Prehospital Blood Transfusion after Combat Injury and Platelet Function in an Animal Model of Complex Military Trauma

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Abstract

The management of trauma has been transformed by recent conflict and by improvements in understanding of the response to injury, particularly coagulopathy. Clotting abnormalities can appear early after trauma and have multiple causes, including tissue injury, shock and the effects of treatment. There is increasing understanding of the contribution of coagulation molecules to these abnormalities. Platelets are recognised as being central to the clotting cascade in the current cell-based model of coagulation but have been incompletely studied after traumatic injury.

There has been widespread adoption of the practice of "haemostatic resuscitation" using blood products rather than crystalloid fluids with a high ratio of plasma to red blood cells to correct the coagulopathy of trauma while restoring the patient's physiology. The British Armed Forces have pushed this forward of the hospital using physician-led medical teams. However, this practice has unquantified benefit and potential for harm, and is a logistical challenge.

The first study in this thesis assesses whether available clinical data support the use of prehospital transfusion. It compared recipients of the treatment with similarly injured controls. Although mortality was halved, confounding changes in hospital transfusion practice made it impossible to rely on these data to establish the efficacy of prehospital transfusion.

To allow further study, an animal model of complex military trauma was developed. The author focused on developing a flow cytometry assay for the assessment of platelet activation and response to *in vitro* stimulation. This was successful. The performance of the assay was assessed in the context of the animal model. Surgical preparation of the model appeared to affect the expression of the relevant activation marker. While the assay proved incompatible with unavoidable constraints of the model, its development acted as the basis for the establishment of a human platelet study of combat casualties in Afghanistan.

For Jemima

The reason I always have something to smile about

Acknowledgements

I have been extremely fortunate in the preparation of this thesis to have worked as part of two very capable teams. The first is that led at Dstl by Dr Emrys Kirkman and including Sarah Watts, Charles Masey, Callie Doran and Keith Male. My deepest thanks go to Alun Carter, the team's immunologist, who has been by turns teacher, collaborator and friend, and without whom I would have achieved very little. I am also grateful to my military colleagues at Dstl, Lt Col Giles Nordmann, Lt Col Will Eardley and Lt Col Hugo Guthrie, with whom I have shared many frustrations and consolations.

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Intellectual Property

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Chapter 1. Introduction

The period from 2001 to 2014 saw the longest sustained period of medium- and high-intensity conflict involving UK and US armed forces since the Second World War. During this period there has been a revolution in many aspects of the care of trauma patients, both in military (Blackbourne *et al.*, 2012) and civilian practice (Howell, 2014). For the UK Armed Forces, the deployment to Helmand Province, Afghanistan saw the highest rate of casualties and the most intense medical effort. A combination of the type of combat, dominated by the use of improvised explosive devices, the protective equipment worn by servicemen and the advances in the speed and quality of immediate medical care produced a patient population characterised by a high incidence of severe injury comprising blast exposure, extensive soft tissue injury and traumatic amputations.

Haemorrhage remains one of the most serious threats to the lives of such patients. It is the leading cause of preventable battlefield death (Hodgetts, Mahoney and Kirkman, 2007; Kelly *et al.*, 2008). The use of tourniquets and other prehospital interventions has had a large impact on the initial fatality of such injuries, meaning that severely injured patients who have had significant bleeding are now more likely to be recovered to medical care. The challenge of managing such patients has been met through new practices, including prehospital transfusion (O'Reilly *et al.*, 2014) and changes to the way blood products are used (Borgman *et al.*, 2007), which are themselves based on a new understanding of the mechanisms underlying traumatic bleeding and coagulopathy. That new understanding began in the civilian sphere (Brohi *et al.*, 2003) and the entire process has been characterised by a free exchange of ideas and practice between military and civilian clinicians and researchers.

While prehospital transfusion has a long history, its introduction into British military practice is seen as one of the key changes in trauma care during this long decade of conflict. However, that change was made on the basis of expert opinion rather than evidence. The first study detailed in this thesis arose from an attempt to correct this by examining the available clinical information. An assessment of the impact of

prehospital transfusion on mortality using human data from Afghanistan was performed. This demonstrated that confounding changes in clinical practice meant that the independent effect of prehospital transfusion could not be established. This supported the use of an animal model to examine the issue.

The use of an animal model provided the opportunity to study aspects of coagulopathy in far more detail than could be achieved in human subjects. Assessment of several aspects of coagulation was an existing part of animal models of trauma in use at Dstl Porton Down. However, the role of platelets, which lie at the heart of the coagulation process, had not been addressed. As a suitable model was set up, the author (under guidance and supervision) therefore developed an assay to assess platelet function. Implementation of this assay in the animal model demonstrated unexpected effects of the surgical preparation of the model on platelet activation.

There are therefore two distinct components to the work described: first, a human data study, which demonstrated the need for an animal model and, second, the development and validation of a platelet flow cytometry assay which was undertaken by the author in the context of that model.

Chapter 2 begins with an exploration of the mechanisms of battlefield injury, including blast, followed by an examination of the body's pathophysiological response, with a particular emphasis on coagulation. The development and evidence base of the resuscitation of trauma patients is examined in Chapter 3. Chapter 4 is a study of the available human data on patients who receive prehospital transfusion.

In Chapter 5 there is discussion of important elements of the techniques that are used in the remainder thesis: coagulation assays, flow cytometry and animal models. Chapter 6 describes the development of a flow cytometry assay of platelet function. Chapter 7 describes an assessment of the functioning of this assay in an experimental setting. Chapter 8 concludes with a description of how work in this area should progress.

Chapter 2. Mechanisms and Consequences of injury

Mechanisms of injury determine the pathophysiological consequences for the patient. This chapter begins with an overview of these mechanisms in order to give context to a more detailed examination of blast injury. Thereafter the chapter turns to the physiology of the cardiovascular response to injury, again with emphasis on the reaction to blast. There is then an examination of the linked systems of inflammation and coagulation before a description of the disordered coagulation seen after severe injury; the role of platelets in these is emphasised.

2.1 Non-Blast Injury

Irrespective of mechanism, tissue injury is caused by the deposition of energy (Hunt, Marr and Stuke, 2012). In thermal and chemical burns, molecular damage is generated, often without actually disrupting the tissue. However, in most cases, kinetic energy does work in tissue, disrupting the bonds of cellular and extracellular structures. This is usually due to the interaction between the host and a solid object moving relative to it. Primary blast injuries are the major exception to this. In any case, damage is generated when kinetic energy does work in the tissues to disrupt intra- and intermolecular bonds. The amount of damage done is a function of the kinetic energy deposited.

The forces generated in any collision are applied to the materials of the objects involved. The force may be in tension or compression or it may involve shear, where forces act in opposing directions on different parts of an object, an example being the differential acceleration of tethered and mobile parts of a viscus. As forces act on a material (stress—force per unit area) they deform it (strain—relative size of the deformation). Depending on the material and the type (tensile, compressive or shear) and magnitude of the strain, the deformation will either be elastic or plastic. Elastic strain is fully reversed by removal of the stress; plastic is not. Kinetic energy may still be dissipated by elastic deformation due to the generation of heat and noise. Finally, at the point of maximum plastic strain, the material fails and is disrupted. The tensile, compressive or shear strength is the stress at which this occurs. A special case is fluid

filled objects subjected to compression. Due to Boyle's law, the reduction in volume caused by the compression increases the pressure of the fluid. This increases the tensile stress on the wall of the fluid-filled structure. If the increase exceeds its tensile strength, the wall will rupture.

Penetrating injuries are also caused by collisions. Sharp objects serve to concentrate the force of the collision in a small area, such that the blade or point produces very large and very local increases in stress on the material through which it thereby cuts. Bullets and other projectiles concentrate the forces less but have more available kinetic energy to do work on the material. Low energy projectiles cause only very local damage to material cut and crushed as they pass through. High energy projectiles, such as high velocity military bullets, occasionally pass through tissue with little loss of energy. (The determinants of this are a complex interaction between the nature of the materials traversed by the projectile and the material and kinetic properties of the latter.) More commonly, their large available kinetic energy is dissipated by generating heat, crushing local tissue, accelerating tissue away from the passage of the projectile and deforming the bullet itself. The consequence is that high velocity projectiles generate a larger permanent cavity than low energy ones. They also generate a much larger temporary cavity. The temporary cavitation does not, in the main, devitalise the affected tissue, most of which is only elastically deformed (hence it is temporary). However, tissue is a mixed medium and some of the material will have less capacity for elastic strain and will either permanently deform or will fail. In particular, bone is weak under tension and temporary cavitation can generate fractures remote from the permanent wound track.

The clinical impact of an injury is not just a function of the kinetic energy that was dissipated in causing it. The site of injury is crucial. Local injury to adipose tissue is of far less significance than destruction of central nervous tissue. Systemically, injuries that cause significant bleeding, disrupt the airway or otherwise embarrass the delivery of oxygenated blood to the tissues (e.g. cardiac tamponade, pneumothorax, vascular occlusion) may be fatal despite relatively minor tissue disruption.

2.2 Blast

2.2.1 Physics of Blast

Explosions liberate large amounts of energy in very short spaces of time (Wolf *et al.*, 2009); e.g. the velocity of detonation (the speed at which the detonation wave propagates through the material) of trinitrotoluene is 6,900m/s (Cooper, 1996). A high velocity, high pressure blast wave is produced which moves out quickly through the surrounding medium. Initially, this wave, known as the peak over pressure, is contiguous with an expanding cloud of the hot gaseous products of the explosion. Since it is moving faster than the speed of sound in the medium, the blast wave soon decouples from the slowing expansion of the gases (Figure 2.1)

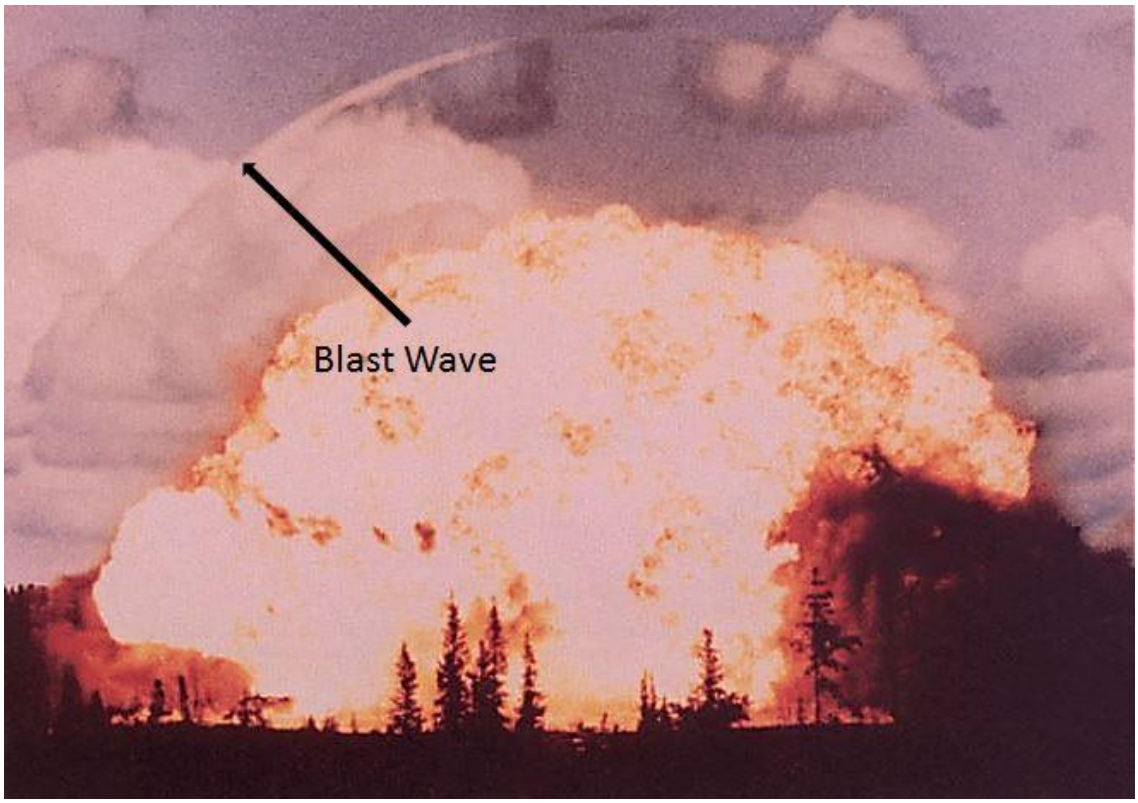


Figure 2.1 A large explosion under (broadly) free-field conditions. The blast wave is clearly visible and has moved away from the expanding gases.

The peak overpressure is short-lived since it decays rapidly as it moves away from the point of the explosion, particularly where the medium is air rather than water. The passage of the blast waves accelerates the air, producing a blast wind (dynamic

overpressure), which carries with it energised fragments of the explosive, its container and other nearby objects. The outward movement of the blast wind eventually dissipates, leaving (in air) a volume of rarefied, low pressure gas near the point of explosion. The medium therefore reverses its movement to correct this underpressure, oscillating until uniform pressure is restored throughout (Figure 2.2).

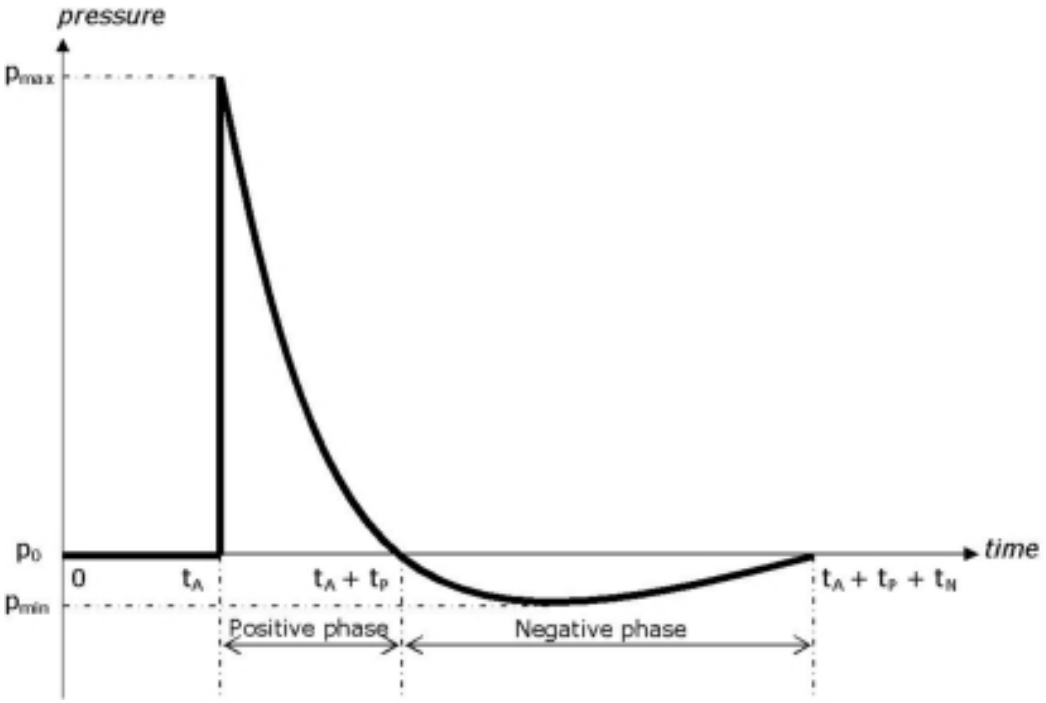


Figure 2.2 A Frielander curve showing the rapid rise to peak overpressure and subsequent negative pressure after an explosion. In this idealised curve, oscillation is not shown. (Mediavilla Varas *et al.*, 2011)

The relative importance of the peak overpressure and the blast wind (and fragments) depends upon the environment of the explosion. In an incompressible liquid medium, as with underwater explosions, the blast wave propagates much further than in a gas, where the compressibility of the medium allows its energy to accelerate and heat the air. Conversely, dense liquid media reduce the movement of the dynamic overpressure and fragments, which consequently have a much reduced radius of effect. Interaction of the blast wave with solid surfaces can cause it to be reflected. In complex environments this can allow reflected and non-reflected blast waves to meet at particular points where the peak overpressure will be summed (Wolf *et al.*, 2009). Consequently, blast waves can cause damage further from the point of explosion in confined spaces and cause more severe injury.

2.2.2 Blast Injuries

Injuries from explosions are categorised as primary, secondary, tertiary and quaternary (Zuckerman, 1940).

- **Primary blast injuries** are those caused by the peak overpressure and are unique to explosions. When the blast wave interacts with the body it causes differential acceleration of structures according to their density and other characteristics. Consequently energy is deposited at interfaces between materials of different density, as may be found at the internal surface of hollow viscera. The propagation of the blast wave though the different tissues of the body generates a high-velocity, low-amplitude stress wave and a low-velocity, high-amplitude shear wave. The stress wave is compressive but when reflected at a density interface pulls tissue apart (tension). This leads to damage at a microscopic level (albeit potentially affecting a considerable volume of tissue) rather than large lacerations (Figure 2.3). Shear waves cause gross relative movement of tissues, particularly due to the movement of the body wall. This causes tearing of structures from their attachments and disruption of solid organs (Horrocks, 2001).
- **Secondary blast injuries** are those caused by the impact of fragments energised by the blast wind. Professionally manufactured military munitions contain preformed fragments or have casings which are designed to break up along lines of weakness producing fragments with predictable ballistic properties (Dearden, 2001). The size, mass and shape of the fragments dictate how far and fast they move through the air and how they interact with the body. This allows the manufacturer to control the wounding effects and radius of the weapon. Munitions may be optimised to typically injure rather than kill and to produce predictable chances of injury and death at given distances from detonation. Most improvised explosive devices (IEDs) lack this sophistication. Their casings fracture randomly and their wounding effects are highly unpredictable. Large fragments may be energised causing severe injuries. However, in some cases secondary blast from an IED may be chiefly due to the impact of energised soil and sand, in which the device has been disguised, which (depending on proximity to the device and the quantity of explosive)

may cause a diffuse, shallow destruction of tissue with devitalisation and tattooing of tissue beyond.

- **Tertiary injuries** are caused by the body being moved by the blast wind and striking against other objects.

- All other effects, including burns, inhalational injury and psychological sequelae are classified as **quaternary**. Crush due to building collapse is classified as a tertiary or quaternary injury by different authors. A hyperinflammatory response seen in some Israeli victims of improvised explosive devices and caused by absorption of pentaerythritol-tetranitrate has been described and the term quinary blast injury proposed (Kluger *et al.*, 2007). The Quaternary group is already disparate and the burden of proof to justify the establishment of a fifth category of injury must lie on Kluger *et al*. No clear reason not to include this novel mechanism as part of the quaternary group is given.

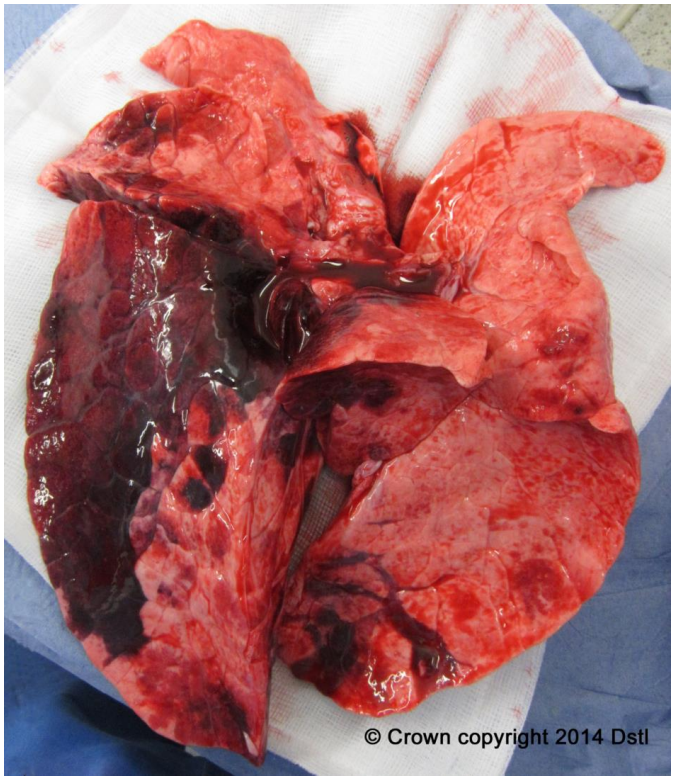


Figure 2.3 *Post mortem* image of primary blast injury in a pig. There is a large area of contusion on the side that faced the explosive but no significant laceration.

Traumatic amputation after blast injury is an interesting phenomenon. These injuries have been common in Afghanistan: Morrison *et al* found that 169 of 656 IED casualties in a four year period sustained 278 lower extremity amputations (Morrison, Hunt, *et al.*, 2012). Amputations were traditionally thought to be caused by differential acceleration of different parts of the limb by the blast wind. This should cause disruption at the joints where those parts interact. However, Hull *et al* (1994), examining injuries sustained in Northern Ireland, found that the level of bony transection was usually in the middle of the shaft of long bones. Experiments on sheep limbs have shown that the bone is in fact fractured by the action of the peak overpressure. The blast wind then accelerates the limb distal to the fracture, tearing it off (Hull and Cooper, 1996). Pure limb flail remains a relevant mechanism: Singleton *et al* (2014) recently found that 17.3% of lower limb amputations were through knee, most without a nearby fracture. They also found no evidence of an association of traumatic amputation with primary blast lung injury, a past finding which had supported Hull and Cooper's hypothesis.

2.2.3 Impact and Implications of Primary Blast Injury

Compared with the general population of trauma victims, those injured by explosive devices are more severely injured, with worse physiology and more need for surgery and critical care. Mortality is also higher (Frykberg, Tepas and Alexander, 1989; Kluger *et al.*, 2004). The relative contribution of the components of blast injury is the subject of some debate. There is considerable variation by bombing type, but penetrating (secondary) injuries undoubtedly predominate (Arnold *et al.*, 2003). In a large review of terrorist incidents in diverse environments, 47% of immediate fatalities were found to have evidence of primary lung injury (Frykberg and Tepas, 1988). Primary blast lung injury is unquestionably an important cause of early death. As may be expected, primary blast lung injury is more common after explosions in confined spaces among patients admitted to hospital (77.5% confined space v 24.5% open space explosion) (Leibovici *et al.*, 1996). These Israeli experiences compare with the Madrid bombing: 18.7% of patients who were admitted to the nearest hospital had evidence of blast lung injury; these 17 patients made up 63% of the patients admitted to the critical care unit (Gutierrez de Ceballos *et al.*, 2005). On 7 July 2005, 4 suicide bombers killed 52

others and injured more than 700 people. The Royal London Hospital received the largest number of casualties. Seven required intensive care admission, three of whom had traumatic amputations. Despite this, and the enclosed space nature of all the incidents, none had primary blast lung injury (Shirley, 2006). The incidence of primary blast lung injury among hospitalised patients varied between 0 and 44% in one review of many incidents (Arnold *et al.*, 2003). This variation may reflect differences in data quality and definitions between incidents as well as the realities of the device, environment and casualties.

From the civilian clinical perspective, the important lesson is that these injuries occur, particularly in the critically ill population. A different point of view is held by a military healthcare system. It must expect to manage blast-exposed patients, rather than simply plan against a low probability, high impact event. It must design clinical facilities and prioritise research efforts on the basis of data about the incidence and consequence of primary lung injury. If this is common and has high immediate lethality, there must be a concentration on prevention and protection. If the immediate lethality is lower but the overall morbidity and mortality still high, there may be an opportunity for therapeutic intervention. In that case there must be more emphasis on clinical care and research. The crucial question is: how common is blast injury in military patients surviving to reach medical care?

Champion and colleagues have argued that the lethal range of fragments from most explosives exceeds that of the primary blast effect (Champion, Holcomb and Young, 2009). Many injury models require exposure to large peak overpressures to induce primary lung injury. This is equivalent to close proximity to typical military munitions, well within the radius of flight of fragments. Three factors may allow contemporary military casualties to sustain primary blast injury while surviving the effect of fragmentation. First, explosions in complex urban or other environments may involve the reflection and summation of blast waves, allowing primary blast effect beyond its free-field range, while fragments are impeded by other structures. Second, the lethality of penetrating fragments is related to the structures they traverse. The advent of advanced body armour for servicemen means they are more likely to survive explosions, albeit often with multiple amputations (Morrison, Hunt, *et al.*, 2012). This

is due to enhanced protection from secondary injury, which could make it possible to be exposed to injurious but sub-lethal primary blast without dying from concomitant fragment injury. Third, improvised explosives do not have the carefully engineered preformed fragments seen in military munitions. Indeed, they may not contain metal fragments at all to avoid detection. Chance variation in fragment behaviour would again allow immediate survivors to be blast-exposed.

Data from Iraq and Afghanistan have fuelled this debate. Ramasamy *et al* (2009) found that 2 (3.7%) of 53 IED injured patients admitted to a British field hospital had significant primary blast injury. Ritenour *et al* (2010) have since shown that 172 (3.6%) of 4765 US patients admitted after becoming victims of explosions in Iraq and Afghanistan had evidence of primary blast injury. Smith (2010) reviewed 1678 British cases (immediate deaths and those admitted to hospital) injured by blast and found that 113 (6.7%) had evidence of blast injury. Smith also found that 44% of these survived to reach medical care but that 24% of those died of their wounds.

These papers, covering heterogeneous time periods, environments and threats, demonstrate that primary blast injury is a significant threat to military patients, including a small group of initial survivors (perhaps 1 in 30 of all casualties in recent conflict, but a higher proportion of the severely injured). Preventative research is required but blast-injured patients will continue to present. Since it can be difficult to detect initially, military resuscitation practice should be optimised to the needs of patients with and without primary blast injury. Consequently, research must also address both patient groups.

2.3 Physiological Response to Injury

The physiological response to injury has several components: the cardiovascular and neurohumoral systems, inflammation, coagulation, metabolism and behaviour are all affected. These responses are intimately related to one another, sharing neural and molecular pathways, initiators and feedback mechanisms. The neurohumoral, metabolic and behavioural aspects are not relevant to this thesis and will not be explored further.

2.4 Cardiovascular Response

2.4.1 Cardiovascular Response to Conventional Trauma

The cardiovascular response to isolated haemorrhage involves two phases of sympathetically mediated changes (Little *et al.*, 1995).

- Initially, reduced venous return leads to reduced stroke volume due to Starling's law. This leads to a reduction in pulse pressure and consequently of arterial baroreceptor output. Through centrally mediated reflex arcs, this causes a loss of vagal output to the heart and then sympathetic activation. The former causes tachycardia. The latter is chronotropic, enhancing the tachycardia, and inotropic, increasing cardiac contractility and consequently stroke volume. These serve to ameliorate the fall in cardiac output. Peripheral sympathetic activation increases vascular resistance through arteriolar constriction. Increased total peripheral resistance and the relative preservation of cardiac output serve to maintain blood pressure. Since the arterioles of certain vital tissues beds (such as the brain) are not constricted by this process, their perfusion is maintained at the expense of others. Perfusion of local tissue beds is also modulated by the production of nitric oxide, its derivatives, carbon monoxide and potassium (Atkins, Johnson and Pearce, 2006).
- In the absence of other stimuli, further haemorrhage will eventually lead to the activation of a second, depressor reflex mechanism. This mechanism supersedes the baroreceptor reflex, activating the vagus and inhibiting sympathetic flow. The consequence is profound bradycardia and hypotension. This reflex response may be compounded by the production of a large amount of the vasodilator nitric oxide by the endothelium. Slowing the heart increases the diastolic interval, which may improve cardiac filling and coronary perfusion. Decreased afterload due to the fall in peripheral resistance may reduce cardiac work. The depressor response may therefore be protective. However, the reduced cerebral perfusion leads to depressed consciousness and fluid resuscitation is required to restore the blood pressure. Atropine will correct the bradycardia, but may endanger a patient's life (Little *et al.*, 1995).

Tissue injury or ischaemia leads to a quite different response. Increased sympathetic activation leads to increased total peripheral resistance with consequent increased blood pressure and redistribution of blood flow towards muscle and away from the gut and kidneys (Overman and Wang, 1947). It also stimulates a tachycardia. This would be opposed by a normal baroreceptor response. However, within a few hours of injury that response is altered, becoming less sensitive to hypotension and recalibrated to maintain a higher blood pressure. One consequence is a persistent tachycardia often seen after injury.

When tissue injury coexists with haemorrhage it attenuates the response to the latter. The early tachycardia is reduced by 10 to 15% and the late bradycardia abolished (Little, Marshall and Kirkman, 1989). This appears to be due to central inhibition of the efferent limb of the response to haemorrhage. The vasoconstriction caused by injury leads to better preservation of blood pressure. However, this seems to be at the expense of cardiac output, and consequently of oxygen delivery (Rady *et al.*, 1993).

Somatic nerve stimulation can simulate much of the modulation of cardiovascular response by musculoskeletal injury. Animals subjected to haemorrhage with nerve stimulation have higher mortality than those without stimulation (Overman and Wang, 1947). Normally oxygen uptake by the tissues is governed by metabolic demands, the supply of oxygen being more than adequate. In haemorrhage with musculoskeletal injury, local blood supply can become inadequate as total body oxygen delivery falls (DO_2) since blood is diverted away from some vascular beds. When this occurs, their oxygen consumption ceases to be independent of DO_2 and this is reflected in a fall in total body consumption of O_2 (VO_2). The level of DO_2 at which VO_2 becomes dependent on DO_2 is called the critical oxygen delivery. It is higher in haemorrhage with brachial nerve stimulation than without (Kirkman *et al.*, 1995).

It is interesting to speculate upon why this modulation is adaptive. It may be that it gives some protection at lesser degrees of blood loss (which were perhaps more common than severe haemorrhage in the lives of our ancestors). The cardiovascular effects of musculoskeletal injury mimic the visceral alerting response of the defence reaction, which exists to prepare the body for fight or flight and activates in response

to fear and other stimuli. Preparing the body for the muscular work that is required to avoid further injury may very well be an adaptive response to musculoskeletal damage.

2.4.2 Cardiovascular Response to Blast Injury

Exposure to thoracic blast produces a characteristic cardiovascular response (Kirkman and Watts, 2010). This is characterised by hypotension and bradycardia, which begin to develop after 2s and 4s respectively (Ohnishi *et al.*, 2001). This is consistent with its being a reflex response, not due to direct effects on the heart or CNS. The initial bradycardia is profound: in rats, an increase in the mean heart period/beat-to-beat interval from 133ms to 489ms. This recovers after 5 minutes with a more moderate bradycardia (heart period around 160ms) persisting for several hours. Mean blood pressure recovers from a nadir of 40mmHg to 70mmHg, also over five minutes, but further recovery to 100mmHg takes 1hr and some hypotension persists for 24hrs. Blast also causes apnoea, which in this model lasted 28s.

The efferent pathway mediating the bradycardia is the vagus nerve. Vagotomy abolishes the bradycardia but (in the Ohnishi model) only partially corrects the hypotension. Atropine blocks the bradycardia without modulating the hypotension. Thus the two are separately mediated: the bradycardia's afferent and efferent loops are vagal; the hypotension has vagal and either non-vagal elements or a non-reflex component. Systemic vascular resistance is reduced for some time after blast (Irwin *et al.*, 1999). The vagally mediated components of these changes are consistent with the pulmonary defence reflex mediated by the pulmonary afferent C fibres (Ohnishi *et al.*, 2001). The fall in SVR may therefore be due to the sympathoinhibition which is a feature of this reflex (Daly and Kirkman, 1988). In addition, blast is known to induce the production of large amounts of NO (Žunić *et al.*, 2000), which causes vasodilatation. Finally, embarrassment of the cardiac musculature by air embolism or contusion may be the cause of the hypotension.

Sawdon, Ohnishi *et al* combined haemorrhage and blast in a rat model (Sawdon *et al.*, 2002). Haemorrhage began 10 min after thoracic blast exposure (i.e. after resolution

of the initial extreme bradycardia). Blasted animals had a lower blood pressure at the onset of haemorrhage and were unable to maintain it at that level as blood was lost; sham blasted animals initially preserved normal MAP. The bradycardia due to haemorrhage appears to have occurred earlier (after less blood loss) in blasted than in non-blasted animals. At the extreme of blood loss, cardiovascular parameters in the two groups converged, presumably reflecting a narrower range of possible combinations available in the context of marked hypovolaemia.

2.5 Inflammatory Response

Inflammation is fundamental to the pathophysiology of trauma. While most deaths occur early, due to head injury and haemorrhage, (Trunkey, 1983) sepsis causes 3.1-17% of deaths and organ failure 1.6-9% (Pfeifer *et al.*, 2009). Perhaps 25% of trauma victims develop multiple organ dysfunction syndrome (MODS) (Ciesla *et al.*, 2005). Critical illness accounts for 42 to 52% of acute care costs for injured patients(Christensen *et al.*, 2008) and has significant long term sequelae (Ulvik *et al.*, 2007). Inflammatory processes underlie all of these problems.

The processes described in this section are associated with characteristic clinical phenotypes. Widespread activation of inflammation is recognised in the Systemic Inflammatory Response Syndrome (SIRS). SIRS is not necessarily a pathological state and is characterised by (at least two of) pyrexia (or hypothermia), tachycardia, tachypnoea and leucocytosis (Bone *et al.*, 1992). SIRS arises in response to both injury and infection, and consequently the manifestation in trauma and sepsis can be indistinguishable. Initial injury, systemic inflammation and subsequent sepsis all interact to lead to MODS. SIRS is accompanied by a simultaneous upregulation of immunosuppressive mechanisms which particularly affect the adaptive immune system. The compensatory anti-inflammatory syndrome (CARS) or immunoparalysis, leaves the patient vulnerable to subsequent infection (Tschoeke and Ertel, 2007). CARS appears to be a misnomer: the immunosuppression was originally thought to be a delayed reaction to the initial inflammation but studies that block the latter (Remick *et al.*, 1995) or look for genomic correlates of both phenomena (Xiao *et al.*, 2011) suggest that this is not the case. In recent years, the supportive management of MODS

and the identification and treatment of sepsis has greatly improved. However, there may be a group of patients in whom this has uncovered a late syndrome of persistent inflammation, immunosuppression and catabolism (PICS) (Gentile *et al.*, 2012).

The mechanisms of dysfunction in individual organs, of the failure to terminate inflammation and of immune suppression are beyond the scope of this discussion. This section will give a "bottom-up" description of the inflammatory response, which underlies all of these, describing its initiation and early mediation. This will allow later sections to draw connections between inflammation and coagulation.

The innate immune system is a highly conserved homeostatic mechanism that protects the body from injury and infection. Under normal conditions, inflammation is followed by resolution and repair. Components of inflammation are involved in other homeostatic mechanisms (e.g. tissue repair, control of metabolism, hormonal regulation) which are not discussed. It is useful to divide the elements of the inflammatory response into functional groups (Medzhitov, 2008). The threat to tissue homeostasis, or insult, generates effects at levels ranging from the cell to the whole organism. These in turn generate or reveal molecular signals that are the **initiators** of inflammation. These are detected by a series of non-cellular and cellular **sensors**. The sensors then activate the (non-cellular) **mediators** and (cellular) **effectors** of inflammation (see Figure 2.4). In order to reach and combat the threat, the principle effectors—neutrophils and macrophages—are able to cause local tissue destruction through the release of enzymes and reactive oxygen species (ROS) (Nathan, 2002). Since many of the mediators and effectors are circulating molecules and cells, the key first stage in inflammation is an increase in blood flow (*calor, rubor*) and an increased, selective permeability of the endothelium (*tumor*). This allows the mediators and effectors to be recruited to the site of the insult. Swelling and the presence of certain initiators and mediators in the tissue leads to activation of nociceptors (*dolor*).

The inflammatory response uses positive feedback mechanisms to promote quick response to threats, and negative feedback to localise and control the response (Nathan, 2002; Chen and Nuñez, 2010). Without microorganisms, damaged tissue quickly moves on to repair—the weal from a slapped wrist quickly fades, the

inflammation of a surgical wound is mild and resolves if it does not become infected. Equally, colonisation by commensal bacteria is not a threat that requires constant inflammation of all the skin and gut wall. These mechanisms of discrimination and control can be overstimulated by large insults to which they are not adapted, causing, respectively, organ injury and immunosuppression.

2.5.1 Initiators and Sensors in the Activation of Inflammation

Trauma has direct effects at the cellular, tissue, organ and organism levels. At the cellular level, it leads to the disruption of cell membranes, causing necrosis. Injury to tissues can disrupt tissue architecture, expose subendothelial molecules and allow toxic substances, such as haem, to enter the interstices. Injured organs can become dysfunctional or disrupted. This discussion will concentrate on the effect of injury to respiratory and circulatory structures. These lead to hypoxaemia, haemorrhage and impaired perfusion which in turn cause local or systemic ischaemia, the latter being shock. Adaptive processes and treatment of local ischaemia or shock leads to reperfusion (locally: ischaemia-reperfusion—I/R; systemically: haemorrhagic shock-resuscitation—HS/R) which can actually worsen the insult. These in turn lead to further cellular stress, which can lead to apoptosis or necrosis. It is this interplay of events that generates the diverse initiators of inflammation seen after severe injury.

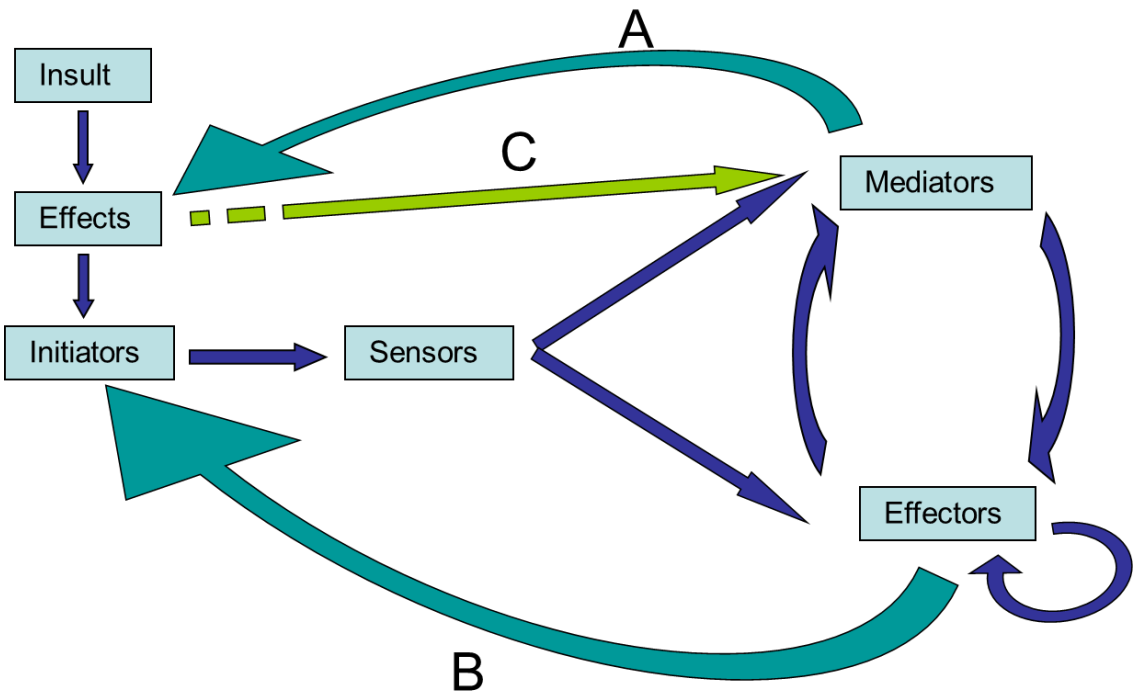


Figure 2.4 Initiation of inflammation. (Derived from Medzhitov, 2008) Insults, such as trauma, lead to effects at the cellular/extracellular level. These lead to the production of endogenous initiators. (Exogenous initiators [not shown] include molecules produced by microorganisms [PAMPs – see Subsection 2.5.3] and others such as allergens.) Sensors for these initiators may be extracellular molecules such as natural antibodies, or cellular receptors, such as TLRs. Mediators include the protease cascades, which are principally initiated by extracellular sensors, and lipid mediators, cytokines and chemokines, which are released by effectors stimulated by cellular sensors. There is then crosstalk between mediators and effectors, producing both positive and negative feedback loops depending on the situation. Effectors also communicate directly with one another, e.g. via adhesion molecule interactions.

However, mediators are able to cause damage to cellular and extracellular molecules (i.e. “effects”) by, for example, the action of proteases and alterations in oxygen delivery due to coagulation and vasoactive mediators (arrow A). This leads to the formation of further initiators, which combines with active production by mediators (arrow B, an example would be release of HMGB-1 by macrophages), forming positive feedback loops. Additionally, some effects can imitate mediators, e.g. through the peroxidation of lipids to generate PAF-like lipids that can ligate the PAF receptor; arrow C). (This could equally be regarded as an initiator-sensor pathway which is then harnessed to be used by a mediator, PAF.)

The key points are redundancy, which is a reflection of the complexity of the system, and the existence of feed-forward and feed-back loops. The latter include inhibitory mechanisms which regulate inflammation and are not shown here.

PAMP—Pathogen-associated molecular pattern; TLR—Toll-like receptor; HMGB-1—High Mobility Growth Box 1; PAF—Platelet-activating factor.

2.5.2 Ischaemia/Reperfusion

During ischaemia, low oxygen tension causes dysfunction in the oxygen transport chain in mitochondria, leading to the release of highly reactive free radicals: reactive oxygen species (ROS) (Fischer, 2006). As ROS levels rise they overcome cellular defence mechanisms to cause damage to functionally vital structures such as membrane molecules, which can lead directly to cell dysfunction or lysis. ATP production falls and the existing supply is consumed, producing ADP and then hypoxanthine. Cell membrane ion pumps begin to fail, leading to swelling and membrane dysfunction. Mitochondrial contents are released, which can initiate cell death. While restoration of oxygen supply is necessary for cell survival, it also leads to the accumulation of more destructive substances: accumulated hypoxanthine reacts with oxygen to produce more ROS. Reactive nitrogen oxygen species (RNOS) derived from nitric oxide (NO) also play an important role.

Finally, ischaemic injury causes endothelium to expose a non-muscle myosin heavy chain antigen (Zhang, Alicot, *et al.*, 2006). A natural antibody exists to this antigen, i.e. a circulating, specific, monoclonal IgM not produced by prior pathogen exposure (Zhang *et al.*, 2004); this activates complement (Zhang, Takahashi, *et al.*, 2006). Since complement deficient animals are protected, this is one of the most important mechanisms of I/R injury (Chan *et al.*, 2003).

2.5.3 Products Released by Cellular Disruption and Extracellular Damage

In normal tissue and cells there is extensive compartmentalisation which separates, for example, nuclear contents from the cytoplasm and blood from the extracellular matrix (Nathan, 2002). After injury this compartmentalisation is broken down, whether by direct trauma, indirectly through I/R or as a result of inflammatory processes. Previously separated receptors and their ligands, or enzymes and their activators or substrates, can now interact. This is one of the principle mechanisms which generates endogenous initiators and exposes them to their counterpart sensors.

Cellular disruption releases many substances including ATP, uric acid, heat shock proteins and high-mobility growth box-1 (HMGB-1) (Bianchi, 2007). These molecules are part of a heterogeneous group known as damage-associated molecular patterns (DAMPs). DAMPS are highly conserved cell molecules which are detected by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). TLRs are expressed by a variety of innate and adaptive immune cells and by epithelial and parenchymal cells (Kaczorowski *et al.*, 2008). Their activation leads to increased production of proinflammatory gene products. (DAMPs have their exogenous counterparts, pathogen-associated molecular patterns [PAMPs]; examples include lipopolysaccharide [LPS] and formyl-methionyl-leucyl-phenylalanine [fMLP].)

HMGB1 is a nuclear protein that binds DNA, and is one of the most studied DAMPs. As well as being released by necrotic cells (Scaffidi, Misteli and Bianchi, 2002) it is actively secreted by a variety of cells including monocyte/macrophages and endothelial cells (Mullins *et al.*, 2004; Qin *et al.*, 2006). Systemic inflammation is induced in mice by bilateral femur fracture (Levy *et al.*, 2007). This can be almost abolished by blockade of TLR-4 or by use of HMGB-1 neutralising antibodies, but there is no synergy between the two methods of blockade. This indicates a profound importance for the HMGB-1/TLR-4 signalling axis in the pathophysiology of systemic inflammation in this model (Levy *et al.*, 2007). Haemorrhagic shock also induces HMGB-1 mediated systemic inflammation (Kim *et al.*, 2005).

Serum levels of HMGB-1 are raised within 30 minutes of severe injury complicated by hypoperfusion. The rise in HMGB-1 correlates with the severity of injury, tissue hypoperfusion, early posttraumatic coagulopathy and hyperfibrinolysis as well as with systemic inflammatory response and activation of complement (Cohen *et al.*, 2009). The relative contribution of active and passive release of HMGB-1 in these situations is unclear.

2.5.4 Effectors and mediators of inflammation

Inflammation is driven by the action of effectors (cellular components) and mediators (molecular components). The following subsections briefly describe the principle

actions of the most important of these in the initiation of inflammation: complement, which responds to signals of I/R injury and leads to mast cell activation and neutrophil recruitment; mast cells and macrophages, which respond to complement and directly to injury and other stimuli and which serve to activate endothelium and recruit neutrophils; and endothelium, which forms the barrier between blood and tissue but which, once activated, becomes the means by which neutrophils are summoned and guided to the site of injury. In a subsequent subsection, the key role of neutrophils in initiating inflammation will be explored. Before that, there is a brief discussion of the largest class of mediators, cytokines, with emphasis on how they signal for the events which lead to neutrophil recruitment.

2.5.5 Complement Activation

Complement is a highly conserved protease cascade and the principle early mediator of inflammation after I/R (Arumugam *et al.*, 2004). Activation leads to mast cell stimulation (Metz and Maurer, 2007), the release of anaphylatoxins and the formation of the membrane attack complex (MAC), which can lyse both bacteria and host cells. The anaphylatoxin C5a is chemotactic to neutrophils and can increase vascular permeability and upregulate the expression of endothelial adhesion molecules. Complement molecules can also be initiated by other mediators of inflammation such as proteases (Huber-Lang *et al.*, 2006) and thrombin (Markiewski *et al.*, 2007). Deposition of MAC is seen extensively after I/R (Carden, Smith and Korthuis, 1990; Hugo *et al.*, 1990; Weiser *et al.*, 1996) and may be a significant pathway of complement-mediated injury.

Systemic complement activation is seen after trauma within half an hour and correlates with injury severity and tissue hypoperfusion, and with mortality and inflammatory complications (Fosse *et al.*, 1998; Ganter *et al.*, 2007). While the classical/lectin pathway is activated, amplification via the alternate pathway is correlated with severity and outcome (Ganter *et al.*, 2007).

2.5.6 Mast Cells and Macrophages

Mast cells possess large cytosolic granules that contain histamine, other mediators and proteases (Metz and Maurer, 2007). Degranulation follows seconds after stimulation and is accompanied by increased synthesis of TNF- α, of other cytokines and chemokines and of the fatty acid derivatives platelet-activating factor (PAF) and the leukotrienes. Mast cell-deficient mice develop significantly less muscle fibre death (Bortolotto *et al.*, 2004) and lung injury (Goldman *et al.*, 1992) after limb I/R.

Macrophages are also contain granules and able to release cytokines and proteases to induce inflammation. Their reaction to initiators of inflammation is less florid than that of mast cells but it is also more sustained. Macrophages (Medzhitov, 2008) are also able to detect the transition of tissue cells between the discreet states of normality, stress, apoptosis and necrosis; they also appear to be able to distinguish between different causes of apoptosis, though the mechanism is unclear. Macrophages can therefore detect, integrate and report the status of many cells in a tissue bed, altering the inflammatory (and other) responses accordingly.[*]

2.5.7 Endothelium

Mast cell tryptase and other proteases cleave the terminal residues of protease activated receptors (PARs) on mast cells, neurones, endothelium and neutrophils. The neoterminal residues of the PARs self-engage, i.e. engage the PAR's own binding site, activating their cytosolic G-proteins. This leads to further activation of mast cells and neurones. It makes endothelium more adhesive to leukocytes and it causes leukocytes to release PAF. Along with mast cell-derived PAF, this in turn reinforces endothelial activation and attracts leukocytes into the tissue.

Endothelial cells can be activated by other cells using a variety of mediators and express their own TLRs. They also transduce mechanical information related to blood flow via the glycocalyx, altering cell function, particularly NO and prostacyclin

[*] This description excludes the important role that macrophages later play in the resolution of inflammation and the initiation and control of healing.

production (see subsection 2.7.5) (Tarbell, Weinbaum and Kamm, 2005). Catecholamines have an important role in activating endothelium (Johansson *et al.*, 2011b). In the first stage of endothelial activation, cells contract, exposing the subendothelial membrane, which allows fluid, mediators and effectors to pass into the extracellular space (Hunt and Jurd, 1998). (When intravascular hydrostatic pressure is low, e.g. due to haemorrhage, endothelial activation may conversely allow interstitial fluid to pass into the circulation, leading to a form of autoresuscitation.) The Weibel-Palade bodies undergo exocytosis, releasing von Willebrand factor (vWF) into the blood and bringing P-selectin to the surface. The second stage of activation requires *de novo* synthesis of cytokines, chemokines, tissue factor and adhesion molecules. Activation causes endothelium to lose its normal anticoagulant phenotype.

Activated endothelium therefore promotes traffic of molecules and cells between the compartments and promotes coagulation. These are adaptive changes which facilitate the inflammatory response (coagulation helps contain infection as well as limiting blood loss).

Activation of large areas of endothelium affects systemic circulation and leads to massive fluid shifts. It also releases inflammatory mediators into the circulation. In sufficient concentration these may prime circulating neutrophils and activate endothelium at uninjured sites. This ectopic activation then opens the door for neutrophil infiltration and consequent injury to bystander organs. Endothelial activation should be a quickly reversible state. However, sufficient damage can render endothelium unable to step down to the resting state or it may induce apoptosis (Hotchkiss *et al.*, 1999; Guan *et al.*, 2002), with desquamation and loss of vascular integrity.

2.5.8 Cytokines

There is a prodigious literature on cytokine signalling that cannot be summarised here. Some important molecules will be discussed briefly. IL-1β and TNF-α are produced by monocytes/macrophages, mast cells, epithelium and other immune and non-immune cells in response to stimuli that include complement, NO, ROS, TLR ligands and

themselves. Their effects include autocrine induction of other cytokines, priming of neutrophils and activation of endothelium. Both can produce anti-apoptotic effects that may be important in sustaining inflammatory response but TNF is also able to induce apoptosis in target cells under certain circumstances.

The elaboration of chemokines, a subclass of cytokines, is crucial to the recruitment of effector cells, which can migrate up a chemokine concentration gradient to reach the point of release. The particular pattern and effect of this varies according to tissue, stimulus and other factors. Chemokine production in animal models of lung contusion (Raghavendran *et al.*, 2005) serves as a good example. This is not least because bronchoalveolar lavage fluid provides a window onto tissue chemokine levels without the need for biopsy. Neutrophil attractant chemokines (CXCL-1 and -2) rises early and peaks at 24 hours after contusion. The monocyte attractant CCL-2 rises by 24 hours but peaks at 48 hours. All return to normal by 7 days. As one would expect, neutrophils infiltration precedes the increase in lung monocyte/macrophages. Chemokines can be immobilized on endothelium (McDonald *et al.*, 2010), allowing effectors to migrate intravascularly along the gradient so formed until very close to the site of inflammation. While cytokine and chemokine signalling has diverse functions, their particular role in this account of the induction of inflammation is the activation and recruitment of neutrophils.

2.5.9 Neutrophils and the Two-Hit Hypothesis

Neutrophils are the primary early effector cell in innate immunity (Nathan, 2006). After injury there is systemic release of steroids, catecholamines and proinflammatory cytokines. These probably lead to bone marrow neutrophil mobilisation, and (in the blood) to activation.

Neutrophils carry a deadly arsenal of cytotoxic substances (Borregaard *et al.*, 1995), yet circulating neutrophils must pass through capillaries that are narrower than the resting cell without releasing them (Nathan, 2006), Marginated cells must scout for signals indicating injury to local tissue. Weak adherence between the neutrophil's surface molecules (L-selectin and leucocyte adhesion molecule-1 [LAM-1]) and those of

the endothelium (E-selectin, P-selectin and endothelial leucocyte adhesion molecule-1 [ELAM-1]) allow the former to roll along the vessel wall. Activated neutrophils and endothelium express integrins (CD11a/CD18) and intercellular adhesion molecule-1 (ICAM-1), respectively. These allow stable adhesion of the neutrophil to the vessel wall. It subsequently spreads and then passes between endothelial cells by diapedesis into the extravascular space.

Neutrophil degranulation is stimulated by a complex interaction of signals that integrate information about the cell's location and the presence of tissue injury and microorganisms (Nathan, 2006). While both of the latter must usually be detected, activation may occur after trauma alone because some initiating signals (e.g. HMGB-1 or fMLP from disrupted nuclei and mitochondria, respectively) may signal via the same pathways as foreign material. Degranulation releases, sequentially, adhesion molecules, signals to increase vascular permeability and enzymes which can degrade the extracellular matrix (matrix metalloproteases). Neutrophils may then be guided from the point they leave the blood to the site of inflammation by chemokines and other signals, including mitochondrial DAMPs (McDonald *et al.*, 2010; Zhang *et al.*, 2010). This process takes less than an hour (Nathan, 2006; McDonald *et al.*, 2010) at which point the neutrophil will attempt to phagocytose bacteria found at the site of inflammation. In their absence, full activation ensues (Nathan, 1987), leading to a respiratory burst and degranulation. In the respiratory burst, iNOS and NADPH oxidase cause a massive production of NO and superoxide, which in turn form other highly damaging ROS and RNOS. The remaining granules release proteinases, other enzymes, antibiotic molecules and oxidants. The latter activate MMPs. In combination these acts give rise to destruction of the extracellular matrix and damage to cells. Neutrophils may also undergo a specialised form of cell death (NETosis) in which they eject their DNA to form neutrophil extracellular traps (NETs) (Kaplan and Radic, 2012). These are coated in many of the cell's antimicrobial effectors.

These changes make the tissue more accessible to immune cells and inhospitable to microorganisms, but at some cost to the structure and function of the tissue. Natural wound healing without these actions, as in immune deficiencies, is prevented by infection (Lekstrom-Himes and Gallin, 2000). However, where micro-organisms are

absent, neutrophils slow wound healing (Dovi, Szpaderska and DiPietro, 2004; Shaw and Martin, 2009). Through activation in normal tissue, they also cause injury at distant sites.

The neutrophil predominates in early systemic inflammation just as it does locally (Weiss, 1989; Baue, 1992). The importance of the neutrophil is not just as the effector of tissue defence and tissue damage but, before that, as the decision-maker of the innate immune system. It integrates different signal types through the multiple stimulation required for full activation. After severe injury, multiple initiators generate redundant contributions to that stimulation, leading to widespread and inappropriate migration and activation.

The following is a summary of likely events. There is a marked early neutrophilia (Botha *et al.*, 1995) (at about 3 hours post injury). Subsequently there is a drop in the neutrophil count (by 6 to 12 hours). This is significantly greater in patients who go on to develop MOF than in those who do not. This presumably represents the sequestration of activated neutrophils in the tissues. So it appears the activated cells are sequestered and are replaced by immature cells from the marrow (Hietbrink *et al.*, 2006). The activation of circulating neutrophils is matched by an increase in their response to *in vitro* stimulation by LPS or fMLP, known as "priming" (Moore *et al.*, 2005; Hietbrink *et al.*, 2006). This is also reduced later after injury.

Neutrophil priming has been implicated in the "two-hit" hypothesis of the development of post-injury MODS (Moore *et al.*, 2005). This holds that the initial trauma with its associated tissue injury and physiological disturbance constitute a "first hit", leading to neutrophil priming. The period during which primed neutrophils circulate, or even enter the tissues without causing damage (Anderson *et al.*, 1991), is then a vulnerable window during which a "second hit" will lead to an excessive and systemic inflammatory response by the neutrophils. Second hits include such immunologically potent events as surgery (Lasanianos *et al.*, 2011), blood transfusion (Bilgin and Brand, 2008) and infection (Moore *et al.*, 2005). Importantly, this hypothesis suggests that the inflammatory reaction after the second hit is more than

simply the addition of the responses which would be expected to the two insults separately.

While some individual patients are seen to have a florid inflammatory response to a particular second hit, a more usual situation may be a series of priming stimuli and a series of activating "second hits". This makes confirmation of the two-hit hypothesis difficult both because of the heterogeneity of patient studies and the difficulty of designing models that reflect the complex interaction between trauma pathophysiology and therapy. Genomic studies have shown no correlation between potential second hits and changes in leucocyte gene expression (Xiao *et al.*, 2011). (This alone is not a sufficient rebuttal of the hypothesis: the profound initial leucocyte genome expression changes after injury may give rise to the "primed" phenotype, while subsequent activation may not have a genomic signature.)

In addition, studies of the two-hit hypothesis have concentrated on the *ex vi*vo biology of neutrophils (Moore *et al.*, 2005). However, inflammation has redundant pathways. Neutrophil activity and the action of terminal complement elements act independently after I/R (Kyriakides *et al.*, 1999). Blockade of either pathway reduces endothelial permeability after hind limb ischaemia by nearly 50%. Permeability is halved again when both are blocked. Equally, neutrophil injury to bystander organs implies activation of the latter's endothelium in order to attract the neutrophils and permit them to enter the tissue. Modern accounts of the gut hypothesis have begun to make these linkages (Clark and Coopersmith, 2007).

2.5.10 The Gut Hypothesis

The mucosa associated lymphoid tissue (MALT) of the gut is the largest lymphoid "organ" in the body. The splanchnic circulation is the first to be sacrificed and the last to be restored in low flow states (McNeill, Stark and Greenway, 1970). Poor perfusion of the gut is associated with loss of gut barrier function (Fink, 2003). Although no evidence has been found that gut-derived bacteraemia or endotoxaemia is the cause of systemic inflammation after injury (Moore *et al.*, 2005), this does allow altered interaction between luminal molecules and microorganisms and the MALT (Clark and

Coopersmith, 2007). Studies have demonstrated that the gut lymph of animals subjected to HS/R (but not the portal blood) (Magnotti *et al.*, 1998) contains substances that can cause endothelial activation and apoptosis in the lung (the first organ to be perfused by blood carrying the lymph deposited in the subclavian vein) (Davidson *et al.*, 2004; Deitch *et al.*, 2004) and activate circulating PMNs (Gonzalez *et al.*, 2001). Two components have been identified as being of particular importance: a modified albumin and various lipid factors (Kaiser *et al.*, 2005). Division of the gut lymphatics can prevent HS/R induced ALI in the models used and administration of HS/R gut lymph to healthy animals can induce ALI (Senthil *et al.*, 2007). Thus in these models, HS/R gut lymph is necessary and sufficient to cause ALI after HS/R. However, isolated limb I/R can cause ALI without systemic hypoperfusion (Kyriakides *et al.*, 2000). Hindlimb (non-gut) lymph after HS/R can also cause endothelial dysfunction and neutrophil priming (Diebel *et al.*, 2008, 2009). Non-gut lymph may therefore contribute to both ALI after both systemic and isolated hypoperfusion. Additionally, most HS/R models do not simulate the tissue injury of major trauma, thus the relative importance of the hypoperfused gut and the damaged tissues is not known.

It is clear that the gut has an important role in inflammatory initiation after injury. However, the systemic release of DAMPs, complement proteins, cytokines and lipid mediators, along with the circulation of activated mediators, generate profoundly redundant pathways for the systemic activation of inflammation.

2.6 Normal Coagulation

The two principle components of coagulation are platelets and the clotting factor cascade. Vessel injury reveals tissue molecules that activate both elements. The formation of a clot begins with the formation of a platelet plug. The clot then matures and strengthens as fibrin is laid down and cross linked. Crucially, platelets are the principal site of clotting factor activity. This section describes these principal components of coagulation according to the cell-based model of coagulation developed by Hoffman (Monroe and Hoffman, 2006). Assays used to measure platelet and clotting function are described in section 6.3.

2.6.1 Platelets

Platelets are discoid cell fragments 2-4µm by 0.5µm which tend to circulate at the margins of blood vessels, pushed there by large cells which occupy the fast flowing centre (Loscalzo and Schafer, 2003). They normally contact but, unlike leucocytes, do not interact with the intact, unactivated endothelium. They have complex structures (see Figure 2.5) with multiple cytosolic granules (Jurk and Kehrel, 2005). α-granules are the most abundant, containing large adhesive proteins such as vWF, growth factors (e.g. platelet-derived and vascular endothelial growth factors), clotting factors (V, VII, XI, XIII), and regulators of coagulation (protein C, plasminogen activator inhibitor 1 [PAI-1], tissue factor pathway inhibitor [TFPI]). Dense granules contain secondary agonists of platelets, which when released will signal for further platelet activation in an autocrine and paracrine manner. They are also a store of calcium, vital to platelet activation and coagulation, as is the dense canalicular system

Platelets also have an open canalicular system (OCS), a network of conduits lined with cell membrane passing through the body of the platelet (Jurk and Kehrel, 2005). Since the outer membrane itself is inaccessible due to the platelet's actin cytoskeleton, exocytosis actually occurs initially into the OCS, with adhesion molecules then redistributing to the exterior. The OCS also provides a store of cell membrane for use when the platelet spreads or divides. A marginal band of microtubules forms a cytoskeleton; associated microfilaments act on this to permit shape change.

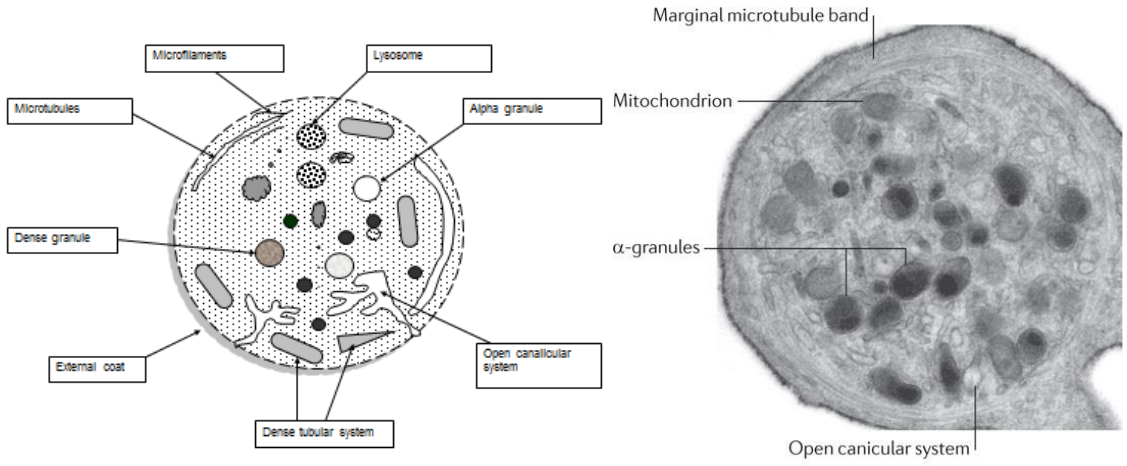


Figure 2.5. Schematic (Mbassa, 2015) (left) and scanning electron micrograph (Semple, Italiano and Freedman, 2011) (right) of the structure of a platelet.

Platelets have multiple roles in clot formation (Monroe and Hoffman, 2006). Coagulation factors are bound to the platelet surface (factors V, VIII and IX) and thus this is the site of most thrombin formation. Vessel wall damage exposes platelets to a variety of stimuli including collagen, fixed vWF, ADP and thrombin, which lead to activation. Initially, Glycoprotein Ib (GPIb) interacts with vWF, tethering the platelet to the area of damage. GPVI then interacts with collagen. Activation of these receptors leads to intracellular signals that cause a conformational change in the GPIIa/IIIb complex (integrin $\alpha_{IIb}\beta_3$, CD41/CD61).* The newly exposed binding site can then bind to vWF (fixing the platelet in place) or fibrinogen (allowing crosslinking). ADP and TXA_2 are also released, leading to autocrine stimulation via $P2Y_{12}$ and TP receptors respectively. ADP serves mostly to potentiate other elements of platelet activation. Thrombin generation (see subsection 4.1.2) strongly activates platelets via PAR-1.

Activation leads to changes in platelet morphology: they sphericise, the surface becomes blebbed and then the platelet begins to spread out across a surface. Granule content is released, bringing P-selectin (CD62P) and platelet-endothelium cell adhesion molecule (PECAM, CD31) to the surface.

Platelets' activation leads to their adherence to damaged vessel wall and their aggregation with each other (and other blood cells). They thereby form a plug which, along with contraction of the blood vessel, begins to block the loss of blood from the vessel. Activation also leads to the expression of negatively charged phospholipids on the platelet surface and the release of small fragments of platelets less than 2µm across known as platelet-derived microvesicles (MV_{plt}) (Martínez *et al.*, 2005). These provide the surface needed for the generation of large amounts of thrombin, which in turn leads to stabilisation of the clot with cross-linked fibrin. The final important role of platelets is contraction, which is mediated by the platelet's actin cytoskeleton, which is linked to the CD41/61 complex at the platelet surface and thereby to the fibrin network (Loscalzo and Schafer, 2003). Clot retraction serves to minimise the size of the clot, particularly the obstruction it may cause to the flow of blood in the vessel.

* Platelets are the only blood component that express this complex which is therefore widely used as a platelet specific marker.(Macey *et al.*, 1999; Jacoby *et al.*, 2001)

2.6.2 Microvesicles

Microvesicles are small cell fragments released by endothelial, circulating and tissue-resident cells. Some are produced constitutively and others in response to stressors or apoptosis. Microvesicles can differ in the nature of their cell wall, the surface markers they express and their cytosolic makeup. The nature of microvesicles is determined both by the parent cell and the stimulus. As such, microvesicles can function as specific mediators or messengers of the parent cell. Microvesicles in the circulation may be important in the crosstalk between coagulation and inflammation (Morel *et al.*, 2011).

Microvesicle production is generated by a variety of mechanisms, whose interaction and relative importance is not clear (Morel *et al.*, 2011). In resting cells, phosphatidylserine (PS) is preferentially expressed on the internal layer of the cell membrane lipid bilayer. Cell stimulation leads to increased intracellular calcium which induces active transport of PS to the external layer. As already noted, the resultant negatively charged membrane is thrombogenic. This change, along with mechanical effects, loss of cytoskeletal anchoring and release of mitochondrial contents, causes instability of the membrane which leads to microvesicle formation.

In normal subjects the largest group of circulating microvesicles are directly derived from megakaryocytes (Italiano, Mairuhu and Flaumenhaft, 2010). Platelets shed MV_{plt} during activation and these are estimated to have procoagulant activity 50 to 100-times greater than the parent platelet. MV_{plt} express large amounts of PS and have a high surface-area-to-volume ratio. MV_{plt} formation takes about 15s and therefore follows initial aggregation and adhesion, serving to promote the clotting cascade (Italiano, Mairuhu and Flaumenhaft, 2010). As clot forms, an early predominance of leucocyte microvesicles is overtaken by their platelet-derived counterparts. The former, which express a considerable amount of tissue factor, may have an important role in clot initiation. The latter certainly are important: Scott's syndrome is a bleeding diathesis that occurs in patients with impaired platelet microvesicle formation. In summary, MV_{plt} appear to after formation of the initial platelet plug and appear to potentiate the formation of fibrin, even outpacing their parent platelets in this regard.

Formation of both procoagulant enzyme complexes and the anticoagulant protein C complex requires PS expression but the latter may require a 10-fold higher concentration (Morel *et al.*, 2011). MV_{plt} may therefore be important in protein C-mediated coagulopathy (see section 2.7). Brain-derived microvesicles have been implicated in coagulopathy after head injury (Zhang *et al.*, 2012).

2.6.3 Fibrin Generation

The understanding of the mechanisms of fibrin formation have been revolutionised by the introduction of Hoffman's cell-based model (Monroe and Hoffman, 2006). This replaced the simplistic notion of the intrinsic and extrinsic clotting cascades, which took no account of the spatial relationship between the molecules involved and failed to explain several aspects of coagulation physiology and pathology. Clot formation proceeds in three stages: **initiation**, **amplification** and **propagation** (Monroe and Hoffman, 2006). These lead to the production of large quantities of thrombin, which in turn leads to the formation and crosslinking of fibrin polymers.

Initiation. Damage to the wall of blood vessels exposes cells that express tissue factor (TF). Factor VII (fVII) binds to the TF and is activated by a variety of proteases to form fVIIa. TF/fVIIa on cell surfaces then activates fX and fXI. fVa, released from platelets as they start to adhere or activated from circulating fV by fXa and other proteases, forms a complex with fXa on the cell surface. This fXa/Va complex then generates a small amount of thrombin. See Figure 2.6. A small amount of fIXa is also generated.

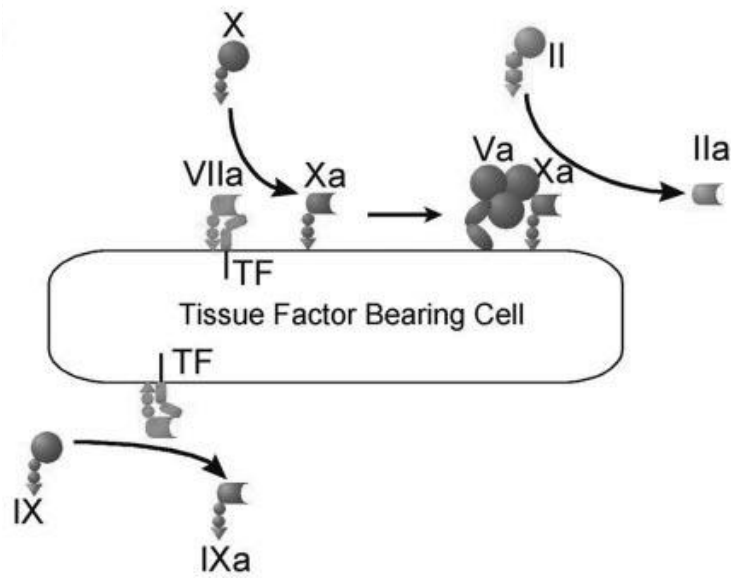


Figure 2.6. Initiation. Modified from Monrow and Hoffman 2006. See text.

Amplification. The thrombin generated during initiation then works in concert with tissue collagen to induce platelets adherent to the site of injury to enter their most activated phenotype. They therefore release their granule contents, adhere and aggregate and alter their surfaces to provide the arena for the clotting factor activity during propagation. The thrombin also activates fV, fVIII (releasing it from vWF) and fXI on the platelet surface.

Propagation. fXIa activates fIX, which (along with fIXa from initiation) then complexes with fVIIIa. fVIIIa/IXa then cleaves fX and the resultant fXa associates with fVa to form the prothrombinase complex. See Figure 2.7. This then produces large amounts of thrombin, sufficient both to cleave fibrinogen and to sustain a positive feedback loop by further activation of upstream factors. All of these reactions are localised to the platelet (or microvesicle) surface. Platelet activation moves negatively charged phospholipids such as phosphatidylserine (PS) to the outer layer of the cell membrane, which allows clotting factors to bind and form complexes. While other cells, such as leucocytes, may express PS and develop a prothrombotic phenotype when activated, platelets and MV_{plt} are far more important.

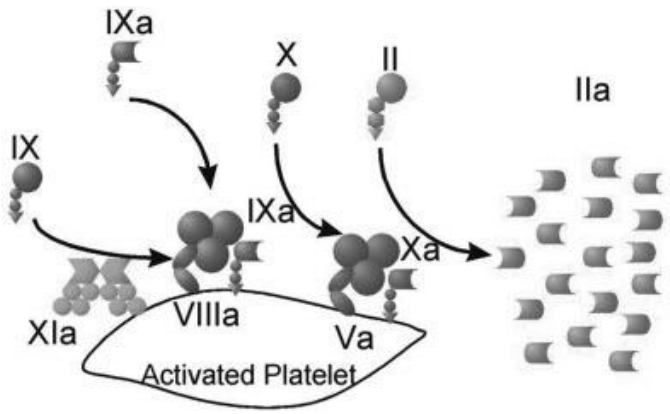


Figure 2.7. Propogation. Modified from Monrow and Hoffman 2006. See text.

Fibrinogen binds to platelet-surface GP IIb/IIIa from where it is cleaved by thrombin to form fibrin, which polymerises. Thrombin, now present in excess of the amount required to clot fibrinogen, is incorporated into the growing clot. It also activates fXIII, leading to cross-linking. Finally, a further stage of platelet activation is initiated by high-concentration thrombin cleavage of PAR-1, which may be the stimulus for platelet contraction.

The preceding subsections have described the activation of platelets by damaged vessel wall, leading to the formation of a plug within which platelets and their microvesicles act as the site for the formation of fibrin. Fibrin then binds the clot together. Small scale vessel injury is commonplace and even large tissue damage usually leads only to local coagulation. What prevents the clotting process spreading?

2.6.4 Natural Anticoagulants and Fibrinolysis.

There are three inhibitory mechanisms: tissue factor pathway inhibitor (TFPI), antithrombin (AT) and protein C. TFPI inhibits fXa once the latter has been released from the cell surface. It is expressed on endothelial surfaces and released by platelets (Lwaleed and Bass, 2006). AT binds to heparin-like glycosaminoglycans, such as endothelial membrane syndecans, and inhibits the action of thrombin and fXa. Thus both TFPI and AT serve to localise clotting activity, particularly at the initiation phase,

to sites of vascular injury and prevent it occurring on normal endothelium (Monroe and Hoffman, 2006).

Protein C is cleaved by the complex of thrombomodulin and thrombin on the endothelial surface. The cleavage of protein C is amplified by binding to the endothelial protein C receptor (EPCR). Activated Protein C (aPC) bound to EPCR can then cleave endothelial PAR-1, with the cytoprotective effects mentioned previously. Most (60% in one mouse model) of aPC is released from EPCR. aPC forms a complex with Protein S and then degrades fVa and fVIIIa. Since these are vital factors for coagulation, thrombomodulin serves to turn thrombin, the lynchpin of coagulation, into an anticoagulant; this is the "thrombin switch". Bound thrombin is also inactivated 20 times faster than free thrombin, stopping both clotting and the production of aPC. Of note, aPC inactivation of thrombin is inhibited at the platelet surface (Oliver *et al.*, 2002) aPC is therefore formed and exerts its effect chiefly on intact endothelium; its physiological role is therefore also to localise clotting at sites of injury (Weiler, 2010) This does not preclude its having a pathological role in coagulopathy after trauma (see subsection 2.7.2).

The last major component of coagulation is the fibrinolytic system. Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) both cleave plasminogen to form plasmin. tPA is the dominant actor and is released by damaged endothelial cells (Maegele, Schöchl and Cohen, 2014). It has low affinity for circulating plasminogen. However, plasminogen becomes incorporated into clot where the fibrin acts as a co-factor for tPA, increasing its activity 100-fold (Rijken and Lijnen, 2009). Plasmin cleaves fibrin, leading to clot dissolution, but also exposing a higher affinity plasminogen-binding site, promoting further fibrinolysis. Plasmin can also cleave other proteins, including elements of the extracellular matrix such as collagen.

The capacity of plasmin to breakdown clot is enormous and has to be kept in check. Thrombin-thrombomodulin complexes cleave thrombin activatable fibrinolysis inhibitor (TAFI). TAFI inhibits thrombolysis by modifying fibrin terminals to remove the higher affinity plasminogen-binding site. Thrombin therefore serves to stabilise clots

against thrombolysis, although TAFI activation may be significantly impaired by the release of platelet factor 4 by activated platelets (Mosnier, 2011). However, the effect of TAFI may be exceeded by thrombin-induced activation of protein C. Excess aPC enhances fibrinolysis by degrading PAI-1. Since aPC generation is also thrombin-dependent, it can be seen that the predominant action of thrombin, pro- or anti-fibrinolytic, will depend on circumstances.

2.6.5 Coagulation-Inflammation Cross-Talk

Having established the normal physiology of inflammation and coagulation (and before moving on to the pathophysiology seen in trauma), this subsection examines the ways in which the two are related, while the next subsection looks at the role of platelets in that interaction.

The mechanism of blood clotting is inextricably bound up with inflammation (Esmon, 2005; Levi and van der Poll, 2010). Its components have evolved alongside those of inflammation and many of them are shared between the two processes (Levi, 2004). Platelets and leucocytes have both evolved from haemocytes found in invertebrate haemolymph. Many of the molecular components, such as the serine proteases of the coagulation and complement systems, also have common antecedents.

However, the linkage is adaptive and not merely vestigial. There is crosstalk between the two processes at many levels. FXa, thrombin and fibrin can stimulate mononuclear and endothelial cells to produce IL-6 and IL-8 (Sower *et al.*, 1995; van der Poll, de Jonge and Levi, 2001). Cleavage of PARs is the key mechanism for the action of the coagulation proteases on inflammation. Thrombin and aPC both cleave PAR-1. However, the resultant signalling is modulated such that they have antagonistic downstream effects on the inflammatory phenotype of endothelial cells: thrombin enhances permeability and cell death while aPC opposes these (Weiler, 2010). In animal models of the therapeutic use of aPC in sepsis, this signalling function has been demonstrated to contribute more to its efficacy than the antithrombotic function. FXa, plasmin and thrombin and can also cleave C3 and C5, thus activating the complement system (Amara *et al.*, 2010). Fibrin also appears to have a direct

proinflammatory effect, stimulating release of cytokines (Perez and Roman, 1995) and chemokines (Harley and Powell, 1999).

2.6.6 Platelets as Immune Cells

Classically considered only to have a role in clotting, the inflammatory role of platelets is now increasingly recognised (Semple, Italiano and Freedman, 2011). Platelet activation leads to the release of a host of substances from its granules and cytoplasm, including cytokines. Platelet production is stimulated by cytokines, especially IL-6. Cytokines also increase the expression and stability of very large vWF multimers released from endothelium, which in turn activate platelets. Calcium ionophores (released from dying cells) and complement, i.e. products of inflammation, are among the most potent stimulators of negative phospholipid expression by platelets (Zwaal and Schroit, 1997).

Platelet selectins cause tethering of neutrophils (Semple, Italiano and Freedman, 2011; Engelmann and Massberg, 2012). Platelets express CD154, the ligand of CD40, by which they can activate neutrophils (and also dendritic cells, thereby promoting activation of the adaptive immune system). NETs (see subsection 2.5.9) are released by neutrophils exposed to TRAP-activated platelets and platelet-induced NETosis has been shown to be important in a mouse model of transfusion-related acute lung injury, a clinical entity that presents similarly to lung injury seen after trauma (Caudrillier *et al.*, 2012). NETs activate coagulation via tissue factor and fXII and recruit platelets by binding vWF. PAMPs and DAMPs can stimulate leucocytes to express tissue factor, thus initiating coagulation. By these and other pathways there is a feedback between coagulation and leucocyte activation that can lead to thrombus formation in response to pathogens, which are thereby trapped—this is immunothrombosis (Engelmann and Massberg, 2012). Platelets express TLR-4 and signalling via this pathway is important to platelet sequestration and cytokine release in sepsis models (Aslam *et al.*, 2006; Cognasse *et al.*, 2006). These interactions are also implicated in thrombotic disease associated with inflammation (e.g. deep vein thrombosis) (Engelmann and Massberg, 2012). There is clearly potential for platelets

to be important mediators of inflammatory activation after trauma, but this requires further study.

2.7 Coagulopathy and Endotheliopathy of Trauma

Disordered coagulation after trauma is well described. It is multifactorial (Hess *et al.*, 2008). Resuscitative dilution of clotting factors, acidosis and hypothermia all play significant roles. However, up to 34% of severely injured patients have been shown to be hypocoagulopathic very early after trauma, before these factors develop (Brohi *et al.*, 2003, 2007; MacLeod *et al.*, 2003; Maegele *et al.*, 2007; Rugeri *et al.*, 2007). This section details the mechanisms producing these changes. Trauma patients also develop a later hypercoagulopathy with a predisposition to thromboembolism (Cap and Hunt, 2014) but this will not be examined here.

2.7.1 Mechanisms of coagulopathy

Hypothermia is common after serious injury, reflecting not just heat loss due to exposure and treatment but, more significantly, reduced heat production due to inactivity and shock. Hypothermia is therefore linked to outcome independent of bleeding (Shafi, Elliott and Gentilello, 2005). Very low temperature (<30C) interferes with clotting function, but this is probably of little significance at commonly encountered levels of hypothermia (33 to 36C) (Hess *et al.*, 2008). Acidosis can directly affect clotting function (Martini and Holcomb, 2007; Darlington *et al.*, 2011). Acidosis both interferes with clotting protease function and promotes degradation of fibrinogen. Correction does not fully correct the coagulopathy, regardless of whether the acidosis was endogenous (due to shock) or exogenous (due to acid administration). Large volume fluid therapy was formerly standard in managing trauma patients, as was red blood cell transfusion without plasma. Both are capable of diluting clotting factors below their critical concentrations (Faringer *et al.*, 1993; Hiippala, Myllyla and Vahtera, 1995; Hirshberg *et al.*, 2003).

2.7.2 Acute Traumatic Coagulopathy

In 2003, Brohi described Acute Traumatic Coagulopathy (ATC; also called Acute Coagulopathy of Trauma Shock [ACoTS]) affecting 24.4% of trauma patients admitted to the Royal London Hospital via the Helicopter Emergency Medical Service (Brohi *et al.*, 2003). These patients had received very little prehospital fluid therapy (median 800mL). Severely injured patients (ISS ≥ 16) were coagulopathic in 33.1% of cases whereas only 10.8% when not severely injured. Macleod independently presented similar data from Florida that year (MacLeod *et al.*, 2003). Other papers have subsequently confirmed the existence of this abnormality.

Brohi and Macleod both suggested that ATC is driven by a combination of shock and tissue trauma. In a subsequent international collaboration they confirmed this interaction (Frith *et al.*, 2010). Patients with a low ISS were unlikely to develop ATC, regardless of base deficit (BD) on admission. Conversely, patients with a low BD were unlikely to develop ATC, regardless of ISS. The extreme groups (ISS<16, BD>12 or ISS>35, BD<0.1) were, unsurprisingly, relatively small (each 1% of the total). However, with n=3646, they still represent modest cohorts. Other studies have confirmed the effect of an interaction between injury severity and shock on the incidence of ATC (Wafaisade *et al.*, 2010).

The aetiology of this has been hotly debated. Brohi, collaborating with Cohen's group in San Francisco, has advanced a mechanism based upon aPC. A fall in circulating protein C was shown in patients with ATC, presumed to represent its activation and consumption (Brohi *et al.*, 2007). A subsequent study with measurement of aPC showed that increases were correlated with coagulopathy and increased fibrinolysis (Cohen *et al.*, 2012). This mechanism was subsequently supported by a mouse model of trauma-shock (Chesebro *et al.*, 2009). The model developed an apparently aPC-driven coagulopathy in response to a combination of trauma and shock. This was abolished by antibody-inhibition of the anticoagulant action of aPC. Of note, blocking aPC's cytoprotective function led to rapid death in all subject, associated with pulmonary thrombosis and perivascular haemorrhage. In contrast to this evidence supporting the role of aPC, a recent study has shown that adding aPC to plasma does

not interfere with clotting except at concentrations far above those seen in ATC (Campbell, Meledeo and Cap, 2014); this may undermine the role of aPC or may mean that the action of aPC is dependent on co-factors and spatial relationships not fully present in the studied assay. A large multi-centre US study has demonstrated a five-fold rise in aPC among trauma patients with evidence of ATC (Cohen *et al.*, 2013).

A paper (Rizoli, Scarpelini, *et al.*, 2011) examining clotting factor deficiency noted that a close association existed between ATC and reduced factor V **activity**. This would be expected in a coagulopathy driven by aPC or by consumption. Reduced activity of the other clotting factors was unusual, making it very unlikely that they had been consumed. More recently Kutcher *et al* (2014) found reduced clotting factor levels when comparing transfused trauma patients with non-transfused ones. However, there was no reduction in fibrinogen on admission. More recently, an international study (Hagemo *et al.*, 2014) has demonstrated that hypofibrinogenaemia at admission is more common than previously thought: 8.2% had plasma levels below 1.5g/L. Low fibrinogen was strongly linked to mortality. This joins other evidence to point towards factor consumption as an important component of ATC (Cap and Hunt, 2014).

2.7.3 Disseminated Intravascular Coagulation

These findings are relevant to the major alternative explanation, advanced by the Japanese group led by Gando (2009). They see nothing novel in Brohi and Macleod's findings (despite the crucial early time point) and assert that ATC is simply disseminated intravascular coagulation (DIC) with fibrinolysis. DIC is a consumptive coagulopathy, most familiar in sepsis, where a massive dysregulation of coagulation is linked to endothelial dysfunction. ATC cases tend to meet the two international sets of diagnostic criteria for DIC (Oshiro *et al.*, 2014). However, these detect the consequences of DIC (evidence of thrombin generation, platelet consumption, fibrinolysis and coagulopathy) and the systemic inflammatory response syndrome rather than addressing the underlying cause. Indeed, it is a widespread failure of the Protein C system leading to consumption of clotting factors, not its overactivity, that is usually advanced as the cause of DIC (Gando, Sawamura and Hayakawa, 2011). Thus, while the DIC criteria may be have some predictive value in trauma (Sawamura *et al.*,

2009), the underlying theory of causation is at odds with the evidence presented by Brohi and others. It is therefore no surprise that histopathological examination of tissue from trauma patients who fulfil the DIC criteria shows no evidence of microthrombi and other markers of DIC (Rizoli, Nascimento, *et al.*, 2011). Of note, DIC implies an uncontrolled consumption of factors due to a specific pathophysiological mechanism. The mere consumption of factors, which in a severely traumatised patient may be fulfilling their proper role of stemming haemorrhage, does not itself imply DIC

2.7.4 Fibrinolysis

If DIC is not the mechanism, then low fibrinogen levels after injury may be due to consumption in clot formation at sites of injury or be due to fibrinogenolysis, although this remains unclear (Davenport, 2013). The latter may be a side-effect of pathways contributing to fibrinolysis, which is another major component of ATC. Once again, Brohi and collaborators are responsible for some of the key papers on this subject (Brohi *et al.*, 2008; Raza *et al.*, 2013). Fibrinolysis is activated in most trauma patients; the degree of activation is correlated with the severity of injury and shock; plasmin-antiplasmin levels on admission, a measure of fibrinolytic activation, are independently correlated with clinical outcome. It may be that the mechanism involves consumption of PAI-1 by aPC, although some authors have argued against this (Cap and Hunt, 2014). There is controversy as to what level of fibrinolysis is pathological; a question complicated by parallel arguments about the use of thromboelastography (TE) in its detection. (TE is discussed in subsection 5.1.2.) In a very large study (n=1,996), Cotton *et al* (2012) defined hyperfibrinolysis (HF) as a 7.5% reduction in r-TEG amplitude 30 minutes after maximum amplitude (LY30>7.5%). HF was present in 2% of patients but was associated with 76% mortality (v 10% in those without HF). The paper by Raxa *et al* (2013) demonstrated that fibrinolysis activation was detectable in patients without severe lysis on TE. Whether all clinically important lysis can be detected by setting a very low threshold for TE detection (LY30>3%) has not been established (Hunt, Raza and Brohi, 2013; Ramos *et al.*, 2013).

This dispute plays into the clinical debate about the treatment of fibrinolysis. Tranexamic acid (TXA) is a lysine derivative which prevents the binding of

plasminogen/plasmin to fibrin and thus inhibits fibrinolysis. The CRASH-2 study (CRASH-2 trial collaborators, 2010) was a large (n=20,211), pragmatic study of TXA use in trauma patients and its use was associated with a 9% reduction in all-cause mortality (relative risk [RR],0.91; 95% confidence interval [CI], 0.85-0.97;p= 0.0035). This was due to a reduction in deaths due to bleeding (Roberts *et al.*, 2011) and occurred despite no significant reduction in blood transfusion. Blood products were not available at many participating sites in this global trial, which might obscure a reduced need. TXA use has been widely adopted, particularly in military practice, (Morrison, Dubose, *et al.*, 2012) but many objections to the trial have been raised, leading to poorer uptake in US civilian practice (Napolitano *et al.*, 2013). An Australian trial may resolve this by assessing the drug's efficacy specifically in the setting of fully resourced Western medicine (Mitra *et al.*, 2014). Of note, TXA also has anti-inflammatory effects which may contribute to its efficacy (Jimenez *et al.*, 2007).

2.7.5 Endotheliopathy of Trauma

The preceding discussion has concentrated on the changes in blood after injury, which are characterised by consumption, fibrinolysis and a possible amplifying and unifying action of aPC. Coagulation is also heavily influenced by the endothelium. Aspects of endothelial dysfunction after trauma were discussed in subsection 2.5.7. This subsection explores recent findings which implicate the endothelium in the coagulopathy of trauma.

While activation leads to the endothelium becoming locally procoagulant, substances released during activation may promote systemic hypocoagulopathy. The endothelium is coated by the glycocalyx, a glycoprotein/proteoglycan layer which sequesters up to a quarter of the plasma volume and modulates the interaction of platelets and leucocytes with the vessel wall. It also contains heparin-like substances. Johansson's group in Copenhagen have produced a series of papers which show the importance of the glycoclayx (Johansson *et al.*, 2011a, 2011b; Ostrowski and Johansson, 2012) and which can be construed to support Brohi's proposed mechanism of coagulopathy (Holcomb, 2011). It has already been noted that ATC occurs when there is a combination of tissue injury and shock and that it is tissue injury, not shock

that leads to sympathetic activation (subsection 2.4.1). High circulating catecholamines after trauma were associated with markers of glycocalyx degradation, endothelial activation, tissue injury and fibrinolysis; they were an independent predictor of death (Johansson *et al.*, 2011b). Syndecan-1 is a major glycocalyx component; circulating levels correlated with catecholamines, protein C degradation and a similar group of markers and, again, predicted mortality (Johansson *et al.*, 2011a). Crucially, patients without a raised Syndecan-1 did not exhibit a correlation between injury severity and catecholamines, inflammation and coagulopathy. Finally, autoheparinisation was detectable in a small proportion of trauma patients, and was associated with high syndecan-1 (Ostrowski and Johansson, 2012). These papers studied a small cohort (75-77 patients) and require verification. In addition, endothelial activation/injury leads to the degranulation of the Weibel-Palade bodies, releasing (*inter alia*) tPA (Maegele, Schöchl and Cohen, 2014).

The implication of these findings is that tissue injury contributes to ATC in several ways: tissue factor exposure activates coagulation, leading to thrombin production; catecholamine release (and presumably direct mechanical effects also) damages endothelial glycocalycx; this releases heparin-like substances; more significantly, it exposes the cell surface molecules that are implicated in ATC (thrombomodulin, EPCR). The low flow state of shock also leads to endothelial damage (Kozar *et al.*, 2011) and may then facilitate the interactions needed to produce the thrombin switch and the activation of protein C. Finally, aPC, as well as deactivating coagulation proteases, may end the inhibition of tPA (which itself has been released as part of endothelial changes).

2.7.6 The Role of Platelets in Coagulopathy

The role of platelets in the coagulopathy of trauma has not been clearly delineated. This subsection reviews the limited number of human studies that are available. These assess platelets using diverse means, which are explored in more detail in Chapter 5. Ogura *et al* (2001) studied a small mixed population of critically ill patients, half of whom had been injured. They demonstrated platelet activation by flow cytometry, reflected in increased CD62P expression, platelet microvesicles and platelet-leucocyte

aggregates compared with blood from normal controls. Jacoby *et al* (2001) studied platelet function in 100 trauma patients. In blood collected on admission, flow cytometry demonstrated increased platelet microvesicles, CD62P expression and binding of PAC-1, a measure of the activation-induced change in GPIIb/IIIa. They also used a PFA-100.* Admission blood showed shorter closing time in trauma patients than controls. Repeat testing at 24, 48 and 72 hours showed the loss of platelet microvesicles and persistent activation. However, there was a marked difference in closing times between survivors and the six non-survivors. The former essentially normalised whereas the latter, which showed a trend to being higher on admission, became notably extended at the later time points. On admission there was a weak inverse correlation between activation and closing time but in non-survivors the activation was associated with prolonged closing time. This would seem to indicate either that platelets have been stimulated to the point of becoming effete or are being inhibited. The PFA-100 is sensitive to haematocrit. While this is an acknowledged limitation of the Jacoby paper (2001), preventing isolated assessment of platelet function, it may actually be a reflection of the contribution of red cells to coagulation *in vivo* (Spoerke *et al.*, 2010).

Solomon *et al* (2011) used multiplate electrode aggregometry to demonstrate a decreased platelet response to ADP and TRAP in non-survivors when compared to survivors along with a reduced platelet contribution to clot strength on ROTEM. While detectable, these deficits were modest. However, there was considerable variability in the results of these tests among survivors, with many exhibiting a phenotype responsive to stimulation. Kutcher *et al* also used multiplate in a more severely injured cohort, finding more hyporesponsiveness at admission than the previous study (below normal response to at least one agonist in 45% of patients) (Kutcher *et al.*, 2012). They followed the time course of platelet hyporesponsiveness, which became nearly universal after 6 hours, with (generally) a slow increase in response up to 120 hours after admission. Wohlauer *et al* used platelet mapping to show a significant fall in ADP-and arachidonic acid-stimulated platelet function after trauma. This was found to be common in a much less severely injured cohort than the two preceding studies, with severely injured patients all exhibiting the deficit (Wohlauer *et al.*, 2012). Time

* The PFA-100 and related nomenclature is discussed in subsection 5.1.3.

from injury to admission was not stated in the paper but the trauma centres involved served rural populations; delayed admission may partly explain the higher rate of platelet dysfunction. Carroll *et al* showed that a reduced clot strength after initiation with ADP correlates with the need for transfusion; unfortunately they did not report the proportion and characteristics of patients with this abnormality (Carroll *et al.*, 2009). Windeløv *et al* (2014) showed no clear relationship between injury severity and platelet function. In a related paper they assessed the contribution of MV_{plt} to coagulopathy (Windeløv, Johansson, *et al.*, 2014). They demonstrated that trauma patients with impaired clot formation had low levels of PS-expressing MV_{plt}.

These papers are heterogenous in methodology and results, but some consistent messages emerge. Platelets from traumatised patients show evidence of activation, e.g. surface marker or MV_{plt} expression. There is evidence of a divergence in platelet function between non-survivors and survivors; there seems to be reduced function and response to stimulation in the non-survivors. There is mixed evidence as to whether such a functional deficit is associated with severity of injury, although most relevant papers support this proposition, and its detection may be sensitive to the timing of assessment.

A low platelet count ($<150x10^9$/L) is uncommon after trauma, occurring in less than 10% of those with ISS<45 (Hess *et al.*, 2009). However, low admission platelet count is correlated with transfusion requirement and outcome, independent of ISS (Hess *et al.*, 2009; Brown *et al.*, 2011). Kutcher *et al* found that platelet responsiveness correlated with platelet count (Kutcher *et al.*, 2012).

A preliminary study using ROTEM in British military casualties showed that 40% of seriously injured patients had significant evidence of hypocoagulopathy (Woolley *et al.*, 2013). Comparison of EXTEM (ROTEM stimulated by thromboplastin) and FIBTEM (EXTEM with cytochalasin D) traces for these patients revealed relative preservation of the FIBTEM trace, indicating that platelet dysfunction plays a significant role. (See sections 5.2 and 5.3 for further explanation of these terms).

In summary, studies with varied patient groups and methods of platelet analysis have produced divergent findings. It is difficult to determine the true incidence of platelet dysfunction, its severity or its contribution to coagulopathy. However, dysfunction certainly occurs, it is linked to the platelet count, it develops over several hours and may persist for days.

3 Resuscitation after Trauma

3.1 Damage Control

There has been an extensive debate about the best fluid regime for the resuscitation of trauma victims. The endpoints of resuscitation, the choice of fluids and the timing and volume of fluid administered have all been the subject of controversy. Until recently, the practice of high volume crystalloid infusion before the use of blood and blood products was an accepted standard of care; the aim was to restore normotension. Studies in animals and humans have led to a re-evaluation of this practice. Small volume, hypotensive resuscitation is now commonplace, as is a greater emphasis on the use of blood and blood products, especially the latter. Resuscitation is now aimed at preventing or relieving the lethal triad of coagulopathy, acidosis and hypothermia. As part of this, surgery is now integrated into the practice of what has become known as Damage Control Resuscitation (DCR)

Damage Control Surgery (DCS) was a term introduced by Rotondo *et al* in 1993, based on naval practice in the preservation of the fighting capacity of warships (Rotondo *et al.*, 1993), although the concept is in fact much older. DCS involves abbreviated surgery to stop haemorrhage and contamination followed by a period of intensive care to restore physiology and later, planned reoperation for definitive management. Initially described for abdominal trauma it has now been implemented for other injuries (Pape *et al.*, 2002) and is widely practiced and endorsed in guidelines.

The move to DCR came in the last decade (Holcomb *et al.*, 2007; Jansen *et al.*, 2009; Duchesne *et al.*, 2010). It combines DCS techniques with simultaneous medical therapy to correct physiology. The principle components are:

- DCS
- Hypotensive resuscitation
- Limited use of crystalloid fluids
- “Balanced” or “haemostatic” resuscitation, using a high ratio of plasma and platelets relative to red blood cells.

These are practiced concurrently. DCS is thus now considered just one part of the DCR process and the former, staged concept has been abandoned. After considering some of the basic physiology of resuscitation, this discussion will examine the three non-surgical elements of DCR. The process of the development of evidence and practice in this area has also influenced the development of the animal model used in this thesis, and will be explored in some detail.

3.2 Physiological Considerations

Fundamentally, resuscitation is intended to restore tissue oxygen delivery without deleterious effects such as increased bleeding or inflammation. Global oxygen delivery (DO_2) is governed by the arterial oxygen content (C_aO_2) and the cardiac output (CO) (Marino, 2013):

$$DO_2 = CaO_2 \times CO$$

CaO_2 is governed by the haemoglobin concentration ([Hb]) and the oxygen saturation S_aO_2, with a negligible contribution from dissolved oxygen.

$$CaO_2 \approx [Hb] \times S_aO_2 \times 1.34$$

Fluid therapy will predominantly affect CO and [Hb] (although changes in lung perfusion may alter S_aO_2). Fluid therapy increases CO by increasing the venous return to the heart, and consequently the filling pressure and end diastolic volume of the heart, leading to an increase in the stroke volume. PRBCs can combine this effect with an increase in [Hb]. It would seem to follow that PRBCs are the ideal resuscitation fluid. However, blood is a non-Newtonian viscous liquid, i.e. one that reduces its viscosity under shear stress, including flow through vessels (Merrill, 1969). (The phenomenon is familiar to anyone who has been the victim of the sudden exit of previously unmoving ketchup from its bottle.) The viscosity is related to the haematocrit. Consequently, dilutional therapy can raise CO by reducing viscosity. The effect is exaggerated by the fact that the blood will become even less viscous at it flows, creating a diminishing positive feedback effect. In consequence, a 38%

reduction in haematocrit in a polycythaemic patient can cause a 225% increase in CO (LeVeen *et al.*, 1980). PRBCs have a very poor ability to increase CO when compared with whole blood, crystalloids and colloids (Shoemaker, 1987). More viscous blood may also be less able to perfuse the microcirculation, leading to alterations in the distribution of flow.

3.3 Evidence for Normotensive and Hypotensive Resuscitation

Evidence in this area is complicated by the fact that some investigators have compared early fluid resuscitation with late, some have looked at different volumes and infusion rates of fluids (from none to slow infusion to large boluses) and some have compared resuscitation to different blood pressure endpoints. In clinical practice, fluid administration will be tailored to an individual patient's needs which may make the practical difference between these concepts small. However, in human trials this leads to protocol violations. Animal studies are more likely to keep to protocol, but the difference between, for example, the blood pressure profile in the large volume infusion arm of one study and that in the normotensive arm of another may be small.

3.3.1 Origins of Conventional Resuscitation Practice

The orthodoxy of normotensive resuscitation was established in the American College of Surgeons' Advanced Trauma Life Support (ATLS) guidelines. These guidelines and the didactic course that promoted them were established by an orthopaedic surgeon horrified by the poor care received by his family after an aircraft crash. They quickly became the mainstay of trauma care training in the USA and throughout the world. Their methodical approach to the care of the injured has unquestionably saved many lives.

Until the ninth edition, the ATLS recommendations on fluid resuscitation for patients with signs of hypovolaemia called for the rapid infusion of 2l of warmed Ringer's lactate solution, followed by an assessment of cardiovascular response and further infusion as indicated to normalise blood pressure (American College of Surgeons, 2008). These recommendations derived chiefly for the work of scientists using a

model of haemorrhage developed by CJ Wiggers (1950). The Wiggers model was designed to investigate the phenomenon of irreversible shock, a state after haemorrhage in which the subject will die regardless of any resuscitation. In this model, blood was withdrawn from experimental animals through an intravascular cannula to achieve a desired level of hypotension and this was then maintained for many hours by withdrawing or infusing blood to compensate for any change in blood pressure. Other investigators modified this model, with less profound and more short-lived hypotension, to make the insult potentially survivable. These investigators then found that resuscitation with crystalloid fluid required three times the volume of shed blood in order to restore normotension or prevent death (Shires T *et al.*, 1964; Dillon *et al.*, 1966).

3:1 resuscitation became widespread, with the explicit intention of filling a depleted extracellular space with fluid. This it did, causing massive pulmonary oedema in patients with non-thoracic injury during the Vietnam War, known as “Da Nang lung” (Dutton, 2007). Fluid resuscitation was a lifesaving intervention in Vietnam but the idea that there may be reason for caution had been born.

A potentially significant fault can be identified in the Wiggers model. Since the tap on the cannula is closed when not in active use, there is no possibility of the animal bleeding. In contrast, an injured patient depends upon maintaining the integrity of a clot to prevent the reactivation of haemorrhage. An increase in blood pressure could disturb a clot, leading to further blood loss.

Despite this, modified Wiggers model experiments continued into the 1980s, confirming the status quo. In parallel with this, a debate began between those who, in accordance with this paradigm, detected a benefit from prehospital fluid administration and those who found it to be pointless, often because it seemed to take longer to gain the necessary intravenous access than it would to take the patient directly to hospital. It is noteworthy that prehospital and in-hospital high volume fluid resuscitation were adopted without any clinical trials. This is despite the existence of clinical evidence against them dating back to the First and Second World Wars (Cannon, 1918; Beecher, 1949). There were even early animal experiments using

uncontrolled haemorrhage: in 1965 a study by Shaftan and colleagues using femoral artery transection showed that elevation of blood pressure by infusion of blood or crystalloid or by vasopressors caused haemorrhage to restart.

3.3.2 Uncontrolled Haemorrhage

From the late 1980s, reports have increasingly been published on animal trials including an element of uncontrolled haemorrhage. The first (Gross *et al.*, 1988) was a study comparing resuscitation after controlled haemorrhage and after haemorrhage from division of three branches of the ileocolic artery. Animals received no treatment or one of two regimes of fluid resuscitation. As one would expect, aggressive resuscitation benefitted the controlled haemorrhage group. However, it caused intraabdominal bleeding and death in the uncontrolled haemorrhage animals. The same group from Haifa found similar results using rat tail transection to model the uncontrolled haemorrhage (Gross *et al.*, 1989).

William Bickell and his colleagues made one of the most significant contributions to this debate with a series of experiments and, importantly, the major randomised trial of delayed resuscitation (see subsection 5.3.3). Their pig model involved open splenectomy (to prevent autotransfusion) followed by placement of a steel wire in the aorta such that when pulled it would produce a 5mm longitudinal aortotomy. The abdomen was closed with the wire ends exteriorised. In an early study, aortotomy did not lead to exsanguination in unresuscitated animals. The nadir of cardiac physiology was reached at 5 minutes, after which animals began to recover. At post mortem the aortotomies were sealed with a dense adherent clot (Bickell, Bruttig and Wade, 1989). In a subsequent study (Bickell *et al.*, 1991), animals were randomly assigned to receive either no fluid or crystalloid at three times the calculated rate of blood loss, beginning 6 min after aortotomy. At 2 hours, all the former group were alive and all the latter group dead ($p<0.05$). The resuscitated group had significantly more intra-abdominal haemorrhage. Initially they exhibited a high blood pressure, but this putative advantage had disappeared after 30min, by which time they were significantly more acidotic than the unresuscitated animals. The resuscitated group had soft, gelatinous, nonadherent clot at post mortem. Bickell suggested that as well as increased pressure

dislodging the thrombus (what is now widely termed "popping the clot"), dilution of clotting factors and altered rheology also add to the degree of haemorrhage.

Stern's group published two important papers in the early 90s which involved a more significant haemorrhage. Immature swine were bled through a cannula to a mean arterial pressure (MAP) of 30mmHg before pulling of the aortotomy wire (Kowalenko *et al.*, 1992; Stern *et al.*, 1993). This was considered to better simulate a potentially lethal haemorrhage. Animals were then resuscitated to a MAP of 40, 60 (in the latter study) or 80mmHg (using a limited volume of crystaloid then reinfusion of shed blood). Animals in the 80mmHg group died significantly more often and earlier than the others in both studies. Volume of haemorrhage into the abdomen was related to the resuscitation endpoint and, congruently, the volume of resuscitation fluid given. The mildly under-resuscitated group (60mmHg) had the best biochemical profile. Stern noted that an effect of the less aggressive resuscitation regimes was a delay before reaching maximal pulse pressure (80mmHg—10min, 60mmHg—35min, 40mmHg—45min). Allowing the clot to mature before increasing blood pressure may be beneficial.

Marshall (1997) modelled uncontrolled haemorrhage by using a cannula to bleed rats at a constant rate for 15 min and then transected their tails at 30 min. Simulated prehospital resuscitation was performed until 90min, when the tail bleed was stopped surgically and resuscitation then continued to a MAP of 80 mmHg and a Hct of 30% using Ringer's lactate and shed blood. The animals were allowed to recover from anaesthesia and monitored for three days. Animals were randomised to receive different prehospital regimes in the period from 30 to 60 min. A group resuscitated to a MAP of 80 mmHg with Ringer's lactate all died before 60 min (group I), whereas 75% of animals survived when the endpoint was 40 mmHg (group III). There were two other groups where shed blood and Ringer's lactate were used in a 1:5 ratio as the prehopital fluid, with the same two MAP endpoints (groups II—80 mmHg, group IV—40 mmHg). These groups had similar survival, base deficit and arterial pH to group III throughout the experiment. Prehospital volume requirements were (means): group I, 151 mL; group II, 69 mL; group III, 29 mL; group IV 20 mL. Group I's needs were significantly greater than the other groups and group II's were significantly greater

than the two groups resuscitated to 40mmHg. Multivariate analysis suggested that MAP, Hct and the interaction between them were significant predictors of mortality. As well as confirming the deleterious effect of a high MAP endpoint in uncontrolled haemorrhage (in the context of surgical control being exercised soon after), this study suggested additional benefit from early blood transfusion and revealed the marked reduction in volume required when even a small proportion of blood is used.

David Burris and colleagues from the Uniformed Services University of the Health Sciences used a rat model with two puncture wounds to the abdominal aorta to investigate the outcome of hypotensive resuscitation in situations where surgery is very delayed, as may occur in battle or after a disaster.(Burris *et al.*, 1999) Animals were resuscitated for two hours after injury and monitored up to 72 hours. At 2 hours, 3 of 11 unresuscitated animals were alive and all died within 4 hours. All resuscitated groups had better survival at 2 hours, but this advantage only persisted to 72 hours in two groups: one given Ringer's lactate to a MAP of 80mmHg and one given hypertonic saline in hetastarch (HH) to a MAP of 40mmHg. Ringer's lactate to a MAP of 100 or 40mmHg and HH to a MAP of 80mmHg had poorer survival. The authors concluded that moderate resuscitation was required in these circumstances.

These models have concentrated on injury to major vascular structures, rather than to the viscera, which is more common in patients. Matsuoka and colleagues addressed this with a model involving sharp removal of part of the liver in rats. (This is a visceral injury, but not a blunt injury.) They concluded that visceral injuries behave differently and that massive fluid infusion is safe after injury to the abdominal viscera (Matsuoka, Hildreth and Wisner, 1995, 1996). However, their studies either used low MAP endpoints, which would not properly reveal the disadvantage of high volume resuscitation, or were confounded by the use of hypertonic solutions, which make it difficult to interpret the implications for pressure endpoints alone. Krausz's group reported studies involving one ("moderate") or two ("severe") incisions to the spleen of rats (Abu-Hatum, Y. Bashenko, *et al.*, 2002; Abu-Hatum, Yulia Bashenko, *et al.*, 2002). In both studies, large volume Ringer's lactate infusion increased bleeding compared to untreated animals; in the severe model, it also worsened survival. These studies used a single bolus fluid infusion.

Krausz's group then investigated fluid infusion at different rates, for the period 15 to 60min after massive splenic injury. Even the fastest infusion of Ringer's lactate used had little effect on MAP. Despite this, increasing rate of infusion was associated with increasing blood loss, although not worsening survival (Abu-Hatum, Yulia Bashenko, *et al.*, 2002). However, in a similar study with the addition of splenectomy at 45min, they did find a shorter mean survival time after operation in the large volume Ringer's lactate group (Krausz and Hirsh, 2003). This could imply that this group had already bled enough by 45 min that splenectomy could not salvage them, whereas more conservative resuscitation delivered the "patient" to the "surgeon" in a better state to be able to benefit from control of haemorrhage. While sharp incision of the spleen may not fully reproduce a laceration, Krausz's group are to be particularly commended for investigating these injuries with variations in severity and management.

This is a necessarily incomplete account of animal trials in this area. In 2003, Mapstone and colleagues published a systematic review of these (Mapstone, Roberts and Evans, 2003). They identified 44 trials comparing fluids with no fluid and 9 comparing hypotensive with normotensive resuscitation. They found that fluids were beneficial in animals with severe haemorrhage but detrimental in those with moderate haemorrhage. Hypotensive resuscitation was beneficial with a pooled relative risk of death 0.37 (95% CI, 0.27–0.50).

Animal work has continued in this area with concordant results. In an interesting paper in 2011, Li *et al* looked to determine the optimum hypotensive resuscitation regime (Li *et al.*, 2011). Using a rat model with a mixed splenic and vascular injury with later surgical haemostasis they evaluated the targeting of several MAP endpoints in the hour before surgery. The found that 50mmHg gave the best outcome, both for mortality at 4 hours post-surgery and for measures of tissue function. They then performed a second evaluation, varying the time for which the 50mmHg MAP was maintained. They found a sharp drop in survival between groups given the hypotensive regime for 90 and 120 minutes. The body's capacity to tolerate hypotensive resuscitation is therefore a function of both its depth and duration.

3.3.3 Human Data

This extensive investigation in animal models has been highly influential on clinical practice since there has been only poor quality human data. The most important human trial in this area is that of Bickell and colleagues (1994). They assigned patients with penetrating injury to receive standard care (i.e. prehospital fluid infusion) or no fluids until reaching the operating theatre. Bickell reported a statistically significant improvement in survival to hospital discharge in the delayed resuscitation group (70% v 62% survival, p=0.04, Chi Square). The trial had several flaws. "Randomisation" was by day of the week and as such there was no allocation concealment, which can have a large effect on trial outcome in either direction.(Higgins *et al.*, 2011) There was also no reported blinding of hospital staff or data collectors. 8% of patients in the delayed arm received prehospital fluids, which was a protocol violation. However, it is unclear whether this was inadvertent or a desperate measure in the face of profound hypotension—an action that might usefully have been permitted in the protocol. The study took place in an urban environment with short prehospital times and excluded patients with blunt injury. Consequently, its general applicability in a mixed trauma population or in the context of prolonged prehospital times (as in rural, military and disaster situations) is moot.

The other major trial comparing fluids with no fluids (Turner *et al.*, 2000) showed no benefit but had so many protocol violations that the proportion of patients actually being given fluids in the two arms of the trial was nearly the same. Three trials comparing large and small volume resuscitation showed no mortality benefit and there have been no completed trials comparing hypotensive and normotensive strategies. A trial comparing two different low MAP endpoints during surgery for trauma has published preliminary data (Morrison *et al.*, 2011). In preparing for this trial the investigators have established that an intraoperative MAP endpoint of 65mmHg represents a consensus of current practice among US trauma surgeons.

A Cochrane Review concluded that there was no reliable evidence to support either a liberal or restricted resuscitation strategy (Kwan *et al.*, 2014). They eschewed meta-analysis because of the heterogeneity of the trials. Wang and colleagues did perform meta-analysis, both of trials and observational studies, and concluded that there was

strong evidence of benefit for a restricted resuscitation strategy (Wang *et al.*, 2014). The Cochrane review's position is more rigorous. Clinicians should regard the benefit of hypotensive resuscitation as supported but not definitively proven by the available evidence.

In parallel with these investigations of the amount and timing of fluid resuscitation there has been extensive examination of which clear fluid (colloids or crystalloids, isotonic or hypertonic) should be used. These lie beyond the scope of this discussion. It should be noted that clinical trials have not realised the promise of these alternative fluid types (Bulger *et al.*, 2010, 2011), that the preponderance of evidence favours isotonic crystalloids (Perel, Roberts and Ker, 2013), that colloids may induce coagulopathy (Kozek-Langenecker, 2015) and that crystalloids predominate in UK civilian practise. While the US military makes extensive use of colloids, the UK Armed Forces have largely abandoned them.

3.3.4 Resuscitation Research at Dstl Porton Down

In the context of these findings about hypotensive resuscitation, which was the UK military standard of care, Kirkman and colleagues at DSTL, Porton Down have sought to examine the effects of different fluid regimes in models of military injury, including prolonged resuscitation and blast injury. In these experiments, blast injury was induced using a bare charge with the subject protected by a Kevlar blanket. Accordingly, blast exposure caused only primary blast injury, allowing the effect of this phenomenon to be studied in detail and preventing wide discrepancies in injury pattern between subjects in the same group, as would occur with exposure to fragmentation injury. They first used a controlled haemorrhage model combined with blast or sham blast to compare extended periods (up to 8 hours) of hypotensive and normotensive resuscitation (Garner *et al.*, 2010). In blast exposed animals, survival times were significantly reduced in the hypotensive groups, with evidence of hypoxaemia induced by the primary blast lung injury combining with poor tissue perfusion to produce inadequate oxygen delivery. In the sham blasted groups, prolonged hypotensive resuscitation produced considerable physiological derangement, including a high base deficit, compared with normotensive

resuscitation. There was a trend towards greater mortality in the hypotensive group but this was not significant. However, given the ethically appropriate constraint on the size of the study, this may represent a type II error. This study clearly demonstrated the possible deleterious effects of prolonged hypotensive resuscitation. However, it did not account for the possibility of rebleeding.

Kirkman and colleagues then developed a model that included the possibility of rebleeding (Kirkman, Watts and Cooper, 2010). To do this they added a grade IV liver injury, which was induced by a surgically preplaced wire which was pulled after controlled haemorrhage and blast or sham blast. Liver injury followed haemorrhage to ensure that the known cardiovascular effects of blast would not have a differential effect on the degree of blood loss from the liver—after 30% blood volume haemorrhage, cardiovascular physiology should be similar between blast and sham blast groups, as already discussed in subsection 2.4.2.

Having demonstrated that hypotensive resuscitation causes problems through poor tissue perfusion and oxygenation, they studied strategies to improve these through fluid therapy and supplemental oxygen. For one trial they designed a novel hybrid resuscitation regime involving one hour of hypotensive resuscitation followed by normotensive resuscitation (Doran *et al.*, 2012). This was designed to allow any clot to gain 80% of its strength during the hour before the increase in perfusion pressure (Shaftan *et al.*, 1965). Thereafter, the improved cardiac output, potentially combined with improved microvascular flow due to decreased blood viscosity, would restore oxygen delivery to the tissue. Hypotensive resuscitation, being the UK military practice at the time, remained the comparator.

In blast exposed animals, the novel hybrid strategy was associated with a significant fall in mortality compared with hypotensive resuscitation (from 5 out of 6 to 2 out of 6, p=0.02, Kaplan-Meier analysis, Peto's log rank test). No difference in mortality was seen in the two sham blasted groups. In all groups, the oxygen extraction ratio (OER), which reflects the adequacy of oxygen delivery, rose from the normal of 25% to the physiological maximum of 80%. All groups also developed a significant acidosis. Restoration of perfusion in the novel hybrid groups associated with an improvement in

the acidosis and a fall in the OER. OER remained maximal in the two hypotensive groups. In the blast-hypotensive group acidosis worsened until the animals succumbed. In the sham blast-hypotensive group, oxygen delivery appears to have been adequate to sustain life but, despite continued maximal oxygen extraction, not to correct the acidosis. There was no evidence of increased bleeding in the novel hybrid groups.

In a separate trial, blast exposed animals were treated with hypotensive resuscitation and either oxygen supplementation titrated to maintain SaO_2 of 95% or air. Oxygen therapy successfully improved the SaO_2, improved delivery of oxygen and markedly improved mortality (6/6 v 2/6, p=0.014) (Kirkman, Watts and Cooper, 2010).

Following a review of all available evidence, novel hybrid resuscitation has been adopted as standard British military practice. Logistical and tactical considerations make supplemental oxygen therapy impractical in many military settings. Nonetheless oxygen is made available and used wherever practicable.

3.3 Haemostatic Resuscitation

The description by Brohi and then others of a hypocoagulopathy that affects a significant proportion of trauma patients (Brohi *et al.*, 2003; MacLeod *et al.*, 2003) and predicts adverse outcome coincided with the large-scale military engagements of the USA and her allies in Iraq and Afghanistan. In-hospital resuscitation practice in the US Armed Forces developed over the subsequent decade in an attempt to find better ways to address the physiological derangements of injured servicemen. Military reports proved highly influential on civilian practice. Two trends were particularly noteworthy: the use of recombinant activated factor VII (rFVIIa) and the use of high ratios of fresh frozen plasma and platelets to red blood cells. rFVIIa was developed for the treatment of bleeding in patients who have a genetic deficiency for the clotting factor. Case reports of its use in traumatic bleeding began in Israel (Kenet *et al.*, 1999), and the practice became widespread in the US military (Spinella *et al.*, 2008). Two civilian randomised control trials failed to provide convincing evidence of its safety and efficacy (Boffard *et al.*, 2005; Hauser *et al.*, 2010). This potent drug has the potential

to cause significant thromboembolic complications (O'Connell *et al.*, 2006). The drug is most effective when given to patients without hypothermia or acidosis (Meng *et al.*, 2003). Defining the correct circumstances for its use therefore remains problematic. Following initial enthusiasm there has been a decrease in its use in the British military (Smith, 2013).

3.3.1 Plasma Therapy in Resuscitation with Blood Components

Blood component therapy replaced whole blood transfusion in the decades after the Second World War as it became technically feasible and was perceived to offer more efficient use of donated blood and rational, targeted use of components to treat specific problems. In the context of trauma and haemorrhage, the practice developed of giving FFP only once a coagulopathy had been documented or a certain amount of PRBC had been transfused (Holcomb and Pati, 2013). Recognising the incidence of coagulopathy early after injury clearly undermines this practice. A series of cohort studies of both military and civilian patients has appeared to show decreased mortality in trauma patients who receive a high ratio of FFP to PRBC during massive transfusion.

The first was the study by Borgmann *et al* (2007). They demonstrated much lower (65 v 19%) mortality in patients receiving high (mean 1:1.4, $p<0.001$) ratios of FFP to PRBC compared to those receiving low ratios (1:8). By 2011, a systematic review included eleven retrospective studies, nine of which found evidence of benefit for high or 1:1 FFP:PRBC ratios (Rajasekhar *et al.*, 2011). More recently, Bhangu *et al* (2013) analysed six studies where the ISS did not differ between low- and high-ratio groups (OR 0.56, 95% CI 0.40–0.78, $p < 0.001$ where high was defined as >1:2) but no additional benefit of 1:1 resuscitation.

However, all these studies are potentially confounded by survival bias. It has been typical practice for PRBC to be immediately (in the ED) or quickly (in the laboratory) available for transfusion to trauma patients. PRBC is kept refrigerated and delivered through a warming circuit to enter the patient at an appropriate temperature. FFP has not been kept thawed and ready because of its short shelf life when not frozen. The current vogue for early, high-dose FFP may mean that some high volume units,

including military hospitals, may have changed this policy. However, in the period covered by these studies, it meant that there was a delay before administration of FFP could begin. Accordingly, patients requiring the largest amount/highest rate of PRBC transfusion will quickly develop a low FFP:PRBC ratio. These patients are those with active haemorrhage and consequently the highest risk of death. It follows that patients who require the highest PRBC volumes will have the lowest FFP:PRBC ratios unless they survive long enough to "catch up". Early death, the outcome measure, can therefore govern FFP:PRBC ratio, the treatment measure, confounding the analysis (Snyder *et al.*, 2009; del Junco, Fox, *et al.*, 2013).

There is other evidence in support of high ratio resuscitation, in the form of studies comparing similar patient cohorts before and after the instigation of high ratio policies. For example, the group at Vanderbilt (Cotton *et al.*, 2008) demonstrated improved mortality after the introduction of such a policy. However, their study had very different cohorts before and after the policy change and such studies are easily confounded by other changes in management. They also claimed to demonstrate a reduction in blood product usage. However, the treatment group was defined by protocol activation (a clinical judgement) and many appear to have received less than 10 units, but the latter was an inclusion criterion for the control cohort. No such reduction was seen in a similar study from Copenhagen which did not have this design flaw. This group confirmed the apparent mortality benefit (Johansson and Stensballe, 2009).

Despite these evidential shortcomings, clinicians have to respond to the reality of early coagulopathy in a substantial proportion of the most severely injured patients. Is high ratio FFP the answer? A careful study by Brohi's group in London has recently demonstrated this (Davenport, Curry, *et al.*, 2011). They examined coagulation status in patients before and after receiving PRBC and FFP, recording the ratio received for each 4 units of PRBC transfused. In coagulopathic patients, FFP transfusion improved coagulation parameters. Interestingly, this effect was maximal at an FFP:PRBC ratio between 1:2 and 3:4. This accords with the maximal mortality benefit seen in some studies (Kashuk *et al.*, 2008; Teixeira *et al.*, 2009). However, this was described as a preliminary report of an ongoing study; multivariate and subgroup analysis on a larger

cohort should yield more information on whether this is an independent effect of the ratio or is confounded by other factors, such as injury severity, physiology, timing and other treatments. Thus it is sensible to predict some benefit from giving early FFP, at least in this range of ratios. As a practical matter, some authors have suggested that, given the logistic constraints of FFP readiness, aiming for a 1:1 ratio may have the effect of achieving a 1:2 ratio (Holcomb *et al.*, 2008).

Others retain an emphasis on achieving 1:1 transfusion. Is there any reason to believe FFP may be harmful? At a mechanistic level, FFP is about as "dirty" as a drug could be (Pandey and Vyas, 2012). FFP contains a multitude of biologically active molecules ranging from pro- and anticoagulant factors to immunoglobulins and cytokines. Any blood transfusion risks infection and other complications, despite donor screening, leucodepletion and other measures. The study of FFP:PRBC ratio in the German Trauma Registry indicated that high ratios may be associated with increased septic complications and multiple organ failure (Maegele *et al.*, 2008). Johnson and colleagues in Denver demonstrated in a retrospective study that volume of FFP transfusion is an independent predictor of multiple organ failure and that this effect is greater when the patient has required more than 6u of PRBC (Johnson JL *et al.*, 2010). However, all such studies suffer from the possibility of selection bias. This works in the opposite direction to survival bias: receiving high doses of FFP may indicate more severe pathology, not measured or accounted for in the multivariate analysis; these patients may be more likely to develop MOF because of these underlying changes rather than the FFP.

Holcomb and colleagues completed a large (n=905) multi-centre prospective study of transfusion ratios which was specifically designed to address the problems seen in previous observational studies (PROMMTT) (Holcomb JB *et al.*, 2013). By using appropriate inclusion criteria, collecting detailed data on the timing as well as amount of blood products given and using time-dependent statistical modelling they sought to remove the influence of survival bias. They concluded that achieving a high ratio of FFP to PRBC during the first 6 hours of resuscitation was associated with a much reduced mortality during the first 6 hours. They demonstrated that received ratios changed over time with most patients initially receiving a low ratio (i.e. PRBCs first)

and then reaching a high ratio over time (as FFP is administered). Subgroup analysis suggested that mortality benefit may have been confined to those patients who were given a high ratio initially (del Junco, Holcomb, *et al.*, 2013). This group then conducted a large randomised control trial (Holcomb *et al.*, 2015) comparing PRBC:FFP:Plt ratios of 1:1:1[*] to 2:1:1. No difference in the primary endpoints (mortality at 24 hours and 30 days) was found, but this may have been a type 2 error. 1:1:1 appeared to be superior in the analysis of secondary endpoints including exsanguination and achievement of haemostasis and was not associated with an increase in adverse events.

More recently, there has been considerable interest in the role of plasma products in addressing the endotheliopathy of trauma. *In vitro* studies demonstrate that plasma, unlike crystalloid, can reduce the permeability of endothelial cells induced by hypoxia and other insults (Pati *et al.*, 2010; Wataha *et al.*, 2013). Plasma also restored the glycocalyx in rat models (Kozar *et al.*, 2011; Torres *et al.*, 2013). Clinical confirmation of these effects has not yet been demonstrated.

3.3.2 Platelet Therapy in Resuscitation with Blood Components

Evidence and practice relating to the administration of platelets has followed a parallel course to that for plasma over the last decade. Initial military reports of benefit of high platelet:PRBC ratios (Perkins *et al.*, 2009) were followed by civilian retrospective studies (Holcomb *et al.*, 2008). A systematic review of studies of platelet:PRBC ratio found seven studies that used regression or propensity scoring to address confounders (Hallet *et al.*, 2013). Those reporting on massively transfused patients found a survival benefit for high ratios, those studying all transfused patients did not. Two studies used a before and after design (either side of the introduction a high ratio policy) to avoid survival bias; one found a benefit. PROMMTT also considered platelet therapy and similarly found a survival benefit associated with achieving a high ratio during the first 6 hours (Holcomb JB *et al.*, 2013).

[*] 1:1:1 implies one unit PRBC, one unit FFP and a volume of platelets equivalent to a single donor's contribution to a pool of platelets. In UK practice, this would equate to one pool for every six units of PRBC and of FFP.(Lowes, 2014) This combination is referred to as reconstituted blood.

The use of platelets in military resuscitation practice carries considerable difficulties. Platelet pools are generated by combining the Buffy coat (the platelet-rich layer between cells and plasma in centrifuged blood) from several donated units of blood. A pool must contain >240x10^9 platelets (UK Blood Transfusion & Tissue Transplantation Services, 2013) and this is achieved by combining platelets from six donors. A pool of platelets must be mechanically agitated during storage and maintained at a temperature of 22 +/- 2°C, presenting logistical challenges in transporting pools to remote parts of the world. A pool must be used by the end of the fifth (exceptionally the seventh) day after collection. Given the time necessary to collect, pool and transport platelets to Afghanistan, their shelf life after arriving at the point of use during the recent operation there was very short. Pools generated in the USA were even more severely affected by this constraint.

An alternative source of platelets is apheresis in which platelets are removed from donor blood which is return to the bloodstream. Platelets were in this way harvested from pre-screened volunteers at Camp BASTION and other bases with military hospitals (Jansen *et al.*, 2014). Generation of platelets for transfusion in this way may take 4 hours, so is not a complete substitute for stored pools (Lowes, 2014). Apheresis platelets were used when stored pools were unavailable in the UK hospital but were the mainstay of platelet component therapy in US units (Perkins *et al.*, 2009).

3.3.3 Fresh Whole Blood

The drivers of the adoption of component therapy were touched on in subsection 5.4.1. However, operations in Iraq and Afghanistan have seen a return to the use of fresh whole blood (FWB), chiefly in US practice. FWB can also be collected from local donors, and is available much quicker than apheresis platelets. It is therefore a useful supply in emergencies and a means of ameliorating the logistical problems alluded to above. However, use of emergency FWB precludes virology testing, leucocyte depletion and other safety measures. Reconstituted blood (see footnote to subsection 5.4.1) is not equivalent to FWB. The storage media used in the components mean that (using US products) reconstituted blood has a haematocrit of 29%, with low platelet and clotting factor concentrations (Kauvar *et al.*, 2006). FWB may have other potential

benefits; e.g. leucocyte microvesicles, which express TF, are preserved and may support clot initiation (see subsection 2.6.2). Use of freshly donated blood prevents any storage lesion.

Extensive US military experience of FWB usage has raised few safety issues: viral contamination rates may be slightly higher than in domestic component therapy but this is considered to be offset by the life-saving indications for which FWB is used. There is some retrospective evidence that FWB usage has been associated with improved outcome relative to those managed with component therapy alone. In particular, Spinella *et al* showed that FWB was associated with less total transfusion and improved mortality when compared with component therapy in an unmatched retrospective study of experience in Iraq and Afghanistan (Spinella *et al.*, 2009). A civilian pilot study with a randomised design showed no reduction in transfusion with FWB use but may have been confounded by uneven distribution of head inured patients between treatment arms: further studies are planned (Cotton *et al.*, 2013).

3.4 Prehospital Use of Blood Products.

3.4.1 Background to Prehospital Transfusion

Blood transfusion became a safe treatment with the development of storage solutions and the understanding of blood groups, work led by Peyton Rous in the US (Hess and Thomas, 2003). Rous's student Robertson developed a practical method and apparatus for collecting, storing and administering blood. He described 44 successful transfusions given in a casualty clearing station on the Western Front (Robertson, 1918). Thus, transfusion has been used to sustain patients forward of surgical care since the earliest days of the treatment. During the Spanish Civil War (1936-1939), Canadian surgeon Norman Bethune revolutionised the use of blood in prehospital care by organising mobile transfusion services and advocating his concept of 'taking the blood to the wounded and not the wounded to the blood' (Franco *et al.*, 1996). Subsequent military prehospital use of blood transfusion has been highly variable. Some civilian experience also exists, including in the context of air ambulances (Dalton,

1993; Berns and Zietlow, 1998; Green *et al.*, 2013; Lockey, Weaver and Davies, 2013) but these accounts do not include a rigorous examination of efficacy.

The decade of conflict in the Middle East and Central Asia has driven a significant number of improvements in the medical care of military casualties (Penn-Barwell *et al.*, 2015). Since exsanguination remains a potentially preventable cause of battlefield mortality (Kelly *et al.*, 2008), the treatment of hemorrhage has been a significant focus of trauma care innovation. The last decade has seen the rehabilitation of tourniquets (Kragh *et al.*, 2009), the development of hemostatic dressings (Lawton, Granville-Chapman and Parker, 2009), improvements in paramedical training and the development of deployed trauma systems (Blackbourne *et al.*, 2012).

There has also been an attempt to move the perceived benefits of damage control resuscitation into the prehospital arena. The UK Defence Medical Services have, for some time, projected advanced resuscitation capability forward of the hospital (O'Reilly, König and Tai, 2008), with demonstrable improvements in mortality (Morrison, Oh, *et al.*, 2013). The UK Medical Emergency Response Team (MERT) is an Advanced Medical Retrieval platform (Morrison, Oh, *et al.*, 2013). In addition to standard *en route* care, the physician-led team can administer resuscitation fluids using the intraosseous and central or peripheral intravenous routes, perform chest decompression (thoracotomy, tube or open or needle decompression) and drug-assisted airway management. The capability was further enhanced by carrying four units of PRBC and thawed FFP, for prehospital transfusion.

Patients who might benefit from prehospital blood transfusion (PHBTx) include those who would not reach hospital alive without support to cardiac output and oxygen transport. Other patients may reach hospital alive but with profound shock, which is associated with adverse effects on coagulation, inflammation and survival. Earlier transfusion may ameliorate these. Detrimental effects may occur in patients in whom the treatment is given unnecessarily but the current clinical consensus is that these are outweighed by the benefits in others.

3.4.2 Evidence for prehospital transfusion

Chapter 4 of this thesis includes the first comparative analysis of the benefits of this strategy in the context of armed conflict. Two recent US civilian papers are worth noting. Brown *et al* (2015) on behalf of the Glue Grant group demonstrated a reduced 24-hour mortality among patients who received blood transfusion prior to admission to a Trauma Centre. There were 50 of these in a cohort of 1415 trauma patients. However, 24 of these received blood after admission to a peripheral hospital. There was no explanation of the circumstances or indications for transfusion among the remaining 26 patients so the findings of this study are hard to generalise. Holcomb *et al* (2014) have published a comparison of outcomes between patients admitted via two helicopter services, one of which had deployed PRBC and FFP for prehospital admission. Early outcomes such as admission base deficit and transfusion requirements were better in patients for whom PHBTx had been available. However, no mortality benefit was seen at 24 hours or 30 days. Multivariate analysis suggested a trend to lower mortality (e.g. at 6 hours OR 0.23, p=0.088). However, there was considerable risk of error due to multiple testing and the analysis had included variables that were confounded by the treatment, such as admission coagulation assays. These two studies have not established an evidence base for the practice of PHBTx. An animal study that should be mentioned is that by Sondeen *et al* (2011). They used a swine model of uncontrolled splenic bleeding to demonstrate increased blood loss after resuscitation with Hextend (a colloid) when compared with blood products. However, animals resuscitated with Ringer's lactate did not develop a coagulopathy (as the Hextend subjects did) or the (statistically significant) increased blood loss, so the model may have simply demonstrated the deleterious effects of colloids on coagulation.(Kozek-Langenecker, 2015)

3.4.3 British Military Indications for and Practice of Prehospital Transfusion

Prehospital transfusion is considered for patients without a palpable radial pulse or who have a non-invasive systolic blood pressure of less than 80mmHg. The aim is to restore these parameters, rather than to resuscitate to normotension, unless a traumatic brain injury is suspected, or time to definitive care is anticipated to exceed

one hour (Doran *et al.*, 2012). If more than one unit of PBRC is required, plasma is co-administered, to achieve a ratio of 1:1. A loading dose of 1g of tranexamic is given to all transfused patients. Platelets are not normally used in prehospital practice.

Blood products are carried in sealed containers consisting of a fabric outer case and a vacuum insulated chamber with an inner, removable thermal isolation compartment. These devices are capable of maintaining storage temperatures of 2-8°C for up to 24 hours, in external temperatures of 45°C. The storage temperature is monitored using a temperature indicator tag together with single-use time-temperature indicators (WarmMark® and ColdMark®, ShockWatch, Dallas, Tx). Blood products are only administered if the tag indicates a green light. Components are warmed during administration, using a portable battery operated fluid warming system (enFLow™, GE Healthcare, Waukesha, WI). The hospital is informed during flight if a transfusion occurs so that the blood container may be promptly exchanged for a new one with a full supply of products. This process has the clinical benefit of alerting clinical teams to the likely arrival of a hemodynamically compromised casualty.

UK guidelines allow red cells stored in saline, adenine, glucose and mannitol to be used for up to 35 days (UK Blood Transfusion & Tissue Transplantation Services, 2013). There is evidence that as red cell age increases, a storage lesion develops which is associated with adverse outcomes in transfused trauma patients (Weinberg, Barnum and Patel, 2011). Consequently, current UK military trauma transfusion practice is to use units less than 14 days old, which has the effect of increasing overall wastage at times of low usage. Plasma is newly thawed for prehospital use following which unused units are available for use in the base hospital for up to five days. The approach provides non labile clotting factors and reduces wastage (Evans *et al.*, 2013). The logistic challenges of maintaining a blood supply to austere settings, and the impact of shortage, necessitates a much greater degree of redundancy than in civilian systems. Despite the challenges, wastage of blood products in the UK system has been managed, with 65% of PRBC and 80% of FFP units sent to Afghanistan used, which compares favorably with US experience (Rentas *et al.*, 2012).

Blood directives mandate haemovigilance and the traceability of all blood components. The documentation of prehospital transfusion is as per the UK military hospital protocol and consists of a double labelling system. One label remains in the patients' records, the other is returned to the pathology laboratory. The exigencies of administering a transfusion in a cramped aircraft, sometimes under attack, can make this challenging, especially if there are multiple casualties. Nonetheless, the MERT prehospital transfusion program achieved 100% traceability. Furthermore, no serious side effects or complications of transfusion attributable to units transfused in the prehospital setting have been reported.

Chapter 4. Prehospital Transfusion Outcomes

4.1 Introduction

Section 3.4 outlined the introduction of PHBTx into UK military resuscitation practice. As already suggested, the purpose was to sustain the lives of exsanguinating patients and to draw the practice of DCR into the prehospital arena (O'Reilly *et al.*, 2014). It was intended principally to reduce mortality among injured servicemen but it was also suggested that it might reduce morbidity, blood product usage and length of stay in ITU. However, there were concerns that PHBTx could be deleterious under certain conditions (e.g. unnecessary transfusions with immune sequelae or efficient volume expansion leading to "popping the clot") and that it was logistically burdensome.

The Flight Intervention and Resuscitation in the Military (FIRM) project was initiated by the author under the authority of the Defence Professor of Surgery to assess whether routinely collected clinical data could be used to retrospectively assess the impact of PHBTx on mortality and other outcomes. This chapter sets out the process and main findings of the original FIRM project.

The introduction of PHBTx usage by the UK MERT coincided with the increasing presence of US troops in Helmand province that began in 2008 and was extended by an 11,000 troop surge in 2009. Consequently, there were three main groups of patients treated in Camp Bastion: local nationals, UK servicemen and US servicemen. The project was intended to assess mortality, morbidity and ITU length of stay but this information was not likely to be always available for local nationals as they are moved to local facilities once this is clinically suitable and then lost to follow up. Since UK and US servicemen were routinely evacuated while still intubated and ventilated, it was essential to have full follow up data for these patients. The project therefore required a unique US-UK data sharing collaboration if it was to proceed. This was set up as a joint project between the Royal Centre for Defence Medicine, Birmingham and the US Army Institute of Surgical Research (USAISR), San Antonio, Tx.

Other projects based on this data collaboration were quickly established (Morrison, Oh, *et al.*, 2013) but do not form part of this thesis. The author initiated the FIRM project and created the initial study design. The author collaborated with others in obtaining the necessary ethical approvals and data access. The author is particularly indebted to Maj J Morrison for his work with colleagues at USAISR to match records from the three source databases to produce the FIRM dataset. Preliminary analysis carried out by the author with statistical staff at USAISR revealed that deficiencies in the available data would mandate a novel study design to achieve the desired matched cohorts of PHBTx recipients and controls. The author produced the initial version of this which was then modified on advice from the project statistician. The matched cohorts were produced according to this new methodology (for which credit is due to Dr A Apodaca-Morrison) and the author then carried out the statistical analysis. In order to properly differentiate the author's contribution to the work being reported, the methods section of this chapter will detail the author's proposals and the ways these were altered.

4.2 Aim and Hypothesis

The purpose of this work was to assess what information about the clinical benefits (or risks) of PHBTx could be gleaned from the available human data. To facilitate this, the study was designed to evaluate the hypothesis that the provision of PHBTx to injured personnel led to a reduction in mortality when compared with similarly injured patients who did not receive PHBTx

4.3 Methodology

4.3.1 Sources of Data

This study was approved by the Royal Centre for Defence Medicine Academic Unit and the US Army Medical Research and Materiel Command. Data was obtained from three sources: the UK Joint Theatre Trauma Registry (JTTR), the US Department of Defence Trauma Registry (DoDTR) and the UK prehospital MERT database. The JTTR includes demographic, injury, treatment and outcome data on all UK personnel hospitalised

due to injury while deployed and on all non-UK personnel treated in UK facilities. Injuries are coded using the Abbreviated Injury Score (AIS) system. (See the Appendix for further discussion of injury coding.) The DoDTR has a similar remit for US personnel and facilities. In both cases, data collection on foreign nationals ceases at the point that they leave the national treatment facility or evacuation chain, whereas data on own-nation personnel is continually updated (and corrected) until after the serviceman has been discharged from medical care. As such, DoDTR data about US servicemen will be more accurate than JTTR data, as it will capture late mortality and morbidity, length of stay and corrections to injury data in the light of fuller evaluation. JTTR and DoDTR records were completed by dedicated trauma nurse co-ordinators (TNCs); at Camp BASTION the UK and US TNCs shared an office but used separate systems. The MERT database covers all patients treated by the MERT, including those evacuated to sites other than BASTION. It was completed by MERT personnel and includes mission data (e.g. flight times) and treatment details.

Each database used its own patient identifiers, albeit with some inconsistent cross-referencing. To resolve this, an algorithm was used to match patients' time and date of injury, demographics and injuries. (The author is grateful to Dr J Jones, USAISR for this work.) This produced a single FIRM dataset.

4.3.2 Initial Study Design

The initial proposal was to use a propensity score-based matching process to establish matched cohorts of PHBTx recipients and non-recipients. Propensity scoring is used when a study population includes a mixture of persons who were and were not exposed to a treatment (or other factor of interest). The concept is that exposed cases are matched with similar non-exposed cases such that known confounders of the outcome of interest may be evenly distributed. This is intended to mimic the benefits of using randomisation in prospective studies (albeit the latter can also distribute unknown confounders). A multivariate logistic regression is used to build a model that calculates the chance that any given subject would receive the treatment if it were available; this is the propensity score. A propensity score is calculated for each treatment recipient and a control case is selected with a similar propensity score. This

produces pairs of recipients and controls, necessitating the use of appropriate statistical tests (Austin, 2008).

The important data fields in the FIRM dataset were:

- Demographics (age, sex, national status)
- Mechanism of injury
- Injury details (AIS codes of all identified injuries)
- Initial prehospital observations (HR, RR, GCS, SpO_2) recorded by MERT/PEDRO/DUSTOFF personnel
- Prehospital treatment (PHBTs, airway, vascular access, chest decompression)
- Timings (Time and date of incident, helicopter arrival and departure, arrival at hospital, start of surgery)
- Observations on arrival at hospital (HR, RR, GCS, SpO_2, BP)
- In-hospital treatment (blood products, rFVIIa, TXA and others)
- Hospital (i.e. BASTION) length of stay
- Disposal (e.g. local national patients transferred to host nation facilities)
- Mortality outcome

The patients were divided into three groups: Those brought in by MERT before PHBTx was available (pre-PHB), those brought in by MERT after PHBTx was available (post-PHB) and those brought in by platforms that were never equipped with PHBTx. The latter group will not be considered further until the discussion. Only those data fields that could not be affected by the intervention could be used in generating the propensity score. This includes demographic and injury data and prehospital observations. It excludes observations on admission to hospital. Many severely injured patients in both groups, including several PHBTx recipients had incomplete or non-existent prehospital observations. Indeed there appeared to be a relationship between the severity of injury and the likelihood of prehospital observations being absent and a change in the chance of their being recorded over time. This is understandable: the constrained, dark, moving and sometimes dangerous environment aboard a helicopter in a combat area makes observations difficult. The more severely injured a patient is, the more important it is to concentrate on

immediately life-saving interventions rather than on accurate data recording. Recording may have become more common as familiarity with the environment grew and training improved. However, this potentially created a confounding relationship between the recording of these data and outcome. To use the prehospital observations might therefore have introduced bias into the results. It was concluded that prehospital observations could not be used as a basis of the propensity score.

Logistic regression on the dependent variable of PHBTx usage in the Post-PHB group was carried out. ISS was used as a summary statistic for the AIS codes. The other independent variables were the patient demographics, the mechanism of injury and the time from injury to arrival at hospital. Given the lack of direct data about transfusion triggers (i.e. prehospital observations), the model generated was heavily dominated by the ISS. Given the limitations of ISS laid out in the Appendix, it was thought likely the model would generate pairs that were well matched for ISS but not necessarily well matched for the underlying factor of interest, i.e. their injury pattern. Ideally, prehospital observations would have compensated for this by matching patients according to their physiological response to injury, but these were unavailable. An alternative method of generating matches was therefore required.

4.3.3 Proposed revised methodology

The author proposed a novel matching methodology designed to produce close matching of injury profile. The three most severe AIS codes for each patient were to be used, regardless of body region (following a convention derived from the method of calculating NISS). Since age and sex were somewhat consistent among US and UK recipients (i.e. mostly young men), the analysis would exclude local nationals and ignore these demographic factors. Each recipient would then be matched with a control from the pre-PHB group with the same:

- Mechanism of injury
- Three most severe injuries as defined by their severity and body region
    - e.g. matching AIS4 lower limb, AIS3 lower limb and AIS3 abdomen

Where such an exact match was not possible, an algorithm would be required to select close matches, ranking these and setting a minimum acceptable level of matching. The algorithm would also need to ensure that each potential control was only selected once and attempt to maximise the closeness of matching overall. This outline was submitted to the project's statistical advisors who then worked on generating a usable methodology. Discussions led to agreement that it would be desirable to include local nationals and account for demographic factors. This led to the development of the final methodology set out below.[*]

4.3.4 Final Matching Methodology

Patients who were administered prehospital blood products (recipients) were paired with patients transported before 1 July 2008 who would have been expected to receive prehospital transfusion if it had been available. A logistic regression was conducted in the Post-PHB cohort with receipt of PHBTx as the dependant variable using the variables sex, age, patient category (i.e. national status), mechanism of injury, and detailed injury data. This was used to provide a propensity score for the second stage of the matching process laid out below. The three most severe Abbreviated Injury Scale (AIS) codes for each patient, irrespective of body region, were included as variables in the regression model. An algorithm was then created to parse the first three digits of the AIS code for each injury to determine the fundamental injury descriptors: (1) body region of injury (head, face, neck, thorax, abdomen, spine, upper extremity, lower extremity, and other), (2) anatomic region (whole area, vessels, nerves, organs/muscles, skeletal/joints, and loss of consciousness), and (3) specific forms of injury (abrasions, lacerations, contusions, amputations, burns, etc).

Matching proceeded in two stages. First, Recipients patients were matched with one or more Pre-PHB patients with similar detailed injury data. Preference was given to exact injury code matches for all three injuries. Where this was not possible an algorithm was used to progressively expand the range of acceptable AIS matches. Second order matches were based on severity and descriptors 1-3 above, third order

[*] The author was responsible for the proposed methodology as set out in the preceding paragraph. The changes made to produce the final methodology are largely credited to Dr Apodaca-Morrison.

matches were based on severity and descriptors 1 and 2 above, and so on. The algorithm stopped when a selection of potential matches was available with equivalent goodness-of-fit, e.g. two potential matches with a single first-order AIS match and two second order AIS matches. Greedy matching[20] was then used to select the potential control with the most closely matched propensity score (as calculated based on the described logistic regression). Once a match was made it was not reconsidered; in the event that more than one unmatched control fitted to a case with equal propensity score, the control chosen for pairing was selected at random by the algorithm. This process is illustrated in Figure 4.1.[*]

This process produced groups of patients, matched recipients and matched non-recipients, consisting of matched pairs (Austin, 2008), Consequently, categorical data were analysed using McNemar's test, non-parametric data using Wilcoxon's signed-rank test.[†] The primary outcome was mortality. Admission observations and total PRBC and FFP use were secondary outcomes. $P<0.05$ on two-tailed testing was determined to be significant.

Mortality for UK and US personnel was defined as death within 30 days of wounding. For other patients, follow-up was limited to the in-hospital stay in Afghanistan. This approach is pragmatic, and has been employed previously (Morrison, Oh, *et al.*, 2013), as it is impossible to track the progress of casualties from other countries through their respective evacuation chains. The chance of this introducing bias should be small since patients were only transferred to local facilities once their clinical progress was such that they were thought no longer to need the advanced facilities at BASTION. Transfusion volumes pertain to the first 24 hours.

[*] This process was conducted by Dr Apodaca-Morrison; all other steps of the analysis were conducted by the author.

[†] Inspection and graphing of the data showed a great deal of skewed data, so non-parametric tests were used throughout.

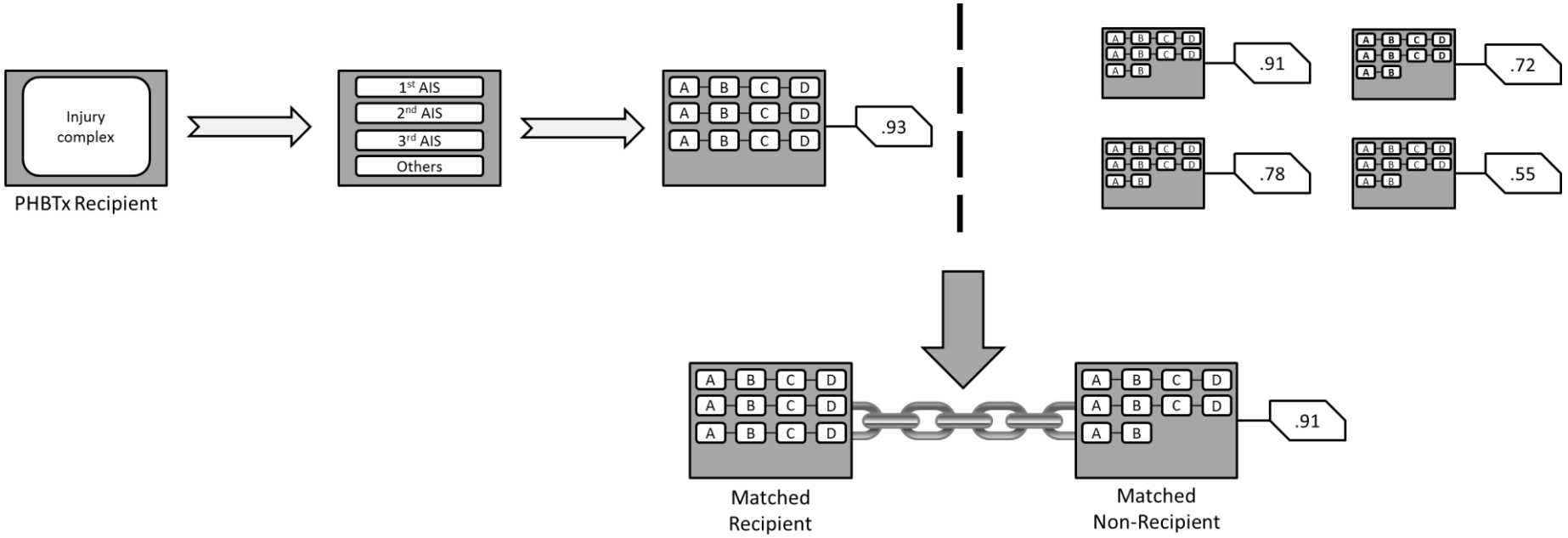


Figure 4.1 Matching process. Each cartouche represents a patient. On the left, a PHBTx patient's injury complex is broken down and the 1st, 2nd and 3rd most severe AIS codes are used for matching. Each AIS code is broken down to its four principle components A to D: A—Severity; B—Body region, C—Organ, D—Form of injury. On the right, four patients from the period before PHBTx became available are selected as possible matches. In this case, the potential matches have two exactly matching injuries but the third injury is only matched for severity and body region. Each potential match has been labelled with its propensity score, i.e. the probability that the patient would have been administered PHBTx had it been available. The potential match with the closest propensity score match is chosen to become the matched non-recipient.

4.4 Results

A total of 1592 patients were included: 439 before 1 July 2008 (Pre-PHB) and 1153 thereafter (Post-PHB). Table 4.1 compares the two cohorts. The rate of severe injury (ISS 16 or greater) rose from 28% to 42.5% ($p<0.001$, Chi-square) between the two cohorts. Mortality was higher in the Post-PHB group. However, mortality among severely injured Post-PHB patients was 134/485 (27.6%) whereas it was 39/122 (32.0%) in the Pre-PHB cohort ($p=0.343$, Chi-square). 310 of the later group (26.9%) received a prehospital blood transfusion during transfer. Table 4.2 shows the characteristics of the 310 PHBTx recipients. Prehospital transfusion was given to 48% of severely injured patients (ISS of 16 or more). 76.7% of recipients were severely injured, mostly as a result of explosions. Almost one-half of patients required intra-osseous circulatory access and/or advanced airway management. The median (interquartile range [IQR]) number of units of PRBC and FFP transfused prehospital was 2 (1-2). The median (IQR) total transfusion volume at 24 hours was 8u (range 3-18) PRBC and 7u (range 2-16) FFP. Nearly one-half of patients required a massive transfusion (>10 units while at Camp Bastion), although one-fifth required no further transfusion after admission.

Ninety seven of the 310 patients who received PHBTx were paired with patients from the Pre-PHB cohort (Figure 4.2). Table 4.3 shows the matched characteristics. The groups had excellent matching of injury profiles with 89 in each group being severely injured. Table 4.4 shows the treatments received and outcomes for both groups. The mortality rate in the pre-hospital transfusion recipients was half that of non-recipients (8.2% v 19.6%; $p=0.013$, McNemar). There was also a small improvement in admission heart rate though not in other observations. However, matched recipients received more prehospital airway interventions and reached hospital more quickly than non-recipients. Matched recipients patients received more blood products overall (Figure 4.3) and in higher FFP:PRBC ratios.

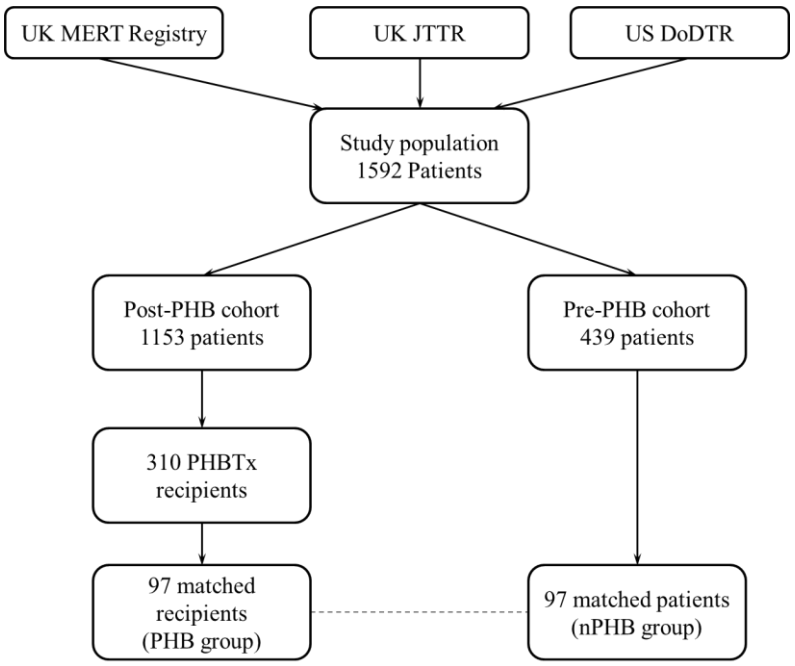


Figure 4.2. Study population flow chart. Pre-PHB/Post-PHB: Study cohorts before and after, respectively, the introduction of prehospital blood transfusion (PHBTx).

Matched recipients: PHBTx recipients successfully paired with patients from the Pre-PHB cohort with similar injury profiles and high propensity scores for receiving PHBTx, had it been available to them.

MERT Medical Emergency Response Team; JTTR Joint Theatre Trauma Registry; DoDTR Department of Defence Trauma Registry

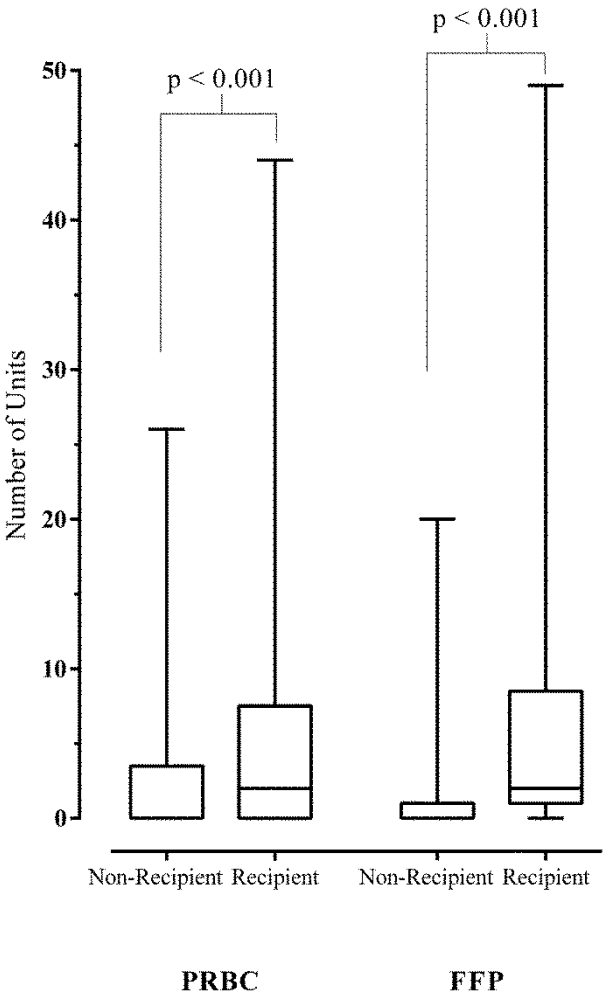


Figure 4.3 Units of PRBC and FFP transfused in the first 24 hours in hospital to matched recipients and non-recipients of prehospital transfusion.
Boxes show interquartile range with median. Whiskers show range. Groups compared with Wilcoxon signed rank test: PRBC Packed red blood cells, FFP Fresh frozen plasma.

		Pre-PHB	Post-PHB	P values
N		439	1592	
Age		23 (21-28)	23 (20-27)	0.277†
Patient Category	UK Military	214 (48.7)	642 (55.7)	**<0.001***
	Coalition Military	129 (29.4)	288 (25.0)	
	Afghan	96 (21.9)	223 (19.3)	
Sex	Male	425 (96.8)	1116 (96.8)	1*
Mechanism of Injury	Blunt	25 (5.7)	43 (3.7)	**<0.001***
	Burn	4 (0.9)	8 (0.7)	
	Explosive	246 (56.0)	809 (70.2)	
	GSW	163 (37.1)	286 (24.8)	
	Other	1 (0.2)	7 (0.6)	
Military ISS		5 (2-16) [1-75]	9 (4-25) [1-75]	**<0.001†**
Military NISS		8 (3-20) [1-75]	14(5-34) [1-75]	**<0.001†**
AIS≥3	Head and Neck	43 (9.8)	119 (10.3)	0.853*
	Face	1 (0.2)	10 (0.9)	0.308*
	Chest	50 (11.4)	151 (13.1)	0.399*
	Abdomen	29 (6.6)	141 (12.2)	**0.001***
	Extremity	95 (21.6)	421 (36.5)	**<0.001***
	External	7 (1.6)	15 (1.3)	0.636*

Table 4.1. Comparison of patients admitted before the introduction of PHBTx (Pre-PHB), those admitted afterwards (Post-PHB). P values are shown for significant results in the comparison of Pre-PHB and Post-PHB. Categorical data are shown as n (%) and compared using Fisher's exact test (*). Ordinal and scale data are shown as median (IQR) with [range] added where relevant and compared using the Mann-Whitney U test (†). Significant results shown in **bold**.

		PHBTx recipients
Age		24 (21-27)
Patient Category	UK Military	131 (42.3)
	Coalition Military	94 (30.3)
	Afghan	85 (27.4)
Sex	Male	302 (97.4)
Mechanism of Injury	Blunt	3 (1.0)
	Burn	1 (0.3)
	Explosive	226 (72.9)
	GSW	80 (25.8)
	Other	0 (0)
Military ISS		20 (16-29) [1-75]
Military NISS		29 (18-48) [1-75]
AIS 3 or greater	Head and Neck	26 (8.4)
	Face	3 (1.0)
	Chest	65 (21.0)
	Abdomen	68 (22.0)
	Extremity	215 (69.6)
	External	4 (1.3)
Prehospital interventions	Intraosseous access	140 (45.2)
	Advanced airway	130 (41.9)
	Chest decompression	38 (12.3)
Prehospital time (min)		61 (47-90) [25-1034]
Admission observations	Prehospital arrest	7 (2.7)*
	Systolic BP	122 (92-142)
	Respiratory rate	18 (12-20)
	Heart rate	105(80-125)
Prehospital transfusion	PRBC	2 (1-2) [0-4]
	FFP	2 (1-2) [0-4]
In hospital transfusion	PRBC	7 (1-15) [0-82]
	FFP	6 (1-14) [0-70]
	Cryoprecipitate	0 (0-1) [0-20]
	Platelets	0 (0-3) [0-18]
Total PRBC		8 (3-18) [0-85]
Total FFP		7 (2-16) [0-74]
Any hospital PRBC transfusion		248 (80)
Massive transfusion		146 (47.1)
Tranexamic acid		119 (38.4)
rFVIIa		33 (10.6)
Mortality		62 (20)

Table 4.2. Injury profile of all patients who received prehospital blood transfusion. Data are displayed as n (%) or median (IQR) with [range] in some cases. Hospital blood product usage shown for the first 24 hours. Massive transfusion defined as 10 or more units PRBC in 24 hours. PHBTx Prehospital Blood Transfusion; ISS Injury Severity Score; NISS New Injury Severity Score; AIS Abbreviated Injury Scale; PRBC Packed Red Blood Cells; FFP Fresh Frozen Plasma; TXA Tranexamic Acid; rFVIIA Recombinant Activated Factor VII. *Excludes 16 patients with no recorded admission observations.

		Recipients	Non-Recipients	P value
N		97	97	
Age		24 (20-28)	23 (21-28)	0.975
Patient Category	UK Military	45 (46.4)	38 (39.2)	0.248
	Coalition Military	31 (32)	45 (46.4)	
	Afghan Civilian	21 (21.6)	14 (14.4)	
Sex	Male	95 (97.9)	97 (100)	u/t
Mechanism of Injury	Blunt	1 (1)	3 (3.1)	u/t
	Burn	0 (0)	0 (0)	
	Explosive	50 (51.5)	48 (49.5)	
	GSW	46 (47.4)	46 (47.4)	
	Other	0 (0)	0 (0)	
Military ISS		16 (9-25)	16 (9-24.5)	0.686
Military NISS		22 (15-33)	21 (14-34)	1
AIS 3 or greater	Head/Neck	0	1 (1)	1
	Face	0	1 (1)	1
	Chest	24 (24.7)	25 (25.8)	1
	Abdomen	18 (18.6)	18 (18.6)	1
	Extremity	67 (69.1)	65 (67)	0.727
	External	2 (2)	3 (2)	1

Table 4.3. Matched characteristics of paired groups of patients who did and did not receive PHBTx. Categorical data are shown as n (%) and compared using McNemar's test. Ordinal and scale data are shown as median (IQR) and compared using the Wilcoxon signed-rank test.
u/t – Untestable due to limitations of McNemar's test (due to zero value or multiple categories; Patient category was dichotomised to coalition/Afghan to allow testing)
ISS Injury Severity Score; NISS New Injury Severity Score.

		Recipients	Non-Recipients	P value
N		97	97	
Prehospital interventions	Intraosseous access	14 (14.4)	16 (16.5)	0.824
	Advanced airway	19 (19.6)	9 (9.3)	**0.041**
	Chest decompression	19 (19.6)	22 (22.6)	0.629
Prehospital time (min)		68 (50-100)	109.5 (70 – 171)	**0.008**
Admission observations	Cardiac arrest	7 (7.2)	9 (9.3)	1
	Systolic BP	132 (111-145)	131 (114-150)	0.145
	Respiratory rate	19 (15-24)	20 (16-26)	0.173
	Heart rate	92 (74-115)	105(82-128)	**0.041**
TXA		22 (22.6)	0 (0)	u/t
rFVIIa		2 (2)*	10 (10.3)	**0.033**
Prehospital transfusion	PRBC	1 (1-2) [0-4]	n/a	
	FFP	2 (1-2) [0-4]	n/a	
In hospital transfusion	PRBC	2 (1-8.5) [0-49]	0 (0-3.5) [0-26]	**<0.001**
	FFP	2 (0-7.5) [0-44]	0 (0-1) [0-20]	**<0.001**
	Cryoprecipitate	0 (0-0) [0-4]	0 (0-0) [0-3]	0.068
	Platelets	0 (0-0) [0-7]	0 (0-0) [0-6]	**<0.007**
Total PRBC		4 (2-10) [0-53]	0 (0-3.5) [0-26]	**<0.001**
Total FFP		2 (2-9) [1-44]	0 (0-1) [0-20]	**<0.001**
Any in-hospital PRBC transfusion		75 (77)	38 (39)	**<0.001**
Massive transfusion		12 (12)	8 (8)	0.388
FFP:PRBC ratio		1 (0.83-1.23)	0.46 (0-0.72)	**<0.001**
Mortality		8 (8.2)	19 (19.6)	**0.013**

Table 4.4. Treatment and outcomes of matched groups of patients who did and did not receive PHBTx. Categorical data are shown as n (%) and compared using McNemar's test. Ordinal and scale data are shown as median (IQR) with [range] added where relevant and compared using the Wilcoxon signed-rank test.
Significant results shown in **bold**.
u/t – Untestable due to limitations of McNemar's test. (due to zero value)
PRBC Packed Red Blood Cells; FFP Fresh Frozen Plasma; TXA Tranexamic acid; rFVIIa Recombinant Activated Factor VII

4.5 Discussion

4.5.1 Methodological Validity

A matched cohort design was employed, using an effective novel matching technique that produced similar injury complexes within each pair. Thus it minimised the effect of the confounding variables that are included in the process. (The substantial effect of unmeasured confounders is discussed below.) The lack of prehospital observations was undesirable. Close matching of injuries was intended to compensate for this. ISS and NISS have been criticised on many grounds (see the appendix for a more in depth treatment of injury coding) (Lefering, 2002). In particular, they conflate injury patterns, which both loses information about particular injuries and makes very different injuries (e.g. severe head injury and proximal limb amputation) essentially interchangeable (Russell *et al.*, 2004). This method therefore sought to match patients much more closely, making fuller use of the information contained in the AIS coding of the patient's injuries by using the approach described. This is a novel application of established techniques which may have wider applicability in the analysis of injury data, particularly if combined with physiological data. The group of potential historical controls was considerably smaller than the equivalent group after the introduction of PHBTx (439 v 1153). As such, it was inevitable that only a minority of PHBTx recipients would be matched to suitable controls. It is possible that this could introduce some element of selection bias. However, it is inevitable that matching recipients to patients from a cohort with less severe injuries selects for less severely injured recipients. It is conceivable that the recorded effect would have been larger if more severely injured recipients and non-recipients had been included in the comparison. In a related analysis looking at the effect of different medical retrieval platforms, a benefit of the MERT platform (including PHBTx) over others was only demonstrated in the ISS range 16-50, so this may not be the case (Morrison, Oh, *et al.*, 2013).

It is impossible to test in this dataset whether the methodology succeeded in compensating for the missing prehospital observations. Since paired tests were used, significant results could have been generated by either between-group difference due to treatment effects or by poor matching of physiological response to injury within the

pairs. The latter was not seen in the available data: admission observations were similar in the two groups.

A facet of propensity score methodology deserves comment. The conventional practice of selecting the potential control with the propensity score most closely matched to that of the recipient was implemented. This is intended to give controls with similar characteristics to the recipients, thereby reducing the effect of **measured** confounders in the outcome analysis. It is possible for the predictors of exposure (receiving PHBTx) and of outcome (mortality) to be orthogonal to one another. At the extreme, if exposure were determined by hair colour and outcome by age then a propensity score methodology would match on the former alone, potentially leaving differences between the groups and, crucially, within pairs on the latter. More realistically, factors may be very differently weighted in how they contribute to the propensity score and to outcome. Thus, a similar propensity score may be arrived at based on many differing combinations of underlying characteristics, and the predicted outcome of these may vary wildly. An example in this context might be severe head injury: it would have a profound effect on mortality but may have little correlation with whether a patient would receive PHBTx. The present methodology goes some way to ameliorating this problem. A more usual approach is to use multivariate analysis of the matched cohorts, employing generalised estimating equation methodology (Austin, 2008). The number of matched cases was insufficient to allow this in the present study.

4.5.2 Implications of the Results

There was a large increase in total blood product usage between the two matched groups with median PRBC usage increasing from 0 to 4 ($p<0.001$, Wilcoxon) and from 2 to 7 among those with ISS 16 or more ($p<0.001$). This was despite the fact there was no increase in the proportion requiring massive transfusion (over 90% of pairs were concordant as to whether they received 10 or more units PRBC). It is known that there has been a steady increase in blood product usage in recent years (Jansen *et al.*, 2014). There was a significant difference in usage between the pre- and post-PHB time periods (Table 4.1). Severely injured pre-PHB patients who were transfused received a

median (IQR) of 6 (3-12) units PRBC. Equivalent post-PHB patients who did not receive PHBTx received 10.5 (4.5-22) units, a significant increase (p=0.003, Mann-Whitney), see Figure 4.4. The PRBC usage in severely injured post-PHB patients who did receive PHBTx was 12 (4-19) units, which is not significantly higher than the latter group (p=0.9, ISS and NISS were also similar). In addition, the FFP:PRBC ratio differed markedly between the matched groups. The 11 excess survivors in the intervention group are inadequate for survivorship bias to fully explain the increased total volumes and ratios. Therefore there was a large secular change in resuscitation practice between the two groups beyond the provision of a pre-hospital transfusion capability.

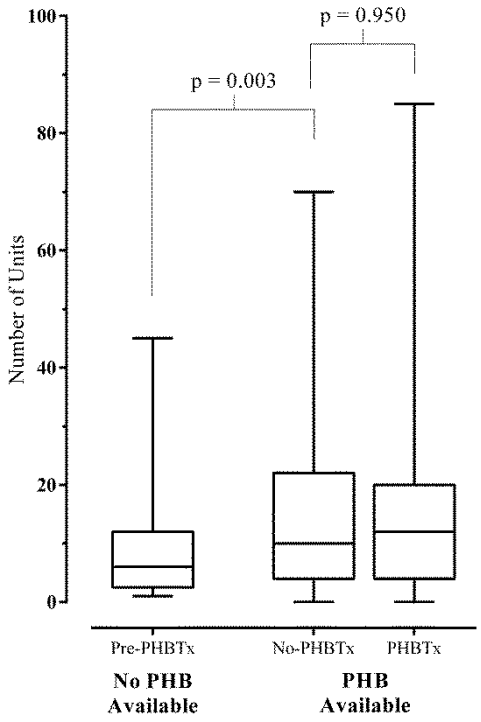


Figure 4.4 Total units of PRBC transfused in the first 24 hours to severely injured (ISS>15) patients who received any PRBC or FFP and:

- Were admitted before PHBTx was available (Pre-PHBTx),
- Were admitted after PHBTx was available but did not receive PHBTx (No-PHBTx),
- Received PHBTx (PHBTx).

Boxes show interquartile range with median. Whiskers show range. Groups compared with Mann-Whitney U test.
ISS Injury severity score, PRBC Packed red blood cells, FFP Fresh frozen plasma, PHBTx Prehospital blood transfusion.

Other factors which may confound the analysis of mortality were identified. The fall in prehospital times probably reflects changes in the deployment of forces in Helmand

Province during the study period, with the arrival of US Marine Corps units accompanied by US aeromedical assets. "Intelligent tasking" of aeromedical assets was implemented, whereby a combination of clinical, operational and logistic considerations drove the choice of which US (Army DUSTOFF or Air Force PEDRO) or UK (MERT) capability was deployed to a given casualty incident. While quick transportation to hospital is no doubt beneficial to the casualty, selection of the larger, more clinically capable MERT platform during the post-PHB period may be an indicator of enhanced casualty burden. This could be individual (i.e. the casualty's injuries and condition) or collective (i.e. the number of casualties). MERT can transport 8 casualties as opposed to 2 for DUSTOFF and PEDRO. Thus the effect of the time from injury to admission is very difficult to assess. The increased rate of prehospital procedures could represent increased need but, anecdotally, there seems to have been a lowered threshold for intervention in combination with the evolution and routine implementation of Standard Operating Procedures. For example, rapid sequence induction is now used more as a definitive form of analgesia or where the requirement for general anaesthesia to facilitate surgery is anticipated ("expected clinical course"). The routine use of TXA and decreased rFVIIa use represent the effects of opposing trends in the evidence base for the two drugs (CRASH-2 trial collaborators, 2010; Hauser *et al.*, 2010; Morrison, Dubose, *et al.*, 2012).

Therefore, no clear assessment of the hypothesis, which relates to the isolated benefit of PHBTx, can be made. However, the data provide evidence that DCR in combination with intensive prehospital intervention including PHBTx is associated with substantial improvements in mortality (although the contributions of the various components cannot be determined and the prehospital time remains a confounding factor).

Haemostatic resuscitation was introduced in the Post-PHB period, in that blood products were used frequently, early and in high FFP:PRBC ratios (Jansen *et al.*, 2014). Rotational thromboelastrometry was deployed to guide coagulopathy management (Doran, Woolley and Midwinter, 2010). Transfusion volumes are often interpreted as a proxy for blood loss. Counter-intuitively, the "haemostatic" regime involved much larger total transfusion volumes. This practice has been demonstrated to be able to correct physiological derangement in very severely injured combat casualties

(Morrison, Ross, *et al.*, 2013). These patients often need extensive debridement which, in the context of normotension with euvolaemia, may require considerable transfusion support. It contrasts with DCR practice as described by others where conservation of blood products has been reported as one of the advantages of haemostatic resuscitation (Cotton *et al.*, 2011).

4.5.3 Policy Response

The data shown here were presented as a preliminary analysis at a meeting chaired by the Medical Director of the UK Joint Medical Command, the senior officer responsible for clinical research. Prior to this, exploratory examination of scanned copies of patients' notes and of the hospital laboratory information system and data available from the deployed ROTEM consoles indicated that it would not be possible to develop adequate coagulation or blood gas data to allow analysis. Critical care follow up data was available for UK patients but would be difficult to obtain for US patients (since it is not held in any central database). In any case, such further outcome analyses would suffer the same confounders as the mortality comparison. The meeting directed that these should not be pursued.

There was considerable debate as to whether a further analysis should be conducted using patients transported by non-MERT platforms as the potential controls. The advantage of this would be that these patients were contemporary with the PHBTx recipients and therefore the analysis would not be confounded by differences in hospital management. However, this analysis would involve differences in prehospital care much more significant than those seen in the present analysis. Given the additional sensitivity of comparison of different national prehospital platforms and practices, the meeting also directed that this analysis should not proceed.

4.5.4 Implications for Further Research

There is very little evidence to support the use of PHBTx. Neither the recent papers by Brown *et al* (2015) or Holcomb *et a l*(2014) nor this analysis establish strong evidence

in its favour. In a separate analysis of UK military data, using a different methodology, Smith *et al* have also found no evidence of benefit (Smith *et al.*, 2014).

Concerns about its safety and logistic implications must therefore remain. While the present analysis may show some support for PHBTx in military practice, it does not follow that this can be transferred into civilian usage. The relative infrequency of severe injury in civilian practice could mean that its carriage by civilian prehospital services would lead to its use among a very different patient population where the risks and benefits may be differently balanced. Where a patient is apparently exsanguinating, there can be little doubt that PHBTx is an appropriate treatment (though secondary to stanching the flow). In all other circumstances, there cannot be certainty as to its usefulness. Accordingly, it should be possible to define a population in which a sufficient degree of equipoise exists to support the randomisation of patients. Randomised control trials are already proposed in the civilian setting: RePHILL (Prof M Midwinter, personal communication) and PAMPer (Brown, Guyette, *et al.*, 2015), and these should be supported.

Randomised research faces additional barriers in military practice, although randomised therapeutic trials have been conducted recently (Fries *et al.*, 2014). However, the withdrawal of UK and US forces from Afghanistan represents the end of the opportunity to carry out such research, which unhappily was not pursued earlier in recent conflicts. Additionally, the perception of the efficacy of PHBTx among military prehospital practitioners is so strong as to make randomisation impossible even if it was a practical option for several years.

This work established that there was insufficient human data to demonstrate a benefit of PHBTx. Given this, and the lack of information about the physiological and coagulation effects, the only option to explore the impact of this treatment in the context of military trauma at present is the use of an animal model.

5 Methodological Background to Work in the Animal Model

The preceding chapter established the need for an animal model to study prehospital transfusion. The remainder of this thesis concerns work carried out in that animal model of severe military trauma, which was designed for the evaluation of haemostatic resuscitation regimes. The model was developed with a view to carrying out a randomised control trial comparing simulated prehospital transfusion with conventional resuscitation. As discussed in Chapters 2 and 3 the roles of platelets in traumatic coagulopathy and of platelet in trauma resuscitation have not been fully elucidated. Consequently, means of evaluating platelet function were required so these could be studied in the proposed RCT and in later work. Therefore, in parallel with the model development, a flow cytometry assay of platelet activation and response to agonist was developed. This became the focus of the author's work and is detailed in the next two chapters.

This chapter begins with a discussion of the coagulation assays, including those that address platelets, which were used in the model. It then focuses on an exploration of the technique of flow cytometry, which is fundamental to understanding the development and assessment of the platelet assay laid out in the next two chapters. Although the development of the animal model is outside the scope of this report, this chapter ends with comments about the role of animal models and about the necessary modifications to the previously discussed Dstl animal model of combat injury.

5.1 Coagulation Assays

The design and validation of the platelet assay was part of the wider selection of assays of the coagulation system. These included standard laboratory tests and thromboelastography (TE).

5.1.1 Laboratory Clotting Assays

Standard laboratory assessments of coagulation, prothrombin time (PT) and activated partial thromboplastin time (APTT), were used. PT (or its derivative, INR) and APTT have been shown to correlate with outcomes after trauma (MacLeod *et al.*, 2003; Dirks *et al.*, 2010; Johansson *et al.*, 2011b). However, these tests were designed to detect deficiencies of procoagulant factors and therefore do not reflect abnormalities of natural anticoagulants (Tripodi, Chantarangkul and Mannucci, 2009). They are also unaffected by platelet number and function. These facts, and the clinical advantages of near patient testing, have led to increased interest in assessment by rotational TE.

5.1.2 Thromboelastometry

Two commercial systems are available, each involving the stimulated clotting of blood in a cup while assessing the forces applied by the clot on a wire suspended centrally within it:

- ROTEM—the wire (in fact a pin) rotates within the cup.
- TEG—the cup rotates around the wire.

The force is recorded as a deflection in the TE trace (see Figure 5.1). Compared with laboratory tests, TE is more clinically convenient, rapid and informative: Table 5.1 shows how different components of a TEG trace are thought to correlate with components of the coagulation system.

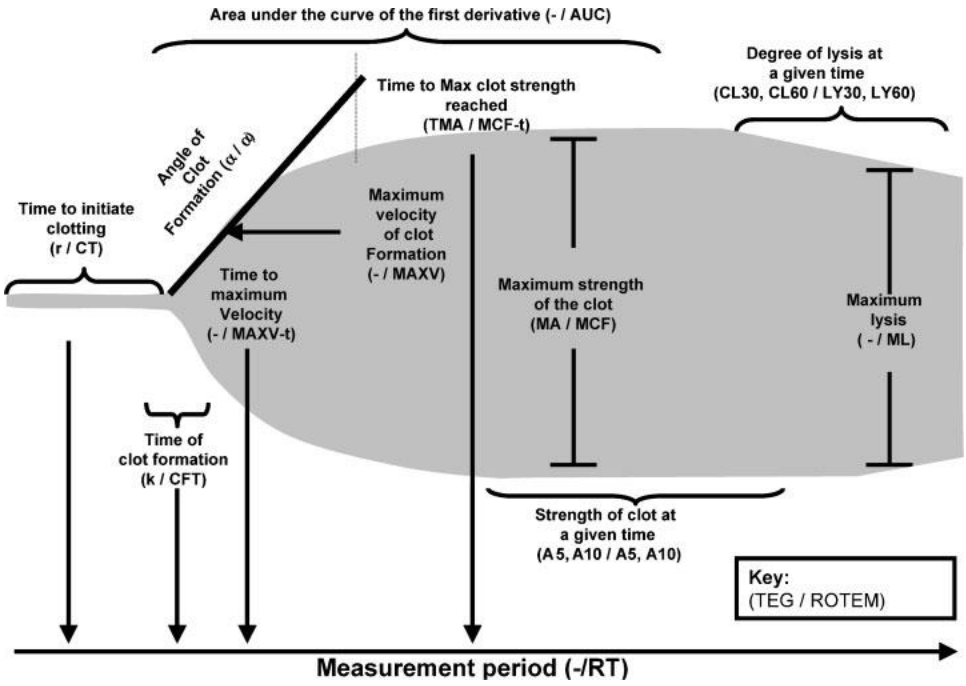


Figure 5.1 Viscoelastic haemostatic assays terminology and parameters

α, alpha angle; AUC, area under the curve; CFT, clot formation time; CL (t), clot lysis (at time t); CT, clot time; k, rate of clot formation; LY (t), lysis (at time t); MA, maximum amplitude; MAXV, maximum velocity; MAXV-t, time to maximum velocity; MCF, maximum clot firmness; MCF-t, time to maximum clot firmness; ML, maximum lysis; r, time to clot initiation; ROTEM, Rotational Thromboelastogram; RT, reaction time; TEG, Thromboelastograph; TMA, time to maximum amplitude; '-'; no equivalent parameter.

Reproduced by kind permission of Dr Roger Luddington, Addenbrooke's Hospital, UK. See Table 5.1 for explanation of those elements used in this thesis.

Table 5.1 Key TEG and ROTEM terms used in this thesis, with attention to the contribution of platelets to these.

TEG term	ROTEM term	Physiological correlate	Platelet contribution
R	CT	Time from activation until the formation of thrombin adequate to lyse fibrinogen, i.e. this represents the action of clotting factors upstream of thombin and is related to the processes of initiation and amplification.	Platelets provide the site on which clotting factors come together, many of them being bound to the platelet surface during physiological clotting.
A	α	Predominantly the speed of generation of fibrin which, except in profound hypofibrinogenaemia, is usually determined by the quantity of thrombin generated.	In viscoelastic tests, clotting factor activation precedes and causes platelet activation, which then contributes to clot strength through platelet aggregation, as the site of fibrin formation and polymerisation, and by binding (anchoring) fibrin polymers. Since it contributes to the overall strength, it also effects the rate of clot formation.
MA	MCF	Maximum strength of the clot is determined by the amount of fibrin generated (and its polymerisation and crosslinking) and by platelet aggregation. The FIBTEM assay gives an MCF due only to the contribution of fibrin (although see text for further discussion).	As above. The difference between EXTEM and FIBTEM trace MCFs is held to represent the platelet contribution.

The use of TE-guided coagulation therapy has been explored in detail in cardiac and liver surgery, including using randomised control trials (Craig *et al.*, 2008; Johansson *et al.*, 2009). The transfusion protocols in these studies typically use TE to detect clot abnormalities and respond by giving procoagulants (FFP, platelets) or antifibrinolytics (Shore-Lesserson *et al.*, 1999). The possible contribution of endogenous anticoagulants, which may be particularly important after trauma (Brohi *et al.*, 2007), is not addressed.

In recent years there has been much attention to TE's applicability in trauma and ROTEM is now in routine clinical use in the British armed forces (Doran, Woolley and Midwinter, 2010). Kauffman *et al* (1997) demonstrated that TEG detected a hypercoagulable state in mildly injured patients on admission but that severely injured patients were hypocoagulopathic. TEG, unlike laboratory tests, was an independent predictor of transfusion requirement after blunt injury. Plotkin *et al* (2008) found that low clot strength was associated with increased transfusion in combat casualties sustaining penetrating injury. Again, TEG was superior to laboratory tests at predicting blood product use. Carroll *et al* (2009) found that TE parameters on admission predicted fatality while Leeman *et al* demonstrated the use of TE to predict massive transfusion (Leemann *et al.*, 2010). Brohi's group published a series (Davenport, Manson, *et al.*, 2011), confirming the superior predictive value of TE over laboratory tests, now confirmed in a large international collaboration (n=808) (Hagemo *et al.*, 2015). Johansson and Stensballe (2009) successfully implemented a haemostatic resuscitation regime using TEG to guide therapy with FFP and other blood products. Schöchl *et al* (2010, 2011) had similar results using factor concentrates. TEG was the established technique in the Dstl laboratory and was therefore used in this study.

5.1.3 Platelet Assays

Having selected TE as the basis of the assessment of coagulation, it was necessary to select a means for assessing platelet function. The assessment of platelet activity is complex because of the variety of functions that can be measured and the multitude of devices available to do this. This variety reflects the fact that none is ideal.

Components of platelet function include activation (i.e. the expression of stored adhesion molecules), microvesicle formation, adhesion, and aggregation. The latter can be static (how many platelet or platelet-leukocyte aggregates are there in the sample with or without *in vitro* stimulation) or dynamic (real time assessment of aggregate formation in response to a stimulus). In addition, it is possible to determine the responsiveness of platelets to a variety of stimulants at different concentrations, which may be termed activatability. No single platform provides for testing all of these. In addition, the focus of many commercially available devices is the monitoring of antiplatelet therapy (Harrison *et al.*, 2007).

Platelets contribute to the TE trace in three principal ways (Table 5.1): as the site of rapid thrombin formation during propagation, by incorporation in the clot through binding to fibrin and by binding to one another (aggregation). Cytochalasin D inhibits the conformational changes of the actin cytoskeleton that are crucial to platelet activation. Use of cytochalasin D abolishes most of the contribution of platelets to clot strength. This assay (FIBTEM in ROTEM nomenclature) isolates the contribution of fibrin to the clot, allowing detection of fibrinogen deficiency. Comparison with the uninhibited assays gives a crude measure of the platelet contribution to clot strength. A poor EXTEM with a normal or near-normal FIBTEM implies inadequate platelet function. When the FIBTEM is also abnormal, it is difficult to distinguish coexistent platelet dysfunction. The functional fibrinogen TEG (FF TEG) removes the contribution of platelets using a GPIIa/IIIb antagonist and can be interpreted in a similar way. In fact, these two methods of platelet inhibition (via the cytoskeleton and GPIIa/IIIb) have different and additive effects on the TE trace (Lang *et al.*, 2004). TE can also be modified to examine platelet function by using platelet agonists (ADP, arachidonic acid) as initiators alongside an exogenous thrombin mimetic and FXIIIa (to remove the influence of all clotting factors upstream of fibrinogen) (Craft *et al.*, 2004). This is known as platelet mapping.

Flow cytometry allows measurement of MV_{plt}, of the expression of activation markers, and of platelet and platelet-leukocyte aggregates. These can be assessed with and without stimulation. The technique can be used to study platelets in enriched samples: platelet-rich plasma (PRP) can be produced by a variety of techniques.

However, Shattil *et al* (1987) introduced whole blood flow cytometry, in which the platelets remain in the physiological milieu of whole blood and artefacts due to agitation during processing are minimised. Platelet flow cytometry has the facility to detect multiple activation markers and to change between them easily (Michelson, 1996). Thus flow cytometry is a versatile technique allowing assessment of several aspects of platelet function.

A variety of other methods are available (Harrison *et al.*, 2007). The bleeding time, the time taken for a standardised wound to stop bleeding, has declined in popularity (Harker and Slichter, 1972). However, given its insensitivity and poor reproducibility (Lind, 1991), this is a greater loss to comedy (Thomas, 1955) than haematology. Light transmission aggregometry and its more user-friendly successor, impedance whole blood aggregometry, measure platelet aggregation under low shear in response to various agonists. While the older systems require time and skill to operate, newer models have largely automated the process. Since they are designed to monitor antiplatelet therapy, they do not allow an assessment of aggregation without *in vitro* stimulation, as might be seen in blood taken from patients after injury. The PFA-100 (Dade International Inc., Miami, FL) tests platelet aggregation and adhesion in conditions of shear intended to simulate blood flow (Kundu *et al.*, 1996). Platelets are activated by either collagen/epinephrine or collagen/ADP and the time taken to occlude a micropore in the wall of a simulated capillary is measured (closing time).

Flow cytometry was chosen because of its inherent versatility and the facts that the equipment was available and the technique was already being employed to assess leukocytes.

5.2 Flow Cytometry

5.2.1 Principles of Flow Cytometry

Having selected flow cytometry for measuring platelet function, a new assay had to be designed and validated. An understanding of the principles of flow cytometry is necessary to discuss this process. Flow cytometry measures characteristics of

individual cells by passing them through a laser beam past a set of sensors (Shapiro, 2003; Macey, 2007). The cells scatter the light. The amount of light scattered forward (FSC) at low angles (0.5-10°) is a function of the size of the particle. As light is reflected and refracted by structures within the cell it is scattered at larger angles. The amount of side scattered light (SSC) is therefore a measure of the internal complexity of the cell. These basic principles were set out by Coulter (1956) and Kamentsky *et al* (1965) in the 1950s and 60s and allowed for cell counting and sorting devices to be developed. However, in the 1990s, the availability of monoclonal antibodies (mAb) conjugated to fluorophores allowed flow cytometers to detect much more detailed information about cells. mAb bind to cell surface epitopes. When excited by the laser, the fluorophores emit characteristic wavelengths of light. Alternatively, membrane, cytosolic and nuclear dyes may be employed.

The cells must pass through the sensing area one at a time. To achieve this most cytometers use a sheath flow technique. Sample containing the cells enters the chamber through a small aperture. This is surrounded by sheath fluid which is under pressure and flowing. Sample fluid is drawn out and focused by hydrodynamic effects which arise from the shaping of the chamber. This moves cells to the centre of the stream. Pressure adjustment allows cells to be drawn out singly, which allows individual examination and prevents clumping. Flow cytometers can process many thousands of detection events (cells, fragments and artefacts) per second. The speed at which the stream passes the sensing area is fixed. Consequently, the event rate is determined by the concentration of cells in the sample fluid and the width of the stream. The laser beam has an oval cross-section. Consequently, it is wider at the centre of the stream than at the edges. If the sample stream is wide, small events passing through the edge of the stream are exposed to the laser for a shorter period at a lower intensity. In consequence they scatter and fluoresce less light. Conversely, the stream may be wide enough to accommodate more than one event passing simultaneously, their total scatter and fluorescence being summed. When the cytometer senses an event it detects all light scattered or fluoresced by the contents of the sensing area. Unbound dyes, for example, can contribute to the light detected.

5.2.2 Platelet Flow Cytometry

The preparation of platelet samples for flow cytometry is a multiple-step process which requires careful standardisation. Steps such as preparing platelet-rich plasma and fixation can affect the detection of surface antigen and attention must be paid to the necessity and ordering of these steps during the design of an assay (Cahill, Macey and Newland, 1993; Michelson, 1996; Shapiro, 2003). The choice of anticoagulant is important as this can also cause activation. Citrate, as used in this case, is recommended over alternatives such as lithium/heparin and EDTA for this reason (Michelson, 1996). Markers must be selected for the detection of platelets and of platelet activation. Platelet populations can be detected by light scatter characteristics alone but under certain circumstances other particles may fall in the platelet area of the scatter plot and not bind platelet-specific mAb (Michelson, 1996). Such an mAb is normally included to allow positive identification. Several markers are available. CD61 was selected because it is a widely used platelet-specific marker (Macey *et al.*, 1999; Jacoby *et al.*, 2001) and a porcine mAb was commercially available.

There are multiple ways of detecting platelet activation. PAC-1 mAb are specific for the binding site exposed by the activation-dependant conformational change in GPIIa/IIIb. CD61 is part of GPIIa/IIb, so use of PAC-1 and anti-CD61 in the same assay could lead to interference at the binding sites. While there is considerable species cross-reactivity, no PAC-1 mAb had been validated for use in pigs so this conflict was avoided. The surface expression of the GPIb-IX-V complex falls during activation as the epitopes relocate within the OCS (Figure 2.5). While this fall can be used as a marker of activation by measuring the fluorescence intensity of the platelets as a whole, it is not possible to define activated and unactivated populations. CD62P and CD31 are widely used activation markers and validated mAb for porcine use were available. CD62P has been shown (in baboons) to be lost from the surface of activated platelets within 2 hours (Michelson *et al.*, 1996). These platelets continue to circulate and function in other respects. For many applications this has the disadvantage that platelets must be drawn soon after any stimulus and processed quickly. However, in this study, serial measurement of CD62P expression allows the detection of ongoing activation: one would expect a single activating stimulus to be associated with a rise

and fall in CD62P expression, whereas sustained expression (subject to assuming constant behaviour between baboon and pig platelets) would imply further recruitment of platelets to the CD62P+ population. Measurement of platelet-neutrophil aggregates (for which the assay could in future be adapted) would enhance this since platelets that have lost their CD62P following initial activation can still form aggregates (Michelson *et al.*, 1996) and these circulate only for short periods (less than 30min) (Michelson *et al.*, 2001). CD62P and CD31 were examined during the design of the assay.

The stimulated activation of platelets in a flow cytometry assay can be used to provide a positive control, i.e. platelet activation may be stimulated and detected, allowing the experimenter to be confident that a failure to detect activation in platelets which have not been exposed to an exogenous agonist reflects a physiological reality. Additionally, it can be used to detect platelets that are inhibited or effete. Several substances are used to stimulate the activation of platelets *in vitro*.

- Thrombin activates platelets by cleaving the free terminal of a protease-activated receptor (PAR). The neoterminal then ligates the receptor's bonding site (Grand, Turnell and Grabham, 1996). While thrombin cannot be used alone *in vitro* due to clot formation, Thrombin Receptor Activating Peptide (TRAP), a short peptide consisting of the same residues as the PAR's neoterminal, is a potent activator.
- ADP has a more selective effect, causing partial degranulation but not aggregation of unstimulated platelets (Janes *et al.*, 1994). Impaired platelet response to ADP after injury is well documented (Kutcher *et al.*, 2012; Wohlauer *et al.*, 2012).
- Collagen is a much weaker agonist unless used in combination.
- Other agonists including epinephrine have been used.

However, human studies indicate that, while there is considerable variation between individuals in their *in vitro* response to these substances, any given individual has broadly equivalent response to all of them (Panzer, Höcker and Koren, 2005). TRAP and ADP were assessed during assay design (see Section 6.4).

5.2.3 Technical issues in flow cytometry

There are a number of further technical issues relating to flow cytometry which underlie the work described in this chapter: compensation, Fc blockade and the use of isotype controls.

Compensation. First, excited fluorophores do not emit light at discreet wavelengths but rather over a range with a prominent peak at the wavelength used to describe them. Hence flourescein has a peak within the range of the green passband (light filter). However, flourescein also emits a small amount of light in the range of the yellow passband. Consequently, any flourescein-conjugated mAb bound to an event will contribute to the phycoeytherin (yellow) signal. Compensation for this phenomenon can be achieved by establishing the contribution of each fluorophore to the other channels of interest and using simultaneous equations to remove this from the reported fluorescence. This process is automated in modern cytometers.

Fc blockade. Second, mAb are used for their ability to bind specifically to the epitope of interest. However, non-specific binding also occurs. Partly, this is due to the Fc domain (a major part of the structure of mAb which is constant and allows interaction with leucocytes) binding to Fc receptors. These are expressed on many cell types but on leukocytes particularly and, to a lesser extent, platelets. To prevent this, samples can be incubated with unconjugated, non-specific serum, which will saturate the Fc receptors.

Isotype controls. Some non-specific binding will still occur and this leads to the third aspect, the use of controls. No stain controls (assays using vehicle without antibody) are used to detect the small amount of autofluorescence which cells exhibit. It is then necessary to appreciate how much fluorescence will be caused by the binding of conjugated mAb to molecules other than the epitope of interest. This is determined by running parallel assays using antibodies specific for irrelevant epitopes (i.e. ones that will not be present; this is achieved by raising the antibodies against a different species) and the same characteristics as the test Ab, i.e. of the same source species, the same isotype (light and heavy chain combination) and same fluorophore. The

highest intensity of fluorescence emitted by isotype-bound events can be used to set a threshold. Events in the test assay which fluoresce above this level are then taken to be positive for expression of the epitope. This is the standard approach for discriminating positive from negative events in most applications of flow cytometry (O'Gorman and Thomas, 1999).

5.3 Animal Models

Chapter 4 of this thesis outlines the findings of the first comparative study of the effectiveness of PHBTx in a military context. Although associated with a survival benefit, confounding changes in other aspects of treatment make it impossible to determine how much of this is attributable to PHBTx. Since PHBTx was not a standard part of UK military practice and is associated with a considerable logistic burden (O'Reilly *et al.*, 2014), further study was warranted to determine its efficacy. There was no prospect of establishing clinical equipoise to allow a randomised trial of PHBTx in the current military environment. Also, it would be extremely difficult to obtain suitably and consistently timed samples and the diversity of injuries would obscure much of the effect. Despite this, understanding how, when and to what extent PHBTx is useful is highly important. An animal model was the only means of achieving this. Use of an animal model had the additional benefit of allowing far more detailed monitoring and analysis of response to injury and treatment than would be possible in a human trial. A suitable model would mimic the response to trauma seen in patients injured in contemporary conflict.

This section will briefly consider some general issues surrounding the use of animal models, examine some of the relevant available models of trauma and describe the development of the existing large animal model of military trauma at Dstl. Finally, it will introduce the process of developing a new model which took place in parallel with the work presented in this thesis.

5.3.1 Use of Animals in Research

The use of animal models remains controversial (Rawstorne, 2010). Animal models are useful because they allow subjects to be monitored and treated in ways that are not possible in human subjects. Information about disease at its earliest stages or about treatment in the absence of the confounding influence of other therapy is gained. Objections range from absolute ethical ones, i.e. that there can be no justification for using animals (sometimes in ways that make them suffer) as the means to furthering man's goals, to practical ones, i.e. that the information gathered is flawed and not helpful. Interspecies differences can undermine the applicability of findings to human disease as can poor modelling of the disease of interest (Frith, Cohen and Brohi, 2012). Animal trials may be poorly designed or reported (Kilkenny *et al.*, 2009). Animal trials suffer from the same potential sources of bias as human studies, both internally (need for randomisation, blinding and allocation concealment) (Bebarta, Luyten and Heard, 2003) and externally (need to assess all available evidence and avoid publication bias) (Roberts *et al.*, 2002).

The three principles of "replacement, reduction, and refinement" are intended to address these issues (Russell and Burch, 1956): Replacement means using other means where possible; reduction means minimising the number of animals used; refinement means ensuring the highest scientific standards so that the use of each animal contributes as much as possible. However, as Roberts has pointed out, these principles may conflict. For example, using inbred, single-sex animals which all have disease induced in the same way with the intent of achieving the same severity and which then receive the same treatment (save for the item of interest) reduces within- and between-group variability, allowing smaller numbers to be used. However, it also reduces the applicability of the results to the varied and dynamic reality of human disease (Roberts *et al.*, 2002). Small group sizes also increase the risk of type 2 errors in animal research.

5.3.2 Models of ATC

Chapter 3 contains a partial description of some of the models which have been used in research about haemorrhagic shock and resuscitation. Controlled haemorrhage can be induced by removal of a set volume of blood or by removing blood so as to reach a predetermined blood pressure. Uncontrolled haemorrhage can be induced by a variety of injuries, including tail transection, aortotomy and liver injury. However, a model of military injury to investigate the effectiveness of prehospital use of haemostatic resuscitation needs to include other aspects of injury and reproduce the pathophysiological response to injury.

Brohi's group recently reviewed available animal models used to investigate trauma induced coagulopathy (Frith, Cohen and Brohi, 2012). They identified 23 models using 5 species. Pigs were the predominant large animal used. Porcine models are held to recapitulate the human cardiovascular responses to injury and haemorrhage (Tsukamoto and Pape, 2009), which was the basis of their selection for the research programme at Dstl. However, it should be noted that porcine inflammatory response has not been well characterised. Pigs are hypercoagulable relative to humans and have less protein C (Siller-Matula *et al.*, 2008). Several models therefore use measures such as haemodilution to "knock down" the pig coagulation system, but it is unclear whether this is a valid manoeuvre. The lack of protein C has clear implications if investigating a coagulopathy that appears dependent on this pathway. None of the large animal models studied developed an endogenous coagulopathy due to combined injury and shock alone, as humans do.

5.3.3 The Dstl Model

The original Dstl pig model (i.e. before the developments explained in subsection 3.3.4) was developed to test the effect of resuscitation on mortality and physiological parameters, such as blood gases, rather than to assess coagulation (Garner *et al.*, 2009; Kirkman, Watts and Cooper, 2010). It was particularly concerned with two aspects of military practice not assessed in other models: blast injury and prolonged evacuation times (Place, Rush and Arrington, 2003). The model therefore contains

both blast and sham blast arms and is designed so that there is potential salvageable mortality among subjects, which can be sustained for several hours with adequate resuscitation.

Terminally anaesthetised large white pigs are surgically prepared, including splenectomy to prevent autotransfusion (Hannon, Bossone and Rodkey, 1985). An adrenaline solution is applied to the spleen before removal, causing it to contract; the intention is to reduce variation by ensuring that the circulation has been similarly bolstered with spleen-sequestered red cells before the organ is removed. The animals are wrapped in a Kevlar blanket before exposure to an explosive charge with no casing; again, these measures reduce variability by preventing secondary and tertiary blast injury. Shock was induced by removing a fixed volume of blood through a preplaced cannula and this was then converted to an uncontrolled haemorrhage. A Grade IV liver injury (i.e. involving disruption of 25-75% of the liver parenchyma) (Moore *et al.*, 1995) was later added to allow an element of uncontrolled bleeding with the possibility of rebleeding. This was induced by placing a heavy suture through the substance of one lobe of the liver during preparation and looping such that it cut through when pulled from outside the closed abdomen. In both cases the controlled haemorrhage was intended to reduce variability in the amount of blood that would be lost, especially between the blast and sham blast groups (see subsection 2.4.2).

The success of these models has been touched on in earlier sections. Several modifications to this original model were required for the proposed study of PHBTx.

- Injury and haemorrhage were to be adjusted to induce a coagulopathy at a time point corresponding to hospital admission.
- The endpoints of the trials were to be based on coagulation parameters rather than mortality. As such, it was necessary for the animals to survive until the end of the experiment.
- The liver injury had face validity as a potentially survivable wound, had a mixture of arterial and venous branches involved and was capable of rebleeding. However, since the liver was cut rather than injured bluntly, there was relatively little damage to tissue that was not amputated. Since tissue injury can alter the response to haemorrhage (Rady *et al.*, 1993) and is crucial

to ATC (Frith *et al.*, 2010), a limb injury (intended to mimic a fragmentation injury) was substituted.

- Changes in the operational scenario in Afghanistan meant that evacuation times were now much shorter (Morrison, Oh, *et al.*, 2013), and the timing of interventions in the model needed to reflect this.

The next chapter focuses on the development of the flow cytometry assay. The details of animal injury that occurred were largely irrelevant at this stage. Consequently, a full description of the model after the modifications mentioned above is reserved for Chapter 7.

Chapter 6. Platelet Flow Cytometry Assay Development

This chapter describes the establishment of a porcine platelet assay designed to detect the expression of an activation marker and the response to an agonist. Development of the platelet assay proceeded in four stages:

1. An initial protocol was designed to allow the detection of platelets using specific markers and FSC/SSC characteristics.
2. This assay was altered to optimise the detection of platelets.
3. Measurements were made of platelet activation and response to agonist.
4. Some simplifications were made to facilitate use of the assay in the context of the complex trial and animal model detailed in Chapter 7.

6.1 Standard Conditions

Animals mentioned in this discussion have been assigned a sequential code (PA1, PA2 etc) in order to simplify the description. Blood used in the development of the assay was drawn from terminally anaesthetised animals being used in the preparation of the model to be used in trials of haemostatic resuscitation. The materials and methods used in animal preparation, injury and sampling were as set out in section 7.1 except that during development variations were made in the degree of blast loading, the volume of haemorrhage and the details of musculoskeletal injury. The combination of these factors peculiar to each animal was not germane to the development of the FACS assay and will not be detailed in this chapter.

All flow cytometry assays were performed on whole blood collected via the femoral arterial line and immediately aliquoted into citrated tubes. These were placed on a roller for 5 minutes. All antibodies were obtained from AbD Serotec (Oxford, UK), see Table 6.1. The initial antibody mixture contained 10µL CD61 mAb, 10µL CD62P mAb and 20µL buffer (or equivalent isotypes, as appropriate). 10µL buffer was later replaced with 10µL CD45 mAb, as noted below, and this mixture was used throughout the remainder of the experiment unless otherwise noted.

Table 6.1—AbD Serotec mAb used during the development of the platelet flow cytometry assay.

Antibody	Target	Isotype	Flurophore
MCA1222APC	CD45	IgG1	APC
MCA2263F	CD61	IgG1	FITC
MCA2418PE	CD62P	IgG1	PE
MCA1746PE	CD31	IgG1	PE
MCA928F	Control	IgG1	FITC
MCA928APC	Control	IgG1	APC
MCA928PE	Control	IgG1	PE

All incubation, lysis and fixation steps described below took place at 25°C, with the specimens protected from light. Where appropriate, for red blood cell lysis whole blood was incubated for 15min in BD Pharm Lyse solution (BD bioscience, Oxford, UK). Where detailed, samples were washed or resuspended in FACS wash buffer (Delbecos Phosphate Buffer Solution pH 7.4, 0.01% sodium azide, 4% newborn calf serum). Where stated, samples were blocked in Fc blocking serum prior to staining (6µL mouse serum and 4µL FACS wash buffer). Except where specified, samples were refrigerated after preparation and analysed the next day. A no-stain control was performed for each animal (and, where appropriate, each protocol). Isotype controls were performed for every assay.

All samples were analysed on a BD FACS Canto^{tm} II and analysed using BD FACSDiva^{tm} software. Positive populations were gated using the relevant isotype control, the gate being set with less than 5% positive events in the isotype population.

6.2 Initial Protocol Design

A porcine FACS leucocyte assay had previously been developed at Dstl Porton Down (Carter, unpublished). Development of the platelet assay began with modifications to this protocol. The initial considerations were the ability to identify the platelet population using FCS, SCC and a platelet-specific marker and secondly to ensure that processing the samples 24hrs after initial fixation would not inadvertently change the platelet population.

The initial protocol, used for analysing the first samples, is detailed in Figure 6.1. Note that no red cell lysis step was included (one had been used in the earlier leucocyte assay) as it was anticipated that this step would lead to artefactual platelet activation (Michelson, 1996). Furthermore it was anticipated that these events (i.e. red cells) could be gated out on FSC/SSC criteria more easily than in the leukocyte assay due to the physical characteristics of the RBCs relative to the platelets.

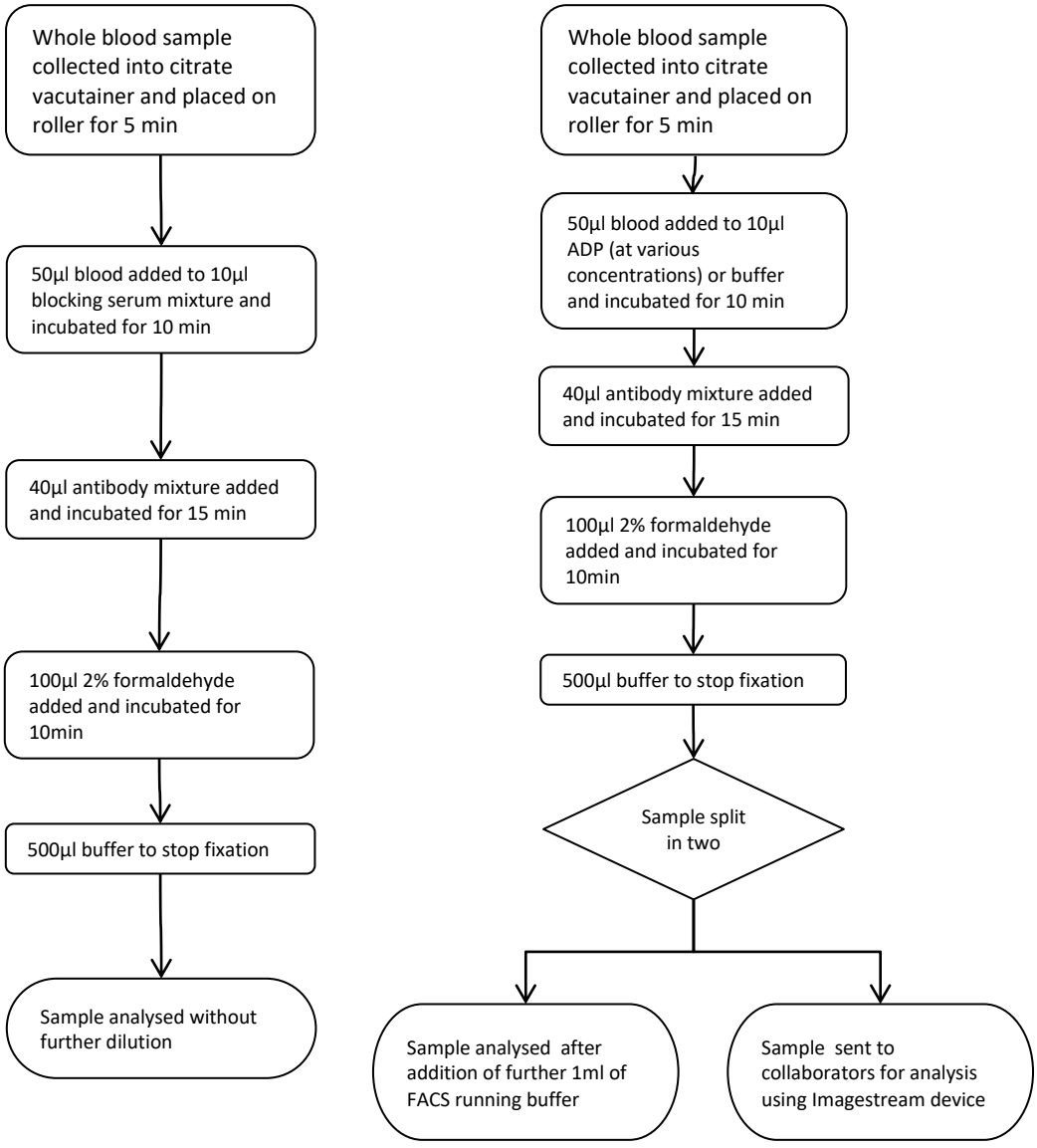


Fig 6.1 Initial (left) and final (right) porcine platelet flow cytometry assay protocols

Samples were single stained for CD61 (Macey *et al.*, 1999; Jacoby *et al.*, 2001) in order to locate the platelet population using fluorescence. The forward and side scatter

parameters were adjusted until the putative platelet population, tagged with CD61, were fully visible in the FCS and SCC scatter plots. Figure 6.2 shows the first scatter plot detecting the CD61+ population.

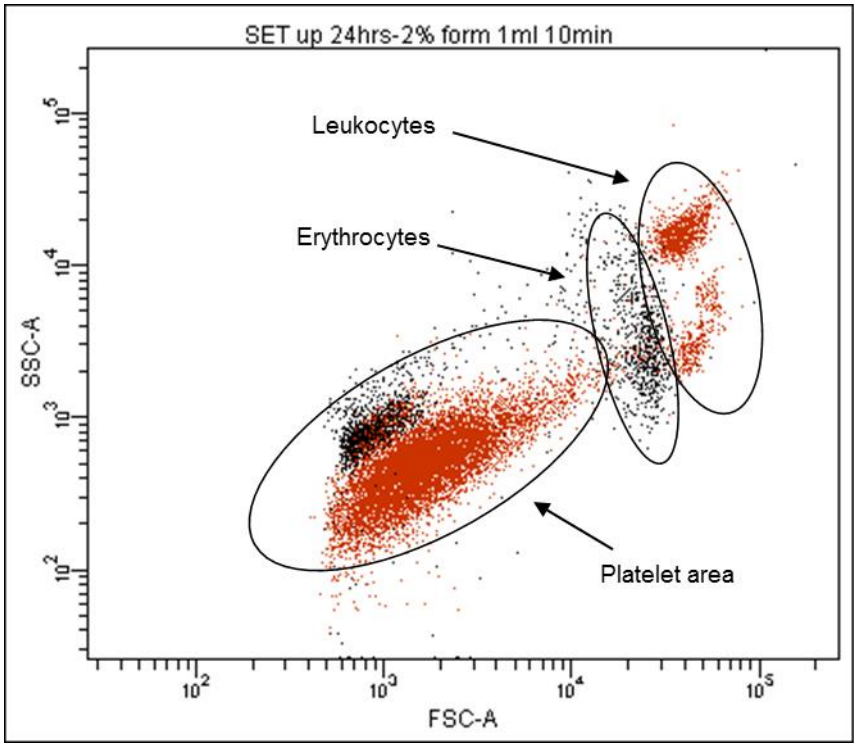


Figure 6.2. PA1. First plt scatterplot

Initial plt assay set up scatter plot. CD 61+ events are in dark red. The large population of low FSC and low SSC events is referred to as the "platelet area". The presumed identities of the other events are also shown. The leukocytes also appear to be CD61+; the likely explanation is that there are platelet-leucocyte aggregates: the flow cytometry equipment was set up to display red data points over black ones in this image.

Representative of a total of 7 samples from 2 animals at 4 time points.

In addition to success in finding this population, which exists below the FSC detection minimum set for previous leucocyte studies, a set of pairwise comparisons of same-day versus next-day analysis on the FACS machine was performed. This revealed no significant changes in FSC, SSC or fluorescence of the population of interest (Figure 6.3). Compensation (see subsection 5.2.3) was also calculated and subsequent experiments used dual staining for CD61 and CD62P.

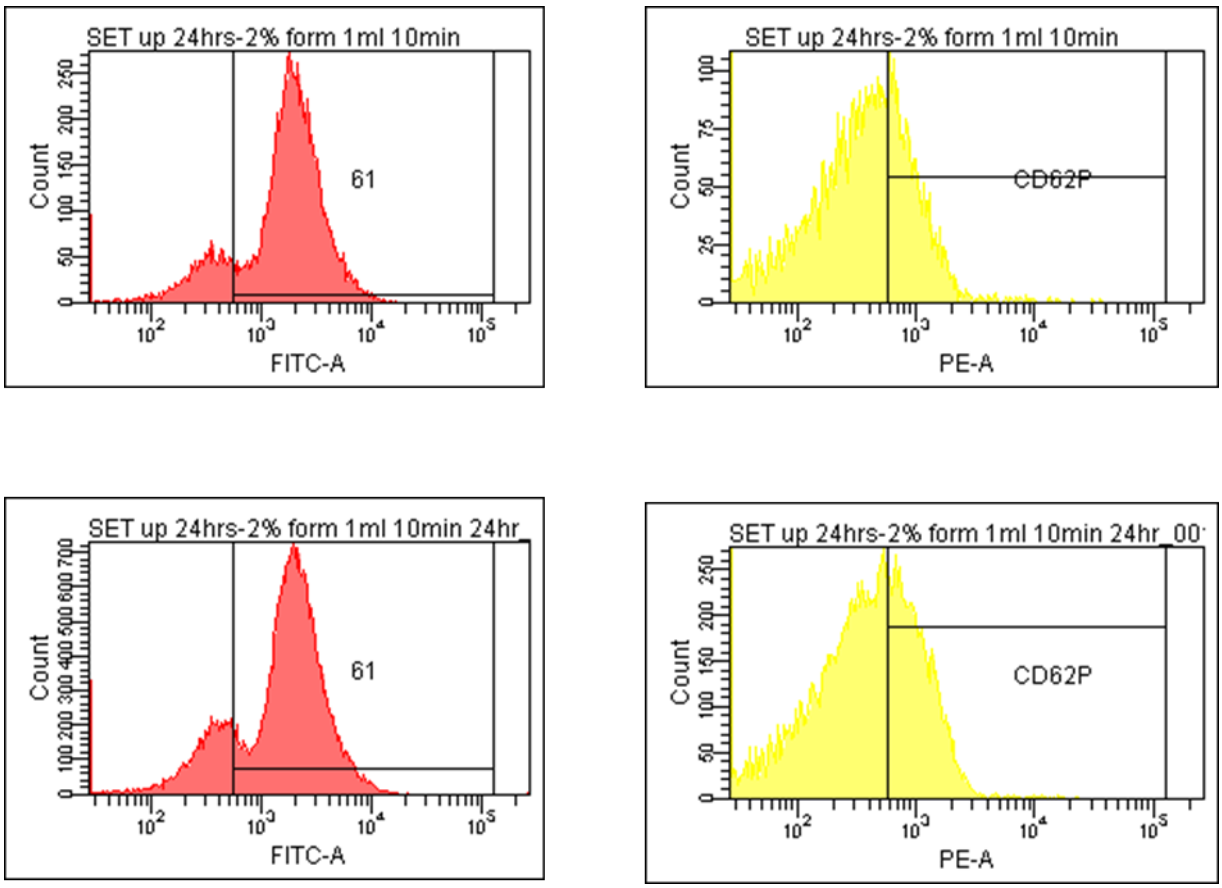


Figure 6.3. PA1. Representative histograms of CD61 FITC and CD62P PE fluorescence processed on the day of preparation (top histograms) and 24 hours later (bottom histograms).
Representative of paired samples from 4 time points in 1 animal.

The next five trials demonstrated consistent flow cytometry findings: Figure 6.4 shows a representative scatter plot from one of these animals (designated PA2) and a diagram labelling the populations seen. It was observed that two populations were present in the scatter plots where the platelets had been identified. The staining of these two populations were not consistent with the expected staining patterns of platelets. The smaller cluster of the two putative platelet populations (Population A) is shifted up and to the left of the larger cluster (population B). The relative complexity of the putative platelets appeared to be greater in population A as compared to population B (as shown by the differences in the SCC of the two populations). CD61+ events occurred in population B. The overall CD61 positivity of the events in the platelet area (i.e. A and B together) was usually 30 to 60%, normally at the lower end. Hence A appears to be a population of more internally complex and more numerous

events than the platelets with a size distribution in the lower part of the CD61+ events. It was not immediately clear what such a population would be.

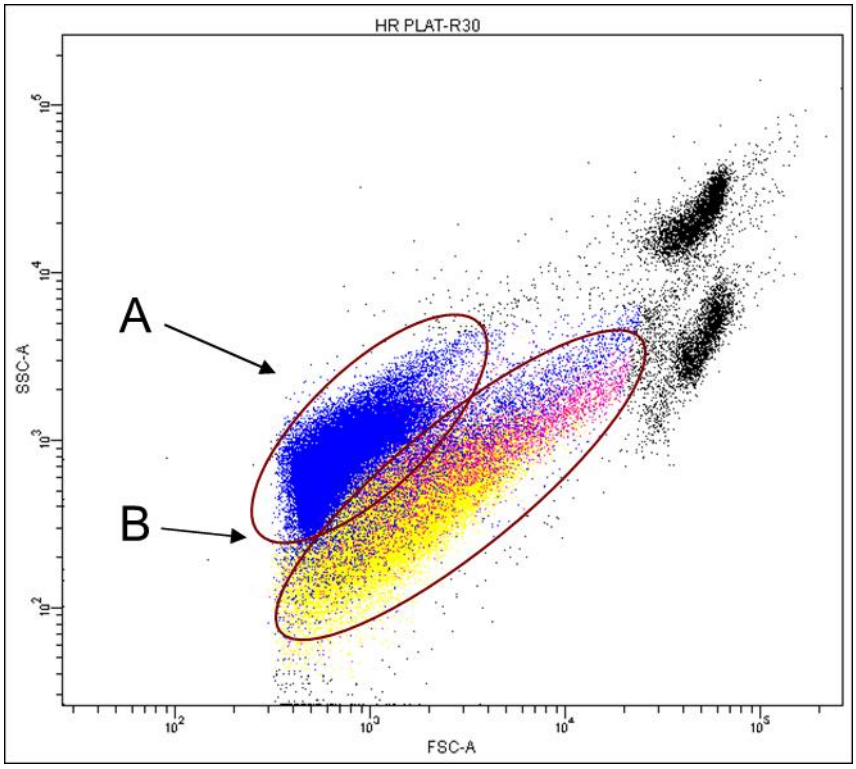


Figure 6.4. PA2 Scatterplot

Only events inside the "platelet area" are coloured.

CD61-/CD62P- Blue

CD61+/CD62P- Yellow

CD61+/CD62P+ Magenta

Representative of 14 samples at 7 time points in 1 animal.

Compounding this, the ratio of CD61+ to CD61- events increased over the course of most experiments. Figure 6.5 shows histograms of CD61 fluorescence of a typical study at two time points. At first there is a distinct positive group that is small compared to the negative one; subsequently, the peaks are more equal. Note that there is not a blending together of the two peaks. The latter might have indicated a lack of saturation of the epitope by antibody, i.e. some events were recorded as negative because not all (or enough) of their surface epitopes had been marked by the antibody, perhaps due to an insufficient concentration.

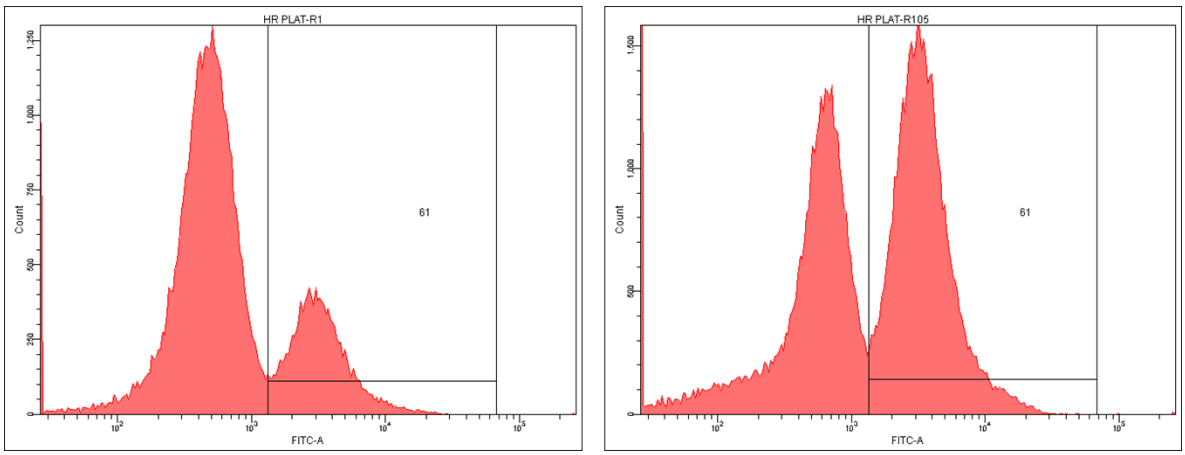


Figure 6.5 PA2 CD61 histograms. Histograms showing FITC-A fluorescence of events in the platelet area at baseline (left) and R105 (right). The gate was set using a 0.1% threshold on the isotype control for the B2 time point.

CD61+ as % of parent: B2—23.7%

R105—51.6%

Representative of the pattern of change seen in duplicate samples from 7 time points in this animal and in 4 other animals.

Thus the initial assay appeared to detect events with the size and complexity of platelets but with poor uptake of the platelet marker, the presence of a second, distinct population of events and a dynamic shift between the two over the course of the experiments. It was now necessary to optimise the detection of platelets and to identify population A.

6.3 Protocol Optimisation

A series of experiments were then conducted to examine all the alterable aspects of the sample preparation process to determine their impact on the results. The following steps were examined:

1. The effect of Fc receptor blockade using mouse serum (which was originally included in the leukocyte assay to prevent non-specific binding).
2. The effect of a red cell lysis step.
3. Comparison of whole blood platelet staining with platelet-rich plasma (PRP) preparation.
4. Antibody and sample titration.

5. The concentration of fixative during the fixation step.
6. The concentration of fixative during storage overnight.
7. The degree of dilution of the sample during analysis on the FACS machine.

A series of pairwise or higher-order comparisons of the first three changes mentioned was conducted during study PA3, a sham blasted animal. The remaining aspects are examined in the next subsection.

Fc receptor blockade and red cell lysis were elements of the original leucocyte assay. Fc receptor blockade with mouse serum interfered with CD61 detection in the platelet area (see Figure 6.6). Use of red cell lysis produced no usable data (Figure 6.7). These steps were removed from the protocol.

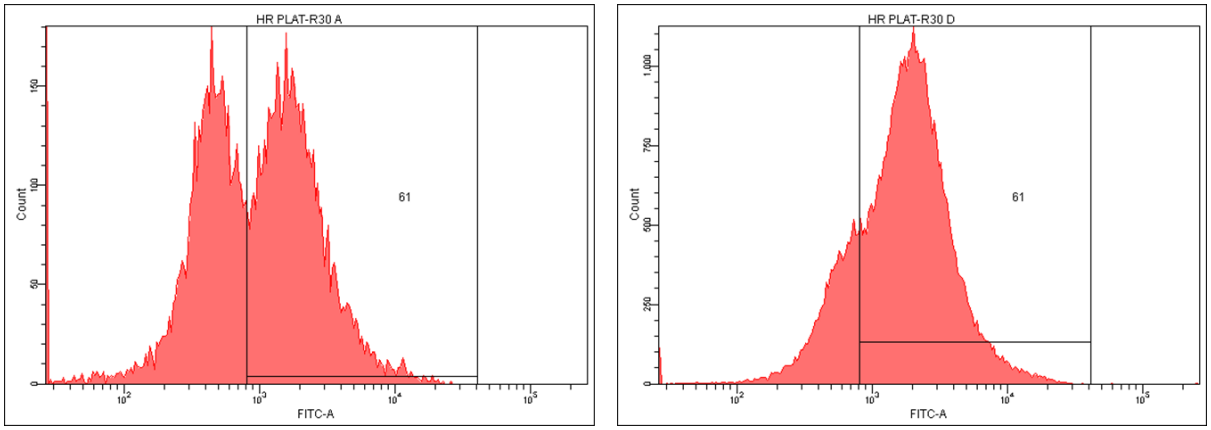


Figure 6.6. PA3 CD61 FITC histograms at B2.

Left—with Fc receptor blockade. Right—without Fc receptor blockade.

No increase in fluorescence was observed with the isotype controls. This implied that the improvement in CD61 positivity was due to improved specific binding to CD61, not non-specific binding to any Fc receptors expressed within the population.

Representative of paired samples at 3 time points in 1 animal.

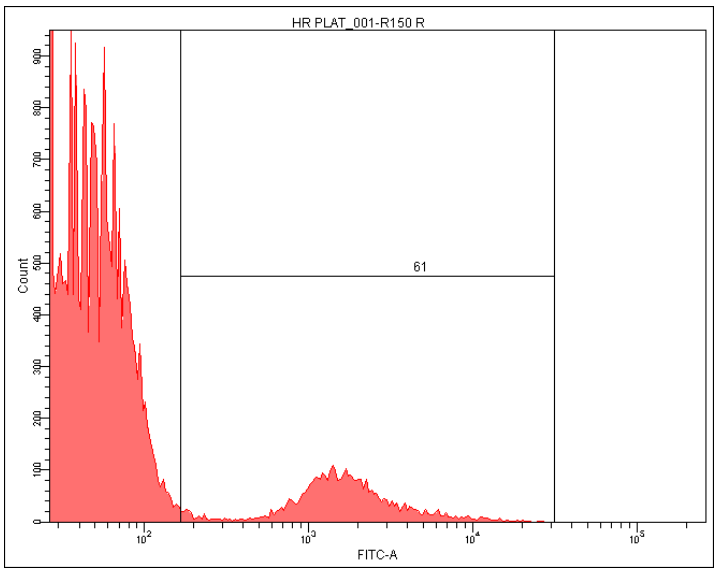


Figure 6.7. PA3 CD61 Histogram at R150.

Post red cell lysis. Note that the gate, set using the isotype control for this protocol, has a much lower threshold than the previous examples. Red cell lysis has had profound effects on both antigenicity and non-specific binding of events in the platelet area.

Representative of 2 samples at one time point in one animal.

Platelet rich plasma was prepared using a low-g centrifugation technique.(Delgado *et al.*, 2003; Lindenblatt *et al.*, 2007) Analysis of these samples (Figure 6.8) showed loss of a considerable part of the CD61+ population, so this approach was abandoned.

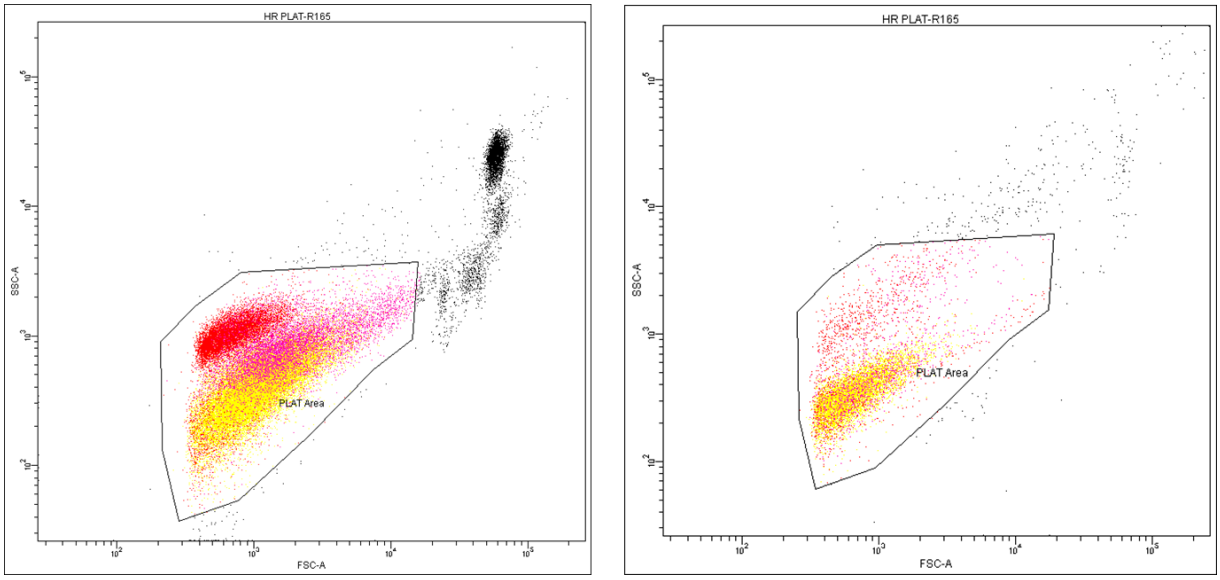


Figure 6.8. PA4. Representative scatter plots at R165 demonstrating the loss of CD61+ subpopulations when comparing the whole blood assay (left) and an assay using PRP (right). Large (possibly aggregated) and activated platelets have been lost. Duplicate assays of paired samples (PRP+ and PRP-) at 8 time points in 1 animal. CD45+,CD61- Red; CD45+,CD61+,CD62P- Yellow; CD45+, CD61+,CD62P+ Magenta.

Addition of the leucocyte marker CD45 demonstrated that it was strongly expressed by population A (as described above), see Figure 6.9. This population was excluded from further analysis.

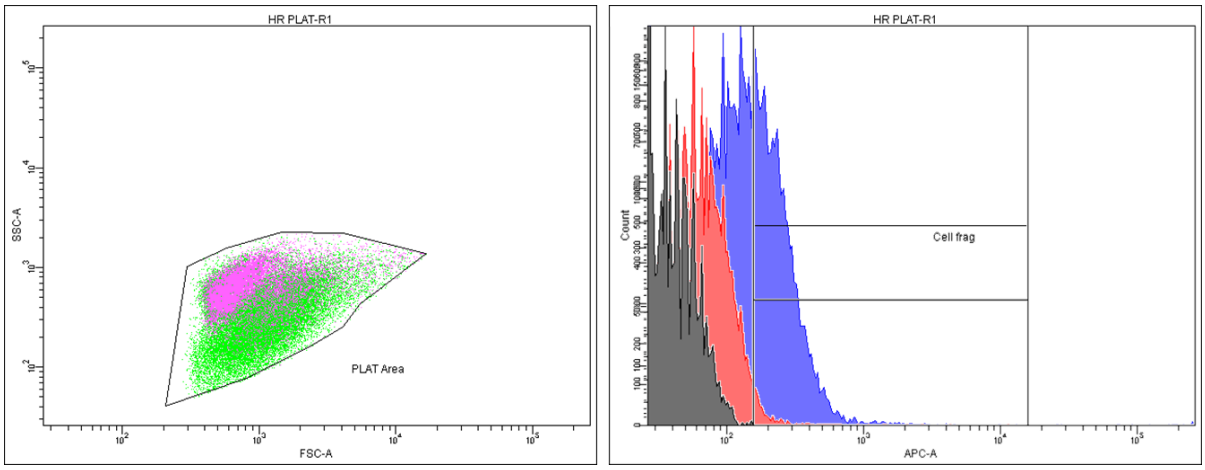


Figure 6.9 PA5 CD45 at B2

Left—CD45+ in magenta and CD45- in green. Most CD45+ events are in the area previously designated population A. Given that they express the leucocyte-specific marker CD45 but are of similar size (judged by forward scatter) as CD61+ events (platelets), it seems likely that these represent fragments of leucocytes).

Right—Gating only on the densely CD45+area in the left figure, APC fluorescence (for CD45) is shown: No stain control in grey, isotype control in red, positive antibody in blue. The marked gate includes 1% of events from the isotype histogram and 48% of those in the positive antibody histogram. Non-specific binding of isotype antibody (possibly due to Fc receptor expression) and wide variation in surface marker expression among fragments would account for these data.

Representative of 50 assays taken from 5 animals at between 3 and 8 time points in each case.

6.3.1 Antibody and Fixative Concentration and Sample Dilution for Analysis

In parallel with analysis using the FACS Canto II, a collaboration was set up with another laboratory to examine the samples with an Imagestream (Amnis, Seattle, WA, USA) imaging cytometer.* The other laboratory requested that sample dilution be minimised so that samples could be processed quickly. However, there are several considerations in manipulating sample and reagent volumes and concentrations in flow cytometry, the effect of which on the assay had to be assessed:

* The Imagestream work affected the development of this protocol but does not itself form a part of this thesis.

- There is a limit to the ability of the cytometer to deal with numerous, small events (such as platelets) which can lead to false negative fluorescence plots.
    - This is compounded by the shape of the laser, as mentioned in subsection 5.2.1.
- High concentrations of fixative can, over time, alter the binding of antibodies to epitopes and cause an endogenous signal to appear in FL1 (Shapiro, 2003).

A series of experiments were undertaken to optimise the assay for use with Imagestream while addressing these issues. First, dilution at the end of fixation was reduced (PA6). As shown in Figure 6.10, this led to loss of positive events. It was not clear whether this was due to the effect of storage in more concentrated fixative or to high events rates during analysis. Recorded event rates in some cases approached the manufacturer's specified maximum of 10,000 per second, so the latter was a possibility.

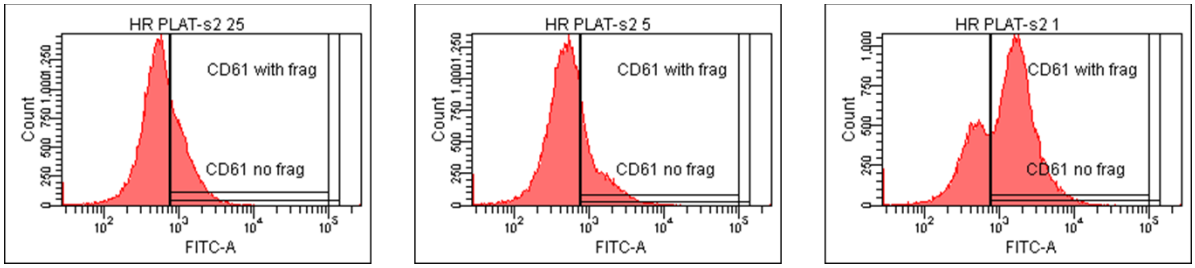


Figure 6.10. PA6. Samples taken at S2 and processed according to the standard protocol except when terminating fixation. Histograms show CD61 FITC fluorescence after termination of fixation using (from left to right) 250µL, 500µL and 1mL of buffer. Note the marked increase in the relative number of positive events as the dilution is increased.
Representative of duplicate assays of each dilution from two time points (twelve assays in total) in 1 animal.

Second, the event rate was increased while holding fixative concentration (during both fixation and storages) constant (PA7). This was achieved by adding two new protocols using more blood and less fixative, albeit at higher concentrations. The protocols are in Table 6.2. This lead to increased fluorescence of unstained controls (Figure 6.11) and to loss of positive events in the assay (Figure 6.12). However, these changes had increased both the concentration of blood (and therefore the event rate during analysis) and the ratio of blood to antibody mix.

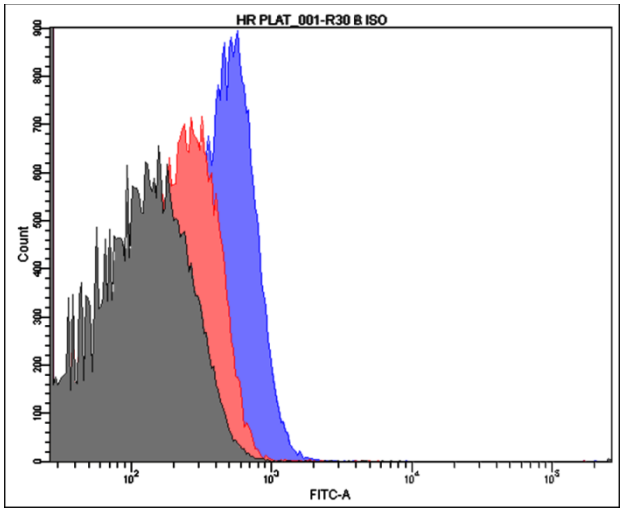


Figure 6.11. PA7. Isotype control histograms at R30 for Protocols A (Black-the standard protocol in earlier assays), B (Red) and C (Blue). B and C represent increasing volumes of blood used with smaller volumes of more concentrated fixative. See Table 6.2.

Representative of duplicate assays of each protocol at 4 time points (24 assays in total) in 1 animal.

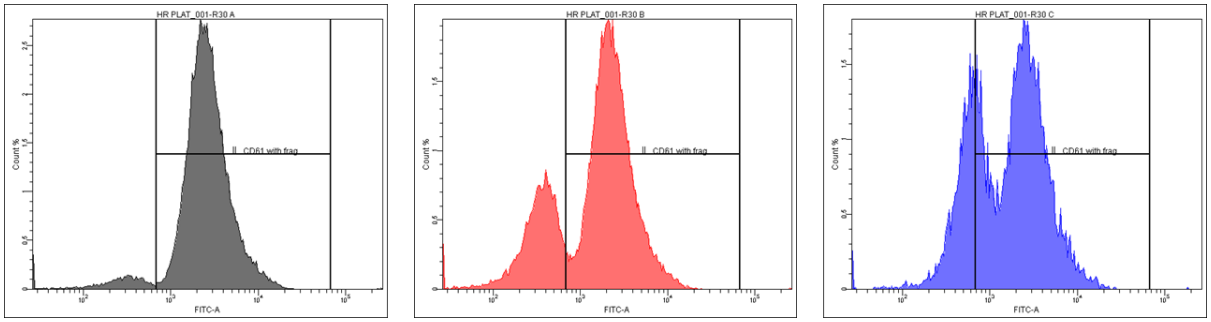


Figure 6.12 PA7 CD61 FITC fluorescence histograms at R30 for Protocols A (Black), B (Red) and C (Blue). See Table 6.2 for protocols.

The gate was set to include 0.7% of events in the protocol B isotype control at R30 (previous figure).

Note the right shift of the negative peaks as we move from A to C, consistent with the previous figure.

In addition, there is a very marked shift from the positive to the negative peak as we move from A to C.

Representative of duplicate assays of each protocol at 4 time points (24 assays in total) in 1 animal.

In the third experiment (PA8), another protocol was added which corrected for this. It did not restore the positive events, indicating that antibody concentration was not the critical issue. To test for the effect of concentration during analysis (i.e. event rate), samples from each protocol were run neat, after the addition of a further 500µL of buffer and after the addition of 1mL (total) of buffer. Protocol C was the "poorest performer" for CD61 positivity in the previous experiment. Figure 6.13 shows analysis of a protocol C sample at the three dilutions. Note the massive shift from a negative to

a positive peak. The dilutions were made immediately before analysis, so the differences seen are due to the performance of the analyser, not due to a storage lesion. Although the effect was less dramatic the "best performer", protocol A, was also enhanced by the additional dilution.

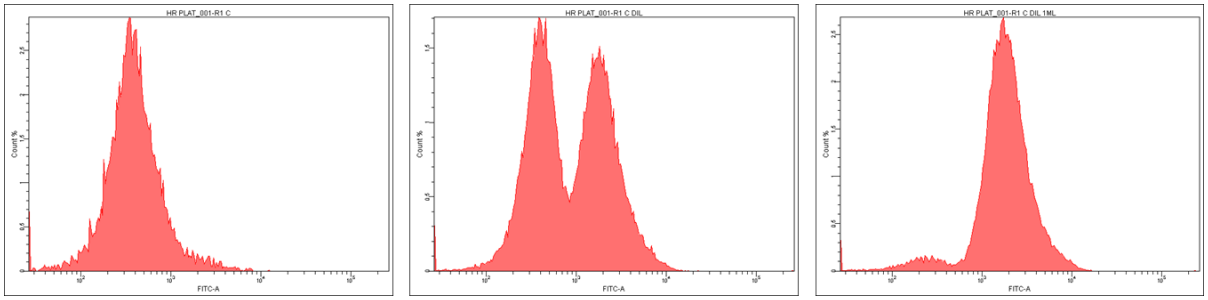


Figure 6.13. PA8. CD61 FITC fluorescence histograms at B2. Protocol C (table n) was used in each case. A) sample analyzed neat. B) sample analyzed after addition of 500µL buffer. C) sample analyzed after addition of 1mL (total) of buffer.Representative of duplicate assays using 4 protocols on samples from 3 time points (24 assays in total) in 1 animal. Each assay analysed with three different dilutions.

It would appear that at high event rates the cytometer is unable to detect many of the positive events. Therefore subsequent experiments used Protocol A (the existing standard protocol) combined with dilution with 1mL buffer, yielding a mean event rate of 700 per second.

Table 6.2—Protocols used during PA7 (A to C) and PA8 (A to D). Protocol A represents the standard protocol in use hitherto. In all cases, fixation was stopped by the addition of 1mL of buffer. PA8 samples were further diluted immediately before analysis.

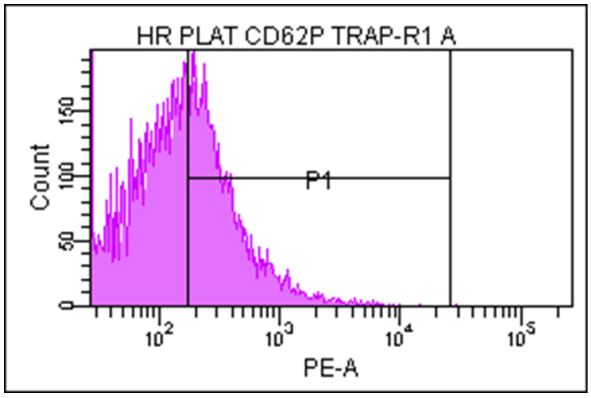
Name	Blood	Ab	Fixative	Fix Volume	Fixative conc	Storage fixative conc
A	50µL	40µL	100µL of 2%	190µL	1.05%	0.17%
B	100µL	40µL	50µL of 4%	190µL	1.05%	0.17%
C	100µL	80µL	40µL of 6%	220µL	1.09%	0.20%
D	50µL	60µL*	100µL of 2%	210µL	0.95%	0.17%

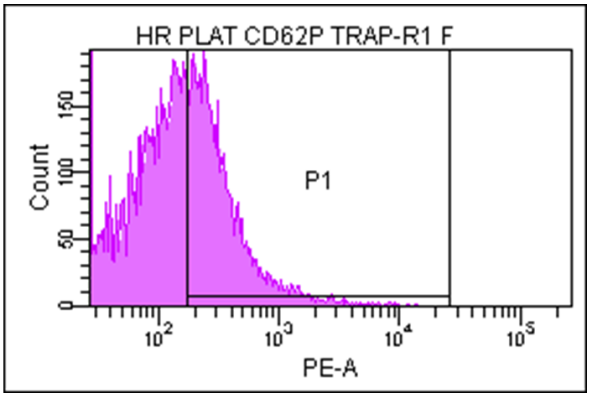
*The antibody mixture for protocol D used 20µL of each mAb without any buffer.

6.4 The Activatability Assay

Subsequent experiments confirmed that the developed protocol gave dependable rates of CD61 positivity. The next stage in refining the assay was to select an appropriate platelet activation marker and validate the assay using platelet activation assays. Hitherto CD62P antibodies had been used but it was now necessary to check that these were useful for detecting platelet activation. This would require a stimulation assay. It was natural to combine this experiment with an assessment of an alternative activation marker (CD31).

Experiments were conducted using a dilution series of TRAP-6* with a maximum concentration of 64µM, which was expected to maximally stimulate the platelets. There was no apparent reaction. Figure 6.14 shows CD62P positivity of platelets at a representative time point after incubation with buffer and with 64µM TRAP-6. This result was repeated in subsequent experiments. The experiment was repeated using TRAP-6 from a different supplier. The results were repeated. As a final check, TRAP-6 from the same batches, prepared in the same way at the same concentrations, was used in a stimulation assay for a study of human platelets that was being developed in parallel. TRAP-6 caused high levels of platelet activation, just as would be expected. A fault with the preparation of TRAP-6 had been excluded. In fact, this confirms the findings of two other groups (Kinlough-Rathbone, Rand and Packham, 1993; Connolly *et al.*, 1994) which have demonstrated that pig platelets show only a partial response to TRAP-6 characterised by shape change and aggregation but not degranulation.





* i.e. a six-amino acid residue version of Thrombin Receptor Activating Peptide

Figure 6.14. PA10. Representative histograms of CD62P PE fluorescence for samples taken at B2 and stimulated with 64µM TRAP-6 (left) and buffer (right) for 10 min. Representative of duplicate assays analysed with 6 concentrations of TRAP-6 (including control) at 4 time points in 3 animals.

TRAP-6 was therefore also abandoned in favour of ADP. PA9 was the first animal used for this purpose. A matrix of assays to be performed at each time point was designed, Table 6.3. The range of ADP concentration used was based on an inspection of the literature (Panzer, Höcker and Koren, 2005). Samples were incubated with one of five concentrations of ADP, or with buffer, for 10minutes. Then they were incubated with the standard antibody mixture, with CD31 Ab in place of CD62P Ab or with isotype controls.

Table 6.3—PA9 ADP concentration series at time points T0, R30, R60, R105. Each concentration was incubated with sample for 10 minutes before staining with Ab mixtures containing mAb for either CD31 or CD62P or with isotype controls.

Name	Volume of concentrate	Volume of PBS	Concentration of solution	Final concentration (with sample)
A	1mL of Stock	Nil	60µM	10µM
B	500µl of A	500µl	30µM	5µM
C	500µl of B	500µl	15µM	2.5µM
D	500µl of C	500µl	7.5µM	1.25µM
E	500µl of D	500µl	3.75µM	0.675µM
F	Nil	1mL	-	Control

Table 6.4 shows percentage positivity for CD31 and CD62P for each ADP concentration of ADP. There was poor positivity for CD31 even at high concentrations of ADP. This was representative of all experiments run using the CD31 mAb and consequently this was abandoned in favour of CD62P. While increased expression of CD31 after platelet stimulation is established in humans (Cramer, Berger and Berndt, 1994) there is no literature to confirm this in swine. Additionally, there is evidence of reduced CD31 expression after *in vivo* stimulation (Metzelaar *et al.*, 1993); the studied platelets had been subject to *in vivo* stimulation and this may have affected their response to *in vitro* stimulation. Finally, ADP may be an unsuitable agonist to use with CD31, given that it only partially stimulates degranulation. (See subsection 5.2.2)

Table 6.4—PA9 % of Platelets (CD45-, CD61+) positive for CD62P or CD31 after incubation with different concentrations of ADP. Gate set to include <5% of events from the respective isotype controls. Representative of a total 5 samples from three time points in three animals. Assays completed in duplicate (i.e. 10 in total).

Tube Name	Final concentration (with sample)	CD62P+ %	CD31+ %
R60 A	10µM	32.5	7.8
R60 B	5µM	30.3	8.6
R60 C	2.5µM	26.4	9.6
R60 D	1.25µM	22.9	8.5
R60 E	0.675µM	23.5	10.2
R60 F	Control	18.7	10.6

6.5 Final Optimisation

The ADP stimulation assay worked and achieved CD62P positivity of the order of 40%, consistent with the literature. Of note, this version of the assay consistently (completed in full for 3 animals) found more than 95% of events in the platelet area to be CD61 positive, with no variation over the course of the experiment, as had been seen earlier (Figure 6.5).

The final step in development was a simplification of the protocol to facilitate its use alongside both the existing leucocyte FACS assay and multiple other aspects of simultaneous sample preparation and analysis required as part of the proposed trial. The platelet FACS protocol remained a developmental tool and a secondary endpoint in the trial, the process outlined above having finished just as model development reached a conclusion. Consequently the assay was optimised to allow its validity and utility to be assessed, as outlined in Chapter 7, while minimising its impact on laboratory and human resources. As such, only unstimulated and maximally stimulated assays were used.

The CD45 mAb was being used to exclude the CD45+ events in area A (Figure 6.4). An analysis was conducted to see whether simply excluding area A from the platelet population would significantly affect the output, i.e. the % CD62P positivity. Figure 6.16 shows a Bland-Altman plot comparing the two methods of excluding the presumed white cell fragments. Gating out area A, rather than using the CD45 marker, reduced the measured CD62P positivity by a mean (SD) of 0.825% (0.198), which is negligible. There was no evidence that the difference in output was affected by the activation state of the platelets (i.e. there is no correlation between the difference and the mean). The CD45 mAb was removed from the assay.

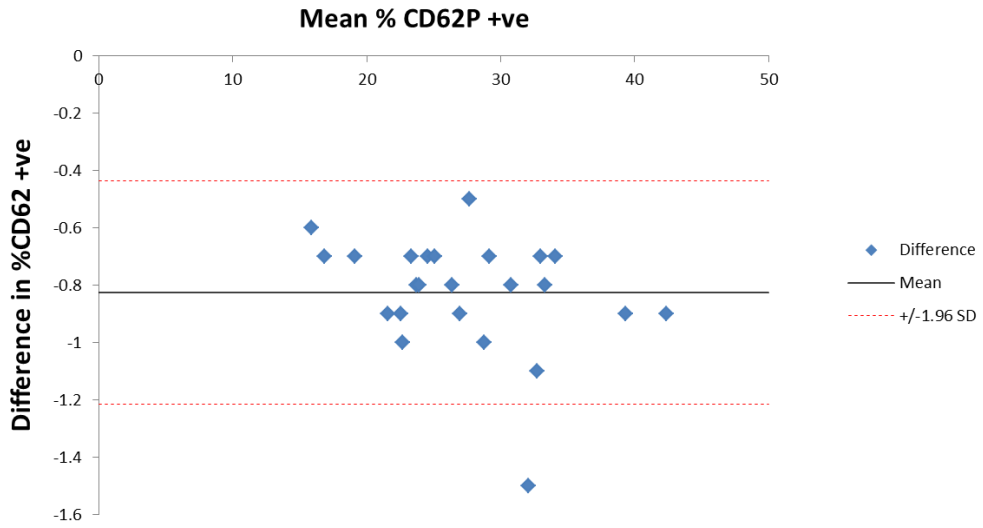


Figure 6.16 Bland-Altman Plot comparing %CD62P positivity among CD61+ platelets identified by (a) excluding all CD45+ events in areas A and B of Fig 4 and (b) only including area B, gating out area A.
X-axis: mean of (a) and (b). Y-axis: (a) – (b). See text above for discussion.

The final protocol for the platelet flow cytometry assay was as shown in Figure 6.1.

Chapter 7. Assay Validation

Development of the flow cytometry assay proceeded in parallel with development of the animal model, such that the assay design detailed in the last chapter was completed just as the model was finalised. The animal work then continued with the start of the randomised control trial. Before using the assay to compare platelet activity between the groups, an assessment of the function of the assay and its interaction with the animal model was required. Since the platelet assay was a secondary outcome of the trial, this was achieved by an analysis of data from six animals from early in two arms of the trial. This chapter sets out the findings of that analysis. This lead to the formulation of a new hypothesis about the effect of surgical preparation of the animals on platelet function. Further assays were conducted to examine this hypothesis. Consequently, methodology and data for this "further analysis" appear in their own subsections below.

7.1 Methods

The study* was conducted on terminally anesthetized cross-bred female Large White pigs and was ethically reviewed and conducted in accordance with the Animals (Scientific Procedures) Act, 1986. The animals were housed indoors and were fed on a complete diet comprising coarse ground mixture of wheat, barley, soya protein, vitamins and minerals. The animals fed ad libitum and consumed approximately 5 kg per day. They were allowed water ad libitum.

7.1.1 Blood bank

Blood was collected by exsanguination from terminally anaesthetised female Large White pigs. Briefly, standard units of blood were collected from a carotid cannula at a flow rate of 65-90 mL/min into citrate, phosphate and dextrose (CPD) blood collection

* The design of the animal model and the trial were not the author's responsibility; descriptions of these matters are based on information provided by Dr E Kirkman and therefore align with the methodology in his team's recent paper based on this work (Watts *et al.*, 2015). The author was on compassionate leave at the time that the six experiments detailed in this chapter were conducted and is grateful to his colleagues for completing these. However, all the analysis presented here is the author's own.

bag (RCB434CCL, Pall Medical, UK), with pauses between bag collection resulting in an overall bleeding rate of 22.5 mL/min. The blood was processed according to standard UK blood transfusion protocols (UK Blood Transfusion & Tissue Transplantation Services, 2013) to separate the red blood cells from the plasma, which was leucodepleted, while the packed red cells were stored in saline, adenine, glucose and mannitol solution (SAG-M). The platelets were discarded. Collected units of blood were processed within 90 min of collection. The resulting units of PRBC were stored at 4°C (LabCold Blood Bank, UK) and used within 14 days of collection. The plasma (FFP) was fast-frozen (MP1100 Plasma Freezer System, Thermogenesis, US), stored at -30°C (LabCold Plasma Freezer, LabCold, UK) and used within 6 months of collection. FFP was thawed at 37°C in a dry plasma thawer (Sahara III Maxitherm, Sarstedt, Germany) immediately before use. Prior to use all donor products were forward and reverse matched to recipient blood. In addition, since PRBC and FFP from different donors were used for resuscitation, they were also cross-matched with each other.

7.1.2 Surgical Preparation

The animals were fasted for 18 hours before the surgical procedure, but allowed water *ad libitum*. After premedication with intramuscular midazolam hydrochloride (0.1 mg/kg) anaesthesia was induced by mask with isoflurane (5%) in a mixture of oxygen and nitrous oxide (1:1) and the animals intubated. Surgical anaesthesia was subsequently maintained with isoflurane (1%–2%) in a mixture of oxygen and nitrous oxide (1:2) and the animals ventilated using positive pressure ventilation (Blease Manley MP3 Anaesthesia Ventilator). Initial monitoring consisted of end-tidal CO_2, pulse oximetry through a tail probe and skin surface electrocardiogram electrodes (Propac 106EL, Protocol Systems Inc, Oregon). With the animal positioned supine surgical preparation took place after skin preparation with povidone-iodine solution (10% wt/vol, Betadine Aqueous Antiseptic Solution, Seaton Healthcare Group plc, UK).

The left carotid artery (Swan Ganz, Edwards Life Sciences Ltd, Newbury, UK), both internal jugular veins, left femoral artery and vein were cannulated (Portex 8FG, Hythe, UK). A balloon tipped flow-directed cannula (744MF75 Swan Ganz, Edwards Life Sciences Ltd, Newbury, UK) was introduced through a right internal jugular vein

cannula introducer sheath, (Desivalve Catheter Introducer, Vygon, Cirencester, UK) and advanced until its tip was in the pulmonary artery. Cannula placement was determined by monitoring pressure changes at the tip.

After venous access had been established, anaesthesia was continued with intravenous alphaxalone to maintain a surgical plane of anaesthesia assessed by palpebral reflex and jaw tone (Alfaxan®, Jurox (UK) Ltd, Malvern Link, UK), and the isoflurane was discontinued.

A midline laparotomy was performed, the spleen contracted by topical application of adrenaline (up to 1.5 mL of a 1 mg/mL solution) before removal and the bladder catheterized by open suprapubic cystostomy. All incisions were closed *en* masse. Animals were allowed to breathe spontaneously for the remainder of the experiment unless they displayed marked respiratory depression, at which stage Synchronized Intermittent Mandatory Ventilation (Drager Evita 2, Draeger Medical UK Ltd, UK) was instituted in an attempt to maintain adequate oxygenation and prevent severe hypercapnoea. The animals recovered from surgery under anaesthesia for 1 hour before baseline measurements were made. Anaesthesia and surgical preparation took place at in a surgical facility collocated with the animal house. After surgery, animals were transported (while anaesthetised) to the experimental facility adjacent to the blast arena.

7.1.3 Cardiovascular Monitoring

Arterial blood pressure was recorded through the carotid artery cannula and pulmonary arterial and central venous pressures were recorded via the flow-directed balloon-tipped flotation catheter, which was also used to determine cardiac output as a 6 minute rolling average (Vigilance Volumetrics CEDV, Edwards Lifesciences, US). Physiological pressure measurements were made using strain gauge manometers (Sensonor 840, SensoNor a.s., Norway) and zero pressure for all transducers was set at heart level. Body temperature was maintained at approximately 38°C using external heating/cooling and blankets as appropriate. The bladder was drained at hourly intervals.

All cardiovascular variables were recorded using a computerized data acquisition system (Maclab 8/s, ADInstruments, UK) and associated software (Chart v4.2.3, ADInstruments) for subsequent analysis.

7.1.4 Blood Gas and Related Chemistry

Arterial and venous blood samples were taken anaerobically into heparinized syringes from the carotid and pulmonary artery catheters respectively for blood gas, base excess, and lactate analysis (Gem Premier 3000 Blood Gas Analyzer, Instrumentation Laboratories, Warrington, UK).

7.1.5 Experimental Protocol

The animals were randomly allocated to one of two treatment groups at the outset (Figure 7.1). The timeline for the experimental protocol is shown in Figure 7.1.

Blast exposure. After baseline measurement the animals were moved outdoors, wrapped in a Kevlar blanket to protect from secondary and tertiary blast effects and positioned on a trolley 2.1 m from a cylindrical charge of EDC1S explosive (2.2 kg) which was then detonated remotely. Animals subjected to sham blast were treated identically but not exposed to blast.

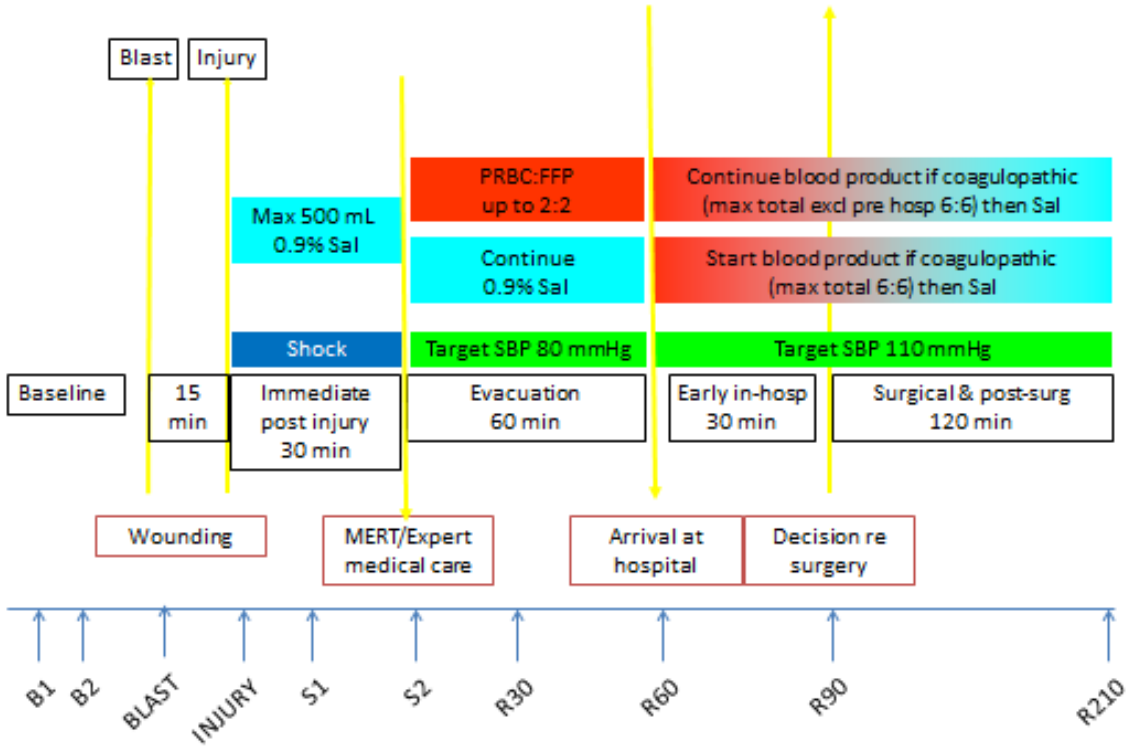


Figure 7.1 Experimental protocol and timeline. Red bordered boxes represent the clinical correlates of experimental events.

Time points: B1—baseline on initial cannulation. SURGERY (not shown)—blood taken immediately after completion of surgery. B2—baseline after completion of surgical preparation and transportation. BLAST—samples taken 15 min after exposure to blast (immediately before injury). INJURY—samples taken after musculoskeletal injury and controlled haemorrhage. S1 and S2—samples taken after 15 and 30 min of shock, respectively. R30, R60 etc—samples taken 30, 60 etc min after onset of resuscitation. R90 coincides with the "decision re surgery" clinical event; this time point was used for the primary endpoint of the study. However, this is not relevant to the discussion in this chapter. Sal—Normal Saline

Tissue injury phase. Immediately after the blast (or sham blast) the animal was returned to the physiological monitoring suite and fifteen minutes later, all animals were subjected to a controlled soft tissue injury using a blunt captive bolt pistol (CASH Special Knocker, Accles & Shelvoke, Sutton Coldfield, UK) delivering 4 standard impacts (using 2 Grain .25 cartridges) to the muscle of the right hindquarter. This resulted in widespread deep contusion in the underlying muscle but no fracture to bone. Five minutes after the tissue injury a haemorrhage of 35% of the estimated total blood volume was performed over 9 min 40 sec via the femoral arterial cannula, using a computer-controlled pump (Masterflex L/S model 7550 –17, Cole Palmer Instrument Company, Chicago, IL). The rate of bleeding reduced exponentially as the

haemorrhage progressed to mimic the rate of haemorrhage from a major arterial lesion. For blood volume and bleeding rate equations see Stern *et al.*, 1993 and Garner *et al.*, 2010

Shock phase. Following haemorrhage, the animals underwent a 30 minute shock period during which a capped volume of 0.9% saline (500 mL maximum) was administered as necessary to maintain a target systolic arterial blood pressure (SBP) of 60 mmHg, reflecting an aspect of current clinical practice by Combat Medical Technicians. Resuscitation infusions were warmed to 37°C and administered at a rate of 200 mL/min (Belmont Rapid Infuser, Belmont Instrument Corporation, US).

Pre-hospital evacuation phase. The next phase of the protocol represented a 60 min pre-hospital evacuation phase. Warmed resuscitation fluid was administered according to the relevant protocol, with a final target SBP of 80 mmHg in each group. Group 1 was given aliquots of 0.9% saline (representing standard of care) to attain and maintain the target SBP, while Group 2 received Packed Red Blood Cells (PRBC) and Fresh Frozen Plasma (1:1 ratio, PRBC and FFP given simultaneously). The volume of blood products in the pre-hospital phase was capped at 4 Units (4x450 mL approximately) per animal (2 units PRBC and 2 Units FFP). Once the maximum amount of blood product had been used resuscitation continued to the same pressure target using 0.9% saline for the reminder of the pre-hospital evacuation phase. All infusion volumes were measured accurately.

In-hospital phase. The in-hospital phase represented more aggressive resuscitation to a normotensive target (SBP 110 mmHg) using PRBC:FFP (1:1) in all groups. Supplementary oxygen (minimum FiO_2 0.3) was given and titrated to attain an arterial oxygen saturation (SaO_2) of 98%. Due to the limitations of the blood bank the total amount of PRBC:FFP used in any one animal was capped at 6 Units of each product (in addition to the PRBC used during the pre-hospital phase in Group 2). Once the total permissible amount of PRBC:FFP had been given resuscitation continued to the same pressure target using 0.9% saline in all groups. In practice few animals received saline in the in-hospital phase (3/9 in Group 1; 2/9 in Group 2).

7.1.6 Sampling and Measurements

Cardiovascular and paired arterial and mixed venous blood gas measurements were made before and after injury/haemorrhage, after 15 and 30 min shock, and at 30 min intervals during the pre-hospital resuscitation phase and in-hospital resuscitation phases. Blood samples for assessment of clotting were also taken at these time points. This included blood for use in the flow cytometry assay, the protocol for which is given in Figure 6.1

Thromboelastography (TEG®). Thromboelastography using a TEG® 5000 Hemostasis Analyzer (Haemonetics Ltd., UK) was performed on fresh, un-citrated whole blood. Arterial blood was taken from the femoral cannula and analysed immediately using dilute Innovin® (1:50887 dilution Dade® Innovin®, Dade Behring; marketed by System UK Ltd, UK) as the initiator (Sørensen *et al.*, 2010). All TEG® analyses were performed in triplicate at 37°C.

Prothrombin and activated partial thromboplastin times (PT and aPTT). Arterial blood samples were taken into citrated vacutainers (9NC 0.105M Vacutainer 367691, Beckton Dickinson, UK) and centrifuged at 1500 x g for 10 min. The plasma was separated and stored at -80°C for determination of PT and aPTT by turbidometry and fibrinogen concentration (Clauss method) using an ACL Elite analyser (Instrumentation Laboratories, Warrington, UK).

Blood Counts. Full blood counts were conducted using an ADVIA 120 (Bayer, Barmen, Germany). The ADVIA 120 is able to measure a parameter known as mean platelet component (MPC), which is related to flow cytometry side scatter and therefore to the internal complexity of platelets. MPC falls when platelets degranulate. To optimise MPC measurement, samples were mixed with ethylenediaminetetraacetic acid and then with citrate-theophylline-adenosine-dipyridamole (EDTA-CTAD) (Macey *et al.*, 2003).

7.1.7 Study Endpoints

The primary end point of the study was the clotting status assessed using thromboelastography (TEG®) after 30 min of in-hospital resuscitation. Secondary endpoints included clotting status throughout the study, physiological status, in particular degree of shock assessed by measuring actual base excess and arterial lactate and volumes of fluids used to attain the pressure-driven resuscitation targets. These endpoints are beyond the scope of this chapter.

At the end of the study all animals were killed humanely using a lethal overdose of sodium pentobarbitone (Euthatal, Merial Animal Health Ltd, Harlow, Essex) given intravenously.

7.2 Results

It should be noted that animal HR3 died at R149. Death was by sudden onset VF with cardiac arrest, at a time when the animal appeared to be recovering.

7.2.1 Physiological Data

Figure 7.2 shows the base excess of each animal, reflecting global adequacy of tissue oxygen delivery, by time point. All animals sustained a significant physiological insult. Figure 7.3 displays the same data but is colourised according to the resuscitation strategy to which the animals were randomised.

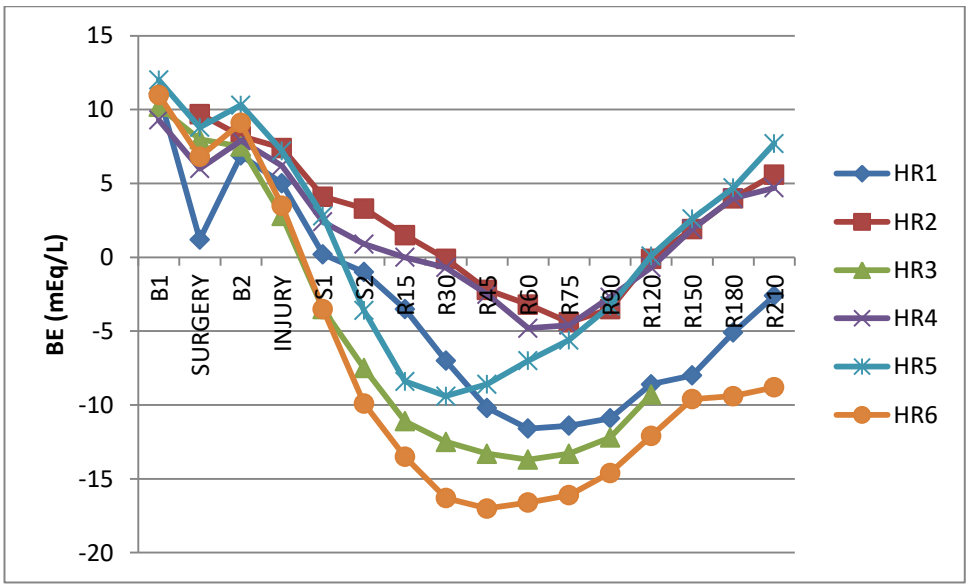


Figure 7.2: Base excess by animal and time point

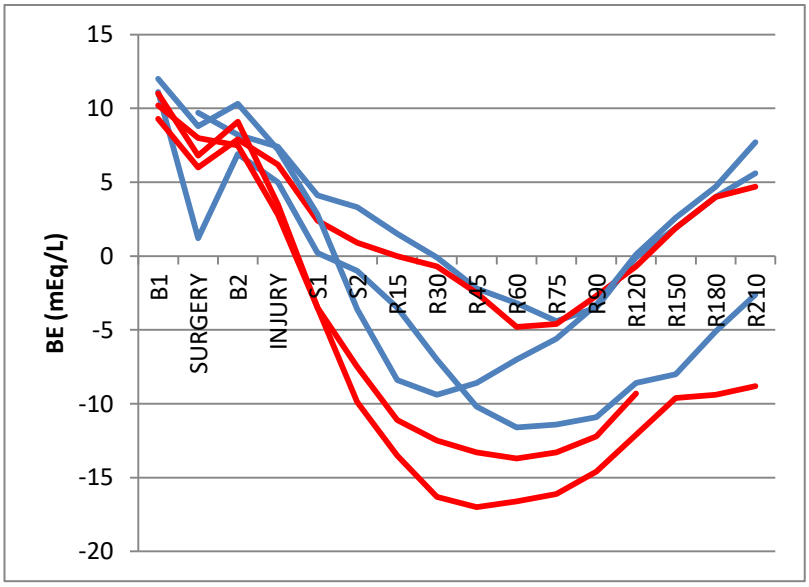


Figure 7.3: Base excess by animal and time point. Blue lines are the animals that received prehospital blood (group 2), the red lines those which did not (group 1).

7.2.2 Platelet Data

Figure 7.4 shows the percentage of CD61 positive events from the platelet area that were positive for CD62P, by animal and time point, in the unstimulated assay.

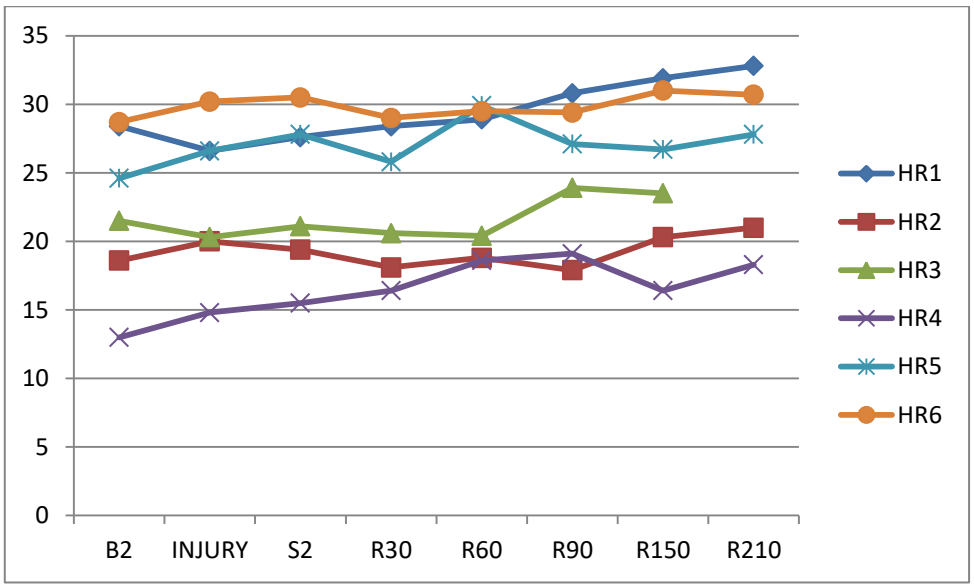


Figure 7.4: % of CD61 positive events (platelets) that were CD62P positive, by animal and time point. No ADP stimulation.

There are two key findings. First, there is a wide variation in CD62P positivity at the initial time point. Second, most animals exhibited little change in C62P expression over the course of the experiment.

Figure 7.5 shows the stimulated and unstimulated CD62P positivity for each animal by time point. These data demonstrate response to ADP stimulation at all time points of the study. Since there is a large variation in the initial unstimulated CD62P positivity (Figure 7.4), relative rather than absolute change on stimulation was used to compare the effect of ADP between animals.

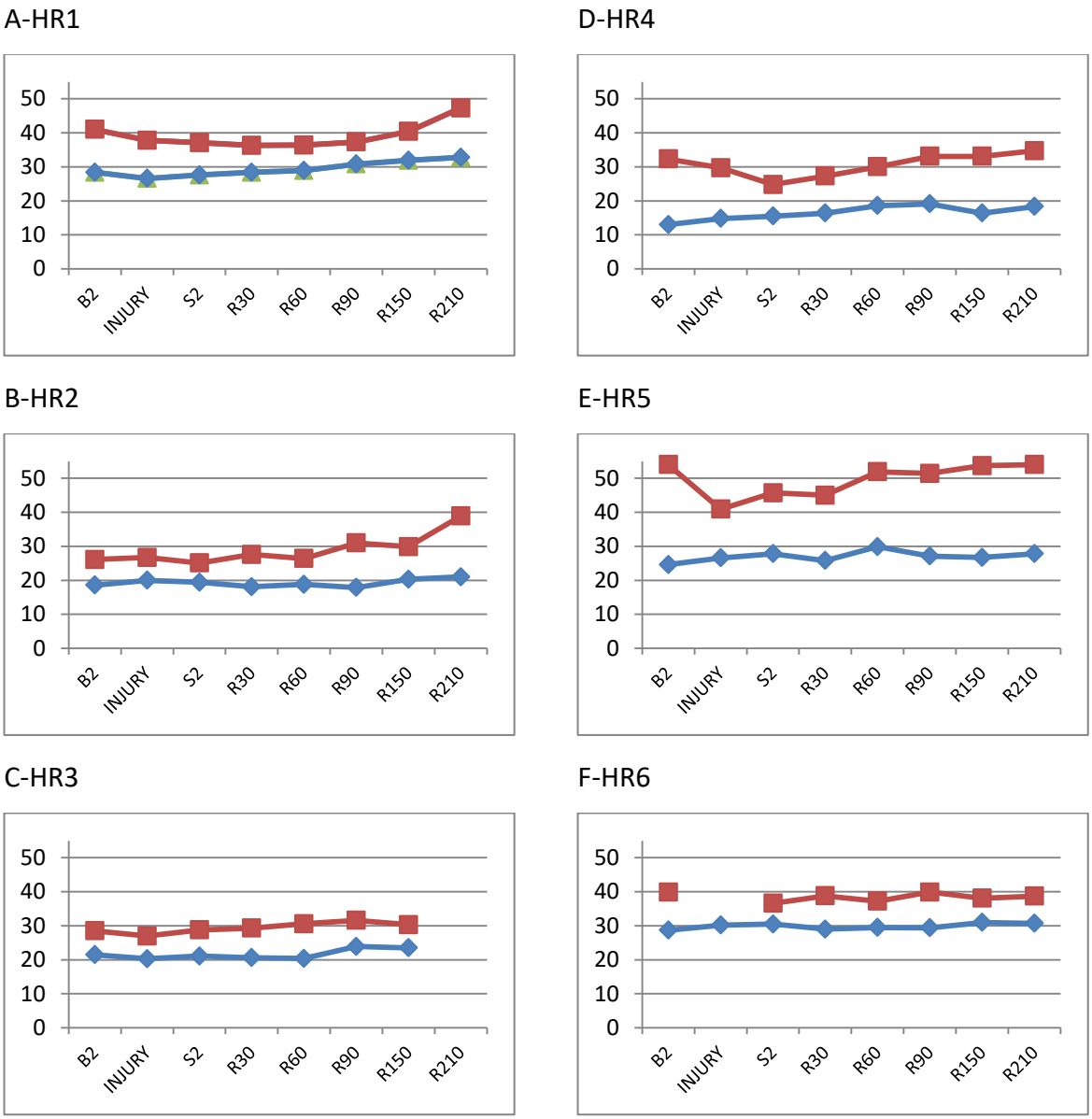


Figure 7.5: % of CD61 positive events (platelets) that were CD62P positive, by time point. Each animal's data are displayed in a separate graph. Data for the unstimulated assay shown in blue. Results after ADP stimulation shown in red.

The foldchange (ratio of CD62P positivity between stimulated and unstimulated assays) was calculated in each case and these data appear in Figure 7.6a. Figure 7.6b shows the mean foldchange.

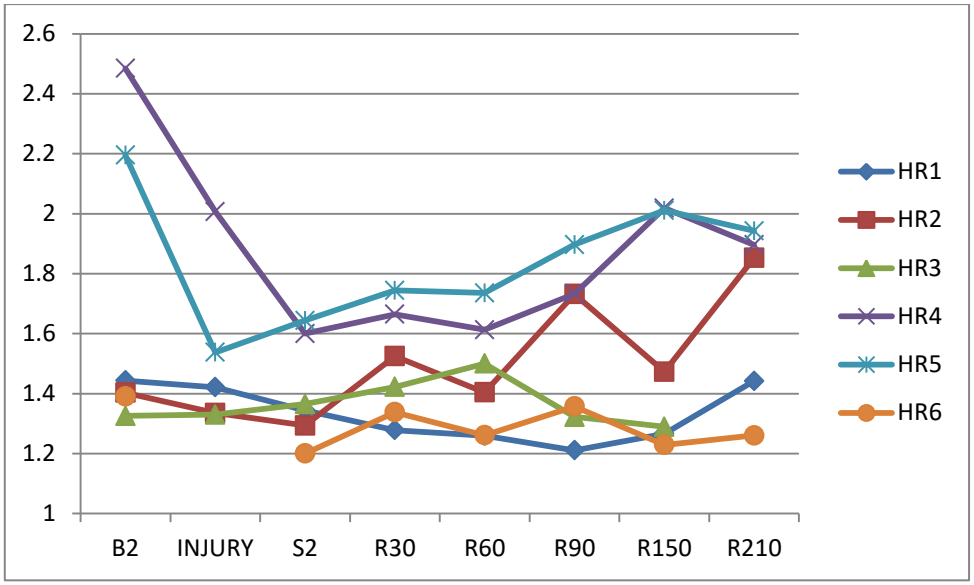


Figure 7.6a: Foldchange in % positivity for CD62P of CD61 positive events (platelets) on stimulation with ADP by animal and time point.

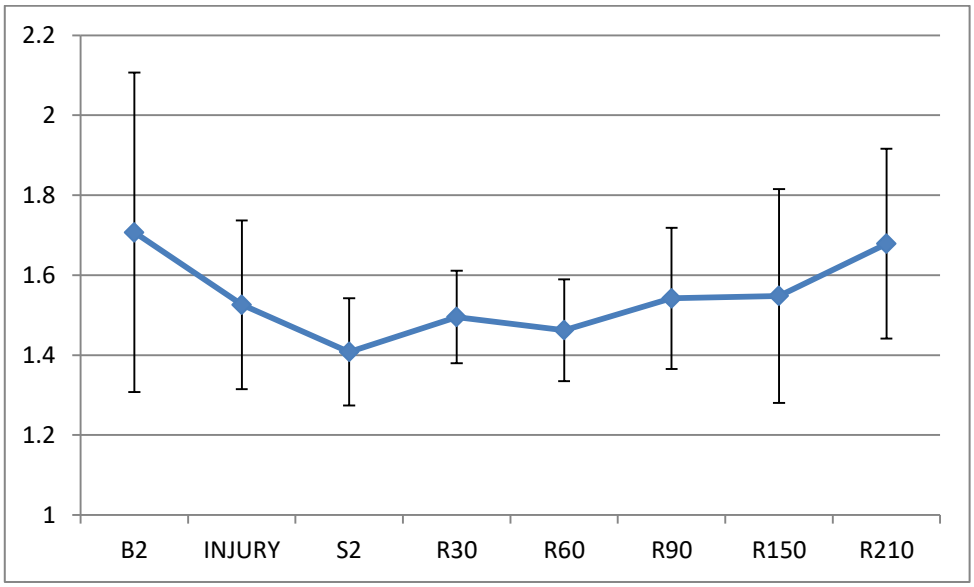


Figure 7.6b: Foldchange in % positivity for CD62P of CD61 positive events (platelets). Data are displayed as mean foldchange; whiskers represent the 95% confidence interval.

There was divergent response to ADP stimulation. Three animals (HR1, HR3 and HR6) began with relatively low activatability (that is ability to respond to agonist by increased expression of the activation marker) and this remained fairly constant throughout the experiment. HR4 and HR5 had high initial activatability, which fell to a

level much closer to the other animals after injury and shock. Activatability was restored in these two cases later in the experiment. HR2 also showed high activatability late in the experiment, having initially followed the pattern of the other three animals. There is no apparent relationship between there different phenotypes and the baseline expression of CD62P.

Figure 7.7 shows platelet counts by animal and time point.

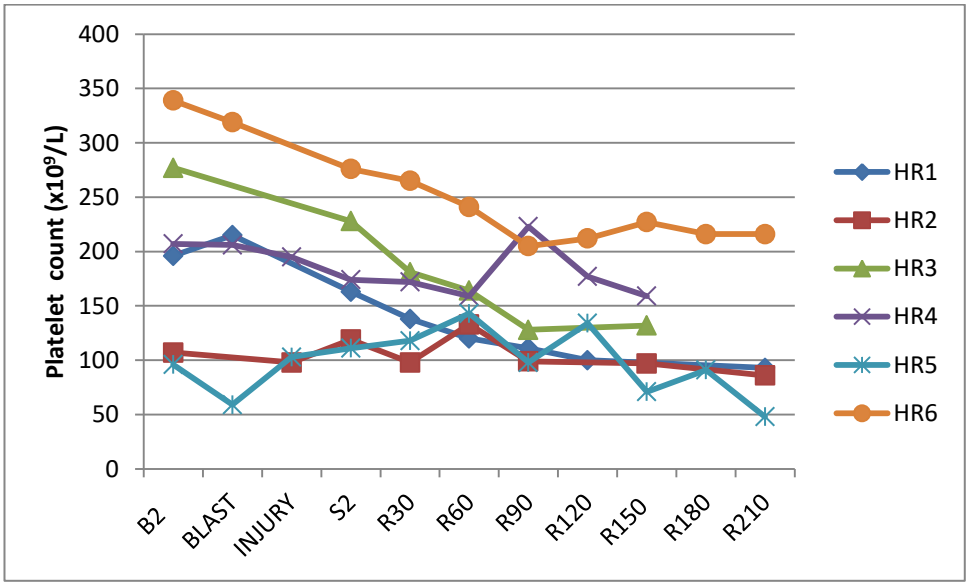


Figure 7.7: Platelet count by animal and time point

Two animals began with and maintained relatively low platelets counts, in the region of $100x10^9$/L. The remainder showed a gradual fall in platelet numbers over the course of the experiment. Platelet counts immediately post-injury are only available in three cases but there was no significant fall at that time point. There is no apparent relationship between platelet number and either CD62P expression or the effect of ADP stimulation.

Figure 7.8 shows the Mean Platelet Component (MPC) data.

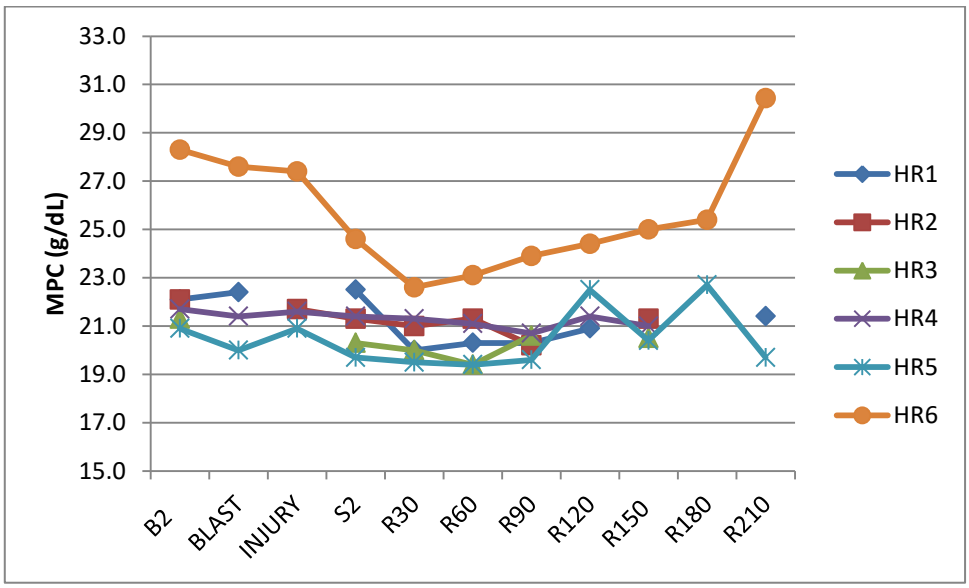


Figure 7.8: Mean Platelet Component by animal and time point.

MPC reflects granularity of platelets. The lack of a fall in MPC over the study period is therefore consistent with the finding that there was no increase in (unstimulated) CD62P expression. Animal HR6 has shown an idiosyncratic pattern of MPC change, one not consistent with its very flat CD62P positivity curve (Figure 7.5E).

In Figures 7.9 and 7.10, the data on platelet activation (CD62P expression and MPC, respectively) are plotted against BE at the same time point. No correlation is observed.

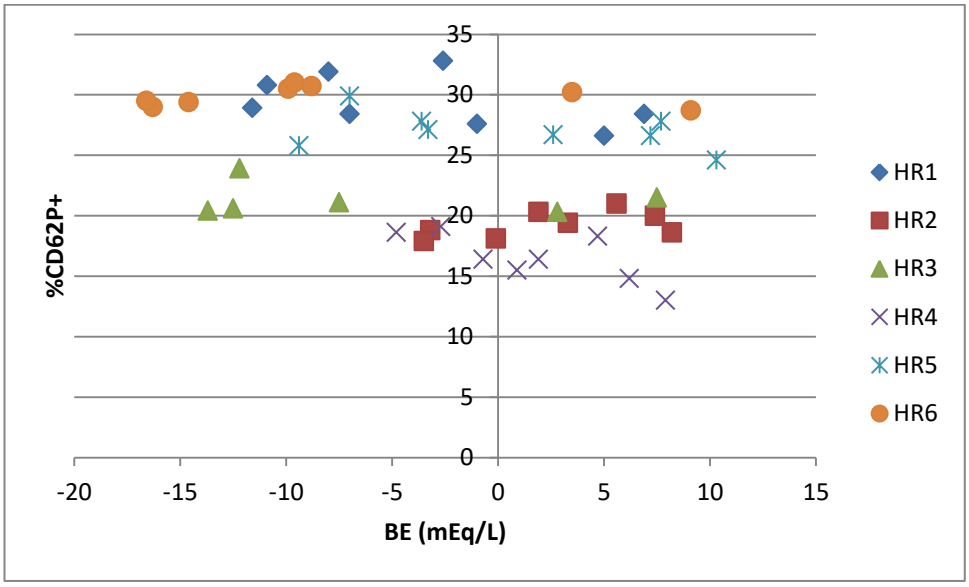


Figure 7.9. Scatter plot of BE versus CD62P expression.

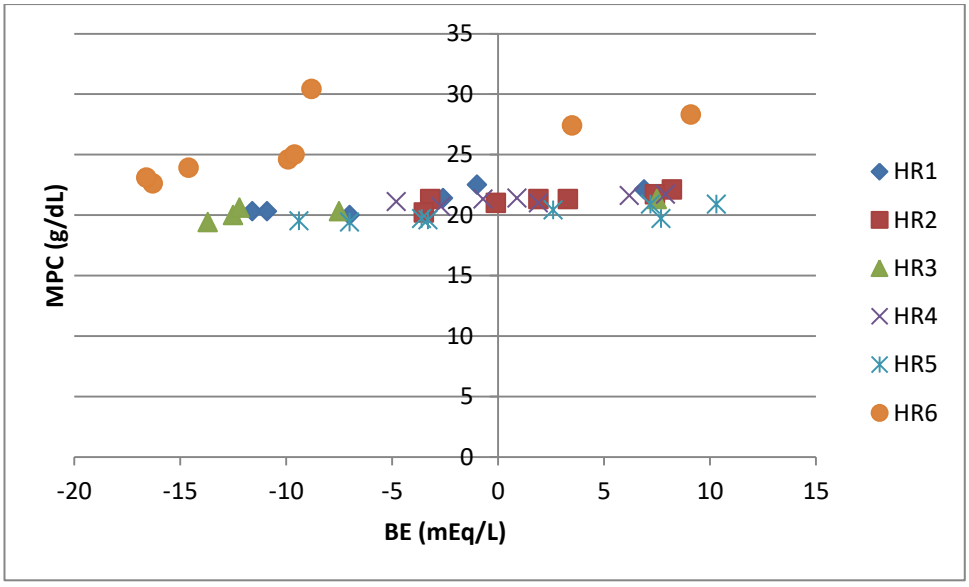


Figure 7.10. Scatter plot of BE versus MPC.

7.2.3 Coagulation Data

Figure 7.11a shows the changes in PT during the experiments. In Figure 7.11b these same data are displayed after normalising each animal's data to the internal control of the first blood sample taken at Line in 1. This will be referred to as normalised PT (nPT)

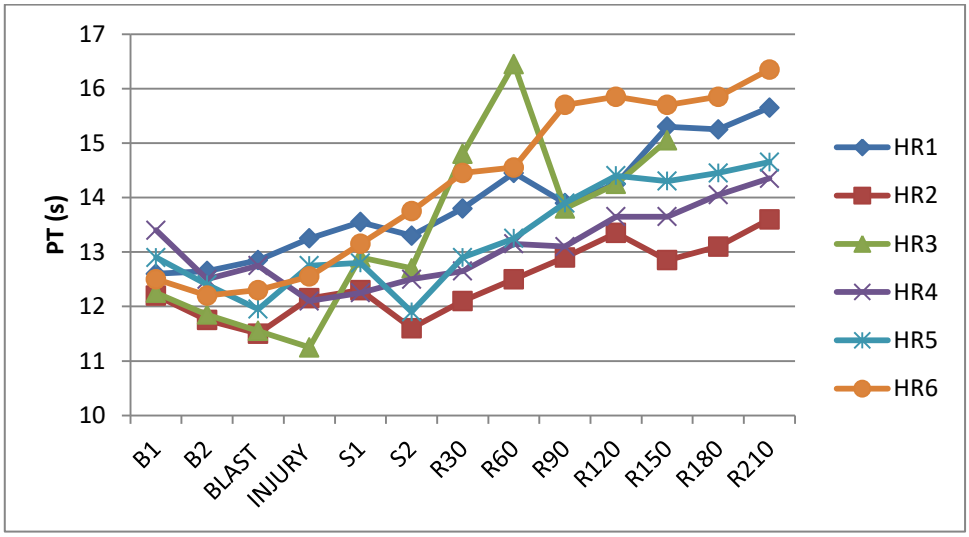


Figure 7.11a: Prothrombin time by animal and time point.

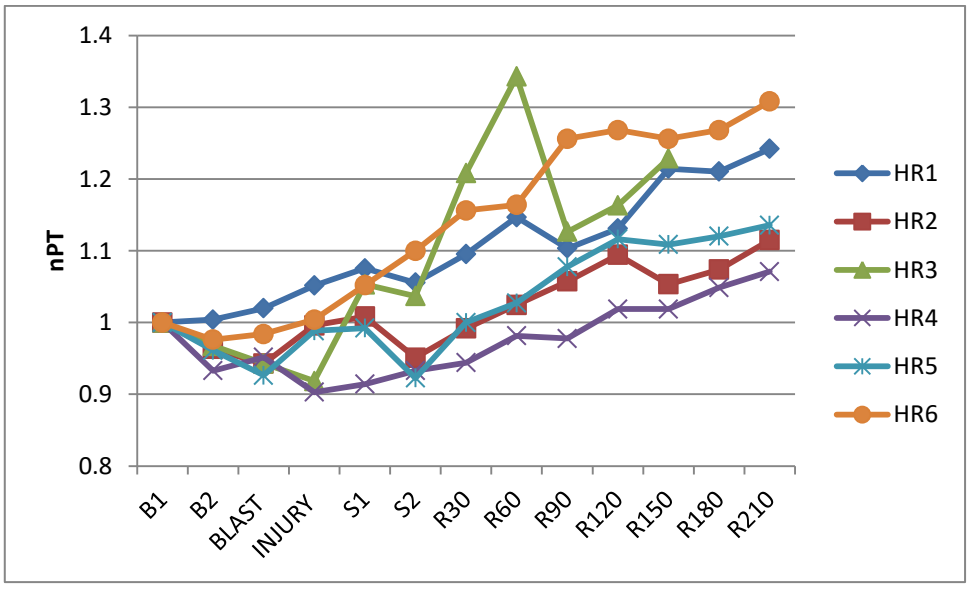


Figure 7.11b: Prothrombin time, normalised to the value at B1, (nPT) by animal and time point.

Immediately after injury there is the suggestion of a slight increase in the inter-animal variability of nPT, but no clear general upwards or downwards change. There is a clear upward trend in PT over the remaining course of the experiment, with some variation

between animals. However, even by R210, three of the animals have only reached an nPT in the region of 1.1. HR3 displayed an unusual peak in PT early during the resuscitation phase, before returning to follow the trend seen in other cases.

In Figure 7.12, the nadir BE achieved by each animal is plotted against the nPT at R150 (the last time point at which data is available on all animals). There is a clear correlation between the depth of the physiological insult and the degree of disturbance of coagulation (Pearson's -0.962, p=0.002).

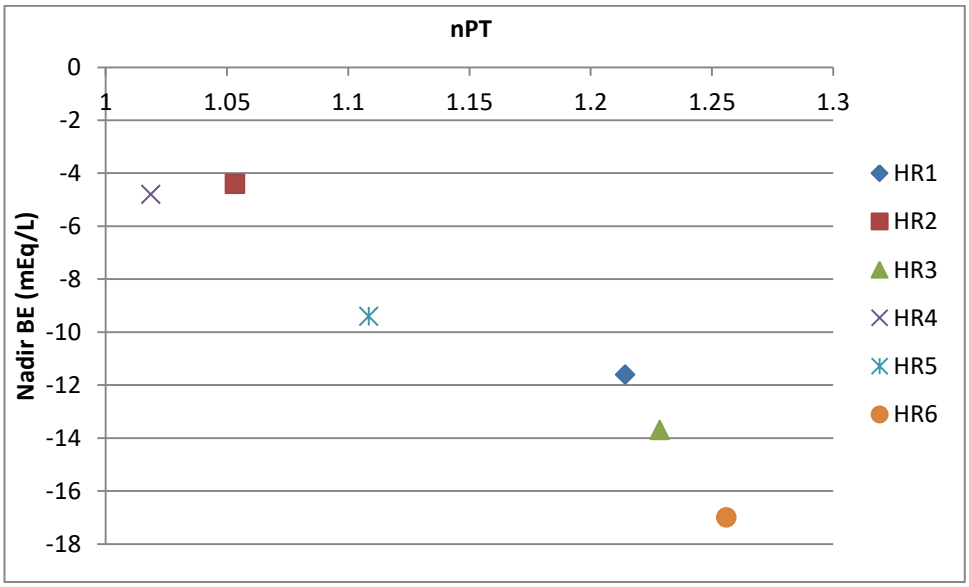


Figure 7.12: Scatter plot showing nadir BE against nPT at R150.

Figure 7.1 demonstrates that all the animals had reached the nadir of BE by R75, but the upwards trend in nPT has only just become established at this point and continues to the end of the experiment.

Figure 7.13 shows aPTT data. Most animals show a shortening of aPTT after surgical preparation and transport and/or after blast. Amid great variability, aPTT measurements then rise again but do not go on to demonstrate any coagulopathy.

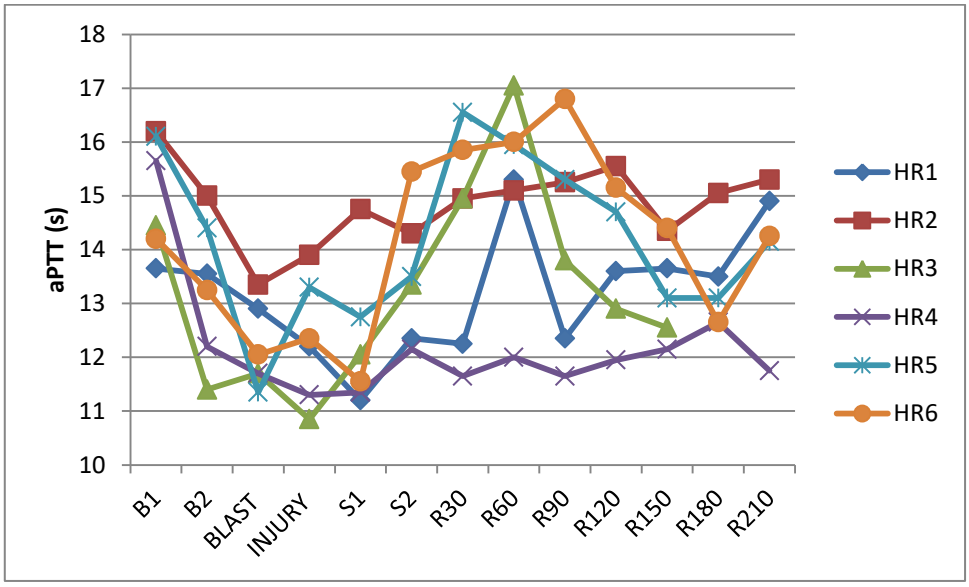


Figure 7.13 aPTT by animal and time point

Fibrinogen data are shown in Figure 7.14. There is an increase in fibrinogen concentration between B1 (mean [IQR]: 2.49 [2.29-2.77]) and B2 (2.78 [2.51-3.41]), which is consistent with the acute phase response to surgery (Wilcoxon signed rank test, p=0.048). Fibrinogen levels then fall to a new low immediately post-injury (2.26 [1.88-2.69], p=0.028). There then appears to be a (non-significant trend) towards increased fibrinogen concentrations during the shock phase. This is followed by divergent behaviour, with sharp falls in HR3 and HR6, the animals with the greatest physiological insult, to levels below the normal range seen in this strain of pig.* More stable values were seen in the others. However, all animals have broadly stable fibrinogen concentrations from R60 inwards. Despite this, abnormalities in PT continue to evolve (Figure 7.11).

* The 95% reference range for plasma fibrinogen in this strain of pigs was 1.77-3.36 g/l. Data based on samples taken after minimal surgery (induction of anaesthesia and insertion of carotid cannula) in Large White pigs of similar age that contributed to this and other studies in the laboratory (n=147). Data distribution was normalized with a 1/square root transformation prior to determining the reference range. (Mrs C Wilson, analysis, and Dr R Gwyther, statistics.)

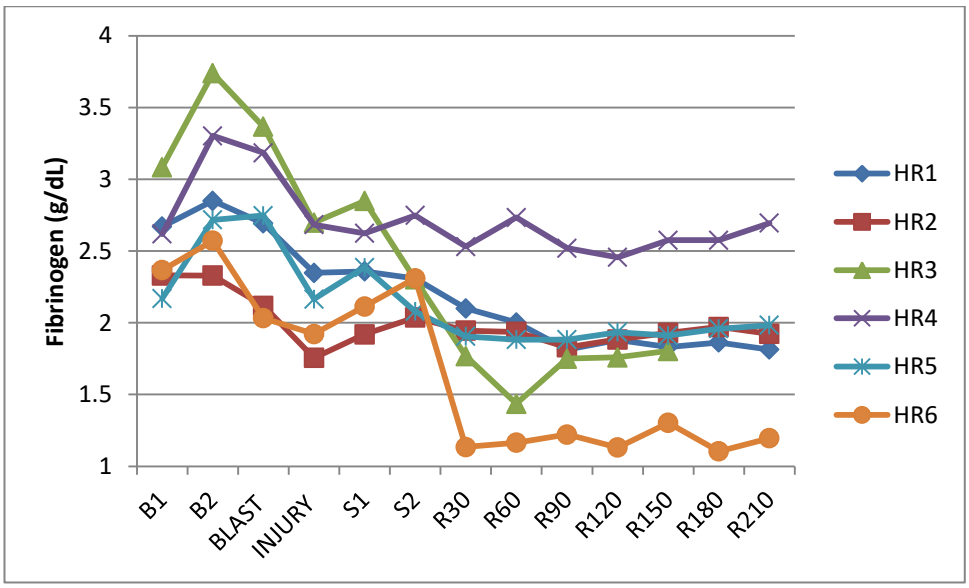


Figure 7.14: Fibrinogen concentration by animal and time point.

Figure 7.15 plots the Fibrinogen concentration at R150 against the nadir BE. There is a correlation between the animals physiological response and the final fibrinogen concentration (Pearson's 0.822, p=0.045).

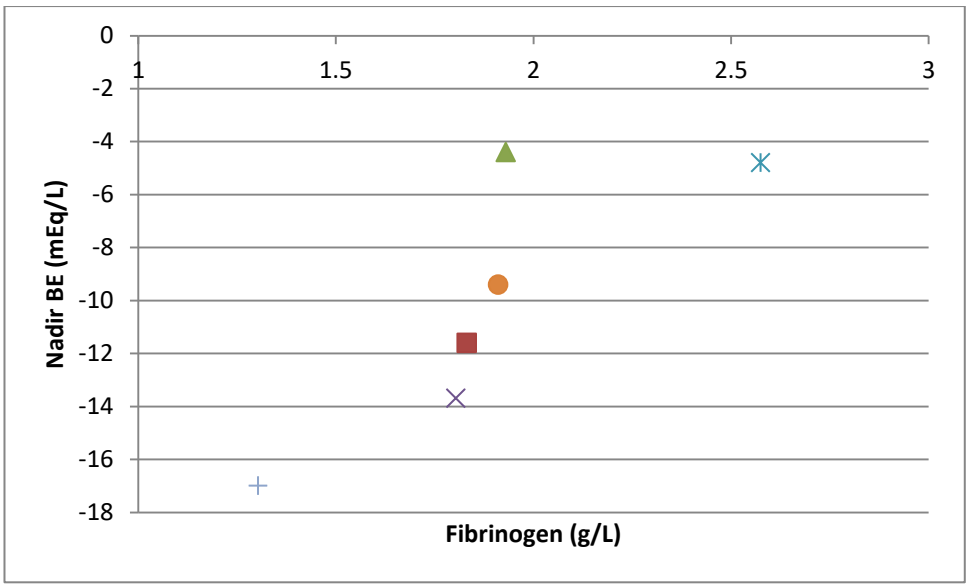


Figure 7.15. Scatter plot of Fibrinogen concentration against nadir BE

Figure 7.16 shows the R time data. At B1, values vary between 6s and 26.6s but by B2 (after surgery and transport to the range) they are much more closely grouped at 11s to 14s. Four animals then show little change in R time over the course of the experiment. The principle exceptions are HR3 and HR6, the two animals which experience the greatest physiological insult. Of note, HR3's early peak in PT is associated with a peak in R time, although subsequent raised PTs in several animals are not reflected in the R time, which normalises.

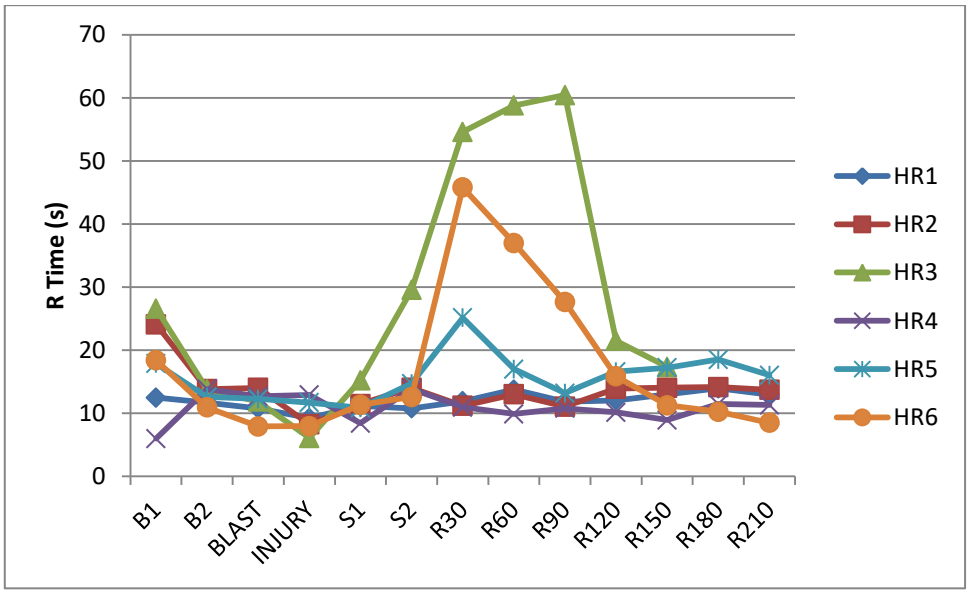


Figure 7.16: R time by animal and time point.

Figure 7.17 shows changes in the alpha angle. Again, the range of values narrows between B1 and B2. There is then a marked rise after exposure to blast (i.e. at the pre injury time point). Animals HR3 and HR6, and to a lesser extent HR5, then show a fall in alpha angle followed by a return to normal values, the nadir approximating the time of the lowest BE (see figure 7.2).

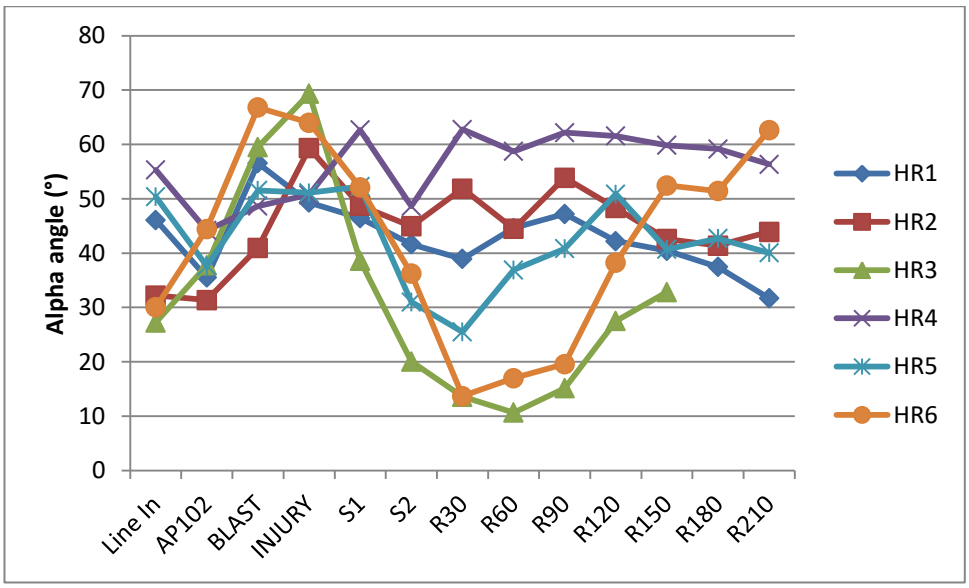


Figure 7.17: Alpha angle is plotted by animal and time point.

Figure 7.18 shows a marked fall in MA between B1 and B2. Five animals show an increase in MA after blast exposure (the pre-injury time point), followed by a return to values broadly in keeping with that seen at B1. HR4 shows a different pattern of MA changes with no significant rise immediately after blast exposure.

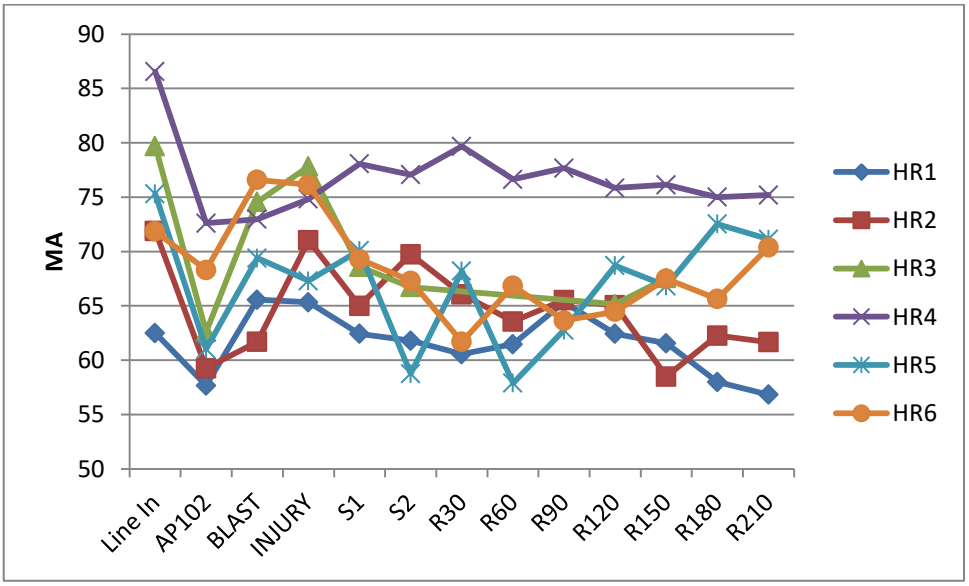


Figure 7.18: MA by animal and time point

Oddly, the highest values of MA at R150 (the last common time point) are seen in animals HR3, HR5 and HR6, despite the fact these animals, to varying degrees, suffered the severest physiological insults and had extended PTs and low fibrinogen at this

stage. Figure 7.19 demonstrates the lack of correlation between Fibrinogen concentration and MA.

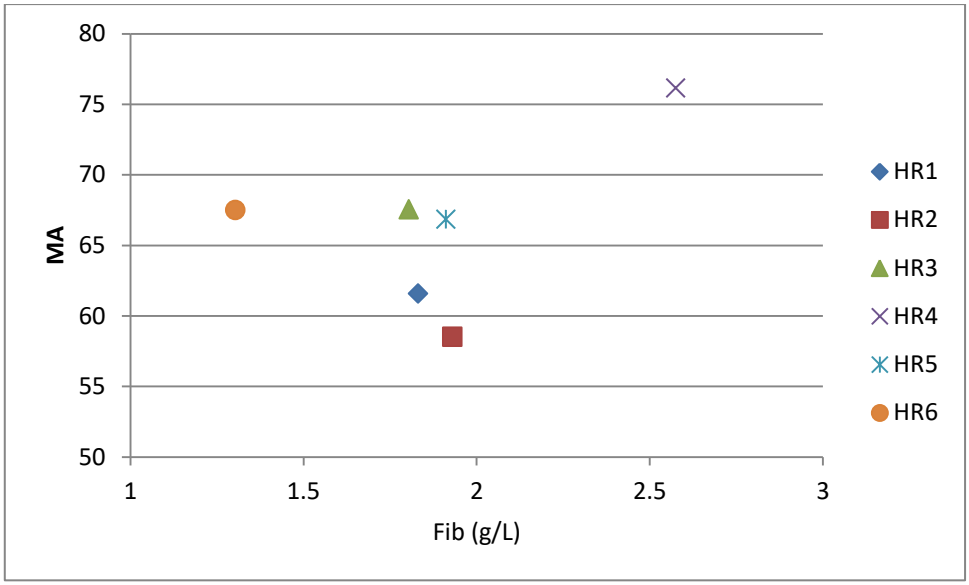


Figure 7.19: Fibrinogen concentration plotted against MA at R150. There is no positive correlation between fibrinogen concentration and MA. Indeed, if HR4 were excluded, there would be something of a negative correlation.

7.3 Discussion

7.3.1 Physiological Response to Injury

Figures 7.2 and 7.3 demonstrate the development of significant shock in all animals. Figure 7.20 gives some idea of the amount of tissue injury generated. There is a notable variation in response to the injury, with BE at the end of the shock phase (S2) ranging from 1.5 to -13.5 me/L. In every case, BE began to rise after 30 to 60 min of resuscitation. This allows an assessment of the function of the assay in a range of physiological conditions. It is not intended to compare the two treatment arms in this chapter. With these small numbers, it is not appropriate to make comparisons between the animals treated with early blood transfusion and the others.

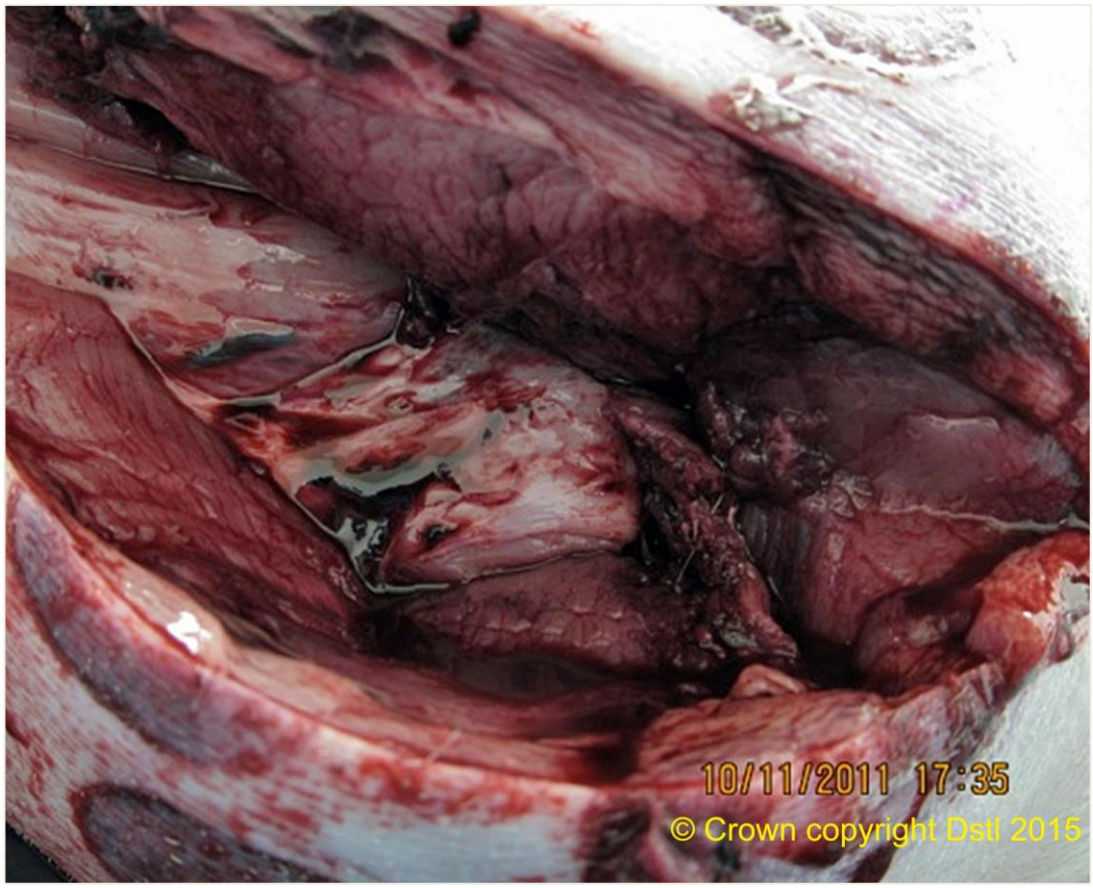


Figure 7.20 Post mortem photograph illustrating the contused and devitalised muscle caused by the action of the captive bolt gun.

7.3.2 Coagulation

Figures 7.11a and b demonstrate a clear upward trend in PT over the course of the experiment. However, the changes would be considered modest in a human polytrauma patient. Additionally, they are only firmly established quite late in the course of the experiment, well after the simulated patient has reached hospital and received resuscitation in both treatment groups. Definitions of acute traumatic coagulopathy have varied. In the initial description by Brohi, coagulopathy was defined as PT over 18 seconds, APTT over 60 seconds, or TT over 15 seconds (1.5 times normal) (Brohi *et al.*, 2003). Other studies have tended to use a Prothrombin Time Ratio (PTr) of 1.2 as a diagnostic threshold for ATC when using laboratory tests (MacLeod *et al.*, 2003; Maegele *et al.*, 2007; Frith *et al.*, 2010; Davenport, Manson, *et al.*, 2011). Caution must be exercised transferring human diagnostic criteria into an animal model and PTr is not directly analogous to nPT (the former using an external control, the latter an internal control).

It is therefore not possible to define a level of PT or nPT that represents coagulopathy in this model. However, it is reasonable to say that small changes in nPT, e.g. nPT remaining near to 1.1 or lower, do not suggest a significant coagulopathy. Three animals achieved nPT>1.2 at any stage. ATC is defined as an early coagulopathy, developing before admission to hospital and in the absence of significant fluid resuscitation (Brohi *et al.*, 2003). The S2 and R30 time points are equivalent to this. There was considerable variation in nPT at S2, but none of the animals could be said to be coagulopathic by this (crude) measure. HR3 did show a large rise in nPT at R30 but this was unique and part of an aberrant pattern of change in nPT. aPTT data showed no evidence of a coagulopathy. Consequently, the coagulation changes seen in this small group is best characterised as displaying mixed but significant disturbances of coagulation. It cannot be said to accurately recapitulate the human syndrome of ATC.

It may be that the animals should not have been expected to develop ATC. ATC was described in civilian patients. Admission coagulation abnormalities have been reported in military patients (Doran, Woolley and Midwinter, 2010; Morrison, Dubose, *et al.*, 2012; Morrison, Ross, *et al.*, 2013). However, the contribution of primary blast

injury to human coagulopathy has not been characterised. The degree of primary blast experienced by patients injured by IEDs is likely to be highly variable. Animal work at Porton Down has previously shown an upregulation of coagulation immediately after blast (Harrisson *et al.*, 2008), a finding recently confirmed by French investigators (Prat *et al.*, 2015). Although not reflected in the PT data, this can also be seen in this experiment. There is a clear rise in both alpha angle and MA at the time point immediately after blast exposure. It may be that subsequent changes in coagulation are altered by this blast-induced change and that this prevents a more profound, early coagulopathy from developing. In keeping with this suggestion, data from animals subjected to this experimental protocol but without blast exposure have subsequently been shown to demonstrate a coagulopathy at the end of the prehospital phase (Watts *et al.*, 2015). An intriguing mechanism for this effect can be hypothesised: blast exposure leads to a bradycardia mediated by vagal efferents (subsection 3.1.2). Recently, a Brazilian group has described attenuation of haemorrhagic shock-induced coagulopathy by efferent vagal nerve stimulation (Rezende-Neto *et al.*, 2014). However, the bradycardia associated with simple models of blast injury is not seen with complex models such as this one, so it is not clear that there is suitable vagal signalling in this context.

The R time and alpha angle changes are well correlated to the animals' physiological response. However, there is a time lag between the nadir of BE and the PT changes. Of the standard mechanisms thought to contribute to trauma induced coagulopathy (Hess *et al.*, 2008), factor consumption or dilution would seem the most plausible causes of these relatively late changes, while acidosis could govern the changes which corrected as the animals recovered. There is also a correlation between the physiological response and the final fibrinogen concentration, as shown in Figure 7.15. This raises the possibility that the degree of change in nPT is driven by the ability of the liver to replenish consumed factors in the face of tissue hypoxia. (The discordance between TEG and laboratory coagulation test results seen in these data is a well-recognized finding [Ågren *et al.*, 2013; Louis *et al.*, 2014].)

Some other aspects of the coagulation data are notable. There is a tightening in the range of the R time and the alpha angle between B1 and B2. There is a fall in MA

between B1 and B2. This suggests that surgical preparation and transport has an effect on coagulation. This accords with the concerns about the effect on platelet function raised earlier. Suggestions of a mechanistic link between these findings must be very tentative: Effete or non-responsive platelets could contribute to a reduction in MA at B2. They could also explain later falls in MA that appear to be independent of fibrinogen (Figure 7.19).

This discussion has been based on data from a very small sample. The interpretations outlined above were constructed *post* hoc. Consequently, only trends can be observed and the data can only serve to generate hypotheses.

7.3.2 Platelet Activation

Figure 7.4 shows that CD62P expression remained fairly constant throughout the experiment in all animals, with some between-animal variation. This lack of change in the face of injury, shock and resuscitation was unexpected (see subsection 2.7.6). No other study has examined platelet activation and response at multiple time points early after trauma, either in animals or humans. However, it is known that there is increased CD62 expression after injury (Jacoby *et al.*, 2001; Ogura *et al.*, 2001) and reduced response to platelet agonists (Tschuor *et al.*, 2008). A primate model has shown that a proportion of expressed CD62P can be shed from the platelet surface within 2 hours, with preservation of platelet function (Michelson *et al.*, 1996). It might be reasonable therefore to expect the following pattern in these data:

- Increased expression of CD62P after injury
- A fall in CD62P expression over the course of the experiment
- A fall in activatability (change in CD62P expression on stimulation) after injury

The first two phenomena were not observed. The last was observed only in those animals which had a high degree of activatability at baseline. Two explanations need to be considered: a) there could be a fault with the assay or b) this finding could reflect the physiological reality.

There are several findings that support the performance of the assay. First, the assay detected changes in CD62P expression in response to ADP. Second, the assay detected changes in the response to ADP over the course of the experiment in some animals (HR1 and HR4, Figure 7.5). Third, Figure 7.8 shows that, with one exception, there was little change in MPC over the course of the experiment. Changes in MPC have been shown to be inversely correlated with changes in CD62P expression (Macey *et al.*, 1999). This lack of change in MPC therefore corresponds with the flat CD62P expression curves in Figure 7.4. Therefore two assays (CD62P expression and MPC) have indicated that there was little variation in the activation of circulating platelets during the experiment. However, it must be emphasised that MPC is not a gold standard against which the assay can be assessed. As noted at the end of Chapter 6, the dose-response curve was removed from the flow cytometry protocol. This has proved unfortunate in the context of these unexpected findings. It is possible that differential response to agonist would have been seen at lower ADP concentrations. Equally, the ADP concentration that was used was based on statements in the literature indicating that it was associated with maximal response in humans (Panzer, Höcker and Koren, 2005). In the light of these data, an assessment of higher concentrations would be justified.

Accepting that the assay has functioned properly, a possible physiological explanation is that the surgical preparation and transport of the animals meant that there had already been substantial CD62P expression before B2. Further experimental work was carried out to address this hypothesis.

7.3.4 Further Analysis – Materials and Methods

15 animals were used, designated X1 to X15. 10 animals being used for blood donation. These animals underwent induction of anaesthesia and cannulation as outlined above. Only their B1 samples were analysed. 5 animals were being prepared for experimental use and therefore completed the full protocol of preparation and transplant as outlined above. B1 and B2 samples were available for assay. All assays were carried out as previously. B1 samples, taken as soon as arterial access is

achieved, are as close as possible to being from normal subjects. The author's colleagues at Dstl kindly carried out these assays on his behalf.

7.3.5 Further Analysis — Results

Figure 7.21 shows CD62P expression with and without stimulation from a) B2 for the six HR subject animals and b) B1 for the 15 other animals (designated X). The HR B2 unstimulated data are statistically significantly lower than the X B1 data. The same is true of the ADP-stimulated results.

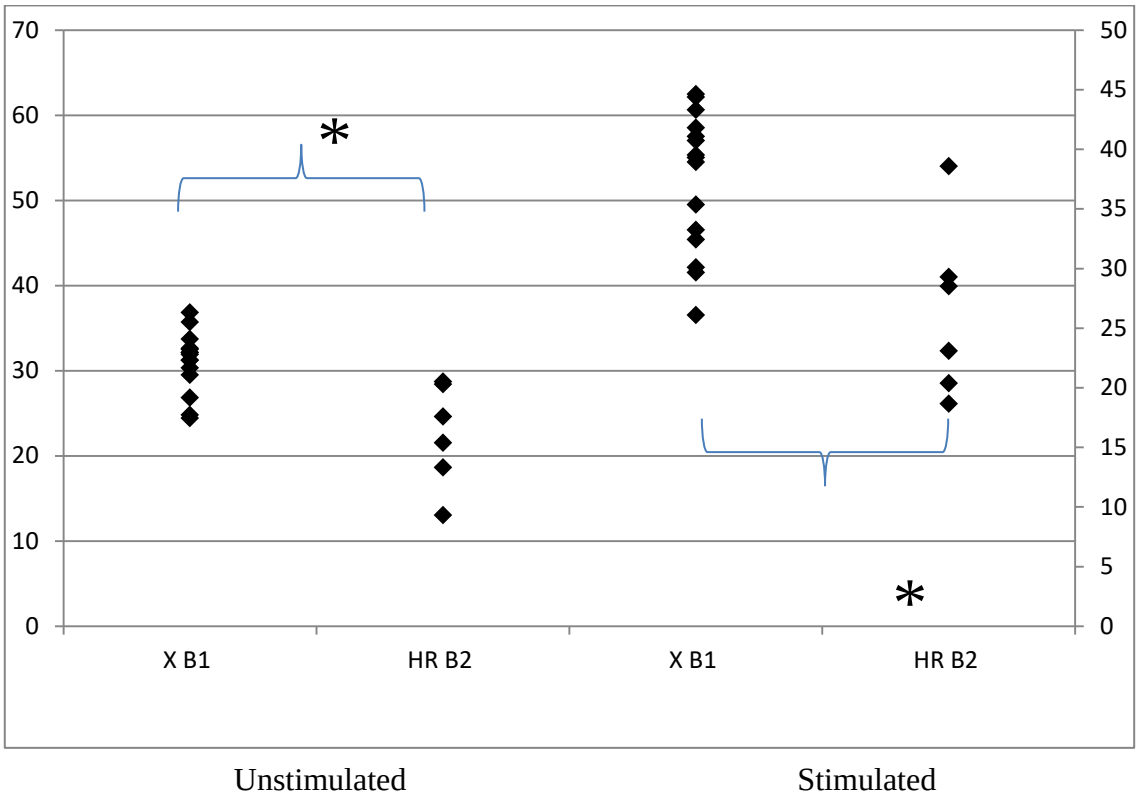


Figure 7.21: %CD62P positivity in unstimulated and stimulated assays from X B1 (normal subjects at true baseline) and HR B2 (experimental subjects after surgical preparation and transport). *The HR B2 results are lower than the X B1 results in both cases (Mann-Whitney U, p=0.003 in both cases).

While 15 is too small a number to allow a normal range to be established, the B1 data in Figure 7.21 indicate where that range would lie. CD62 expression at B2 in the HR animals shown appears to be lower, in both the stimulated and unstimulated assay.

Figure 7.22 A shows CD62P expression in sequential samples taken at B1 and B2 in five animals. These were the five animals mentioned above which were being prepared for

experimental use. Figure 7.22 B shows the results after ADP stimulation. With the exception of unstimulated CD62P expression by platelets taken from X6, all the data show either no change or more commonly a fall in between the two time points.

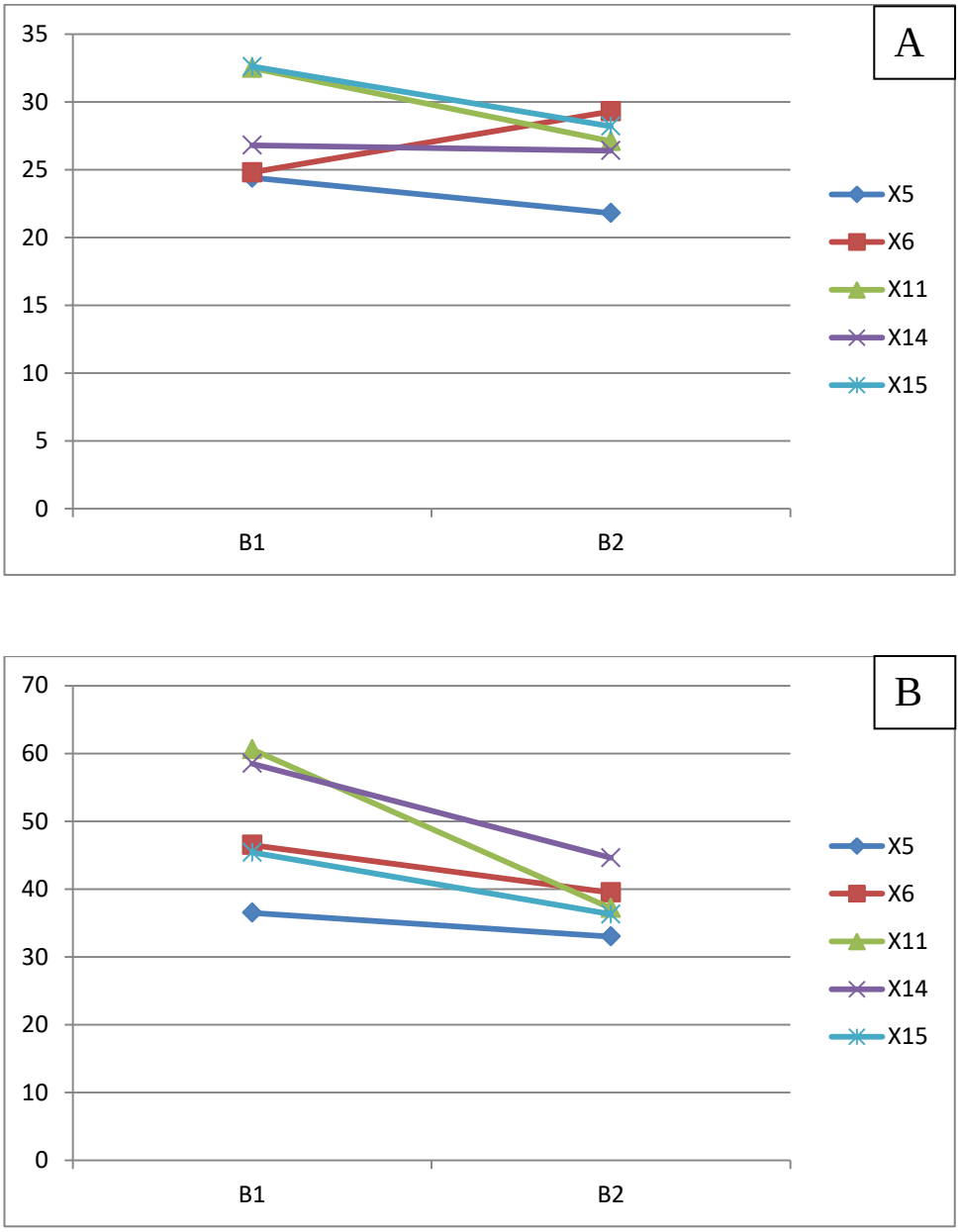


Figure 7.22. A) CD62P expression without ADP stimulation at B1 and B2. B) CD62P expression with ADP stimulation at B1 and B2.

Figure 7.23 expresses these data as foldchange in CD62P expression on ADP stimulation.

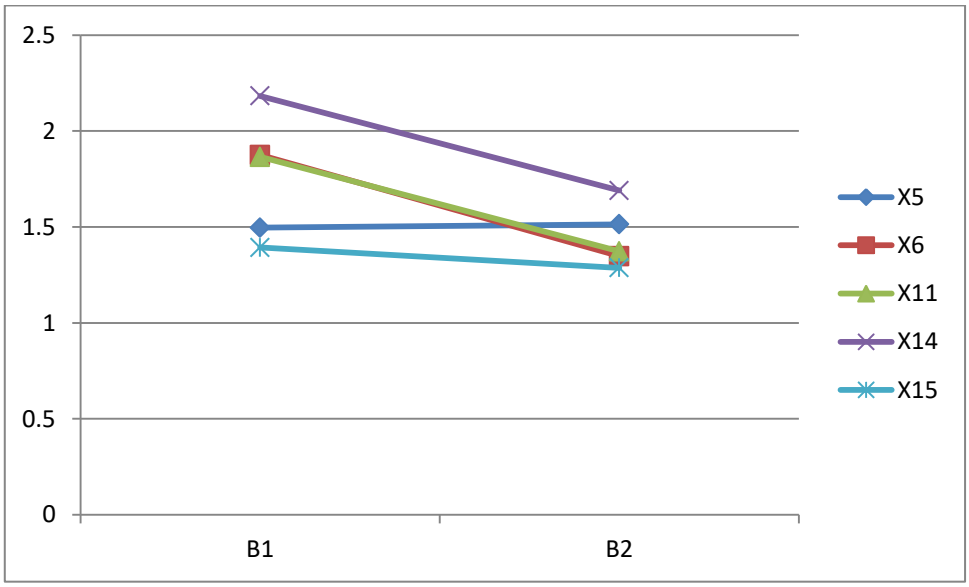


Figure 7.23 Foldchange in CD62P expression following ADP stimulation of platelets from animals tested at B1 and B2.

Three of the five animals show a considerable fall in their response to ADP stimulation.

7.3.6 Further Analysis – Discussion

These data (Figures 7.21 to 7.23) suggest that there is an effect of surgical preparation and transport on the results of the CD62P assay in some animals. However, the effect is not consistent. In particular, two HR subject animals had a preserved response to ADP at B2. These were among the three animals which exhibited high activatability at the end of the experiment. This could suggest an inter-subject variability in the ability to restore responsiveness to ADP stimulation.

In the light of these data, it was concluded that it would not be appropriate to carry out a comparison between the arms of the trial of CD62P expression. Equally, it is not possible to comment on what would otherwise be interesting findings in the present data, such as the lack of correlation between physiological derangement and platelet activation seen in Figure 7.9. Further work to establish an assay (or assays) of platelet function and a compatible model of coagulopathy after military injury would be required. This is discussed further in Chapter 8.

Chapter 8. Implications for Further Research

8.1 Animal Experiments

Platelets are fundamental to coagulation. The practical difficulties of studying platelets and the complexity of their actions (relative to clotting factors) has meant that their role in trauma induced coagulopathy remains unclear. They are implicated both by their functional centrality and by some human data. Translational research into platelet function after trauma must lead to an understanding of how platelets contribute to coagulopathy and to the identification of potential therapeutic targets. However, it is not yet clear which aspects of platelet function are involved and consequently which assays will be useful. Further work in this area must begin with broadly based assessment of multiple aspects of:

- platelet number,
- stimulation (multiple agonists),
- degranulation and epitope conformational change (use of multiple expression markers),
- aggregation and adhesion

both in animal models and humans. This will make it possible to confirm that animal models accurately reflect the human disease and allow identification of the important pathways.

The present model and platelet assay may have a role in this process. The data suggest several lines for the possible improvement of both. This assay can be adapted and expanded in several ways. With optimisation of the cytometer settings and of the assay preparation and the addition of uniform sized microspheres, platelet microparticles could be detected. By reintroducing the CD45 specific mAb, platelet-leucocyte aggregates can be detected. Given the wide variation in detected platelet forward scatter (see Figure 6.8), it seems likely that platelet-platelet aggregates are included in the platelet population. An imaging flow cytometer could be used to demonstrate these. As noted during assay development, porcine platelets do not respond to TRAP in the same way as humans (Kinlough-Rathbone, Rand and Packham,

1993; Connolly *et al.*, 1994). Since thrombin is a crucial platelet activator, it would be desirable to circumvent this problem. TRAP does induce a change in platelet shape and platelet-platelet aggregation; an imaging flow cytometer would be useful to observe these also. Thrombin itself fully activates porcine platelets, possibly through a receptor other than PAR-1. Thrombin could be used as an agonist if a further modification were made to prevent it acting on fibrinogen in the sample (Michelson, 1994).

Only two CD62P assays were conducted at each time point: unstimulated and stimulated. The concentration of ADP used (10µM) has been shown to be sufficient to stimulate significant platelet activation, including CD62P expression, in several studies (Janes *et al.*, 1994; Daniel *et al.*, 1998; Panzer, Höcker and Koren, 2005). However, these all used human subjects. Further work to establish whether higher concentrations of ADP would have a further effect on porcine platelets, particularly in those showing little response at 10µM, is required.

Extending this, dose-response curves for ADP stimulation could be used. Figure 8.1 illustrates data from two of the developmental animals.[*] It shows the percentage CD62P positivity of platelets at each ADP concentration at three time points: B2—before injury; S1—during the shock phase; R30—30 min into resuscitation. There was a differential response to progressively increasing concentrations of ADP and this reversed the relationship. At maximal ADP concentration, there was a greater CD62P positivity in the samples from the uninjured animal than the later samples. In the first animal, there is the suggestion that response to ADP after injury was maximal at a lower agonist concentration than before injury (i.e. the lines plateau).

[*] Dose response curves were not included after initial assay development (i.e. in Chapter 6) as it would not have been physically possible to run these in parallel with other assays using available staff and equipment.

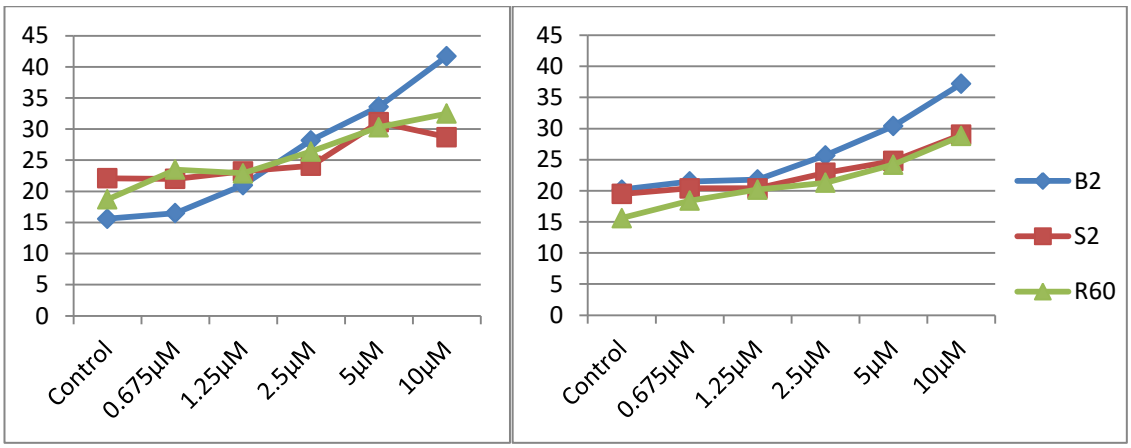


Figure 8.1. PA9 (left) and PA10 (right). % of platelets (CD45-, CD61+) positive for CD62P. Gates were set to include <5% of events from the relevant isotype control. Samples from 3 time points are shown. Each sample was incubated with ADP at the concentration shown for 10min before Ab staining.

No conclusion can be drawn from this finding where n=2, and indeed it is not consistent with subsequent findings. However, it indicates how the use of dose-response curves could enhance the assay. Figure 8.2 illustrates how these could identify subtle changes in the effect of ADP.

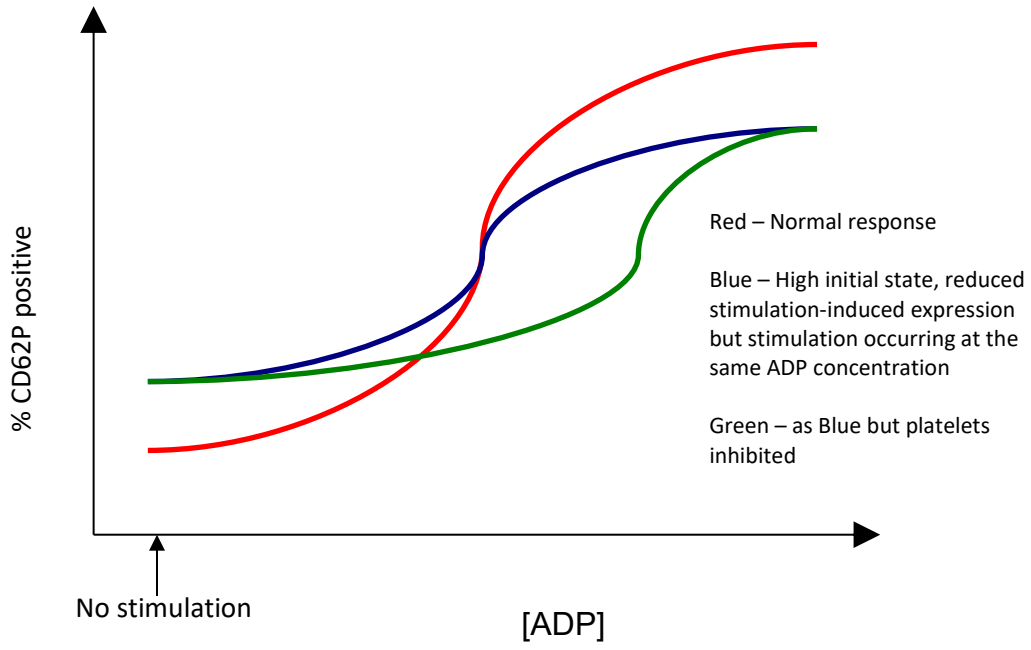


Figure 8.2. Hypothetical dose-response curves for possible post-injury platelet phenotypes.

The assay uses a single marker of platelet activation, CD62P. The use of additional markers of other aspects of activation would be useful. In particular, it would be desirable to establish a marker of GPIIa/IIIb conformational change equivalent to PAC-

1 (see subsection 5.2.2). In parallel with the continued use of flow cytometry, it would also be desirable to use other means of detecting disparate aspects of platelet function, such as platelet-inhibited thromboelastography and functional analysers such as aggregometers.

The preparation of the animal subject appears to have an effect on platelet and coagulation function. This should be minimised. At present, the splenectomy and suprapubic catheterisation are conducted via a full length laparotomy. All could be achieved by laparoscopy (Morrison *et al.*, 2014), which is known to cause less inflammatory activation (Jacobi *et al.*, 1998; Pascual *et al.*, 2011). Topical adrenaline, a platelet agonist, is applied to the spleen before removal (to make it contract, with the intention of reducing variation in haemoglobin concentration between subjects); this may promote platelet activation. An assessment should be made as to whether removing this step would adversely affect the model in other ways. Vascular access, particularly in the neck, is achieved by surgical cut down. Porcine anatomy means that this is an extensive procedure. Minimal access techniques are possible (Fudge, Coleman and Parker, 2002), could be enhanced with ultrasound guidance and would reduce the amount of tissue trauma. While possibly desirable, it is not practical to house animals at the experimental location on the range at Porton Down, so remote preparation and transportation will continue. Despite these possible improvements, it may be that pigs are not a suitable model for studying this aspect of trauma-induced coagulopathy and that alternative animal models are required.

8.2 Human Studies

The results of the study in Chapter 5 demonstrate some of the difficulties that attend research involving trauma patients and of retrospective studies. It would have been more appropriate for a prospective study of the impact of PHBTx to have been conducted. In particular, this could have improved the ascertainment of pre-hospital physiology data, by allowing feedback from data collectors to clinicians in real time. This would not have altered the confounding changes in hospital resuscitation practice but would have allowed an assessment of the effect of PHBTx on physiology between initial medical care and hospitalisation. The implication for future major innovations in

care is clear. Where possible, high level evidence (i.e. randomised trials) should be established to support changes. Where this is impractical, high quality, prospective observational evaluations should be instituted.[*]

More narrowly, the investigation of the platelet contribution to coagulopathy requires human observational and, in due course, interventional studies. The high throughput of severely injured patients at the hospital in BASTION provided the Defence Medical Services with an opportunity to contribute to this process. Studies using ROTEM data have already been cited (Doran, Woolley and Midwinter, 2010; Woolley *et al.*, 2013). The Platelet Function in Acute Traumatic Coagulopathy study (PATCH)[†] was initiated to supplement the existing ROTEM work with a flow cytometry-based assay of platelet function. This used a PAC-1 mAb to measure platelet activation using admission blood samples. TRAP-6 was used to produce stimulation dose-response curves. A Cyflow flow cytometer (Partec UK Ltd, Canterbury, UK) was acquired and deployed to Afghanistan where it was used alongside a Multiplate analyser (Roche International Diagnostics, Rotkreuz, Switzerland) by a team of Dstl and military researchers. Analysis is ongoing.

[*] None of this should be taken as criticism of the enormous hard work of the trauma nurse coordinators in Afghanistan, Iraq, the USA and Birmingham who maintain the trauma registries and whose efforts made Chapter 5 possible.

[†] This study will need to be renamed since the previously mentioned Australian study of TXA usage has the same acronym (Mitra *et al.*, 2014).

Appendix. Injury Scoring

In Afghanistan, the UK Joint Theatre Trauma Register (JTTR) and the US JTTR, now known as the Department of Defence Trauma Register (DoDTR) record injury, treatment and outcome data on a) all injured military personnel of the respective nations, wherever they were treated and b) all injured patients treated in medical facilities staffed by the military medical services of the respective nations, regardless of the nationality of the casualty. Since the hospital at Camp BASTION was staffed by both countries for much of the period covered by the study in Chapter 4, patients were entered into both systems. Local nationals were soon lost to follow up but UK and US casualties were followed throughout their care to, where appropriate, discharge in the home nation. Consequently, the databases contain more accurate injury and follow up data about servicemen of their own nationality.

In both cases, injuries are recoded using the abbreviated injury score (AIS) of the American Association for the Advancement of Automotive Medicine. This is a codification of all injuries which assigns each a 7 digit code. For example a femoral shaft fracture is coded as 851814.3. "8" represents the body region (lower limb), "518" represents the anatomical structure ("5"-skeletal, "18"-femur), "14" the particular injury, in this case its level (shaft) and "3" is a measure of the severity and appears after the dot. There are 9 body regions in AIS.

Severity is coded from 1 to 6, where 6 is unsurvivable. This is an ordinal not an interval scale; i.e. AIS 5 injuries are supposed to be all roughly equivalent and to be worse than AIS 4 injuries but the difference between AIS 5 and AIS 4 is not the same as between AIS 4 and AIS 3. Severity in AIS codes is similar to that found in the injury grade system of the American Association for the Surgery of Trauma.

The AIS code book has been repeatedly updated since its introduction in 1969. The 2005 edition contained a separate military AIS coding to account for difference between military and civilian trauma (Champion *et al.*, 2010). This version was used in the work presented in Chapter n.

AIS codes are used to calculate the Injury Severity Score (ISS). AIS codes are regrouped into 6 body regions and the highest severity in the three most severely injured regions are used. The three numbers are squared and summed. Any AIS 6 leads to an ISS of 75, which is the limit of the scale. ISS is open to criticism on several counts (Lefering, 2002). First, it ignores severe injuries coexisting in the same body region; the New ISS (NISS) ignores the body region to use the three most severe codes in order to address this. Second, it takes data from an ordinal scale, transforms them and purports to produce an interval scale. Third, it conflates injury patterns, which both loses information about particular injuries and makes very different injuries (e.g. severe head injury and proximal limb amputation) essentially interchangeable (Russell *et al.*, 2004). Despite these concerns, use of the ISS is almost universal in trauma research

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