
<td>0.70****</td>
<td>0.57*</td>
<td>-0.69***</td>
<td>-0.83***</td>
<td>-0.75***</td>
<td>(-)</td>
<td>(-)</td>
<td>0.91****</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Protein C</td>
<td>-0.54*</td>
<td>-0.59*</td>
<td>(-)</td>
<td>0.78****</td>
<td>0.71***</td>
<td>0.79***</td>
<td>-0.89****</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
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</tr>
<tr>
<td>aPC</td>
<td>0.31</td>
<td>(-)</td>
<td>-0.59*</td>
<td>-0.57*</td>
<td>-0.25</td>
<td>-0.72**</td>
<td>0.62**</td>
<td>0.63*</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Lactate</td>
<td>(-)</td>
<td>0.31</td>
<td>-0.54*</td>
<td>(-)</td>
<td>(-)</td>
<td>0.79***</td>
<td>0.62</td>
<td>0.59*</td>
<td>(-)</td>
<td>-0.58***</td>
<td>(-)</td>
</tr>
<tr>
<td>PAI-1</td>
<td>(-)</td>
<td>-0.72**</td>
<td>0.79***</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>0.73***</td>
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<tr>
<td>INTEM a angle</td>
<td>(-)</td>
<td>-0.29</td>
<td>0.66**</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>-0.69*</td>
<td>(-)</td>
<td>(-)</td>
<td>0.66****</td>
</tr>
</tbody>
</table>

aPTT = activated partial thromboplastin time, aPC = activated protein C, PAI-1 = plasminogen activator inhibitor-1, F VIII = factor VIII, F V = Factor V, sTM = soluble thrombomodulin (-) = correlation not assessed. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001
4.3 Discussion of accepted manuscript

At the commencement of this thesis the ability of sheep to demonstrate coagulopathy in response to a combination of trauma and haemorrhage had not been described in any available publications. The primary aim of the laboratory work performed in this thesis was therefore to design and develop a survivable ovine model of trauma and haemorrhage that demonstrated altered coagulation function in response to these insults. The observational assessment of proposed pathophysiological mechanisms was a secondary aim, and was undertaken using correlation analysis to facilitate identification of pathways, processes or therapeutics that may benefit from mechanistic evaluation in future studies.

The type of tissue trauma and degree of haemorrhage was designed using information obtained from the systematic review described in Chapter 3. As the response of the sheep to such insults was unknown the severity of trauma was increased in a step-wise fashion during the model development process. This was primarily to improve the ethical acceptability of the proposed project, as a significant degree of animal mortality would have precluded the model development studies from continuing. A 6 hour monitoring period was included for extended assessment of changes in coagulation function, as this information would facilitate future model refinement. All animals included in the study were from the same herd of origin and demonstrated no statistically significant differences in baseline physiological parameters.

To facilitate evaluation of ATC in this model attempts were made to minimise the impact of the physiological derangements of haemodilution, hypothermia and acidosis. Temperature was monitored throughout and normothermia maintained with the use of heating mats when required. A degree of endogenous haemodilution secondary to fluid shifts from extravascular sources was expected in the severe trauma groups secondary to hypovolaemia. However this was not compounded by iatrogenic haemodilution as the trauma animals received no fluid resuscitation, with crystalloid administration post injury minimised to 10ml/hr in all animals. The trauma animals also failed to demonstrate a significant acidosis despite the observed rise in lactate levels, most likely due to the development of a co-existing metabolic alkalosis of unknown cause.

Sheep demonstrate a significantly lower resting haemoglobin (Hb) in comparison to humans (59) which results from physiological differences in splenic function. The ovine
spleen stores up to 26% of the circulating red cell mass and boosts circulating Hb levels during periods of physiological stress (236), a function that is lacking in the human spleen (235). All animals in this thesis underwent a splenectomy to attenuate this difference in auto-transfusive capacity. The splenectomy was preceded by intra-splenic administration of adrenaline to induce splenic contracture (237) to increase the pre-injury Hb levels to the equivalent of human values. Splenectomy is a realistic component of an injury scenario and was considered for inclusion during the injury phase in this model without induced splenic contracture. However in the sheep this would also remove a variable amount of circulating red cell mass, which may increase variability in response and influence the reproducibility of the model. As a result it was included as part of the instrumentation phase in this thesis.

The severe trauma animals in this thesis demonstrated changes in INR, aPTT and EXTEM A10 at 2-4 hours post injury that were consistent with current definitions of ATC. This thesis therefore achieved the aim of producing an ovine model of trauma haemorrhage that demonstrated clinically relevant coagulation changes. However in human trauma patients ATC is usually evident within 30 minutes of injury (22, 89). The proposed model therefore has limitations, such as the time taken to achieve a clinically relevant coagulopathy, that indicate the need for ongoing refinement. The overall findings, limitations and future research directions for this model are discussed in further detail in Chapter 5.
CHAPTER 5: DISCUSSION

5.1 Key Findings

The ultimate aim of this thesis was achieved, with the creation of the first ovine model of trauma and haemorrhage to achieve proposed definitions of ATC. The design of the model was based upon a reproducible and survivable tissue injury that was equivalent to an ISS of 25, and was combined with fixed volume haemorrhage as it was deemed more clinically significant and facilitated assessment of the haemodynamic responses to hypovolaemia. The human dimensions of the sheep enabled more detailed observational assessment of proposed pathophysiological contributors than has previously been published in any other experimental model. This has provided further evidence of the importance of tissue hypoperfusion in the development of ATC, with the onset of coagulopathy strongly correlated with arterial lactate levels. It has also further implicated the protein C pathway as a key component of pathophysiology, and provided additional evidence of the possible contribution of endothelial glycocalyx degradation to ATC development. The findings of this thesis have therefore added to the understanding of ATC pathogenesis, and produced an experimental model that with ongoing refinement may be useful for further investigation of ATC and its management. These findings are discussed in further detail in the following sections.

5.1.1 The suitability of sheep as a model of acute traumatic coagulopathy

The success of ovine models in biomedical research is well recognised, with established models of critical illness and haemostatic diseases widely published. This success combined with established similarities with humans in coagulation parameters (58, 59), and haemodynamic, microcirculatory and immunological function (75-77) made them an attractive species in which to develop a model of ATC. A validated ovine model of blood transfusion also exists and demonstrates good comparability with humans in terms of red cell preparation, lifespan and storage lesions (244). Future evaluation of different haemostatic resuscitation regimens in an ovine model of ATC may therefore improve the translatability of results and better inform subsequent human studies.

The model described in this thesis adds to the list of successful ovine models, as it is the first described ovine model of trauma and haemorrhage to achieve ATC as defined by INR, aPTT and EXTEM A10. It is also the first large animal of trauma and haemorrhage to achieve proposed clinical definitions of ATC without associated mortality in the injury
group(s). The aim of creating a survivable tissue injury that was equivalent to an ISS of 25 was therefore achieved. This may have been facilitated by the established similarities between human and ovine coagulation function (58, 59). The severity of tissue injury and degree of haemorrhage required to produce a clinically relevant response in sheep may have been less than in other species that demonstrate established differences with human coagulation function.

The similarities in size between humans and sheep allowed standard human monitoring equipment to be utilised, which provided detailed information on the haemodynamic responses to trauma and haemorrhage in this model. Collection of multiple blood samples throughout the study period was also possible due to animal size, allowing more in depth evaluation of proposed pathophysiological mechanisms. As a result the model utilised in this thesis is the only animal model of ATC to have evaluated aspects of all major hypothesised pathophysiological contributors (table 4).

**Table 4. Hypothesised pathophysiological mechanisms evaluated in animal models of trauma and haemorrhage that achieve clinical definitions of ATC.**

<table>
<thead>
<tr>
<th>Author</th>
<th>Animal Species</th>
<th>Coagulation findings</th>
<th>Pathophysiological features evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>van Zyl et al</td>
<td>Sheep</td>
<td>20% increase in INR, aPTT &gt; 34s, EXTEM A10 &lt; 40mm</td>
<td>Protein C pathway (aPC, FV, FVIII, PAI-1, sTM), Glycocalyx (syndecan-1, hyaluronan), Fibrinolysis (D-dimer, FIBTEM, Fibrinogen, Histology), Platelet function (Multiplate induced aggregation testing), Hypoperfusion (Lactate, Base deficit)</td>
</tr>
<tr>
<td>Duan et al</td>
<td>Pig</td>
<td>20% increase in INR/PT, Prolonged R value</td>
<td>Fibrinolysis (ATIII, Fibrinogen, D-dimer), Hypoperfusion (Lactate, Base deficit)</td>
</tr>
<tr>
<td>Darlington et al</td>
<td>Rat</td>
<td>PT &gt; 18s, aPTT &gt; 24s, Decreased MCF</td>
<td>Hypoperfusion (Lactate, Base deficit), Fibrinolysis (Fibrinogen, FIBTEM)</td>
</tr>
<tr>
<td>Frith et al</td>
<td>Rat</td>
<td>PTr = 1.3</td>
<td>Hypoperfusion (Lactate, Base deficit)</td>
</tr>
<tr>
<td>Chesebro et al</td>
<td>Mouse</td>
<td>aPTT &gt; 34s</td>
<td>Hypoperfusion (Lactate, Base deficit), Protein C pathway (Monoclonal protein C antibodies)</td>
</tr>
</tbody>
</table>
5.1.2 Tissue hypoperfusion, blood lactate levels, base deficit and coagulopathy.

Epidemiological work strongly suggests that the development of ATC requires a combination of tissue injury and tissue hypoperfusion., with coagulopathy most likely to occur if an ISS >15 is combined with a base deficit of > 6mmol/L (29). Retrospective studies and experimental models support this finding, with coagulopathy not developing in the presence of normal base deficit, regardless of the injury severity score (29, 37, 92, 93).

The findings of this thesis further support the role of hypoperfusion in the development of coagulopathy. Tissue hypoperfusion causes inadequate oxygen delivery to the tissues, forcing the onset of anaerobic metabolism that leads to the accumulation of lactic acid (245). The severe trauma group in this thesis demonstrated a strong correlation between coagulopathy as defined by both aPTT and EXTEM A10 and arterial lactate levels. However the severe trauma animals did not develop an associated base deficit or acidosis despite the significant rise in arterial lactate. Base deficit is mechanistically linked to arterial lactate levels, and is often used as a surrogate marker for the accumulation of lactic acid as it has been shown to correlate with arterial lactate in animal models of shock (246). However clinical studies evaluating trauma and critical illness have shown poor correlation between base deficit and arterial lactate levels (245, 247, 248). Clinical studies have also shown arterial lactate to be a strong predictor of outcomes such as mortality and need for resuscitation in trauma (249, 250), and arterial lactate was a better predictor of haemorrhage severity than base deficit in an LD50 model of penetrating trauma (251).

There are no publications that assess the sensitivity of arterial lactate as a predictor of ATC. However in the trauma animals in this thesis arterial lactate appeared to be a better predictor of coagulopathy than base deficit.

All animals in this thesis (including controls) demonstrated a significant increase in bicarbonate levels during the experimental period, suggesting the development of a metabolic alkalosis. It is likely that a co-existing metabolic alkalosis in the trauma sheep blunted the changes in base deficit expected to accompany the observed rise in blood lactate levels. The cause of the metabolic alkalosis in the animals used in this thesis is unknown. The development of a metabolic alkalosis has been seen previously in sheep using alternate forms of total intravenous anaesthesia (252, 253), and while there are no published reports associated with alfaxalone it is possible it may be secondary to the anaesthetic agent. Sheep do also demonstrate significant salivary and ruminal losses during anaesthesia (254). Ruminant foregut physiology would suggest that these losses
should be pH neutral or high in bicarbonate, which should not trigger the development of a metabolic alkalosis (254, 255). However no analysis of the gastric losses from the animals used in this thesis was undertaken. Movement of acid from the abomasum towards the rumen may have occurred, resulting in acid losses and triggering the development of alkalosis. Further investigation of these changes should be considered in future studies.

5.1.3 Changes in coagulation function

Coagulation function within this model was assessed using both traditional plasma based assays and viscoelastic assays of coagulation function. This was in keeping with the multiple proposed definitions of ATC, and the traditional and contemporary assessment of coagulation dysfunction in the trauma patient (22, 23, 29-34). ATC was originally characterised using PT, aPTT and/or INR, and these tests remain the current standard for establishing a definitive diagnosis of coagulopathy. Studies suggest PT/INR to be more sensitive indicators of coagulopathy in trauma (256, 257), whilst aPTT appears to be more specific (98). However these traditional assays were originally developed to identify specific factor deficiencies within the extrinsic or intrinsic pathways of coagulation resulting from heritable coagulopathies or anticoagulant therapy (258). They have been shown to correlate poorly with bleeding risk in patients undergoing elective surgical procedures (259), and as they require 30-60 minutes for processing they may not accurately reflect the rapidly evolving coagulation status of the trauma patient (33). This has led to the increasing use of viscoelastic testing in the trauma setting, as EXTEM A5 and A10 and FIBTEM A5 have all shown to be sensitive predictors of coagulopathy (30, 32, 34).

Assessment of INR within this model was performed using both formal laboratory testing and a point of care (POC) measuring device (Coaguchek XS System, Roche Diagnostics, NSW, Australia). The accuracy of POC devices in the evaluation of traumatic coagulopathy in the emergency setting has been assessed in retrospective and prospective studies (154-158). In the majority of these studies the results from the POC device were well correlated with formal laboratory tests, allowed rapid availability of results and appeared cost effective (154-157). The rationale behind the use of POC devices in this thesis was twofold. Firstly it was to provide some immediate feedback with regards to INR levels during the experimental period, which assisted with the model development process. Secondly it was to evaluate the correlation between POC and laboratory INR values in this experimental model. The results from the POC device were well correlated with the results from the formal laboratory tests within this model (figure 7). This is
consistent with findings in human studies and suggests that POC INR devices may play a role in the early identification of the coagulopathic trauma patient, particularly in the pre-hospital setting or in facilities that are unable to access viscoelastic point of care testing.

![Correlation of INR (formal vs POC)](image)

**Figure 7.** A strong correlation between laboratory and point of care INR measurements was evident in the severe trauma group \((R=0.8746, p<0.0001)\)

The severe trauma animals in this thesis demonstrated significant changes in ROTEM EXTEM A10 values compared to controls, with proposed definitions of ATC achieved at 3 hours post injury \((30)\). Changes in EXTEM A10, EXTEM A5 and FIBTEM A5 values on ROTEM have been shown to be strongly predictive of massive transfusion in trauma in observational studies \((30, 32, 34)\). FIBTEM A5 appeared the most sensitive predictive measure in one study, leading to suggestions it should be used in combination with EXTEM A10 or A5 in the detection and resuscitation of the coagulopathic trauma patient. \((34)\). However it is recognised that significant fibrinolysis appears to develop in a subset of patients with ATC \((39, 96, 111, 112)\), and the sensitivity of FIBTEM A5 is likely to be highlighted in this group. In this thesis there were no significant changes in any FIBTEM parameters or D-dimer levels, suggesting fibrinolysis did not make a significant contribution to the coagulopathy observed in the proposed model. The presence of fibrinolysis in trauma has been associated with a higher ISS and base deficit \((89, 111)\), and it is possible that the aim of developing a survivable injury in this thesis inhibited the development of a model that demonstrated significant fibrinolysis. It has also been demonstrated that FIBTEM assays are relatively insensitive to fibrinolysis, detecting only 10% of patients with plasmin-antiplasmin complex confirmed fibrinolysis \((112)\). Additional
testing is therefore warranted in future studies to better characterise fibrinolysis and its contribution to ATC.

5.1.4 The contribution of the protein C pathway.
A strong association between coagulopathy and the protein C pathway was evident in the model described in this thesis. The degree of coagulopathy was correlated with an increase in sTM and aPC, and a decrease in protein C, factor V, factor VIII and PAI-1 levels in the severe trauma animals. This suggests protein C activation, inhibition of the coagulation cascade and the onset of global anti-coagulation (37-39). These findings support existing evidence suggesting that activation of the protein C pathway may play a significant role in the development of ATC (37-40).

Protein C is a systemic antiocoagulant that is proteolytically converted to an active form by thrombin bound concurrently to TM and the EPCR (16, 37). Once activated it proceeds to inactivate factors Va and VIIIa and deplete PAI-1 (16, 260). Activation of the protein C pathway serves two primary protective functions. The first is inhibition of systemic thrombosis following tissue injury through the binding of thrombin to constitutively expressed thrombomodulin on undamaged endothelial cells (17, 260). The second is inhibition of local thrombosis during periods of hypoperfusion via the increased expression of thrombomodulin on activated endothelial cells (16, 17).

The proposed trigger for the pathological activation of protein C in ATC is the widespread release of thrombomodulin from endothelial cells in response to tissue hypoperfusion (37). Clinical evidence has demonstrated that systemic hypoperfusion is associated with an increase in sTM levels (37, 89). This was supported by the findings of this thesis, with the severe trauma animals demonstrating a significant rise in sTM levels that was correlated with arterial lactate measurements. Furthermore the rise in sTM observed in the severe trauma animals was correlated with protein C depletion, aPC rise and degree of coagulopathy. There is some debate in the literature regarding the relationship between sTM and endothelial bound thrombomodulin (eTM), with studies variously suggesting that sTM may reflect overall TM activity (261, 262), inhibit the action of eTM (263, 264) or act simply as a marker of endothelial injury (265). However the association between sTM and aPC levels in this thesis suggests that sTM levels reflect overall thrombomodulin activity.
Widespread activation of protein C impairs thrombin utilisation, resulting in impaired clot formation. ROTEM EXTEM A10 provides a quantitative assessment of mean clot firmness (164, 165), and the reduction in EXTEM A10 in this ovine model provides evidence of impaired clot formation. The correlation between aPC and EXTEM A10 values in this thesis suggests that aPC may have contributed to this finding. Protein C activation also results in consumption of PAI-1, which may cause uninhibited tPA mediated conversion of plasminogen to plasma. In this thesis a decrease in PAI-1 was correlated with the increase in sTM and aPC levels, suggesting that activation of protein C may have triggered PAI-1 inhibition. However there was no evidence of overt fibrinolysis in this thesis, which is consistent with suggestions that aPC is unable to PAI-1 to the extent required to produce a clinically relevant fibrinolysis (41).

5.1.5 The contribution of the endothelial glycocalyx
The endothelial glycocalyx is a layer of cell bound proteoglycans and glycoproteins that acts as a key regulator of vascular permeability, cell adhesion and inflammation (130, 266, 267). The major constituents of the endothelial glycocalyx are syndecan, hyaluronic acid, chondroitin sulphate and heparin sulphate (266, 267). Disruption of the endothelial glycocalyx leads to endothelial cell activation, platelet adhesion and the expression of anticoagulant or pro-fibrinolytic proteins (130, 268, 269).

An increase in syndecan-1 has been associated with coagulopathy and mortality in trauma patients, suggesting that degradation of the endothelial glycocalyx may contribute to the development of ATC (45, 46, 132). The findings of this thesis further support this hypothesis, with both syndecan-1 and hyaluronon correlated with the degree of coagulopathy as measured by EXTEM A10 and aPTT. However the mechanism by which endothelial glycocalyx shedding contributes to coagulopathy is still unclear. A volume of plasma containing heparin like substances is also held within the glycocalyx, and release of this following degradation may have direct anticoagulant effects from endogenous heparinisation (46). An alternative hypothesis is that shedding triggers increased fibrinolysis and thrombin generation, which in the presence of increased thrombomodulin from endothelial cell damage may trigger protein C activation and global anticoagulation (45, 135). Further work to better evaluate the mechanistic contribution of the microvasculature in ATC is therefore indicated.
Traumatic injury and haemorrhagic shock result in tissue ischaemia, activation of the inflammatory system and stimulation of the neuro-humoral axis with a subsequent catecholamine surge (45, 131-134). Changes in the endothelial glycocalyx in trauma have predominately been attributed to this catecholamine surge, with sympathetic blockade effective in preventing coagulopathy and endothelial glycocalyx shedding in rodent models of haemorrhagic shock (241, 242). However this finding was not supported in this thesis. No elevation in plasma catecholamine levels was evident in either trauma group, and there was no association between glycocalyx degradation and catecholamine levels evident on correlation analysis. Instead the increase in both syndecan-1 and hyaluronon was strongly correlated with arterial lactate levels. Ischaemia has been shown to be a significant trigger for endothelial glycocalyx degradation in patients undergoing vascular surgery (134). The findings of this thesis suggest that ischaemia secondary to hypoperfusion may have been the trigger for endothelial glycocalyx degradation in the severe trauma animals. It is therefore possible that tissue hypoperfusion rather than catecholamine excess is the trigger for endothelial glycocalyx degradation in trauma patients.

5.1.6 Altered platelet function did not contribute to coagulopathy in this model

The cell based model of haemostasis recognises the fundamental role of platelets in the balanced assembly of a stable fibrin clot (14). Trauma patients demonstrate decreased responsiveness to platelet aggregation testing, leading to speculation that dysfunction of platelet activation and adhesion pathways may contribute to ATC (47, 48, 140). However differences in platelet function may exist between platelets at the site of injury and the circulating platelet pool (270), with further evaluation required to ascertain if circulating platelets truly reflect active platelet function.

In this thesis the severe trauma animals developed significant thrombocytopaenia immediately post injury which persisted throughout the monitoring period. This is consistent with human findings in ATC, in which minor reductions in the admission platelet count are predictive of mortality (138, 139). Thrombocytopaenia in the severe trauma animals was correlated with the reduction in EXTEM A10 and INTEM alpha angle, which suggests that thrombocytopaenia may have contributed to reductions in clot formation and clot strength. However there was no associated change in induced platelet aggregation parameters to suggest altered platelet function. Constitutional differences in platelet function may have contributed to this finding as the magnitude of ADP and collagen induced platelet aggregation in sheep is less than half that of humans (59). It is possible
that a significant alteration in platelet function did develop, however was unable to be
detected by the tests utilised in this thesis.

5.1.7 Limitations of the model used in this thesis

Pre-clinical animal research will always have associated limitations, and the model used in
this thesis is no different. The lack of catecholamine release by the trauma animals in this
thesis suggests that the ethical necessities of sedation and anaesthesia may have
significantly altered the compensatory physiological responses to injury (211), impacting
on the overall response that was observed. In contrast to human trauma patients who
simultaneously experience tissue injury and uncontrolled haemorrhage, this thesis applied
standardised insults in a staggered fashion to control the degree of trauma and
haemorrhage experienced by the animals. This non-pathophysiological sequence of
events may have also influenced the overall response observed by further impacting on
the compensatory response to injury in the trauma animals.

Sheep are genetically distinct from humans, and despite demonstrable similarities in
coagulation function also have acknowledged differences in primary and secondary
haemostasis that may impact the translatability of findings (58, 59). These differences
include constitutively different concentrations of coagulation factors, with mean protein C
activity that is 60% of human values and mean factor VIII activity that is three times those
of humans (59) (table 5). Sheep also demonstrate significant differences in platelet
response to induced aggregation testing. Ovine platelets have proven unresponsive to
TRAP-6, arachidonic acid and risocetin agonists and show less than 50% of the human
response to ADP and collagen agonists (59, 62) (table 5), which may make it difficult to
accurately assess the contribution of platelet dysfunction to coagulopathy in this model.

### Table 5. Ovine ranges for selected coagulation parameters compared to human reference ranges.

<table>
<thead>
<tr>
<th>Coagulation test</th>
<th>Sheep (SBL) Mean ± SD</th>
<th>Human Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein C activity (%)</td>
<td>48.6±9.3</td>
<td>70-140</td>
</tr>
<tr>
<td>Factor VIII activity (%)</td>
<td>816.9±201.4</td>
<td>50-150</td>
</tr>
<tr>
<td>ADP induced platelet aggregation (U)</td>
<td>38±20</td>
<td>57-113</td>
</tr>
<tr>
<td>Collagen induced platelet aggregation (U)</td>
<td>41±19</td>
<td>72-125</td>
</tr>
</tbody>
</table>

ADP=adenosine diphosphate, SBL = Samm Border Leisceter. All values taken from Foley et al 2014 (54)
ATC develops within 30 minutes of injury in human patients (22, 37, 89). In this thesis a significant prolongation of aPTT was evident within 30 minutes of injury in the severe trauma animals, however proposed definitions of ATC were only achieved at 2-4 hours following injury. The severe trauma animals also demonstrated variability in haemostatic and metabolic responses to injury. One animal became noticeably more unwell than the other animals in the group, demonstrating a much higher rise in plasma lactate concentration and INR and greater reduction in EXTEM A10 (see appendix 7.1.1). The results reported for this study included this animal because power calculations required a minimum of 4 animals and it was felt 3 animals were insufficient to give valid quantitative results. However the impact of this animal on overall results was assessed by performing a basic secondary statistical analysis with this animal removed (see appendix 7.1.2). A statistically significant difference in EXTEM A10, aPTT and INR was maintained following removal of this animal, although a difference in the time point at which statistical significance was achieved was evident. Variability of response can be expected in any in vivo study and is taken into account when performing power calculations, particularly given the need to minimise the number of animals required for statistical validity (271). However the degree of variability in the severe trauma animals in this study was unexpected. Both the variability in response and the time taken to achieve clinical definitions of ATC indicate that further refinement of the model is required to improve clinical relevance and reproducibility for future studies.

5.2 Future research direction

5.2.1 Refining the proposed model
The overarching aim of this thesis was the development of an ovine model of trauma and haemorrhage that demonstrated coagulopathy consistent with current definitions of ATC. This aim was achieved with the severe trauma group demonstrating an increase in aPTT to 34.25 ± 0.85 s at 2 hours post injury, decrease in EXTEM A10 to 40.75 ± 4.21mm at 3 hours post injury and increase in INR by 20.50 ± 6.08% at 4 hours post injury (23, 29, 30). This confirms our supposition that sheep may be a suitable species for the development of a large animal model of ATC. However as mentioned in the previous section ATC is usually evident in human patients within 30 minutes of injury. The time taken for a clinically relevant coagulopathy to develop in the model used in this thesis, and the variability of response evident in the severe trauma animals indicates that ongoing model refinement is required to improve reproducibility and clinical relevance.
Fixed volume haemorrhage was used in this model as it was felt it more closely mimicked the clinical situation, and facilitated the assessment of the haemodynamic responses to hypovolaemia (215). However haemorrhage of 30% blood volume in this model failed to produce a significant change in pH or base deficit which suggests that an increase in volume loss is indicated. However estimating the degree by which volume loss needs to be increased is difficult given the variability in response that was observed in the severe trauma animals. As the volume removed was based upon body weight the pre-experimental state of the animal influenced the degree of blood loss. All animals used in this study were fasted for 12 hours prior to surgical intervention, which resulted in loss of 2.4±1.9kg of body weight from pre-fasting measurements. Although the difference in fasting weight loss is small, it is possible that it may have contributed to the variability in physiologic and metabolic response that was observed. Fixed pressure haemorrhage could be used as an alternative as it would allow the degree and duration of hypotension to be controlled (215). However the need for repeated blood withdrawal or volume administration to maintain target pressure is not reflective of the clinical situation and introduces potential confounders. Maintaining pressure with crystalloids may result in iatrogenic haemodilution (272) whilst administration of anti-coagulated blood may cause iatrogenic anti-coagulation (67).

Epidemiological work in human patients has shown that ATC is most likely to develop if an ISS of 25 or greater is combined with a minimum base deficit of 6mmol/L (29). The model used in this thesis was designed to simulate an ISS of 25 based upon this work. As a result it may be worthwhile targeting haemorrhage to a variable predictive of ATC, rather than a target blood pressure or volume as these have not proven to predict the onset of coagulopathy. While a significant rise in base deficit was not evident in this thesis the severe trauma animals did demonstrate a significant rise in blood lactate levels, which have proven to be a sensitive predictor of mortality and need for resuscitation in clinical studies (249, 250). Qualitative evaluation of data from this study and other published experimental models suggests that animals that develop a lactate value of 4mmol/L or more following trauma and haemorrhage were more likely to develop a clinically relevant coagulopathy (table 6) (29, 40, 65, 67). Therefore it may be more appropriate to target haemorrhage to a minimum blood lactate level of 4mmol/L, rather than a fixed volume based upon body weight or a fixed pressure. This may improve the reproducibility of the proposed model, whilst also accelerating the time point at which a clinically relevant coagulopathy becomes evident.
Table 6. Lactate values and coagulation changes in published animal trauma and haemorrhage models.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Mean lactate values post trauma and haemorrhage</th>
<th>Clinically relevant coagulopathy</th>
<th>Coagulation findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>van Zyl et al</td>
<td>1.88±0.37mmol/L at 1hr (Moderate)</td>
<td>Borderline</td>
<td>aPTT 34.75±2.50s (4hrs) INR ↑22.5±1.0% (4hrs)</td>
</tr>
<tr>
<td></td>
<td>4.15±0.70mmol/L at 1hr (Severe)</td>
<td>Yes</td>
<td>aPTT 34.25±1.71s (2hrs) INR ↑22.5±6.0% (4hrs) EXTEM A10 40.75±8.42 (3hrs)</td>
</tr>
<tr>
<td>Duan et al</td>
<td>4.98±1.4mmol/L at 10min</td>
<td>Yes</td>
<td>INR ↑ 23±0.7% (40 min)</td>
</tr>
<tr>
<td>Darlington et al</td>
<td>5.6±0.4mmol/L at 40min</td>
<td>Yes</td>
<td>PT 21.6±0.3s (1hr)</td>
</tr>
<tr>
<td>Frith et al</td>
<td>8.6±2.0mmol/L</td>
<td>Yes</td>
<td>PT 23.5±2.4s</td>
</tr>
<tr>
<td>Chesebro et al</td>
<td>10.7±3.6mmol/L at 1hr</td>
<td>Yes</td>
<td>aPTT 35.3±3.1s (1hr)</td>
</tr>
<tr>
<td>Mulier et al</td>
<td>2.0±1.3mmol/L at 45min</td>
<td>No</td>
<td>Increased CFT on TEG.</td>
</tr>
</tbody>
</table>

5.2.2 Further investigation of proposed pathophysiological mechanisms

This thesis evaluated changes in platelet function, markers of endothelial glycocalyx degradation, components of the protein C pathway and fibrinolytic function that accompanied the onset of coagulopathy in the severe trauma animals. As a result it encompassed aspects of all major hypothesised pathophysiological mechanisms (270). However as this thesis was primarily aimed at model development the examination of proposed pathophysiological mechanisms was purely observational in nature. Calculated correlation coefficients examined the relationships between coagulopathy and various biomarkers, further supporting hypothesised contributions of the endothelial glycocalyx and protein C pathway. However correlation does not equate to causation and further mechanistic studies are indicated to better elucidate the mechanisms underlying the development of ATC.

The ability to evaluate isolated pathways within the coagulation system has been demonstrated in a murine model of ATC, in which the administration of activated protein C antibodies stopped the development of coagulopathy (40). However more detailed investigation in rodent models has been limited by animal size and established differences in coagulation function (58, 224). Refinement of the model used in this thesis may facilitate
mechanistic evaluation of ATC on a larger scale via the interference or manipulation of isolated pathways. The development of ovine antibodies to various aspects of the protein C pathway would facilitate a more detailed assessment of the contribution of various components of the pathway to ATC. Assessment of plasmin-antiplasmin complexes would better reflect the degree of fibrinolysis in the model (112), and manipulation of PAI-1 and tPA may improve understanding of the mechanism underlying fibrinolysis. A better understanding of the microcirculation and endothelial glycocalyx may be elucidated through the use of electron microscopy, organ microdialysis and sidestream dark field camera application (136, 273, 274). The role of endogenous heparinisation could also be better assessed via the use of anti-Xa assays (275). Immunohistochemistry (IHC) to investigate the tissue expression of biomarkers such as thrombomodulin in addition to measuring circulating levels will also contribute to improved understanding, particularly given the controversies regarding the action of sTM. This was considered in this thesis; however no commercially available ovine thrombomodulin antibodies suitable for IHC were able to be located. Cross reactivity with human (thrombomodulin/BDCA antibody, R&D systems), bovine (thrombomodulin antibody, Biorbyt) and caprine (thrombomodulin antibody, Enzo Life Sciences Inc) antibodies was assessed, with none found. Thrombomodulin IHC would therefore require the development of ovine specific antibodies which was beyond the scope of this thesis.

The evaluation of additional pathophysiological theories is also indicated in future work. Coagulation dysfunction triggers a de novo systemic inflammatory response, which may contribute to the increased incidence of sepsis and multiorgan failure in trauma patients (50, 276). ATC has been associated with increased cytokine expression (most notably TNF-α and IL-1) and complement activation via the release of mitochondrial damage-associated molecular patterns (DAMPs) (276). Immune activation may therefore aggravate tissue damage and amplify haemostatic activation. In addition trauma patients have demonstrated an increase in platelet derived microparticles (PMPs) that were associated with increased blood product requirements and mortality (148, 149). These PMPs are thought to also have pro-inflammatory effects and may play an important role in linking coagulation and inflammation; however the precise contribution to ATC is still undetermined. Methodological evaluation of inflammatory markers and PMPs in a controlled fashion using an in vivo model may help elucidate the contribution of these additional factors to ATC.
5.2.3 Investigation of proposed therapeutic strategies.

Haemostatic or ‘damage control’ resuscitation regimens form the current core therapeutic approach to patients with traumatic injury. It is perceived that these regimens restore circulating volume and reverse coagulopathy, resulting in improved patient outcomes (51, 52, 81, 82). However a recent prospective study demonstrated worsening haemostatic function in trauma patients despite the use of haemostatic resuscitation, suggesting factors other than correction of coagulopathy may contribute to the survival benefits associated with these regimens (80).

The composition of haemostatic resuscitation varies between countries and institutions (84-86), reflecting a lack of understanding of the benefits and risks of individual blood components in trauma. There is some evidence to suggest that fresh frozen plasma (FFP) may have a protective and restorative effect on the endothelial glycocalyx (137, 178), a property that has not been described with fibrinogen concentrate or cryoprecipitate. This may make FFP an attractive option for inclusion in resuscitation regimens given the proposed contribution of the glycocalyx to ATC. However a systematic review evaluating the comparative clinical effectiveness of FFP and fibrinogen demonstrated no significant difference in outcomes (277). Refinement of the model proposed by this thesis would allow more detailed evaluation of the effects of FFP and other resuscitation components on the endothelial glycocalyx and haemostatic response. Differing resuscitation regimens could also be compared, which may better inform subsequent human studies.

5.3 Concluding statement

Pre-clinical animal research is a necessary adjunct for improving the understanding and management of ATC given the acknowledged limitations of human research in the emergency setting. Creating a clinically relevant model of ATC that facilitates translation of results to humans is difficult given the complexity of the condition and known limitations of animal research. This thesis describes the development of the first ovine model of trauma and haemorrhage to demonstrate coagulation changes consistent with current definitions of ATC. The degree of coagulopathy was closely correlated with the degree of shock as quantified by arterial lactate levels, further supporting the importance of tissue hypoperfusion in the development of ATC. Observational assessment of proposed pathophysiological mechanisms within the model demonstrated a strong correlation between coagulopathy and activation of the protein C pathway, which supports
suggestions that protein C activation may play a central role in the development of ATC. Coagulopathy was also strongly correlated with an increase in markers of endothelial glycocalyx shedding, providing further evidence that the endothelial glycocalyx may contribute to the development of coagulopathy in trauma. However there was no evidence of fibrinolysis and no significant changes in induced platelet aggregation testing to suggest that fibrinolysis or platelet dysfunction made a significant contribution to coagulopathy in this thesis.

Clinical definitions of ATC were evident 2-4 hours following injury in this thesis and the severe trauma animals did demonstrate variability in the metabolic and haemostatic response to trauma. This indicates the need for ongoing refinement of the proposed model in order to improve reproducibility and clinical relevance. Refinement may facilitate mechanistic evaluation of proposed pathophysiological mechanisms, which may improve understanding of the condition and identify novel therapeutic targets. Refining the proposed model would also provide a platform for the evaluation of targeted and novel haemostatic interventions in a whole biological system, which may reduce exposure to blood products and help improve outcomes for trauma patients.
CHAPTER 6: REFERENCE LIST


28. Practice guidelines for perioperative blood transfusion and adjuvant therapies: an updated report by the American Society of Anesthesiologists Task Force on


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CHAPTER 7: APPENDICES

7.1 Repeat statistical analysis of selected variables.

Variability in response was evident in the severe trauma animals. Animal 3 developed a higher blood lactate level and INR, longer aPTT and lower EXTEM A10 value than the other animals in the group (appendix 7.1.1). The impact of this variability on overall results was assessed by repeat statistical analysis of selected variables with this animal removed (appendix 7.1.2). A statistically significant difference in these variables remained, however the time at which statistical significance was achieved differed in some cases. The time at which the clinical definition of ATC was achieved was also prolonged (most notably for EXTEM A10) however all 3 proposed definitions were still met.

7.1.1 Individual responses of severe trauma group animals to selected variables.

The figures below demonstrate the individual responses of animals in the severe trauma to selected variables. Animal 3 demonstrated a greater rise in INR [A] and lactate [B] than the other animals in the group. A greater prolongation of aPTT [C] and reduction in EXTEM A10 values [D] was also demonstrated by animal 3 compared to the other animals in the group.

![Graphs showing individual responses of severe trauma group animals to selected variables.](image)

**Figure 8. Individual responses of animals in the severe trauma group to selected variables.**
7.1.2 Repeat statistical analysis of selected variables

Repeat statistical analysis of the selected variables presented above was undertaken to ascertain if a statistically significant difference in response remained without the inclusion of animal 3 (table 7). A statistically significant difference in all variables remained following the exclusion of animal 3 (severe (3)). The aPTT based definition of ATC was still met at 2 hours post injury (34.00 ± 2.00s) and a statistically significant difference (p =0.006) remained from 30 minutes post injury. However EXTEM A10 did not drop below 40mm until 6 hours post injury (37.00 ± 10.15mm) following the removal of animal 3, with a statistically significant difference (p=0.072) not evident until 5 hours post injury.

Table 7. Repeat statistical analysis of selected variables following removal of severe trauma animal 3. Values are expressed as mean ± SD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>30 min</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
<th>5 hr</th>
<th>6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPTT (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.25±1.50</td>
<td>23.50±1.92</td>
<td>23.25±1.89</td>
<td>24.00±2.00</td>
<td>24.50±2.08</td>
<td>23.75±2.88</td>
<td>24.25±2.50</td>
<td>24.75±2.50</td>
</tr>
<tr>
<td>Moderate</td>
<td>23.50±2.88</td>
<td>28.00±2.44</td>
<td>30.75±4.50</td>
<td>32.00±4.97</td>
<td>32.75±2.22</td>
<td>34.75±2.50</td>
<td>35.00±2.16</td>
<td>34.50±2.88</td>
</tr>
<tr>
<td>Severe</td>
<td>24.75±1.50</td>
<td>32.50±4.20</td>
<td>33.00±2.58</td>
<td>34.25±1.71</td>
<td>36.25±4.35</td>
<td>39.25±4.99</td>
<td>42.00±8.16</td>
<td>44.50±6.56</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>24.33±1.53</td>
<td>32.00±5.00</td>
<td>33.33±3.01</td>
<td>34.00±2.00</td>
<td>35.00±4.36</td>
<td>37.00±2.65</td>
<td>38.00±2.00</td>
<td>41.33±2.08</td>
</tr>
<tr>
<td>INR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.43±0.06</td>
<td>1.43±0.06</td>
<td>1.43±0.06</td>
<td>1.43±0.06</td>
<td>1.45±0.06</td>
<td>1.45±0.06</td>
<td>1.45±0.06</td>
<td>1.45±0.06</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.33±0.06</td>
<td>1.38±0.10</td>
<td>1.48±0.06</td>
<td>1.48±0.06</td>
<td>1.55±0.06</td>
<td>1.63±0.06</td>
<td>1.65±0.06</td>
<td>1.65±0.06</td>
</tr>
<tr>
<td>Severe</td>
<td>1.33±0.10</td>
<td>1.35±0.06</td>
<td>1.38±0.10</td>
<td>1.43±0.06</td>
<td>1.48±0.10</td>
<td>1.60±0.20</td>
<td>1.73±0.32</td>
<td>1.80±0.28</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>1.30±0.10</td>
<td>1.33±0.06</td>
<td>1.33±0.06</td>
<td>1.40±0.06</td>
<td>1.43±0.06</td>
<td>1.50±0.00</td>
<td>1.58±0.06</td>
<td>1.68±0.06</td>
</tr>
<tr>
<td>EXTEM A10 (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>61.00±6.06</td>
<td>55.75±4.44</td>
<td>54.75±4.58</td>
<td>52.00±5.94</td>
<td>53.00±5.72</td>
<td>51.75±8.34</td>
<td>54.00±6.80</td>
<td>52.75±6.60</td>
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<tr>
<td>Moderate</td>
<td>64.50±4.20</td>
<td>54.75±2.22</td>
<td>53.50±2.08</td>
<td>53.50±1.00</td>
<td>52.25±1.50</td>
<td>49.50±2.38</td>
<td>50.25±2.50</td>
<td>49.00±2.16</td>
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<tr>
<td>Severe</td>
<td>59.00±2.70</td>
<td>48.50±3.42</td>
<td>47.25±2.62</td>
<td>45.25±2.40</td>
<td>40.75±6.42</td>
<td>38.25±13.2</td>
<td>35.25±14.72</td>
<td>31.25±14.2</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>57.67±7.09</td>
<td>49.67±3.05</td>
<td>48.00±2.65</td>
<td>46.33±3.22</td>
<td>44.67±3.79</td>
<td>44.67±3.79</td>
<td>42.33±4.93</td>
<td>37.00±10.15</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.23±0.38</td>
<td>0.58±0.20</td>
<td>0.53±0.16</td>
<td>0.53±0.16</td>
<td>0.55±0.20</td>
<td>0.50±0.14</td>
<td>0.55±0.14</td>
<td>0.55±0.09</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.58±0.56</td>
<td>1.80±0.72</td>
<td>1.88±0.74</td>
<td>1.80±0.80</td>
<td>1.63±0.48</td>
<td>1.75±0.91</td>
<td>1.80±1.02</td>
<td>1.90±0.82</td>
</tr>
<tr>
<td>Severe</td>
<td>1.48±0.26</td>
<td>3.50±1.08</td>
<td>4.15±1.39</td>
<td>4.68±1.93</td>
<td>4.70±2.80</td>
<td>5.08±3.00</td>
<td>6.13±0.80</td>
<td>6.73±0.30</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>1.40±0.27</td>
<td>3.07±0.81</td>
<td>3.50±0.60</td>
<td>3.73±0.50</td>
<td>3.37±1.03</td>
<td>3.53±0.49</td>
<td>3.60±0.69</td>
<td>4.40±0.12</td>
</tr>
</tbody>
</table>

Severe (3) = results with animal 3 removed, aPTT = activated partial thromboplatin time, INR = international normalised ratio, EXTEM A10 = clot amplitude at 10 minutes, * = significant difference between control and severe trauma groups p<0.05. x = significant difference between control and moderate trauma groups p<0.05

The original statistical analysis also demonstrated a significant rise in INR from baseline at 3 hours post injury in both the moderate (p=0.002) and severe (p=0008) trauma groups compared to the control group, with a 20% increase from baseline evident from 4 hours (figure 9 [A]). Following removal of severe trauma animal 3 the statistically significant increase in INR at 3 hours post injury in the severe trauma group compared to the control group was maintained (p=0.02). However the time at which the INR based definition of ATC was met was prolonged, with a 20% increased from baseline not evident until 5 hours post injury (figure 9 [B]).
Figure 9. INR changes in this thesis [A] An increase in INR was evident in the moderate trauma (p=0.002) and severe trauma (p=0.008) groups compared to the control group, with a 20% increase from baseline evident at 4 hours. [B] This increase was maintained in the severe trauma group following removal of animal 3 (p=0.02), however a 20% increase in baseline was not evident until 5 hours.

7.2 List of manuscripts by the candidate included in the thesis

7.3 List of published abstracts relevant to the thesis


7.4 List of oral presentations made by the candidate and relevant to the thesis

- **van Zyl N**, Milford EM, Diab S, Dunster K, McGiffin P, Rayner SG, Staib A, Reade MC, Fraser JF. *Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and haemorrhage* The Royal Australian College of Surgeons (RACS) Annual Scientific Congress (ASC) 4th May 2016 Brisbane, QLD Australia

7.5 List of poster presentations made by the candidate and relevant to the thesis

- **van Zyl N**, Milford E, Diab S, Dunster K, Reade M, Fraser J. 2015. *A reduction in Factor VIII levels is associated with coagulopathy in an ovine model of trauma and haemorrhage*. Australasian Trauma Society Conference 2nd-4th October 2015, Gold Coast, QLD, Australia


haemorrhage. Australasian College for Emergency Medicine (ACEM) Annual Scientific Meeting (ASM) 7-11$^{th}$ December 2014, Melbourne, VIC, Australia