

Perioperative hypercoagulability

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I, Craig Anthony Lyness, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Abstract

Perioperative hypercoagulability may manifest as thromboembolic complications, such as myocardial infarction, deep vein thrombosis and pulmonary thromboembolism, which are critical pathological events that increase postoperative morbidity. Reducing the incidence of both venous and arterial thromboses are key goals of improving perioperative outcomes.

A large observational cohort study comprehensively characterised the alterations in coagulation status following major intra-abdominal surgery. Results confirmed that postoperative hypercoagulability is seen up to at least postoperative day 5. This prothrombotic phenotype was diagnosed by pre-defined thromboelastography criteria and not by conventional coagulation tests. Goal directed fluid therapy in the immediate perioperative phase was associated with excessive fluid administration but no adverse effect in terms of haemodilution-induced TEG-defined hypercoagulability. However, patients receiving goal directed therapy showed evidence of a higher degree of glycocalyx damage.

Inflammation-induced hypercoagulability and scope for potential modification by putative anti-thrombotic agents were further explored using an *ex vivo* experimental model in healthy subjects and preoperative surgical patients. Statin and steroid therapy attenuated, while anti-oxidant N-acetylcysteine exacerbated, the prothrombotic state in the presence of inflammation. The concept of cholinergic autonomic modulation of coagulation was supported by suppression of the hypercoagulable response by nicotine in the same model.

In summary, perioperative hypercoagulability is common in the elective major surgical population and is associated with adverse outcome. Data from this thesis confirm a patient phenotype at increased risk of thromboembolic complications. Thrombotic risk may be modified by existing perioperative therapies which require further mechanistic evaluation.

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Abbreviations

ACh	Acetylcholine
APTT	Activated partial thromboplastin time
CRP	C-reactive protein
DVT	Deep vein thrombosis
EPCR	Endothelial cell protein C receptor
GDT	Goal directed therapy
IL-	Interleukin
INR	International Normalised Ratio
LPS	Lipopolysaccharide
MA	Maximum amplitude
MI	Myocardial infarction
NAC	N-acetylcysteine
nAChR	Nicotinic acetylcholine receptor
PAI	Plasminogen activator inhibitor
POD	Postoperative day
PT	Prothrombin time
PTE	Pulmonary thromboembolism
ROTEM	Rotational thromboelastometry
TAT	Thrombin-antithrombin complex
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGTs	Thrombin generation tests
TLR	Toll-like receptor
TNF	Tumour necrosis factor
vWF	Von Willebrand factor

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Chapter 1

Introduction

1.1 Introduction

Thromboembolic complications are the leading cause of preventable postoperative morbidity and mortality. Of the 312 million surgical procedures performed worldwide each year (Weiser et al., 2016), an estimated 4% are complicated by arterial thrombosis and a further 4% by venous thrombosis (Donze et al., 2014). Postoperative myocardial infarction (MI), ischaemic stroke, deep vein thrombosis (DVT) and pulmonary thromboembolism (PTE) constitute a substantial healthcare burden, despite routine thromboprophylaxis and improvements in perioperative care. Reducing the incidence of both arterial and venous thrombotic events are key goals in improving perioperative outcomes and reducing healthcare expenditure.

Understanding normal haemostasis, the pathogenesis of both arterial and venous thrombosis, the bidirectional relationship between inflammation and coagulation and the precise role of surgical trauma in producing hypercoagulability is key to fully characterise the postoperative pro-thrombotic phenotype and thus explore therapeutic targets to reduce thromboembolic complication rates.

1.2 The normal haemostatic system

Normal haemostasis comprises of primary and secondary haemostasis, two equally important processes that occur concurrently at the site of vascular injury.

Primary haemostasis consists of platelet adhesion, activation and aggregation, which in turn lead to the formation of a platelet plug. Following platelet adhesion to the site of vascular injury, platelet activation is promoted by certain agonists, including thromboxane A₂, thrombin and collagen, stimulating the release of alpha and dense granule contents which promote

further platelet recruitment and then activation and aggregation. Subsequently platelets are linked by fibrinogen through surface glycoprotein IIa/IIIb receptors to form a platelet plug.

Secondary haemostasis consists of a series of consequential reactions, ultimately resulting in the production of thrombin and covalently cross-linked fibrin. This coagulation cascade is triggered in response to vascular injury by the exposure of tissue factor at the site of injury. Tissue factor is expressed on subendothelial fibroblasts, injured vascular endothelium and activated monocytes.

In the initiation phase, tissue factor complexes with circulating activated factor VII (VIIa) to form the extrinsic tenase complex (TF:VIIa), which is responsible for the cleavage and subsequent activation of factors IX and X. Activated factor X (Xa) activates only a small amount of prothrombin to form thrombin because its cofactor, factor Va, is not yet available. This small amount of thrombin, rather than generating fibrin, activates cofactors V and VIII to stimulate further platelet activation. The extrinsic tenase complex is rapidly inactivated by the formation of a complex with factor Xa and tissue factor pathway inhibitor (TFPI). This rapid inactivation means that continued coagulation is dependent on ongoing generation of factor Xa via factors VIIIa and IXa (Figure 1.1).

An amplification phase follows whereby the formation of two enzyme complexes is responsible for the massive amplification in thrombin generation and results in rapid and extensive clot formation. Factors IXa and VIIIa, which have formed during the initiation phase, bind via calcium ions to exposed platelet phospholipid on the surface of the haemostatic plug. This binding creates the intrinsic tenase complex which then prompts the rapid generation of factor Xa. The rapid localised increase in factor Xa concentration leads to the formation of the prothrombinase complex, consisting of factor Xa, calcium ions, platelet phospholipid and factor Va generated from initiation. The

prothrombinase complex cleaves prothrombin to form thrombin. Meanwhile factors Va and VIIIa act as accelerators, promoting the production of both factors Xa and thrombin.

Thrombin then stimulates cleavage of fibrinogen to fibrin. Moreover, thrombin directly activates factors V, VIII and IX. Therefore, thrombin generation allows production of more accelerating cofactors, Va and VIIIa, resulting in a further large scale increase in thrombin production. Fibrin monomers spontaneously form hydrophobic and electrostatic bonds with other fibrin monomers allowing formation of linear fibrin polymers to produce unstable fibrin clot. The stabilisation of the fibrin clot occurs following formation of crosslinks between adjacent linear fibrin molecules, conducted by the transglutaminase, factor XIIIa, produced by the action of thrombin on factor XIII. (Figure 1.2)

Physiological inhibitors of blood coagulation exist to limit coagulation to the site of injury. Tissue factor pathway inhibitor (TFPI) is synthesized by endothelial cells and binds to factor Xa and the TF:VIIa complex thereby limiting their activity. Antithrombin III is a glycoprotein, synthesized by the liver and endothelium, that forms a complex with thrombin and other serine proteases causing inactivation. Complex formation is substantially promoted by the action of heparin. Protein C is a vitamin K dependent protein that both inhibits coagulation and stimulates fibrinolysis. Protein C is activated by thrombin in the presence of the cofactor thrombomodulin. Activated protein C inhibits coagulation by inactivating factors VIIIa and Va, thereby reducing the rate of thrombin generation. Protein S is another vitamin K dependent protein, synthesized in the liver and endothelium, which complexes with activated protein C and calcium ions on platelets and on the endothelial surface, to further amplify the inhibitory activity of activated protein C. Thrombomodulin is present in the vascular endothelium and forms complexes with thrombin. This complexed thrombin activates protein C much faster than free thrombin but does not cleave fibrinogen, activate factors V and VIII or aggregate platelets. Protein C activation by the thrombomodulin-thrombin

complex occurs more efficiently when protein C is bound to endothelial cell protein C receptor (EPCR).

The fibrinolytic system is responsible for the degradation and dissolution of formed fibrin clot within the circulation. This is mainly achieved by the rapid and localized formation of the proteolytic enzyme, plasmin, formed from the activation of plasminogen at the site of clot formation. Plasminogen activators can be subclassified as tissue-type (t-PA), synthesized and stored in vascular endothelium, or urokinase-type (u-PA), synthesized in the kidney. Both convert plasminogen to plasmin, with the presence of fibrin accelerating the process for t-PA but not u-PA.

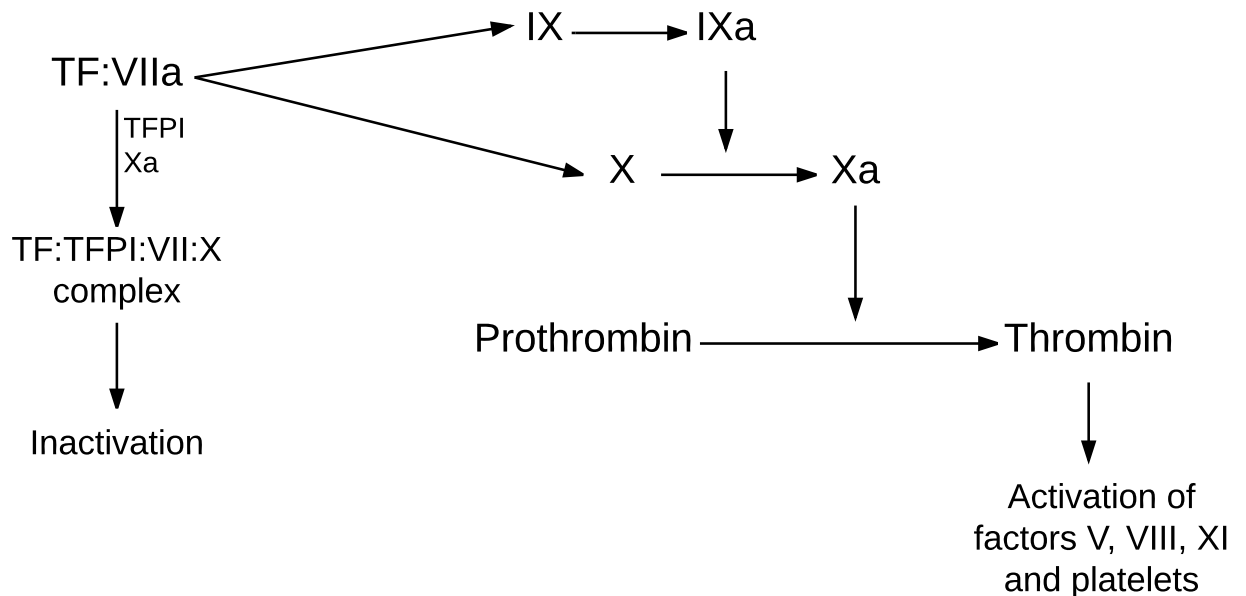


Figure 1.1 Normal haemostasis: Initiation of coagulation

Coagulation is initiated when tissue factor (TF) is exposed to blood. Circulating factor VII binds to tissue factor exposed at site of injury. Tissue factor is expressed on subendothelial fibroblasts, injured vascular endothelium and activated monocytes. The tissue factor bound factor VIIa converts either factor IX or X to their respective enzyme forms. The resulting factor Xa activates a small amount of prothrombin to form thrombin in insufficient amounts to generate significant amounts of fibrin. However initial thrombin production is sufficient to cause localized platelet aggregation and activation of critical cofactors factors V and VIII. Tissue factor bound factor VIIa (extrinsic tenase complex) is rapidly inactivated by the formation of complex with factor Xa and tissue factor pathway inhibitor (TFPI).

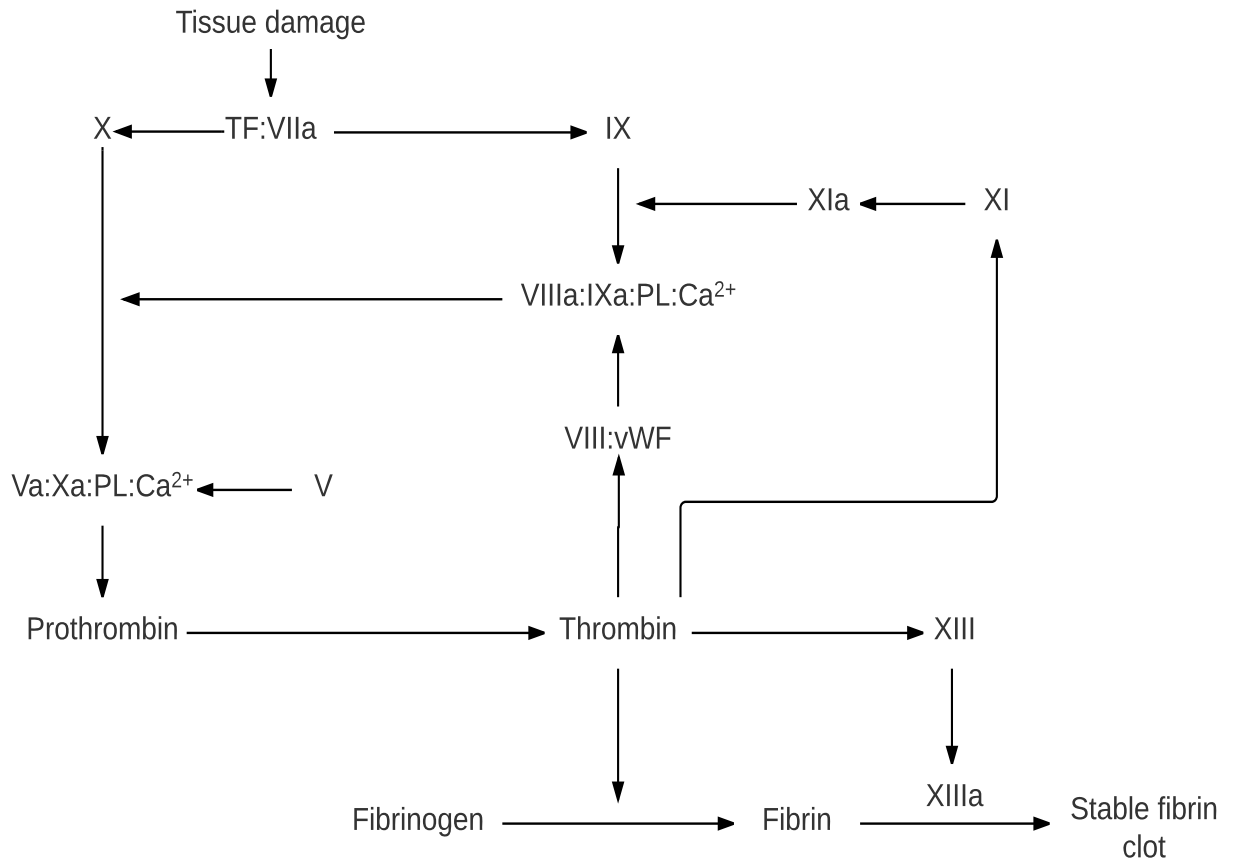


Figure 1.2 Normal haemostasis: Amplification of coagulation

The small amount of thrombin generated during the initiation phase activates platelets and converts factors V to Va, VIII to VIIIa, and XI to XIa. Factor IXa, produced in the initiation phase, forms the intrinsic tenase complex with activated cofactor factor VIIIa on the activated platelet surface, in the presence of calcium. The intrinsic tenase complex (VIIIa:IXa:PL:Ca²⁺) amplifies the activation of factor X. Factor Xa, with its activated cofactor, factor Va, forms the prothrombinase complex on the platelet surface. The intrinsic tenase complex (VIIIa:IXa) and the prothrombinase complex (Va:Xa), co-localised on the platelet membrane in the presence of calcium, create a positive feedback loop, the result of which is the rapid generation of thrombin.

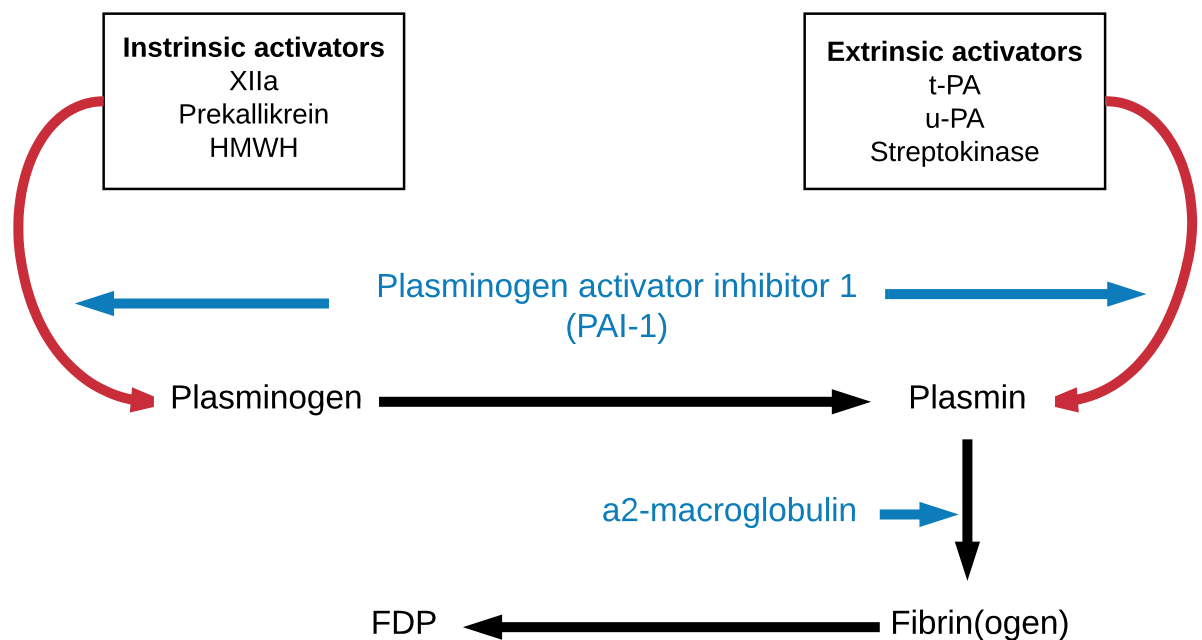


Figure 1.3 Normal haemostasis: Fibrinolytic system

The blood fibrinolytic system comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme, plasmin. Plasmin degrades fibrin into soluble fibrin degradation products (FDPs) by plasminogen activators (PA) including tissue-type PA (t-PA) and urokinase-type PA (u-PA). t-PA mediated plasminogen activation is mainly involved in the dissolution of fibrin in the circulation. u-PA binds to a specific cellular receptor (u-PAR) resulting in enhanced activation of cell bound plasminogen. Inhibition of the fibrinolytic system may occur either at the level of the PA by plasminogen activator inhibitor (PAI)-1 or at the level of plasmin by α 2-macroglobulin.

1.3 Bidirectional cross-talk between inflammation and coagulation

To elucidate the pathogenesis of postoperative hypercoagulability it is crucial to understand the bidirectional relationship between the inflammatory and coagulation processes, in the context of surgical trauma.

The postoperative phase constitutes a pro-inflammatory shift in the haemostatic balance in favour of activation of coagulation. Inflammatory mediators elevate platelet count and reactivity, downregulate natural anticoagulant mechanisms, initiate the coagulation cascade, facilitate propagation of the coagulation response and impair fibrinolysis.

In experimental models of inflammation, levels of coagulation activation, as measured with thrombin-antithrombin complex (TAT), corresponded with interleukin (IL)-8 levels, with the effect attenuated by tissue factor pathway inhibitor (Johnson et al. 1996). Inflammation leads to the induction of TF expression on leucocytes, especially monocytes, as demonstrated experimentally by endotoxin and tumour necrosis factor [TNF α] (Parry and Mackman, 1998). Fibrinogen is an acute phase reactant, elevated during inflammatory processes, with elevated levels associated with increased incidence of thrombotic events (Hantgan et al. 2001). C-reactive protein (CRP), similarly raised in inflammation, has been shown to facilitate monocyte endothelial cell interactions, promoting both plasminogen activator inhibitor-1 (PAI-1) and TF production (Devaraj et al. 2003), with elevated CRP levels correlating with an increased risk of myocardial infarction (Ballantyne et al., 2004). Platelet production is increased by pro-inflammatory mediators such as chemokine interleukin (IL)-6, with newly formed platelets activated at lower concentrations of thrombin, thus being more thrombogenic (Burstein, 1997). Platelet responsiveness is indirectly increased by the inflammatory mediators IL-6, IL-8, histamine and TNF α , via release of ultra-large von Willebrand factor (vWF) multimers from the endothelium (Bernardo et al., 2004).

In terms of natural anticoagulant pathways, antithrombin inhibitory activity is markedly reduced in acute inflammation (Opal, 2000). Similarly concentrations of vascular heparin-like molecules are reduced by inflammatory cytokines and neutrophil activation products (Klein et al., 1992). The protein C pathway is very sensitive to downregulation by inflammation. Endotoxin, IL-1 β and TNF α all reduce thrombomodulin and EPCR levels, and thus ability to generate activated protein C, in experimental models (Fukudome and Esmon, 1994). Thrombomodulin, EPCR and activated protein C levels are similarly reduced in patients with sepsis (Faust et al., 2001). Furthermore, thrombomodulin and EPCR expression is reduced on endothelium overlying atherosclerotic plaque, likely secondary to inflammatory cytokines generated within the lesion (Laszik et al., 2001).

Meanwhile the coagulation system can amplify the inflammatory response via numerous mechanisms. Activated platelets release high concentrations of the pro-inflammatory CD40 ligand, which induces TF synthesis and is associated with increased pro-inflammatory mediators, such as chemokine IL-8 (Henn et al. 1998). The extrinsic tenase complex (TF:VIIa) can activate protease activated receptors (PARs) on endothelium, especially in the presence of factor X, resulting in the expression of adhesion molecules that facilitate leucocyte-mediated injury (Isermann et al., 2001).

Conversely, antithrombin decreases both TF and IL-6 expression in monocytes and endothelium (Souter et al., 2001). Thrombomodulin has direct anti-inflammatory activity via its lectin like N-terminal, which is not involved in protein C activation. In mutant gene mice lacking this lectin domain, leucocyte recruitment is significantly greater following endotoxin challenge (Pixley et al., 1993). Activated protein C inhibits NF- κ B signalling in monocytes conferring anti-inflammatory activity (Yuksel et al., 2002).

The fibrinolytic system has multiple effects on inflammatory processes.

Fibrinogen and fibrin increase production of pro-inflammatory cytokines, including $\text{TNF}\alpha$, $\text{IL1}\beta$, and chemokines. In fibrinogen deficient mice, models of inflammation produce reduced macrophage adhesion and less cytokine production (Szaba and Smiley, 2002). Fibrinolysis inhibitors play a role in modulating cytokine production, with plasminogen activation inhibitor-1 (PAI-1) inhibiting endotoxin-induced $\text{TNF}\alpha$ production by mononuclear cells (Robson et al. 1990).

1.3.1 The integral role of the autonomic nervous system

Given the bidirectional relationship between inflammation and coagulation, the impact of the sympathetic nervous system on this interaction is increasingly recognised. Following physiological stress such as surgical trauma, but also with pain, anaesthesia and prolonged fasting, catecholamines (epinephrine and norepinephrine) levels are increased. It is now well established that catecholamines regulate immune and inflammatory responses such that macrophages and neutrophils, when stimulated, can generate and release catecholamines *de novo*, which regulate mediator release from these phagocytes via interaction with adrenergic receptors (Flierl et al. 2007). Furthermore, literature suggests a dose-dependent stimulation of factor VIII activity, vWF antigen, tissue type plasminogen activator and platelets following infusion of epinephrine (Von Ka È Nel and Dimsdale 2009).

1.4 Shared biological mechanisms in arterial and venous thrombosis

Traditionally arterial and venous thromboses have been considered as separate entities with distinct pathogeneses. However, with more epidemiological data there is increasing evidence of shared mechanistic properties, largely through inflammatory pathways. Reports on the incidence of venous thromboembolic events with subsequent arterial thrombosis show a consistent association (Prandoni et al., 2003; Sørensen et al., 2007), with the relative risk for MI following a DVT or PE being 1.60 and 2.60 respectively, with the risk highest in the first year diagnosis of venous thromboembolism (Sørensen et al., 2007). Moreover, of the agents specifically targeted to prevention of arterial thrombotic disease, statins show promise in reducing the occurrence of venous thrombotic risk (Ageno et al., 2008).

This association has previously been attributed to common risk factors. Increasing age, concomitant cancer, immobility, obesity, smoking, diabetes and metabolic syndrome all increase the likelihood of developing both venous and arterial thrombotic events (Agnelli, 2004). Within these risk factors, inflammation, elevated levels of coagulation factors and inherited thrombophilia are characteristics of both disorders.

Arterial thrombosis mainly occurs at the site of a ruptured, lipid-rich atherosclerotic plaque, as the usual precipitating event in the transition from stable or subclinical atherosclerotic disease to myocardial infarction (MI), stroke or peripheral arterial occlusion. Pathologic studies of coronary arteries in acute MI suggest that the acute thrombosis likely involves activation of both platelets and the coagulation system. Following atherosclerotic plaque rupture, platelets initially adhere to exposed subendothelial vWF and collagen. Platelet adhesion to the vascular subendothelium induces a series of intracellular signalling events that result in platelet activation. The binding of fibrinogen and vWF to the conformationally active form of the glycoprotein

IIb/IIIa receptor results in platelet aggregation, and further propagation of the platelet thrombus. Plaque rupture also results in exposure of subendothelial TF, which initiates the coagulation cascade and leads to the thrombin generation and fibrin clot formation. While arterial thrombi are traditionally considered to be composed predominantly of platelets, accumulating evidence suggests that certain arterial thrombotic disorders are associated with greater activation of the coagulation system, which results in occlusive thrombi that are relatively rich in fibrin (Chan et al. 2008).

Venous thrombosis is more closely associated with stasis and hypercoagulability than changes in vessel wall integrity. Precisely how thrombosis commences is poorly understood but impaired venous blood flow, with valvular sinus stasis and an associated hypoxic hypercoagulable microenvironment, is likely. Venous thrombi have a characteristic laminar structure, rich in fibrin and red blood cells and pervaded by large numbers of leukocytes (Wakefield et al., 1995). Subsequent upregulation of procoagulant activity including TF on endothelium initiates the coagulation response. In a mouse model of DVT, the rapid accumulation of neutrophils and monocytes were demonstrated as indispensable for DVT development with subsequent leucocyte-derived, TF-mediated activation of coagulation (von Brühl et al., 2012).

Although the initial phases of thrombosis are distinct, the pro-inflammatory milieu that propagates each response is shared. A persistent pro-inflammatory response has long been recognised as a major determinant for MI and stroke as well as DVT and PTE. In a large observational cohort of over 2.3 million surgical procedures, preoperative sepsis was an important independent risk factor for both arterial and venous thrombosis, with about 3 times the risk of each complication compared to patients without evidence of preoperative sepsis (Donze et al., 2014). This supports the hypothesis that inflammation contributes significantly to both processes through endothelial injury, platelet aggregation and a procoagulant, anti-fibrinolytic state.

1.5 Pathogenesis of surgery-associated hypercoagulability

In considering perioperative hypercoagulability, it is necessary to examine the effect of both surgical and patient factors.

1.5.1 Surgical trauma

Surgical trauma is associated with tissue injury itself but also additive effects of anaesthesia, pain, hypothermia, bleeding and fasting which are all associated with physiological stress. These factors combine to initiate inflammatory, hypercoagulable, stress and hypoxic states.

The inflammatory state involves increases in $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 and CRP which, as previously discussed, may have a direct role in plaque fissuring prior to arterial thrombosis as well as venous thromboembolism. The hypercoagulable state involves increased platelet number and reactivity, elevated prothrombotic markers such as factor VIII and vWF, accompanied by decreases in coagulation inhibitors, AT and protein C (Bezeaud et al., 2007). Enhanced thrombin generation with increased thrombin-antithrombin (TAT) concentrations is seen following tissue injury (Schreiber et al. 2005) with raised D-dimer concentrations also indicative of recent thrombin burst in surgical patients (Yoo et al., 2009). Hyperfibrinogenaemia is consistently demonstrated following surgery conferring increased risk of thrombosis even with normal markers of thrombin generation. Lison et al. examined a heterogeneous group undergoing major surgery and identified longitudinal changes in procoagulant factors. Factor VIII, vWF and fibrinogen were consistently elevated compared to baseline from postoperative days 2 to 6. The majority of other clotting factors including factors II, VII, X, XI and XII showed a significant decrease from baseline at postoperative day 1 with all except factor VII remaining reduced until postoperative day 3 (Lison et al., 2011).

1.5.2 Surgical subtype

The severity of the surgical trauma and indeed the site of surgery produces a variable degree of hypercoagulability and pro-inflammatory response. In a study comparing open and laparoscopic surgery, both produced elevations in prothrombin fragment F_{1+2} , TAT and fibrinogen, with concordant decreases in AT, protein C and plasminogen but the changes were significantly more marked in the open surgery group. The pro-inflammatory cytokine response, with increased IL-6 and IL-1 β , was similarly more exaggerated following open surgery up to postoperative day 6 (Schietroma et al. 2004).

Different surgical cohorts have demonstrated similar patterns of postoperative hypercoagulability, such as major abdominal surgery including liver resection and pancreatic surgery (Cerutti et al., 2004; De Pietri et al., 2010), joint replacement and major orthopaedic surgery including trauma (Selby et al., 2009), neurosurgery (Goobie et al. 2001), aortic surgery (Gibbs et al., 1992), pelvic cancer surgery (Benyo et al., 2012) and surgery requiring cardiopulmonary bypass (Lison et al., 2011). Without thromboembolic prophylaxis, the incidence of DVT has been shown to be 14% following gynaecological surgery, 22% following neurosurgery, 26% following abdominal surgery and between 44 and 60% following orthopaedic surgery (Bombeli et al. 2004). Although this is likely influenced by the degree of immobilisation it may also reflect differential activation of coagulation related to the operative location. The susceptibility of neurosurgical patients to postoperative thrombosis, for example, may be partly due to parenchymal manipulation of highly TF enriched brain tissue (Abrahams et al., 2002).

Although each cohort is invariably exposed to a determined surgical insult, there are numerous additional perioperative factors which differ in each subgroup including mode of anaesthesia, volume of fluid administration, exposure to tumour in cancer surgery, introduction of biomaterials, relative circulatory stasis following immobilisation and the use of cardiopulmonary bypass (Rafiq et al., 2012).

1.5.3 Mode of anaesthesia

Neuroaxial anaesthesia has been shown to have favourable outcome in terms of thromboembolic events compared to general anaesthesia. Epidural analgesia is associated with lower PAI-1 levels and enhanced release of plasminogen activators, thus diminishing the procoagulant response. This may be mediated by off-target anti-inflammatory properties of local anaesthetics, such as lidocaine, as shown in animal models (Beloeil et al., 2005).

1.5.4 Haemodilution

Intravenous fluid administration is required to a variable degree perioperatively, depending on surgical subtype and the extent of blood loss, fluid shifts and impaired vasomotor tone. With larger intravascular volume resuscitation comes haemodilution, which has differential effects on coagulation depending on the type of fluid administered. Numerous *in vitro* and *in vivo* studies have demonstrated induced hypercoagulability due to haemodilution up to 30%, with crystalloid solutions such as isotonic saline and Ringers lactate (Roche et al. 2006; Ruttmann et al. 1996). The reverse effect has been shown at higher crystalloid dilutions of more than 40%, which are arguably less clinically relevant (Coats et al. 2006). The underlying mechanism is poorly understood but may represent a disproportionate dilution of natural anticoagulants, such as AT, or alternatively an increase in thrombin generation, as suggested by unchanged TAT concentrations despite haemodilution seen *in vivo* (Ng et al., 2002).

The effect of colloid administration is more equivocal and remains controversial. All colloids induce a specific decrease in vWF and factor VIII:c. (De Jonge and Levi 2001). The consensus from multiple *in vivo* studies is that gelatin-induced haemodilution confers a net anticoagulant effect (Evans et al., 1998). The interaction between the negatively charged colloid and the platelet membrane, resulting in impaired platelet adhesion and aggregation is

a plausible mechanism. Similarly, in vitro haemodilution with hydroxyethyl starch solutions produces a hypocoagulable effect, particularly solutions comprised of higher molecular weight starches (Petroianu et al., 2000).

1.5.5 Cancer

The association between cancer and hypercoagulability is well established and first reported in 1865 by Trousseau. Cancer is a major risk factor for the development of thromboembolic disease, increasing risk by 6 to 10-fold (Piccioli et al., 1996), with 20% new diagnoses of thrombosis associated with cancer (Thorson et al., 2014).

The pathogenesis is multifactorial and can be subdivided into tumour-specific and iatrogenic factors. Many tumour cells constitutively express TF, with exposure of TF-rich tumour cell surface or TF-microparticles (MPs) initiating coagulation activation by forming a complex with factor VII. Numerous studies have demonstrated high levels of circulating tumour-derived TF-MPs in patients with active cancer (Geddings and Mackman, 2013). TF expression increases with histologic grade in different cancer types, including pancreatic cancer, and a correlation between the level of TF and venous thromboembolic events has been demonstrated in pancreatic and brain tumours. Cysteine proteases secreted by carcinoma cells can directly activate factor X to produce thrombin. Tumour-derived inflammatory cytokines also serve to activate endothelial and platelet adhesion cells. In examining coagulation changes in thoracoabdominal malignancy, van Haren et al. demonstrated elevated markers of thrombin generation (prothrombin fragment F_{1+2}), endothelial activation (factor VIII) and fibrinolysis (D-dimer) postoperatively, supported by hypercoagulable changes in rotational thromboelastometry (Van Haren et al., 2014). Subjects became hypercoagulable immediately following surgery and did not return to normocoagulable parameters until 9 months after cancer resection, with around 30% deemed hypercoagulable even prior to surgery.

1.5.6 Age

Increasing age is associated with an exponential increase in risk of both arterial and venous thrombotic events (Lowe, 2008). There is a relative pro-inflammatory, hypercoagulable state which may be exacerbated by increased likelihood of prolonged immobilisation and concurrent infections. A persistent pro-thrombotic state is demonstrated by elevated D-dimer and prothrombin fragment F_{1+2} (Lowe et al., 1997), with increased IL-6 and CRP levels, in elderly cohorts, conferring a higher risk of arterial and venous thrombosis (Rosendaal et al. 2007).

1.6 Identification of the hypercoagulable state

The detection of hypercoagulability is difficult because the coagulation mechanism is complex and thus far coagulation tests have not been well characterised for their capacity to detect hypercoagulability.

Standard laboratory tests for coagulation include prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen assay. Each provides assessment of different components of the traditionally described *in vitro* coagulation cascade. PT measures the function of coagulation factors in the extrinsic pathway, APTT measures the intrinsic pathway, TT measures the conversion of fibrinogen to fibrin by thrombin and the fibrinogen assay quantifies the plasma concentration of fibrinogen. These haematological parameters provide a well-established screening tool for *in vivo*, with alterations requiring specific factor assays to identify underlying abnormalities. Despite being widely used to diagnose bleeding diatheses, these routine investigations are unable to reliably detect hypercoagulability.

Fibrinogen is an acute phase reactant and although associated with a pro-thrombotic state is commonly raised in inflammatory states so is non-specific in determining hypercoagulability. D-dimer is commonly used as an adjunct to imaging studies to exclude the presence of DVT or PTE (Aguilar et al., 2002). When a thrombus is degraded by thrombin, D-dimers and other fibrin split products are formed from cross-linked chains of fibrin clot. However, D-dimer is similarly non-specific, with elevated D-dimer levels commonly seen in cancer, sepsis and excessive bleeding.

The identification of more specific biomarkers of hypercoagulability is crucial to better assess thromboembolic risk such that these biomarkers have incremental predictive value for demonstration of the high risk or 'prethrombotic' phenotype. Such biomarkers reflect different functional haemostasis activity groups.

Vascular injury, inflammation and endothelial activation are commonly

assessed by measurement of coagulation factor VIII, vWF, soluble TF, microparticles, thrombomodulin, tissue-type plasminogen activator (t-PA) and soluble P-selectin. vWF is an adhesive protein produced by endothelial cells and plays a crucial role in platelet adhesion to the subendothelial matrix of vessels and in plasma factor VIII stability. Increases in factor VIII:C and vWF have been shown to be independent risk factors for arterial thrombosis (Folsom et al., 1997). Microparticles are formed by outward blebbing of plasma membranes of endothelial cells and leucocytes, subsequent release as small phospholipid vesicles and selective packaging of surface proteins including tissue factor and adhesion molecules. TF-MPs are procoagulant not only due to the presence of TF but also the exposure of negatively charged phospholipids, such as phosphatidylserine, facilitates assembly of positively charged coagulation protein complexes (Figure 1.4). Functional TF-MP assays have been shown to correlate with venous thromboembolism in cancer patients (Geddings and Mackman, 2013). Thrombomodulin and EPCRs are abundant on the arterial endothelial wall and thus are elevated following vascular injury associated with initiation of coagulation.

Thrombin generation can be assessed by biomarkers such as prothrombin F₁₊₂ and thrombin-antithrombin (TAT) complexes, both commonly employed in assessing postoperative changes in coagulation (Bezeaud et al., 2007; Van Haren et al., 2014). Individual clotting factors can be easily measured with activity assessed using clot-based reactions employing modifications of the APTT and PT. Natural anticoagulants such as protein C, protein S and antithrombin (AT) also have readily available commercial assays. Widely used biomarkers for fibrinolysis include D-dimer and plasminogen activation inhibitor (PAI)-1, both routinely used in studies examining coagulation changes perioperatively.

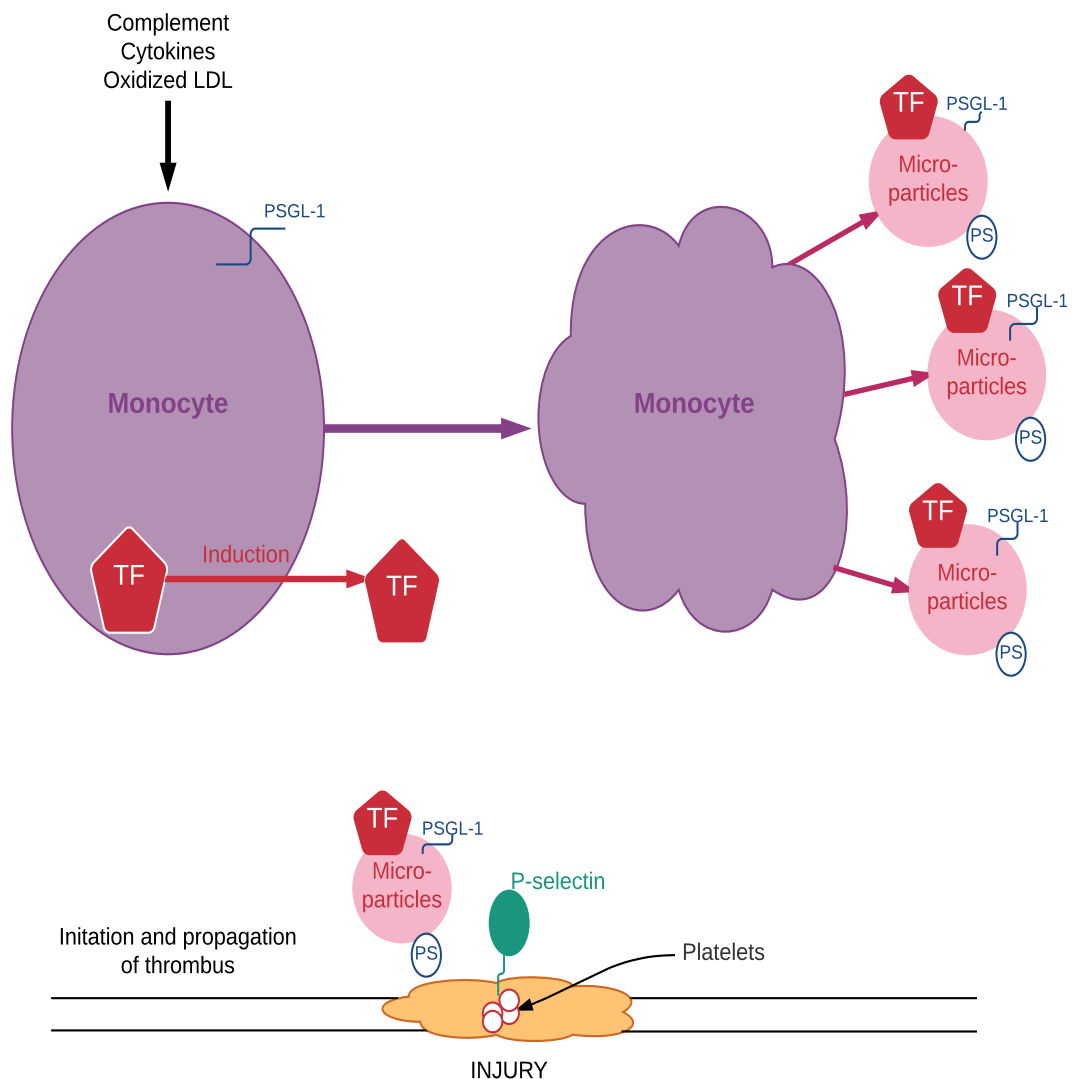


Figure 1.4 Leucocyte-mediated tissue factor release

When monocytes are stimulated by cytokines or endotoxin, it triggers tissue factor synthesis, shape change and release of microparticles, containing adhesion molecule, P-selectin glycoprotein-1 (PSGL-1). Tissue factor rich microparticles bind to activated platelets through the counter receptor P-selectin thereby concentrating tissue factor on the site of injury. Microparticles also have negatively charged surface phosphatidylserine (PS) allowing tissue factor to concentrate at the injury site and express potent coagulant activity.

Tests of global haemostasis have attracted attention in examining not only hypercoagulability but in the management of major haemorrhage.

Viscoelastic modalities are dynamic tests of whole blood which evaluate the kinetics of clot formation, mechanical properties of clot and time to dissolution of clot (Lancé 2015). Tensile strength of clot provides information about the capacity of clot to achieve haemostasis and kinetics determine the adequacy of quantitative factors available for clot to form. Therefore, whole blood viscoelastic testing provides a composite picture reflecting the interaction of plasma, blood cells and platelets, rather than isolated components, thus more closely reflecting the situation *in vivo*. There are three commercially available point-of-care (POC) viscoelastic devices: thromboelastography (TEG[®]), rotational thromboelastometry (ROTEM[®]) and Sonoclot. All devices measure rate of fibrin formation, clot strength and clot lysis.

Viscoelastic testing has been extensively used in surgical cohorts associated with major blood loss, such as liver transplantation and cardiac surgery, to continuously monitor haemostasis and has been shown to effectively limit unnecessary transfusion of blood components (Whiting et al., 2015; Wikkelsø et al., 2016). However, it is now well established in the clinical setting as a rapid means to detect and quantify hypercoagulability and predict thrombotic risk. Postoperative hypercoagulability by viscoelastic criteria has been demonstrated for TEG (Mahla et al., 2001; De Pietri et al., 2010), ROTEM (Van Haren et al., 2014) and Sonoclot (Pivalizza et al. 1997) Viscoelastic defined hypercoagulability has been shown to consistently correlate with corresponding changes in individual prothrombotic biomarkers (Bezeaud et al., 2007; Lison et al., 2011). Moreover, in patients deemed hypercoagulable by viscoelastic criteria, there is a clear association with likelihood of both arterial and venous thrombotic events, in multiple clinical settings (Kashuk et al., 2009; McCrath et al., 2005; Rafiq et al., 2012)

Thrombin generation tests (TGTs) are an alternative global haemostatic

assessment hitherto largely confined to the research setting. TGTs quantify the continuous and dynamic properties of thrombin generation in the presence of fibrin(ogen) following a given tissue factor stimulus. The classic thrombin generation curve has a waveform from which a series of specified parameters can be calculated (lag time, thrombin generation velocity, peak thrombin concentration, time-to-peak thrombin and endogenous thrombin potential). In comparison to viscoelastic tests, which assess the fibrin clot formation in general, the TGT could give more information on the haemostatic capacity in total because the generation of thrombin does not stop at the moment when the fibrin clot has been generated. TGT can be performed in platelet poor plasma (PPP), platelet rich plasma (PRP) or whole blood. The major drawback of TGTs is the time taken to perform which has precluded more widespread use clinically. However, there are numerous studies to validate the use of TGTs in the context of postoperative hypercoagulability (Lison et al., 2011; Potze et al., 2015).

1.7 The perioperative conundrum in managing postoperative hypercoagulability

Surgical intervention is associated with higher thromboembolic risk such that the indication for thromboprophylaxis should be evaluated in all surgical patients. However thrombotic risk needs to be balanced against bleeding risk on an individualised basis taking into account the likelihood of clinically significant intraoperative or immediate postoperative bleeding versus specific patient and surgical risk factors for thromboembolic complications. This net risk is dynamic with the absolute magnitude and duration not equal for all patients or for a given individual over time.

Pharmacological thromboprophylaxis is the mainstay of treatment, typically with low molecular weight heparin (LMWH), together with early ambulation and mechanical thromboprophylaxis, such as compression stockings and intermittent pneumatic calf compression. Numerous guidelines have been published to provide risk stratification in different cohorts and encourage appropriate thromboprophylaxis proportional to the net risk (Farge et al. 2012; Geerts et al. 2008). Since the widespread introduction of pharmacological thromboprophylactic regimes worldwide, the rates of thromboembolic complications have been reduced by up to 80% in high-risk surgical populations (Agnelli, 2004). However, concern remains over bleeding complications, in the context of both perioperative anticoagulant and antiplatelet administration.

Perioperative administration of aspirin has been shown to prevent thromboembolic complications by inhibiting thrombus formation. Although there is strong evidence that aspirin prevents venous thromboembolism after non-cardiac surgery (PEP Trial Collaborative Group 2000), anticoagulant therapy is more commonly used for the prevention of venous thromboembolism. Nevertheless, approximately one third of patients undergoing non-cardiac surgery receive perioperative aspirin (Devereaux et al., 2005). In the POISE-2 study, administration of aspirin preoperatively and

immediately postoperatively had no significant effect on the rate of a composite of death or nonfatal myocardial infarction but did increase the risk of major bleeding (Devereaux et al., 2014a).

Therefore, in detecting and managing postoperative hypercoagulability, challenges remain in mitigating thrombotic risk without exaggerating perceived bleeding risk. By examining the pathogenesis of the perioperative pro-thrombotic phenotype, there are opportunities to identify potential thrombomodulatory targets outside the traditional anticoagulant or antiplatelet regimes. Numerous commonly used perioperative agents have putative anti-inflammatory or anti-thrombotic off-target actions, including sympatholytics (Giebelen et al., 2008) and statins (Steiner et al., 2005), and thus require further evaluation on the potential to ameliorate hypercoagulability without necessarily modifying bleeding risk.

1.8 Conclusion

Thromboembolic complications are a major determinant of postoperative morbidity and mortality. Hypercoagulability promoted by the pro-inflammatory surgical stimulus is implicated in both arterial and venous thrombosis.

Accurate identification and quantification of perioperative hypercoagulability is vital to determine high risk groups with a 'pre-thrombotic' phenotype.

Viscoelastic testing such as thromboelastography allows more dynamic global assessment of changes in coagulation status seen perioperatively compared to standard haematological investigations. Thromboelastography has been demonstrated to reliably detect postoperative hypercoagulability and predict risk of thromboembolic complications in specific surgical cohorts.

Perioperative pharmacological thromboprophylaxis and aspirin significantly reduces risk of postoperative thromboembolic complications but benefits are offset by concern of bleeding in high risk surgery.

Therefore, further work is essential to better characterise the perioperative hypercoagulable state by employing multimodal assessment of both coagulation and inflammation processes and examining the effect of surgical factors, such as surgical subtype and haemodilution, on coagulation status. To understand the aetiology more comprehensively, it is necessary to further explore the precise effect of inflammatory stimuli on coagulation parameters, and robustly determine the thrombomodulatory potential of different perioperative therapies with previously reported anti-inflammatory or anti-thrombotic properties.

1.9 Hypothesis

The presence of an inflammatory stimulus, both clinically in high risk surgical patients, and *in vitro* with an inflammatory model produces a reproducible hypercoagulable state, as defined by thromboelastographic criteria and corroborated by selected haematological biomarkers

1.10 Aims

1. Comprehensively characterise longitudinal alterations in coagulation parameters following high risk major surgery using standard haematological investigations, thromboelastography and selected coagulation and inflammatory biomarkers
2. Examine the effect of different surgical factors such as surgical subtype and the role of different perioperative fluid regimens on the same perioperative coagulation parameters
3. Determine the effect of a standardised inflammatory stimulus on the coagulation status of both healthy volunteers and preoperative surgical patients, using thromboelastography and a validated *in vitro* model of inflammation.
4. Evaluate the putative thrombomodulatory potential of selected perioperative therapies on the inflammation-induced hypercoagulability produced in the same *in vitro* model of inflammation.

Chapter 2

General Methods

2.1 Clinical Study

The clinical study cohort investigated in this thesis was a subset of surgical patients recruited to the POM-O (Post Operative Morbidity Oxygen) trial. Trial patients in this sub-study were recruited exclusively at the Royal Free Hospital, between May 2011 and October 2012 and prospectively studied (n=50).

2.1.1 POM-O trial

The parent POM-O trial (ISCRTN 76894700; UK NIHR Portfolio CSP: 22346) was a multicentre, double-blind, randomised controlled trial undertaken in four London university hospitals. All subjects presented for major elective intra-abdominal surgery, predominantly hepatobiliary at the Royal Free site, associated with a high incidence of postoperative morbidity. The primary objective of the trial was to determine if enhancing oxygen delivery by goal-directed therapy (GDT) to a pre-determined target, immediately postoperatively, reduced morbidity and length of hospital stay in high-risk surgical patients. The secondary objective was to demonstrate whether there was an acquired loss of parasympathetic autonomic function as an unintentional result of goal directed haemodynamic therapy. However, the POM-O thromboelastography (TEG) sub-study detailed here specifically examined alterations in the perioperative coagulation profile in a smaller group of subjects, in addition to the outcome measures for the parent trial.

Adult patients undergoing major elective surgery expected to last for at least 120 minutes were eligible for recruitment, provided the following high-risk criteria were satisfied: American Society of Anesthesiologists classification of 3 or more; surgical procedures with an estimated or documented risk of postoperative morbidity (as defined by the Postoperative Morbidity Survey)

exceeding 50%; modified Revised Cardiac Risk Score of 3 or more, as defined by age 70 years or more, a history of cardiovascular disease (myocardial infarction, coronary artery disease, cerebrovascular accident, electrocardiographic evidence for established cardiac pathology), cardiac failure, poor exercise capacity (anaerobic threshold <11 mL/kg per min assessed by cardiopulmonary exercise testing or Duke Activity Status Index), renal impairment (serum creatinine $\geq 130\mu\text{mol/L}$), or diabetes mellitus.

Exclusion criteria included refusal of consent, pregnancy, lithium therapy or allergy, recent myocardial ischaemia (within the previous 30 days), acute arrhythmia, acute bleeding, and patients receiving palliative treatment only.

Across the four trial sites, 599 subjects were assessed for eligibility but 395 were excluded due to either patient refusal, type of surgery planned or undertaken not fulfilling inclusion criteria, participation in other research trials, anaesthetist refusal or lack of research staff. Therefore, 204 subjects were enrolled and subsequently randomised to either treatment arm, between May 2010 and February 2014.

The POM-O trial was approved by the South London Research Ethics Committee Office (09/H0805/58). Written informed consent was obtained prior to enrolment to POM-O. Potential participants were screened by local investigators having been identified from surgical and pre-admission clinic lists, and by communication with the relevant nursing, surgical and medical staff. There were no financial payments offered to patients. Non-English speakers were provided with full access to NHS translation services to ensure appropriate understanding of the trial and consent process. Patients who lacked capacity to give or withhold informed consent were not recruited.

Anaesthesia was undertaken using a protocolised regimen for all enrolled patients. Before any anaesthetic intervention, an arterial line was inserted into a radial artery under local anaesthetic in the anaesthetic room.

Preoperative oxygen delivery was calculated using the LiDCO cardiac output

monitor, after the lithium-based calibration, by determining preoperative cardiac output. At least three calibration curves were constructed routinely to ensure accuracy of haemodynamic data. Once these calibration data were obtained, anaesthetic preparation (insertion of thoracic epidural catheter, endotracheal intubation) commenced and surgery followed, during which time haemodynamic data were captured throughout, but not revealed to any operating room staff including attending anaesthetists. Alternative haemodynamic monitors were permitted during surgery.

2.1.1.1 Randomisation and masking

Randomisation occurred immediately following completion of surgery. The randomisation list was generated by Stata and then concealed by envelope. Participants were centrally allocated to treatment groups. All principal investigators were masked to study group allocation. To further minimise the possibility of bias, the medical research and nursing staff delivering the haemodynamic protocol did not reveal the study group allocation to critical care staff, attending surgical or physician teams. Every patient enrolled had a central venous catheter inserted after induction of anaesthesia. A syringe with saline or dobutamine was connected via extension tubing to the central venous catheter, with the identity of the syringe contents covered. Patients were followed up by an investigator unaware of the patients' achievement or not of the baseline oxygen delivery target. All participants were admitted as elective postoperative cases to a critical care facility.

2.1.1.2 Postoperative haemodynamic therapy intervention

The intervention period commenced once the patient reached the critical care unit after surgery and continued for 6 hours (Figure 2.1). Both randomisation groups (goal-directed therapy and control patients) were managed by research staff during the postoperative study period. Before the intervention,

the LiDCO lithium-based sensor was recalibrated to determine cardiac output. Research staff were entirely responsible for haemodynamic management within this period, and intensive care unit staff played no part in this aspect of care. All patients received standard measures to maintain oxygenation ($\text{SpO}_2 \geq 94\%$), haemoglobin ($>80 \text{ g/L}$), core temperature ($\geq 36^\circ\text{C}$), and heart rate (<100 beats per min). Supplemental oxygen, packed red cells, and forced warm-air heating were administered when any of these thresholds were breached. Compound sodium lactate (Hartmanns solution) was administered at 1 ml/kg per hour as maintenance fluid, with additional fluid administered only by the study research staff guided by pulse rate, arterial pressure, urine output, and central venous saturation. Mean arterial pressure was maintained between 60 mm Hg and 90 mm Hg using an α_1 -adrenoceptor agonist as needed. Postoperative analgesia was provided by thoracic epidural infusion (low dose mixture of bupivacaine and fentanyl), intravenous infusion, or patient-controlled analgesia (morphine or fentanyl).

The goal-directed therapy group patients received intravenous fluid and inotropic therapy guided by the haemodynamic therapy algorithm targeting the preoperative oxygen delivery value for each individual patient. The first hour of the intervention period was dedicated to achieving maximum stroke volume with the use of the synthetic gelatin-based colloid, Gelofusine[®]. If the preoperative oxygen delivery target was not met after the first hour of stroke volume optimisation, patients in the goal-directed therapy group also received an intravenous infusion of dobutamine ($1\text{--}20 \text{ }\mu\text{g/kg/min}$) through a central venous catheter. Administration and dose of dobutamine were strictly controlled by ensuring that heart rate remained at less than 100 beats per min, or not more than 25% from baseline heart rate at the start of the intervention period. If heart rate exceeded one or more of these thresholds, the dobutamine infusion was discontinued. At the end of the 6 hour intervention period, dobutamine if needed was titrated to stop over the course of the next hour. Cardiac output monitoring was not done in the control group unless specifically requested by clinical staff because of patient deterioration.

Haemodynamic parameters were recorded in the control group, but calculation of oxygen delivery values was delayed until the end of the intervention. All other management decisions were taken by senior clinicians who retained the discretion to alter any aspect of patient care, having informed the site principal or chief investigator if this affected haemodynamic management during the study intervention period.

Achievement of the preoperative oxygen delivery target was determined by analysing mean oxygen delivery throughout the postoperative intervention period, and relating this value to the number of predefined hourly time points during the intervention where postoperative oxygen delivery met, or exceeded, the preoperative value.

2.1.1.3 Data collection

Data entry was performed using a secure university site. Automated validation checks included plausibility ranges and cross-checks between data fields. Further manual data checks were done both centrally and through source data verification.

Preoperative clinical and demographic data were recorded prospectively, including age, sex and comorbidities including hypertension, ischaemic heart disease (IHD), cerebrovascular disease and chronic kidney disease (CKD). Intraoperative clinical variables were recorded, such as duration of surgery, estimated blood loss, volume of fluid administered, patient blood management details, and vasopressor support. Similarly, postoperative haemodynamic variables were collected including volume of fluid administered, presence of dobutamine infusion and patient blood management details.

Postoperative visits were performed on postoperative day (POD) 2, 3, 7 and 14, where appropriate, in addition to the day of hospital discharge, with data and samples collected as indicated (Figure 2.2). Postoperative morbidity was

assessed using both the Postoperative Morbidity Survey (POMS) and the Clavien-Dindo scale, with grade II or more defining a deviation from normal postoperative recovery (Clavien et al., 2009).

Further postoperative outcomes were collected including time to become morbidity free and length of hospital stay. Parasympathetic autonomic function was assessed by specific heart rate variability parameters, measured by Holter ECG recording at pre-determined perioperative time points.

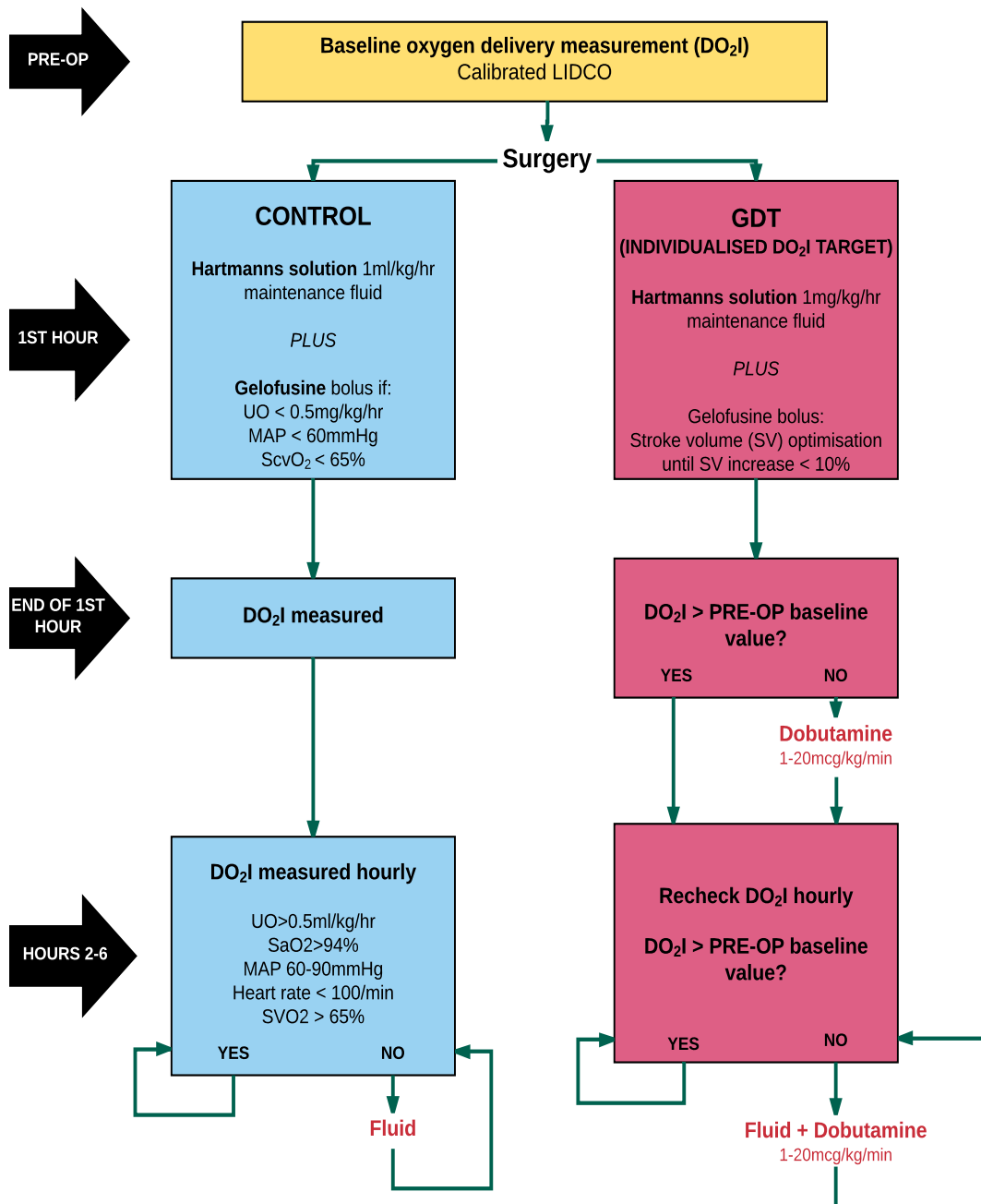


Figure 2.1 POM-O trial postoperative haemodynamic therapy intervention protocol

Treatment algorithm for each trial patient on day of surgery. Patients randomised to postoperative haemodynamic therapy intervention, CONTROL or GDT. DO_2I = oxygen delivery index. UO = urine output. MAP = mean arterial pressure. $ScvO_2$ = central venous oxygen saturation

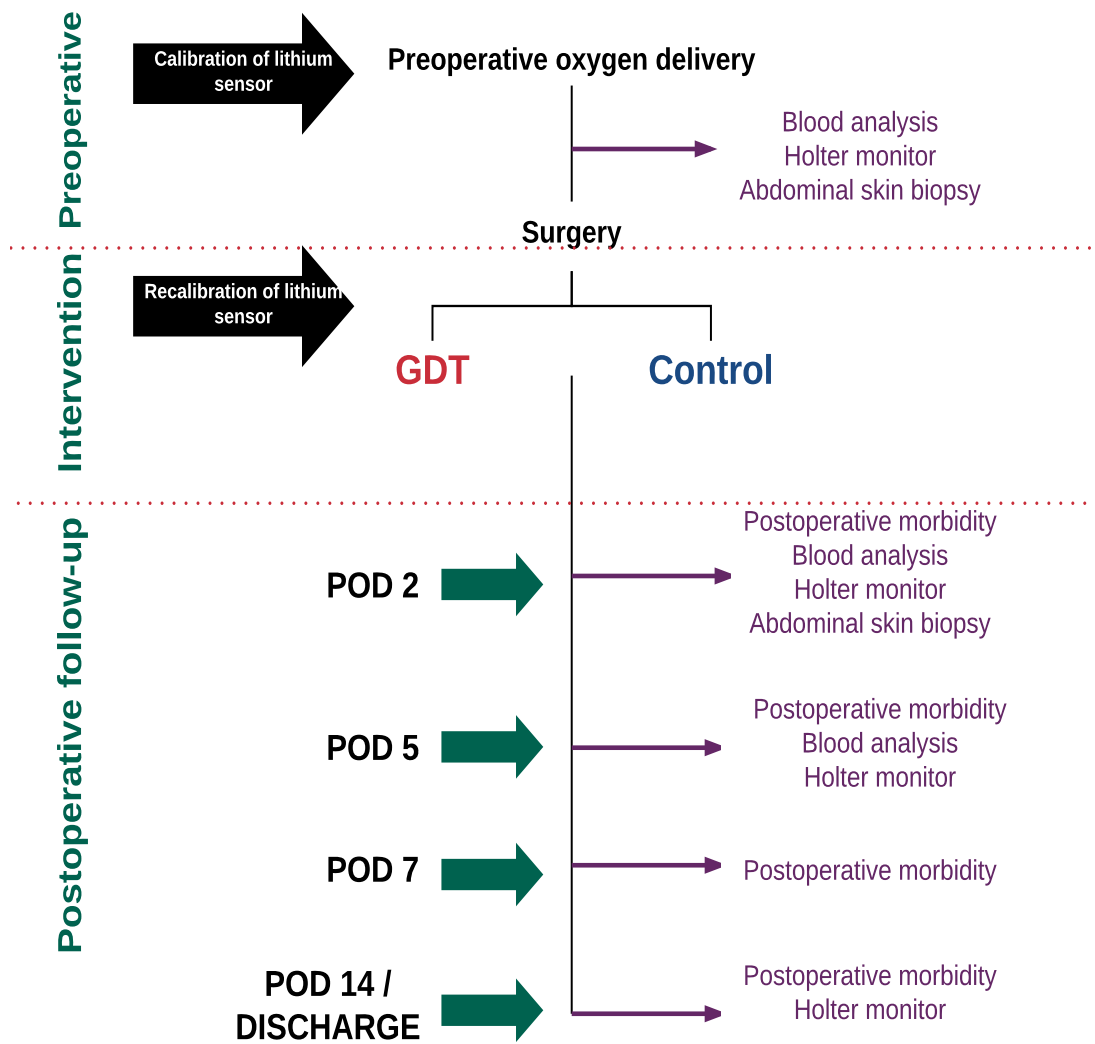


Figure 2.2 POM-O trial data and sample collection summary

Flowchart to demonstrate perioperative time points used for data and sample collection. Blood analysis denotes samples taken for parent POM-O trial and routine blood tests as per standard care. POM-O TEG study blood samples were taken to coincide with the time points shown plus 2 additional time points pre- and post-intervention.

2.1.2 POM-O TEG sub-study

This sub-study evaluated alterations in the coagulation profile in POM-O trial subjects at the Royal Free Hospital site only, between May 2011 and October 2012 (n=50).

Blood samples were collected for analysis by thromboelastography at 5 perioperative time points: immediately postoperatively (prior to POM-O study intervention), 6 hours postoperatively (following POM-O study intervention) and both 2 and 5 days postoperatively (early morning samples).

Samples were obtained preoperatively from 20-gauge arterial cannulae in conscious surgical patients but postoperatively by venepuncture, from the antecubital fossa, using a 19G butterfly needle. Arterial samples were drawn from transducer sets primed with 0.9% NaCl, with the first 5ml discarded. Similarly, the first 5ml of venepuncture samples were discarded to minimise the effects of the tourniquet. Samples were collected into Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing sodium citrate 3.8% (for thromboelastography and laboratory coagulation screen) or EDTA (for full blood count) and gently inverted to ensure mixing of sample.

Thromboelastography with citrated native TEGs was performed, following recalcification of citrated blood, 20 mins after collection at each time point.

Routine haematological parameters were measured, including full blood count, coagulation screen (including prothrombin time and activated partial thromboplastin time) and fibrinogen level at each perioperative time point, as part of routine perioperative care, within the Haematology clinical laboratory at the Royal Free Hospital. Haemoglobin, haematocrit and platelet count were measured using a Sysmex FBC analyser. Prothrombin time (PT), activated partial thrombin time test (APTT), thrombin time (TT), fibrinogen and D-dimers were all measured using an ACL TOP coagulometer

[using Recombiplastin-2G, SynthaSIL, thrombin reagent, QFA reagent and D-dimer reagents respectively (all from Instrumentation Laboratory, USA)].

In a further subset of patients, a more detailed haematological assessment was undertaken to evaluate corroborative haematological data to any alterations in thromboelastographic variables (n=15). Further blood samples were taken at the same perioperative time points with the exception of the post-intervention sample as this usually occurred outside normal laboratory working hours. Therefore, an early morning sample on the first postoperative day was taken as a surrogate for the post-intervention sample.

Additional blood samples were collected into Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing either sodium citrate 3.8% or SST (silica and gel) and a single S-Monovette tube (Sarstedt, Leicester, UK) containing 0.106mmol/l trisodium citrate, with pre-added corn trypsin inhibitor (CTI). CTI is used to abolish contact activation and prevents imprecision in thrombin generation assays, and was added to S-Monovette tubes within 72 hrs prior to sample collection. These samples were analysed in a specialist Haemophilia clinical laboratory at the Royal Free Hospital and processed by a dedicated senior biomedical scientist.

Detailed haematological assessment involved measurement of: individual clotting factor levels; D-dimer and von Willebrand factor antigen (vWF:Ag); natural anticoagulants, such as antithrombin, protein C and protein S; and biomarkers of endothelial injury, such as syndecan-1, thrombomodulin and soluble P-selection. Moreover, thrombin generation assays were performed at each time point. Thus a global assessment of coagulation was undertaken encompassing endothelial activation, activation of coagulation with thrombin generation and fibrinolysis.

Factor VIII (FVIII:C) levels were measured by one stage APTT based clotting assay and factors VII (FVII), factor V (FV), factor VII (FVII) and factor X (FX)

were measured by one stage prothrombin based assays as previously described. vWF antigen was measured by ELISA, as previously described (Agarwal et al., 2012).

Plasma levels of tissue factor activity, tissue factor pathway inhibitor (TFPI), thrombomodulin (all Sekisu, American Diagnostica GmbH, Germany) and syndecan-1 [CD138] (Abcam Ltd, Cambridge, UK) were measured according to the manufacturers' instructions.

Thrombin generation (TG) was assessed at all time-points on PPP by using the Calibrated Automated Thrombography (CAT) method as described (Hemker et al. 2003) TG assays were triggered with 5pM tissue factor (TF) reagent containing 4 μ M Phospholid (Thrombinoscope B.V., Maastricht, Netherlands). Parameters measured were endogenous thrombin potential (ETP) or total thrombin generated, peak height (PH) and lag time (LT) or time to initial thrombin formation, time to peak, ST and Slope. All TG values were normalised using the same normal pooled plasma (NPP) that had been included in each TG run of samples, by dividing the TG parameter with the respective NPP parameter value and multiplying by 100 to obtain a normalised % parameter result.

2.2 Ex vivo incubation experimental study

2.2.1 LPS TEG experimental model

This experimental model was developed on the basis of published work which both validated the use of lipopolysaccharide (LPS) *in vitro* as a surrogate for the systemic inflammatory response syndrome (SIRS) seen following surgery and the use of thromboelastography in the assessment of coagulation activation seen perioperatively (Koch et al. 2009; Spiel et al. 2006; Zacharowski et al. 2006).

Lipopolysaccharide (LPS), or endotoxin, is a key component of the cell membrane of gram negative bacteria. Endotoxin is one of the most potent known activators of innate immunity and the inflammatory response in humans. Endotoxin is hypothesized to enter the systemic circulation by translocation of gut commensal microbes or by LPS fragments moving across the intestinal mucosal barrier during period of relative hypotension and hypoperfusion. LPS is released into sterile compartments of the organism and in turn is detected by pattern recognition receptors (PRRs), like toll-like receptor (TLR)4, resulting in initiation of inflammation and coagulation (Nakamura et al. 2007). TLR4 is the key receptor involved in LPS recognition and signal initiation, with TLR4 mutations in humans associated with hyporesponsiveness to LPS (Arbour et al., 2000). Serum LPS binding protein (LBP) is responsible for extracting LPS from bacterial membranes or vesicles released by bacteria. LBP then transfers LPS to CD14, which can be found either in soluble form or linked to the cell surface by a glycosylphosphatidylinositol anchor. CD14 splits LPS aggregates into monomeric molecules and presents them to the TLR4-MD-2 complex. Aggregation of the TLR4-MD-2 complex after binding LPS leads to activation of multiple signaling components, including NF- κ B, and the subsequent production of pro-inflammatory cytokines. The intracellular signalling mechanism for LPS is complex but LPS stimulation of coagulation has been

shown to be reduced by both specific inhibition of the NF- κ B pathway and p38 MAPK inhibition in an *ex vivo* incubation model using thromboelastography (Koch et al., 2012).

Experimental models characterising the effect of LPS stimulation on both inflammation and coagulation have been described. LPS-stimulated human whole blood is associated with elevated levels of pro-inflammatory cytokines, such as TNF α , IL-6 and IL-8, and increased cytokine gene expression in both *in vivo* and *in vitro* models (De Forge and Remick, 1991). Different experimental paradigms have consistently demonstrated the ability of LPS to initiate coagulation activation via tissue factor (TF) expression on endothelial cells and blood monocytes (Moore et al., 1987), mainly regulated at transcriptional level (Franco et al., 2000). LPS administration to human subjects is associated with elevated TF positive monocytes and biomarkers of coagulation activation, such as prothrombin fragment F₁₊₂, *in vivo* (Pernerstorfer et al., 1999). Endotoxin activity levels have been shown to directly correlate with viscoelastic parameters of coagulation activation in patients with early systemic inflammation, independent of the cause being non-infectious or infectious (Koch et al. 2013)

Viscoelastic testing has been extensively used for the determination of LPS stimulated coagulation activation in whole blood samples. Following addition to whole blood, LPS reduces clotting time (CT) as measured with rotational thromboelastometry, with an 50% effective concentration (EC50) of 18 μ g/mL (Zacharowski et al., 2006). Endotoxaemia *in vivo* produces a hypercoagulable state with shortened CT correlating well with peak plasma levels of prothrombin fragments F₁₊₂ (Spiel et al., 2006). In whole blood stimulated with increasing concentrations of LPS *ex vivo*, alterations in all ROTEM parameters consistent with increased activation of coagulation, were demonstrated with LPS concentration of 100ng/ml (Koch et al., 2009b).

Therefore, on the basis of the validated use of thromboelastography for the assessment of inflammation-induced activation of coagulation, an *ex vivo*

incubation model was chosen for this LPS-TEG sub-study, using LPS at a final concentration of 100ng/ml. The effect of LPS, compared to endotoxin-free saline, on activation of coagulation in whole blood of both surgical patients and healthy volunteers was quantified, using thromboelastography. Furthermore, the hypothesis that various perioperative drugs, associated with potential to reduce thrombotic risk, could attenuate the procoagulant effect of LPS was tested using thromboelastography.

2.2.2 Study population

Subjects included both healthy drug-naïve volunteers, from the Anaesthetics Department at the Royal Free Hospital, and patients recruited to the POM-O TEG clinical sub-study, as described earlier. Enrollment of both healthy volunteers and additional blood sampling from the patients recruited to the POM-O study was undertaken following local Research Ethics Committee approval (UCL Ethics Project Approval ID 4353/001 & 4353/002) and written consent.

Exclusion criteria for healthy volunteers included subjects less than 18 years old, pregnancy, any known history of coagulopathy and concomitant use of all prescription or over the counter medications. POM-O trial surgical patients taking preoperative anticoagulation therapy were excluded and any antiplatelet medications were discontinued 7 days preoperatively. Written informed consent was obtained prior to enrolment. There were no financial payments offered to volunteers.

2.2.3 Study protocol

The study was designed as a prospective case-control study. Following enrolment, blood samples were taken at a single time point (immediately preoperatively in the surgical cohort).

Blood samples were obtained either from 20-gauge arterial cannula in conscious surgical patients or by venepuncture in healthy volunteers, from the antecubital fossa, using a 19G butterfly needle. Arterial samples were drawn from transducer sets primed with 0.9% NaCl, with the first 5ml discarded. Similarly, the first 5ml of venepuncture samples were discarded to minimise the effects of the tourniquet. Samples were collected into 2-3 Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing sodium citrate 3.8% and

gently inverted to ensure mixing of sample. Arterial blood sampling was limited to preoperative collection whereas all other samples were venous blood. Previous studies have highlighted potential differences in TEG parameters between arterial and venous blood, in cardiac surgical patients, with a tendency to shorter R-time and larger MA in native arterial samples (Manspeizer et al., 2001). Therefore, it was important to ensure a standardised sampling technique was used in each subject group.

Aliquots of 1 ml citrated blood were subsequently transferred to Eppendorf (1.5ml) tubes prior to incubation at 37°C for 4 hours. In each subject, 1ml citrated blood was incubated with either 10µl LPS (*Salmonella typhus*) or endotoxin-free sterile 0.9% NaCl (normal saline), added immediately prior to incubation. The final concentration of LPS was 100ng/ml, although a further subset of experiments was conducted with lower concentrations, following dilution of stock LPS solution.

For drug treated samples, only venous whole blood from healthy volunteers was used. Each citrated blood aliquot was pre-treated with a selected drug 20 mins prior to the addition of LPS or sterile saline. Samples were gently inverted following both drug pre-treatment and addition of LPS or sterile saline, to ensure mixing of the sample (Figure 2.3). Drug solutions had sterile normal saline, unless otherwise stated, as vehicle control.

Following incubation, thromboelastography was performed, after recalcification of citrated blood, immediately before 4 hours following the time of blood collection. TEG measurements have been demonstrated to produce accurate and reproducible results up to 4 hours in citrated blood (Camenzind et al, 2000).

In drug treated samples, whole blood was incubated with doses at the upper range of plasma concentrations reported in previous publications. Drug selection was based on existing use in the perioperative scenario, together

with previously reported putative anti-inflammatory or anti-thrombotic effect.

Perioperative drugs tested were categorised broadly into the following categories, with clinically relevant concentrations shown in Table 2.1.

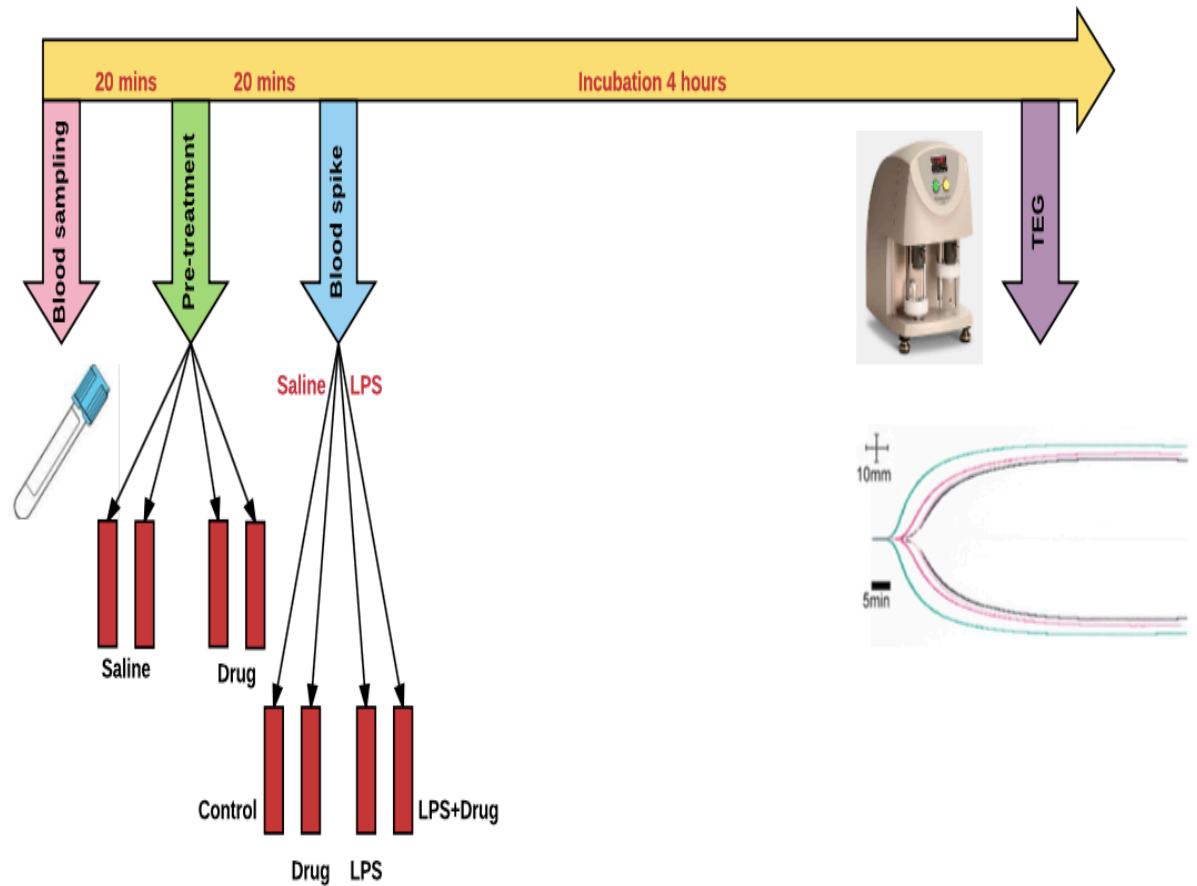


Figure 2.3 LPS TEG study experimental protocol

Blood samples were collected 20 mins prior to pre-treatment with nominated drug or sterile saline (control) in 1ml aliquots of citrated whole blood. Following 20 minutes, pre-treated samples were then treated or 'spiked' with LPS or sterile saline (control) to form 4 corresponding samples. After adequate mixing, samples were transferred for incubation before TEG analysis was performed at 4 hours. A typical TEG trace with 4 superimposed traces for each corresponding sample with own control is shown.

Table 2.1 Selected perioperative drug classes and doses administered in ex vivo LPS incubation model

Drug class	Experimental drug & dose	Reference
Statins (HMG coA reductase inhibitors)	Simvastatin (10-25ng/ml)	(Backman et al., 2000) (Ziviani et al. 2001)
β 1-adrenergic receptor antagonists	Metoprolol (100ng/ml) Atenolol (500ng/ml)	(Aqil et al., 2007) (Spanakis and Niopas 2013)
	Carvedilol (100ng/ml)	(Neugebauer et al., 1990)
α 2-adrenergic receptor agonists	Clonidine (5ng/ml)	(Anavekar et al., 1982)
Steroids	Dexamethasone (40ng/ml or 100nM)	(Dandona et al., 1999)
Local anaesthetics	Lidocaine (5ng/ml)	(Herroeder et al., 2007)
Antioxidants	N-acetylcysteine (500ng/ml)	(Prescott et al., 1989)
Electrolytes	Magnesium sulphate (2mM)	(Maier et al., 2004)

Experimental *in vitro* drug doses based on previous publications of upper range of plasma concentrations (References included)

2.3 Thromboelastography (TEG)

2.3.1 Principles of thromboelastography

Thromboelastography was first described in 1948 (Hartert 1948), as a method to assess the viscoelastic properties of coagulation in whole blood.

Viscoelastic point-of-care haemostatic assays, such as TEG and rotational thromboelastometry (ROTEM), provide a graphical representation of the different stages of the haemostatic process from the initiation of clot formation to subsequent clot lysis. The term ‘thromboelastography’ or TEG[®] was used generically until 1996 when both became registered trademarks of Hemoscope Corp (Niles, IL) and so hitherto describes assays performed by Hemoscope, or latterly, Haemonetics instruments only. Rotational thromboelastometry (ROTEM[®]) is an alternative instrument using modified technology developed by Pentapharm GmbH (Munich, Germany). Both technologies provide a visual assessment of clot formation and subsequent lysis under low shear conditions, similar to the microenvironment of the vena cava and below that seen in venules, large veins, and the arterial system.

Viscoelastic tests are dynamic modalities that provide a composite picture reflecting the interaction of plasma, blood cells and platelets, thereby more closely reflecting the situation *in vivo* than conventional coagulation tests, which are performed solely in plasma and measure only isolated end points. They provide real-time information about the quality of the clot and the kinetics of its formation, thus characterising the interaction between the vascular endothelial wall, platelets, von Willebrand factor (vWF), pro- and anticoagulant factors and blood flow. This global assessment of haemostasis more realistically represents the cell-based model of coagulation (Hoffmann & Munroe 2001), as opposed to the traditional description of intrinsic and extrinsic pathways, by encompassing the entire process of thrombin generation and the role of platelets. For effective haemostasis, there must be sufficient thrombin generation (coagulation factors and platelets), adequate

substrate (fibrinogen) and clot stability. Thus, viscoelastic tests are advantageous in measuring changes in clot tensile strength in real-time, assessing the dynamics of clot formation (coagulation factors and anticoagulant activity), clot strength (platelets and fibrinogen) and clot stability (fibrinolysis and factor XIII).

In thromboelastography blood is incubated in at 37°C in a special stationary cylindrical cup with a pin suspended freely from a torsion wire within the cup. The cup and pin are oscillated relative to each other through an angle of 4°45', with the movement initiated by the cup (compared to the pin in ROTEM), as shown in Figure 2.4. As fibrin forms between the cup and the pin, the transmitted rotation from the cup to pin is detected at the pin and a trace generated. The strength of fibrin-platelet bonding affects the magnitude of pin motion, such that strong clots move the pin directly in phase with cup motion. Therefore, the magnitude of the output is directly related to the strength of the clot. During clot lysis, these bonds are broken and the transfer of cup motion is diminished. This rotational movement of the pin is transduced to an electrical signal that is monitored by computer software. A characteristic TEG trace, as depicted in Figure 2.5, provides a comprehensive haemostasis profile measuring the time taken for the first fibrin strand to be formed, the kinetics of clot formation, the clot strength and the subsequent dissolution of the clot.

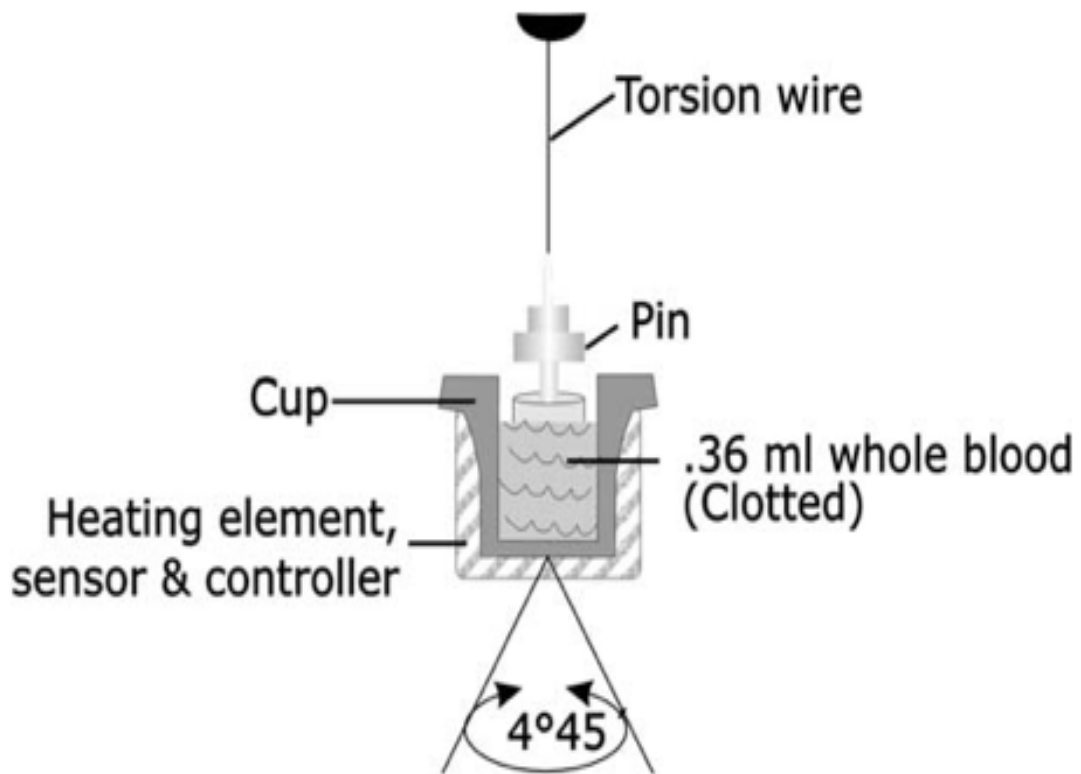


Figure 2.4 Schematic diagram of TEG cup and pin components of the TEG 5000 analyser

A depiction of the TEG device used in which a pin suspended from a torsion wire is immersed in a cup of whole blood. The cup is held in a heating block and continually oscillates through $4^{\circ}45'$ every 5 sec. Changes in viscoelastic clot strength are directly transmitted to the torsion wire and detected by an electromechanical transducer.

TEG® Haemostasis Analyser images and Tracings are used by permission of Haemonetics Corporation

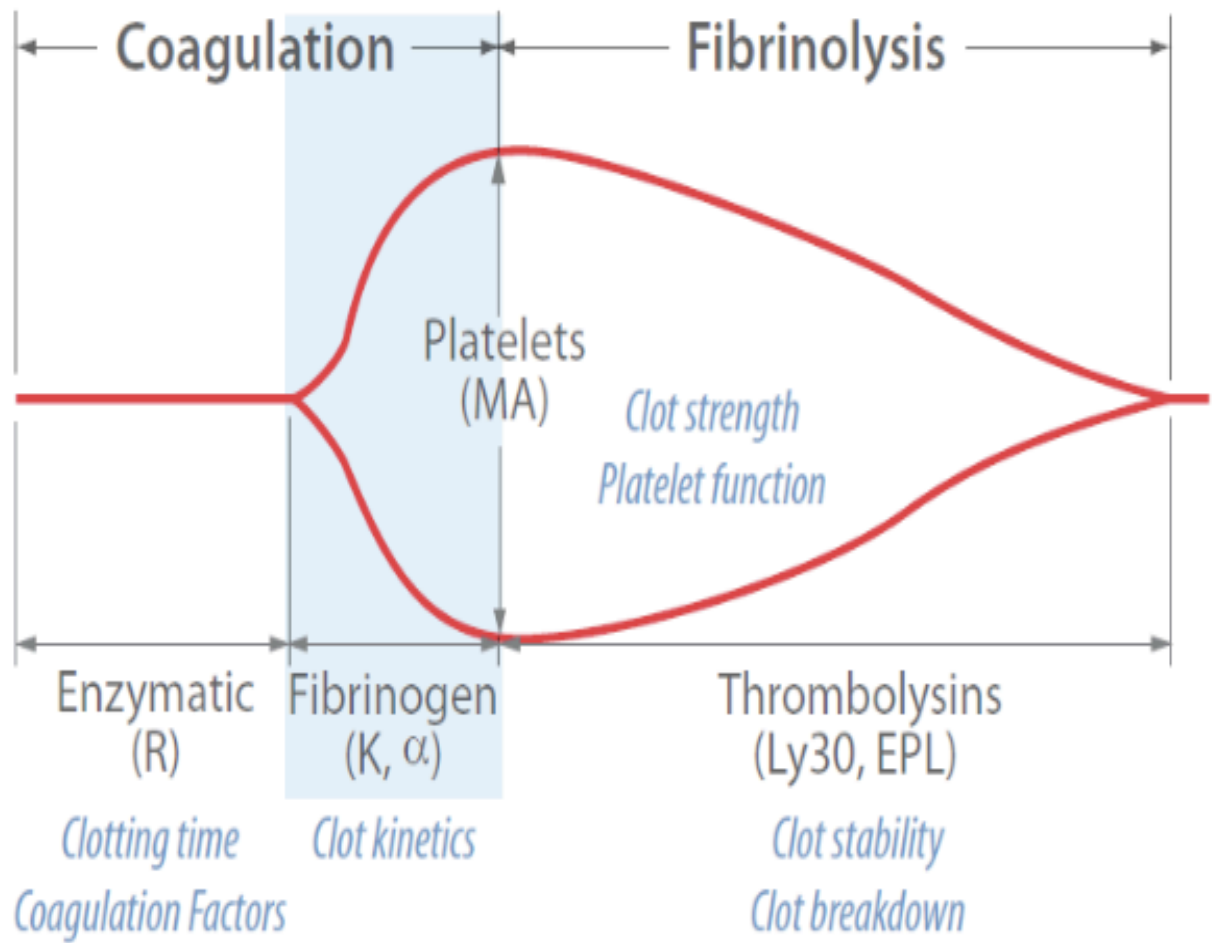


Figure 2.5 Schematic diagram of typical TEG trace demonstrating nomenclature of TEG parameters

A depiction of a TEG output demonstrating clot initiation, propagation, stabilisation and lysis. TEG parameters are defined in the main text (R, R-time; K, K-time; α , angle; MA, maximum amplitude; Ly30, lysis time; EPL, estimated percent lysis)

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2.3.2 TEG parameters

The standard parameters used for viscoelastic testing are depicted in Table 2.2, illustrating the difference in nomenclature between TEG and ROTEM.

TEG parameters relate to either clot formation or clot lysis and are specifically defined as follows:

Clot formation:

- **R-time** is the time from placing the blood in the cuvette until the tracing amplitude reaches 2mm, denoting initial fibrin formation and representing the enzymatic portion of coagulation.
- **K-time** is measured from R to the point where the amplitude of the tracing reaches 20mm, representing clot kinetics.
- The alpha (α) **angle** is the angle formed by the slope of the TEG tracing from the R to K value, thus measuring the rapidity of fibrin build-up and cross-linking (clot strengthening).
- The **maximum amplitude** (MA) is the greatest amplitude of the TEG tracing and is a reflection of the absolute strength of the fibrin clot and platelet function/aggregation.

Clot lysis:

- Lysis 30 and Lysis 60 (**LY30** and **LY60**) are the percent reductions in the area under the TEG curve, assuming MA remains constant, that occur 30 and 60 min after MA is reached.
- Estimated Percent Lysis (**EPL**) is the estimated percent lysis at 30 minutes after MA. This parameter gives an idea of the percent lysis prior to 30 minutes after MA. EPL is computed by finding the slope connecting MA to any point between MA and 30 minutes after and then extrapolating to the amplitude at 30 mins.

Further derived parameters are available in TEG analysis to represent more composite or standardised values and are presented in numerous publications:

- CI (Clotting Index): global assessment of clot formation using a linear combination of the variables: R, K, MA, and Angle (α).
- G (Shear Modulus Strength): Maximum amplitude (MA) is transformed into an actual measure of clot strength. G measured in Kdynes/cm²
- E (Elasticity Constant): normalised value of G such that a normal maximum amplitude (MA) of 50 mm will yield an E value of 100

Various activators and reagents are used with both TEG and ROTEM to either improve speed of analysis, with the addition of tissue factor or kaolin, or provide extra diagnostic capabilities, with heparinase used to reverse heparin and functional fibrinogen (TEG) or FIBTEM (ROTEM) used to present information on fibrinogen levels (Table 2.3).

2.3.3 TEG experimental procedure

Given that the experimental hypothesis centered on the initiation of clot formation, by the release of tissue factor and subsequent activation of coagulation cascades, the TEG parameters chosen to measure in both the clinical and *ex vivo* incubation studies were absolute, non-derived variables of clot formation. Further parameters of clot lysis, such as LY30, were not considered relevant to this experimental paradigm and so are not presented (Table 2.3).

Manufacturer instructions stipulate that native TEGs must be performed within 6 minutes of blood sampling. Therefore, citrated blood samples were required to allow both adequate time for transfer of collected blood samples for the clinical study or for the 4 hour incubation period in the LPS TEG study. In all

experiments, citrated native TEGs were performed using citrated blood in plain TEG cups, under standardised conditions, following recalcification with CaCl_2 . Citrated storage is a routine and validated means to facilitate TEG analysis beyond the timeframe of immediate collection. Although earlier studies comparing native and citrated samples showed a tendency to hypercoagulability, in terms of R-time reduction, in citrated samples with prolonged storage, larger more recent studies demonstrate reproducible citrated TEG parameters both within reference ranges and comparable to non-citrated native samples, up to 4 hours from collection (Dias et al., 2017; Wasowicz et al., 2008) (Whiting and Dinardo, 2014).

The influence of both exogenous and endogenous heparins was accounted for in an experimental subset of the clinical study. Exogenous heparins form the mainstay of pharmacological thromboprophylaxis and are administered in the perioperative period to minimise risk of thromboembolic complications. Endogenous heparinoid activity is increasingly recognized in liver surgery, with both heparinase-modified TEGs and anti-Xa activity demonstrating heparin-like activity in cirrhotic patients and following orthotopic liver transplantation (Kettner et al., 1998; Senzolo et al., 2009). Naturally occurring heparin-like activity, in the form of endogenous heparin and heparan sulphate proteoglycans, have also been shown in normal human plasma. However, the role of endogenous heparinoids in major surgery, particularly liver resection, has hitherto not been investigated, despite the potential impact that heparin-like activity would produce on the postoperative coagulation status. Therefore, in a subset of patients in the clinical study, additional citrated native TEGs with heparinase were undertaken (340 μL blood added to a heparinase TEG cup, recalcified with 20 μL of CaCl_2 0.2M)..

Thromboelastography was performed using a computerized TEG coagulation analyser (Model 5000; Haemonetics, Braintree, MA, USA), using software TEG 5000 Hemostasis System Version 4.2 (Figure 2.6). The TEG analyser was calibrated and maintained according to strict manufacturer guidelines and

institutional laboratory standards. The TEG analysers were located in the Point-of-Care laboratory, within the operating theatre suite at the Royal Free Hospital, which has a dedicated biomedical scientist charged with maintenance of all laboratory equipment. Polypropylene and polyethylene pipettes were used to process blood and reagents.

Quality control (QC) procedures included electronic 'eTest' determination, to ensure electronic function of the analyser was maintained, which was undertaken daily and applied to each individual channel. TEGs would not be undertaken unless all 8 channels in the laboratory had successfully passed eTests on any given day. Use of biological controls served as a further operational check and calibration verification. Level I and II QCs were performed weekly. These controls contain animal citrated whole blood, including platelets and plasma, stabilizers, and buffer. Level I has been formulated to simulate a normal blood sample, while Level II simulates an abnormal sample of a bleeding patient. Data regarding instrument performance was collected and included failed eTests, nonsense/questionable results and analyser eTest errors.

Table 2.2 Comparison of TEG and ROTEM nomenclature

Haemostatic phase	TEG	ROTEM	Description
Clot initiation	R (reaction)-time	CT (clotting time)	Time to initial clot formation
Clot kinetics	K-time	CFT (clot formation time)	Speed to reach defined level of clot strength
	Angle (α)	α	Rate of clot formation (fibrin build up and cross linking)
Clot strength	MA (maximum amplitude)	MCF (maximum clot firmness)	Strength of clot (fibrin and platelet binding)
Clot stability	Lysis (Ly) 30	CLI (clot lysis index)	Fibrinolysis (rate of amplitude reduction of clot strength at 30 mins)

Viscoelastic tests use different nomenclature for parameters representing the same haemostatic process. However, the corresponding nomenclature is not interchangeable due to the different operating characteristics of the individual viscoelastic technology and modality specific reference ranges.

Table 2.3 Commercially available thromboelastography assays

Assay	Activator/Inhibitor	Role
Native	None	Non-activated assay
Kaolin	Kaolin	Assessment of clot formation, fibrin polymerisation and fibrinolysis via the intrinsic pathway.
Heparinase	Kaolin + heparinase	Assessment of clot formation in heparinised patients (both unfractionated and low molecular weight)
Platelet mapping	ADP Arachidonic acid	Assessment of platelet function and monitoring of antiplatelet therapy (such as aspirin or clopidogrel)
Rapid TEG	Kaolin + tissue factor	Provides more rapid results than standard kaolin assay (mean 20 minutes versus 30 minutes for standard TEG with initial results in less than one minute)
Functional fibrinogen assay	Lyophilized tissue factor + platelet inhibitor	Partitions clot strength (MA) into contributions from platelets and fibrin

TEG assays can be modified by addition of reagents to the *in vitro* blood sample. Reagents are broadly classified as activators, such kaolin or tissue factor, or neutralisers such as heparinase, used to eliminate any heparin effect.



Figure 2.6 **TEG® 5000 analyser (Haemonetics)**

TEG® Haemostasis Analyser images and Tracings are used by permission of Haemonetics Corporation

2.4 Reagents

<i>Reagent</i>	<i>Supplier</i>	<i>Address</i>
Lipopolysaccharide (from Salmonella Typhosa) <i>Stock solution in PBS sterile filtered, stored at 4°C</i>	Sigma Aldrich	Dorset, UK
Simvastatin	Sigma Aldrich	Dorset, UK
Metoprolol	Astra Zeneca UK Ltd	Bedfordshire, UK
Atenolol	Sigma Aldrich	Dorset, UK
Clonidine	Boehringer Ingelheim	Berkshire, UK
Dexamethasone	Hameln Pharmaceuticals	Gloucester, UK
Lidocaine	Hameln Pharmaceuticals Ltd.	Gloucester, UK
N-acetylcysteine	Phoenix Laboratories	Co. Meath, Ireland
Magnesium sulphate	Martindale Pharmaceuticals Ltd.	Essex, UK
Nicotine	Sigma Aldrich	Dorset, UK
SB265610	Sigma Aldrich	Dorset, UK
LY294002	Sigma Aldrich	Dorset, UK
ZSTK474	Sigma Aldrich	Dorset, UK
Dobutamine	Hameln Pharmaceuticals Ltd.	Gloucester, UK
Hartmanns solution	Baxter Healthcare	Berkshire, UK
Gelofusine [®] solution	B. Braun Medical	Sheffield, UK

2.5 Equipment and disposables

<i>Equipment</i>	<i>Supplier</i>	<i>Address</i>
TEG 5000 analyser	Haemonetics Corporation	Braintree, MA, USA
TEG cups Plain & heparinise	Haemonetics Corporation	Braintree, MA, USA
Gilson Pipetman® P1000 & P100 pipettes	Gilson Inc	Middleton, WI, USA
Diamond® Tipack DF1000 & DF100 pipette tips	Gilson Inc	Middleton, WI, USA
Eppendorf tubes 1.5ml	Eppendorf UK Ltd	Stevenage, UK
Vacutainer tubes (sodium citrate, EDTA, SST)	BD (Becton, Dickinson & Company)	Plymouth, UK
S-Monovette tube (trisodium citrate)	Sarstedt AG & Co.	Leicester, UK
Butterfly needle	BD Valuset™ (Becton, Dickinson & Company)	Plymouth, UK

2.3 Statistics

2.3.1 General points

Categorical data are presented as absolute values (with percentage) and were compared using Pearson's χ^2 or Fisher's exact test depending on group size.

Continuous data are presented as median with interquartile range (IQR), or mean with standard deviation (SD) for normally distributed data. Normality was assessed using the D'Agostino & Pearson normality test.

In the clinical study, differences were determined either between values measured at different perioperative time point with respect to the baseline preoperative value or between different groups (such as treatment groups or surgical subgroups) at corresponding time points. Data from postoperative time points were compared with preoperative values using one-way ANOVA (with Bonferroni's multiple comparisons test) or Friedman test (with Dunn's multiple comparisons test). In longitudinal series with missing data at postoperative time points, Kruskal-Wallis test (with Dunn's multiple comparisons test) was used as an alternative to Friedman's test. Differences in values at each perioperative time point between groups were compared using unpaired t-tests or Mann Whitney test for parametric and non-parametric data respectively.

In the laboratory study, differences between treatment groups were compared using a 2-tailed paired t-test or one-way ANOVA (with Bonferroni's multiple comparisons test) for parametric datasets of 2 or more groups, as appropriate. Wilcoxon signed rank test or Kruskal Wallis test (with Dunn's multiple comparison test) were used for non-parametric datasets of 2 or more groups, as appropriate.

Statistical significance was set at $p < 0.05$. GraphPad Prism 8 (San Diego, CA, USA) was used for statistical analysis.

2.3.2 Sample size calculations

Sample size for laboratory work was based on the following:

- Previous studies describe ~60% reduction in clotting time CT (R-time equivalent) following LPS incubation (Koch et al. 2009).
- Reversal of LPS-induced decrease in R-time by 50% would be biologically important

Therefore, to address the hypothesis that selected drugs attenuate pre-defined LPS-induced hypercoagulability, at least 6 subjects per experimental comparison would be required to detect this R-time difference at the $\alpha = 0.05$ level (80% power).

Chapter 3

**Postoperative goal directed
haemodynamic therapy and the
perioperative coagulation profile
following major intra-abdominal
surgery**

3.1 Introduction

Thromboembolic complications, such as deep vein thrombosis (DVT), pulmonary embolism (PE), ischaemic stroke and myocardial infarction (MI), are associated with increased postoperative morbidity and higher healthcare costs (Anderson and Spencer 2003; Kakkar et al. 1993). Major abdominal surgery is more commonly associated with a postoperative hypercoagulable state that, in itself, is increasingly recognised as a contributory factor in the occurrence of these thromboembolic complications (Mahla et al., 2001). Mechanisms of hypercoagulability following surgery relate to increased activation of coagulation, reduction of natural anticoagulants or impaired fibrinolysis, in addition to more generic risk factors such as immobilisation and the presence of cancer. The perioperative coagulation status is influenced by specific surgical factors with tissue trauma and haemodilution producing a more hypercoagulable state compared to major haemorrhage and vascular clamping more associated with hypocoagulability.

Goal directed therapy (GDT) constitutes individualised haemodynamic management of high risk surgical patients, using cardiac output monitoring to guide intravenous fluid administration with or without inotropic support, as part of a treatment algorithm. Numerous trials have shown that goal directed therapy is associated with improved patient outcomes in high risk surgical patients. Postoperative complications and death occur more commonly in high risk patient groups, with advancing age and concomitant medical co-morbidities, undergoing major non-cardiac surgery (Khuri et al., 2005; Pearse et al., 2012). Consequently, haemodynamic therapy algorithms using cardiac output monitoring devices are recommended by the National Institute for Health and Care Excellence in this high risk cohort undergoing major surgery. However, a Cochrane review in 2014 suggested the benefit may be more marginal than previously thought and more recent studies investigating goal directed therapy regimens in both sepsis (Peake et al., 2014; Yealy et al.,

2014) and following major gastrointestinal surgery (Pearse et al., 2014) showed no significant benefit over routine care.

The impact of goal directed therapy on either the perioperative coagulation profile or the relative risk of thromboembolic complications has not been specifically studied. In contrast, the components of haemodynamic therapy algorithms, intravenous fluids and inotropic support, have each been demonstrated to affect the perioperative coagulation profile either *in vitro* or *in vivo*. Increasing concern exists whether potential harm is associated with goal directed therapy regimens, potentially attributable to fluid excess (Wei et al., 2008), autonomic dysfunction (Ackland et al., 2015a), myocardial injury or invasive monitoring devices. Potentially deleterious off-target effects of implementing goal directed therapy may be a generic phenomenon or specific to individual regimens given that published goal directed therapy protocols are considerably heterogeneous. There is wide variation between published studies in terms of patient selection, haemodynamic optimisation thresholds and targets, cardiac output monitoring devices and choice of intravenous fluid and/or inotropic therapy administered.

Excessive fluid administration with goal directed therapy leads to haemodilution (Pearse et al., 2014). The degree of haemodilution is highly variable and is related to the fluid regimen within each haemodynamic protocol, with different strategies used to optimise cardiac output, including stroke volume or oxygen delivery optimisation. Protocols invariably use crystalloid solutions as maintenance intravenous fluid as a pre-determined rate with administration of additional fluid boluses determined by specific haemodynamic variables. There is significant variation in the choice of both crystalloid and colloid intravenous fluids used within the myriad of studies published. The *in vitro* and *in vivo* effects of crystalloids, such as isotonic saline or Ringers lactate have consistently demonstrated haemodilution associated hypercoagulability, as determined by biomarkers of coagulation activation and viscoelastic testing (Ruttmann et al. 2002).

In addition to the effect of goal directed therapy and perioperative fluid management, the investigation of perioperative alterations in coagulation must also take into account the influence both exogenous and endogenous heparins. Exogenous heparins form the mainstay of pharmacological thromboprophylaxis and are administered in the perioperative period to minimise risk of thromboembolic complications. Endogenous heparinoid activity is increasingly recognized in liver surgery, with both heparinase-modified TEGs and anti-Xa activity demonstrating heparin-like activity in cirrhotic patients and following orthotopic liver transplantation (Kettner et al., 1998; Senzolo et al., 2009). Naturally occurring heparin-like activity, in the form of endogenous heparin and heparan sulfate proteoglycans, have also been shown in normal human plasma. However, the role of endogenous heparinoids in major surgery, particularly liver resection, has hitherto not been investigated, despite the potential impact that heparin-like activity would produce on the postoperative coagulation status.

The central issue highlighted by studies evaluating perioperative coagulation is how best to measure the global coagulation status of the surgical patient and thus determine the risk of thromboembolic complications. The interaction of coagulation components and time course of coagulation changes are poorly described. This acquired postoperative prothrombotic condition cannot be diagnosed by routine laboratory tests such as prothrombin time (PT) and activated partial thromboplastin time (APTT). However, thromboelastography (TEG) provides a comprehensive evaluation of the viscoelastic properties of blood and thus assesses platelet activation, fibrin formation and clot retraction (Mallett and Cox, 1992). TEG has been shown to be one of the most sensitive methods for detecting and monitoring postoperative hypercoagulability (Kashuk et al., 2009; Lison et al., 2011; Mahla et al., 2001). However, there is no universal method for defining hypercoagulability using TEG criteria, with previous publications requiring TEG parameters relevant to both enzymatic and cellular components of the coagulation cascade to be

GDT & perioperative coagulation following major surgery deranged to be deemed hypercoagulable.

3.1.1. Hypothesis

Thromboelastography (TEG) parameters demonstrate postoperative hypercoagulability following major elective intra-abdominal surgery in a high-risk surgical cohort. Goal directed therapy is associated with excessive intravenous fluid administration and is associated with alterations in the perioperative coagulation profile, as assessed by thromboelastography (TEG).

3.1.2 Aims

The hypothesis was addressed by characterising the perioperative changes in coagulation indices in a defined surgical cohort randomised to 2 treatment arms of postoperative haemodynamic therapy.

Primary outcome

TEG (citratated native TEG) parameters at each pre-defined perioperative time point

Secondary outcome

- Routine haematological data (Haemoglobin, Haematocrit, Platelets, PT, APTT, Fibrinogen) at each pre-defined perioperative time point
- Citratated heparinase TEG parameters at each pre-defined perioperative time point in a subgroup analysis, to assess the potential role of endogenous heparin activity and exclude any influence of exogenous heparin.
- Further haematological parameters (Individual clotting factor levels, selected biomarkers of endothelial injury and fibrinolysis and natural anticoagulants) in a subset of patients, at each pre-defined perioperative time point.

3.2 Methods

3.2.1 Post Operative Morbidity Oxygen (POM-O) trial

Surgical patients recruited to the POM-O (Post Operative Morbidity Oxygen) study, at the Royal Free Hospital, between May 2011 and October 2012, were prospectively studied. This cohort presented for major elective intra-abdominal surgery under general anaesthesia, predominantly hepatic and pancreatic surgery, associated with a high incidence of postoperative morbidity. Subject recruitment, detailed study protocol, data collection and statistical methods are discussed in the General Methods section.

Demographic data and details of indications for surgery and precise surgical procedure undertaken were collected.

Participants were centrally randomised to either postoperative intervention group, GDT (goal directed therapy) or control, as outlined in detail in Chapter 2. All principal investigators were masked to study group allocation. The intervention period commenced once the patient reached the critical care unit after surgery and continued for 6 hours. Both randomised groups (GDT and control) were managed by research staff during the postoperative study period. The goal-directed therapy group patients received intravenous fluid and inotropic therapy guided by the haemodynamic therapy algorithm targeting the preoperative oxygen delivery value for each individual patient. Haemodynamic parameters were recorded in the control group, but calculation of oxygen delivery values was delayed until the end of the intervention.

Patients receiving anticoagulation preoperatively were excluded while aspirin and other antiplatelet medications were discontinued 7 days preoperatively. Low molecular weight heparin (LMWH) was prescribed postoperatively, with a standardized 6pm administration time, and variable administration was recorded.

3.2.2 POM-O thromboelastography sub-study

3.2.2.1 Study protocol

This sub-study evaluated alterations in the coagulation profile in POM-O trial subjects at the Royal Free Hospital site only. Demographic data and details of indications for surgery and precise surgical procedure undertaken were collected.

Blood samples were collected immediately pre-operatively, immediately postoperatively (prior to POM-O study intervention), 6 hours postoperatively (following POM-O study intervention) and both 2 and 5 days postoperatively (early morning samples), as described in the General Methods section.

Routine haematological parameters were measured, including full blood count, coagulation screen (including prothrombin time and activated partial thromboplastin time) and fibrinogen level at each time point.

Thromboelastography with citrated native TEGs was performed using citrated blood, collected at each perioperative time point. The procedure for blood sampling, performing thromboelastography, the TEG parameters used and the reference ranges have been discussed in the Methods section.

Hypercoagulability was defined, in our surgical cohort, as the presence of at least one of the following TEG parameters:

- Shortened R +/- K time
- Increased angle
- Increased maximum amplitude (MA)

The hypercoagulable state was further defined according to the underlying mechanism. Hypercoagulable states may be characterized by rapid fibrin

GDT & perioperative coagulation following major surgery formation, with subsequent clot formation (enzymatic hypercoagulability) or hyperactive platelet function, characterized by rapid clot development and abnormally high clot strength (platelet hypercoagulability). Enzymatic hypercoagulability is detected by shortened R +/- K time, platelet hypercoagulability with increased angle and MA, or combined enzymatic and platelet hypercoagulability with all of these alterations at the same time.

In a further subset of patients, a more detailed haematological assessment was undertaken to include thrombin generation assays; measurement of individual clotting factor levels; D-dimer and von Willebrand factor antigen (vWF:Ag); natural anticoagulants, such as antithrombin, protein C and protein S; and biomarkers of endothelial injury, such as syndecan-1, thrombomodulin and soluble P-selection.

According to local departmental policy, low molecular weight heparin (LMWH) was administered at 1800h (>12hrs prior to POD2 & POD5 blood sample collection), and was not administered when routine laboratory data showed INR >1.5.

Details of the POM-O study intervention period (volume of fluid administered +/- inotropy) were collected (as per POM-O study protocol).

3.2.2.2 Outcome

The primary endpoint was difference in each thromboelastographic parameter at each perioperative time point comparing:

- postoperative time points to the preoperative baseline (each patient acting as own control) within each treatment group
- perioperative time points between treatment groups to detect whether goal directed therapy produced any significant changes in thromboelastographic parameters with respect to the control arm.

Secondary outcomes were differences between perioperative time points and between treatment arms in both routine laboratory haematological investigations and the additional comprehensive research laboratory haematological assessment.

3.2.3 Statistical analysis

Values are expressed as numbers (with percentages), means (with SD) or medians (with interquartile ranges) as appropriate. For continuous data, normality was assessed using the D'Agostino & Pearson normality test. To calculate differences between values measured at each perioperative time point within each treatment arm with respect to the baseline preoperative value, the repeated measures ANOVA or Friedman test were used as appropriate. In longitudinal data sets with missing values, ordinary one-way ANOVA (with Dunnett's multiple comparisons test) or Kruskal-Wallis test (with Dunn's multiple comparisons test) were used as alternatives, in parametric and non-parametric data sets respectively. Differences in values at each perioperative time point between treatment groups were compared using unpaired t-tests or Mann Whitney test for parametric and non-parametric data respectively. Statistical significance was set at $p < 0.05$. GraphPad Prism 8 (San Diego, CA, USA) was used for statistical analysis.

3.3 Results

3.3.1 Baseline patient characteristics

50 consecutive patients were enrolled into this POM-O TEG sub-study from 79 patients recruited into the POM-O parent trial at the Royal Free site. The coagulation sub-study commenced shortly after the parent trial had started, with a dedicated research fellow appointed to oversee the sub-study, and terminated when recruitment ceased at this site.

Baseline patient characteristics are summarised in Table 3.1. Demographic data are similar between treatment groups, control (n=26) and goal directed therapy (n=24). All patients underwent major intra-abdominal surgery however it should be noted that the control group had a higher proportion of pancreatic resections (54%), compared to the goal directed therapy, where hepatic resections were the predominant procedure (79%). This was further reflected by the histological diagnosis with the goal directed therapy group comprised of mostly metastatic liver disease, compared to underlying pancreatic malignancy in the control group. The majority of patients (88%) had confirmed presence of malignancy, either primary or metastatic, at surgical resection.

All subjects fulfilled the high-risk criteria for trial recruitment, as outlined in the General Methods section, with American Society of Anesthesiologists classification of 3 or more. The commonest medical co-morbidities were cardiovascular disease (hypertension, coronary artery disease and valvular heart disease) and diabetes mellitus. Coexisting medical comorbidities were broadly similar between the treatment arms.

Table 3.1 Baseline patient characteristics

	Control (n=26)	GDT (n=24)
Age (years)	69 (54-86)	72 (47-84)
Gender		
Male	16 (62%)	14 (58%)
Female	10 (38%)	10 (42%)
Weight (kg)	77.6 (16.1)	76 (18.0)
Haemoglobin (g/l)	12.8 (1.6)	12.7 (1.9)
Albumin (g/l)	42.3 (5.0)	43.5 (4.0)
Creatinine (μmol/l)	81.4 (21.5)	84.7 (18.6)
Medical co-morbidities		
GFR < 60 ml/min	4 (15%)	4 (17%)
Diabetes mellitus	6 (25%)	5 (21%)
Cardiovascular disease	20 (77%)	18 (75%)
Cerebrovascular disease	0 (0%)	2 (8%)
Surgical procedure		
Hepatic resection	7 (27%)	19 (79%)
Hemihepatectomy	2	8
Wedge resection	4	11
GB fossa resection	1	0
Pancreatic resection	14 (54%)	3 (13%)
Whipples procedure	12	2
Distal pancreatectomy & splenectomy	1	1
Total pancreatectomy	1	0
Other	5 (19%)	2 (8%)
Extrahepatic bile duct reconstruction	1	0
Hepaticojejunostomy	1	0
Gastrojejunostomy	1	1
Excision of retroperitoneal sarcoma	1	1
Excision of GIST	1	0
Histology		
Metastatic liver disease		
Colorectal	5	13
Breast	0	1
GIST	0	1
Hepatocellular carcinoma	1	2
Intrahepatic cholangiocarcinoma	0	1
Extrahepatic cholangiocarcinoma	1	0
Pancreatic adenocarcinoma	9	3
Pancreatic NET	1	1
Pancreatic cystadenoma	0	1
IPMN	2	0
Gallbladder papillary carcinoma	1	0
Bile duct tubulopapillary adenoma	1	0
Indeterminate	1	0
No histological specimen	3	1

Data are median (range), mean (SD) or n (%). GIST = gastrointestinal stromal tumour. IPMN = intrapapillary mucinous neoplasm. NET = neuroendocrine tumour.

3.3.2 Intraoperative management

Intraoperative clinical management is summarized in Table 3.2. In both treatment arms similar volumes of intravenous fluid were administered intraoperatively, with comparable volumes of both crystalloid and colloid solutions. Blood products were transfused in a comparably low proportion of patients in both treatment groups. Postoperative haemoglobin and arterial lactate (at the end of surgery), duration of surgery and mode of anaesthesia were similar for both control and goal directed therapy groups.

Table 3.2 Intraoperative clinical management

	Control n=26	GDT n=24
Duration of surgery (mins)	300 (134)	319 (84)
Mode of anaesthesia		
GA	14 (54%)	10 (42%)
GA + epidural	12 (46%)	14 (58%)
Intravenous fluids		
CSL (ml/kg)	54.7 (10.3)	54.2 (20.3)
Colloid (ml/kg)	16.4 (11.6)	12.3 (9.5)
Blood products		
PRBC transfusion	2 (7.7%)	3 (12.5%)
PRBC (ml/kg)	0.4 (1.3)	0.7 (2.1)
Haemoglobin (end of surgery, g/l)	10.3 (1.4)	10.5 (1.6)
Lactate (end of surgery, mmol/l)	2.05 (1.2)	2.47 (1.3)

Data are mean (SD) or n (%). GDT = goal directed therapy. GA = general anaesthesia. CSL = crystalloid solution. PRBC = packed red blood cell.

3.3.3 Postoperative haemodynamic therapy intervention

Differences between treatment arms in the postoperative intervention period are summarized in Table 3.3. The goal directed therapy group received significantly more colloid during the 6hr intervention ($p = 0.001$). The volume of crystalloid was similar, as defined by the maintenance fluid regime in the interventional protocol (Figure 4.1). The proportion of patients receiving blood transfusion and the volume of packed red blood cells in the goal directed therapy group was not significantly different from the control group. In the goal directed therapy group, 41.7% required dobutamine to achieve baseline oxygen delivery target, as per the haemodynamic therapy algorithm.

Table 3.3 Haemodynamic management during postoperative intervention period

	Control n=26	GDT n=24
Intravenous fluids		
CSL (ml/kg)	6.2 (0.7)	6.6 (1.1)
Colloid (ml/kg)	11.9 (8.2)	22.4 (9.6) [†]
Blood products		
PRBC transfusion	3 (11.5%)	5 (20.8%)
PRBC (ml/kg)	0.6 (1.6)	0.87 (1.8)
Dobutamine infusion	0 (0%)	10 (41.7%)

Data are mean (SD) or n (%). GDT = goal directed therapy. CSL = crystalloid solution. PRBC = packed red blood cell. [†] $p < 0.05$ between GDT and control groups

3.3.4 Longitudinal changes in perioperative thromboelastographic parameters and the effect of goal directed therapy

The pattern of alteration in TEG parameters in both treatment arms over each perioperative time point is depicted in Figure 3.1. R-time was reduced immediately postoperatively (POST) and post-intervention (POST6), compared to baseline preoperative TEG (PRE), in both groups. R-time remained significantly lower than baseline on postoperative day 5 (POD5) in the GDT group only. There were no significant inter-group differences at any perioperative time point. K-time was reduced immediately postoperatively (POST) and on POD2 in the control group but on POD5 only in the GDT group. K-time at POD 5 was significantly lower in the GDT group compared to the control group. The angle was increased immediately postoperatively in the control group and at POD5 in the GDT group, both compared to baseline and the control group. Maximum amplitude (MA) was outside normal range on POD2 and POD5 in both groups, but did not differ significantly compared to baseline preoperative normocoagulable values for either treatment arm. MA was reduced immediately post-intervention compared to preoperative baseline in the control group only.

The relative changes in TEG parameters at each postoperative time point were compared and expressed as a relative percentage of preoperative baseline values, as shown in Figure 3.2. Inter-group differences between control and GDT were demonstrated at POD5 only, with more pronounced reductions in K-time and increased angle in the GDT group.

The coagulation status was defined at each time point for each treatment group, based on normal reference ranges for each TEG parameter, as illustrated in Table 3.3. Both control and GDT groups met criteria for hypercoagulability at each postoperative time point with preoperative values showing baseline normocoagulability. In the early postoperative period, both groups demonstrated enzymatic hypercoagulability whereas at POD2 and

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POD5 both enzymatic and platelet hypercoagulability was seen in each group.

Surgical subgroups

Results were compared, within each treatment arm, depending on the type of surgery undertaken with subgroups for hepatic, pancreatic and other surgery. As illustrated in Table 3.1, the hepatic surgical subgroup was predominant in the GDT group whereas the pancreatic subgroup comprised the majority of the control treatment arm. The perioperative trend for each subgroup is shown in Figure 3.3. Inter-group differences were compared between each surgical subtype for each TEG parameter at all perioperative time points in each treatment arm. Differences were detected between the hepatic and pancreatic subgroups preoperatively with R-time in the GDT group and immediately post intervention with K-time and angle in the control group. The R-time at POD5 was reduced in the other surgical subgroup compared to pancreatic subgroup, in the control arm alone.

Comparison between native and heparinase TEG parameters

Coagulation changes in a subset of the cohort (n=31) were further characterised by measuring heparinase TEG parameters at the same perioperative time points, within each treatment arm. This further subset was limited due to resources, mainly availability of heparinase TEG cups. The treatment arm subset characteristics differed with the control group having a more varied surgical subgroup [hepatic (n=6); pancreatic (n=6), other (n=5)], whereas the GDT group was predominantly hepatic surgical patients [hepatic (n=11; pancreatic (n=1), other (n=1)]. Differences were demonstrated between native parameters, as indicated in Table 3.5. However, for the heparinase TEG to demonstrate any effect of exogenous or endogenous heparins, the heparinase value is lower than the corresponding native variable. Therefore, the only heparinase TEG parameter to show a 'heparin effect' with respect to native parameters was TEG angle immediately post intervention and on POD2 in the GDT group only.

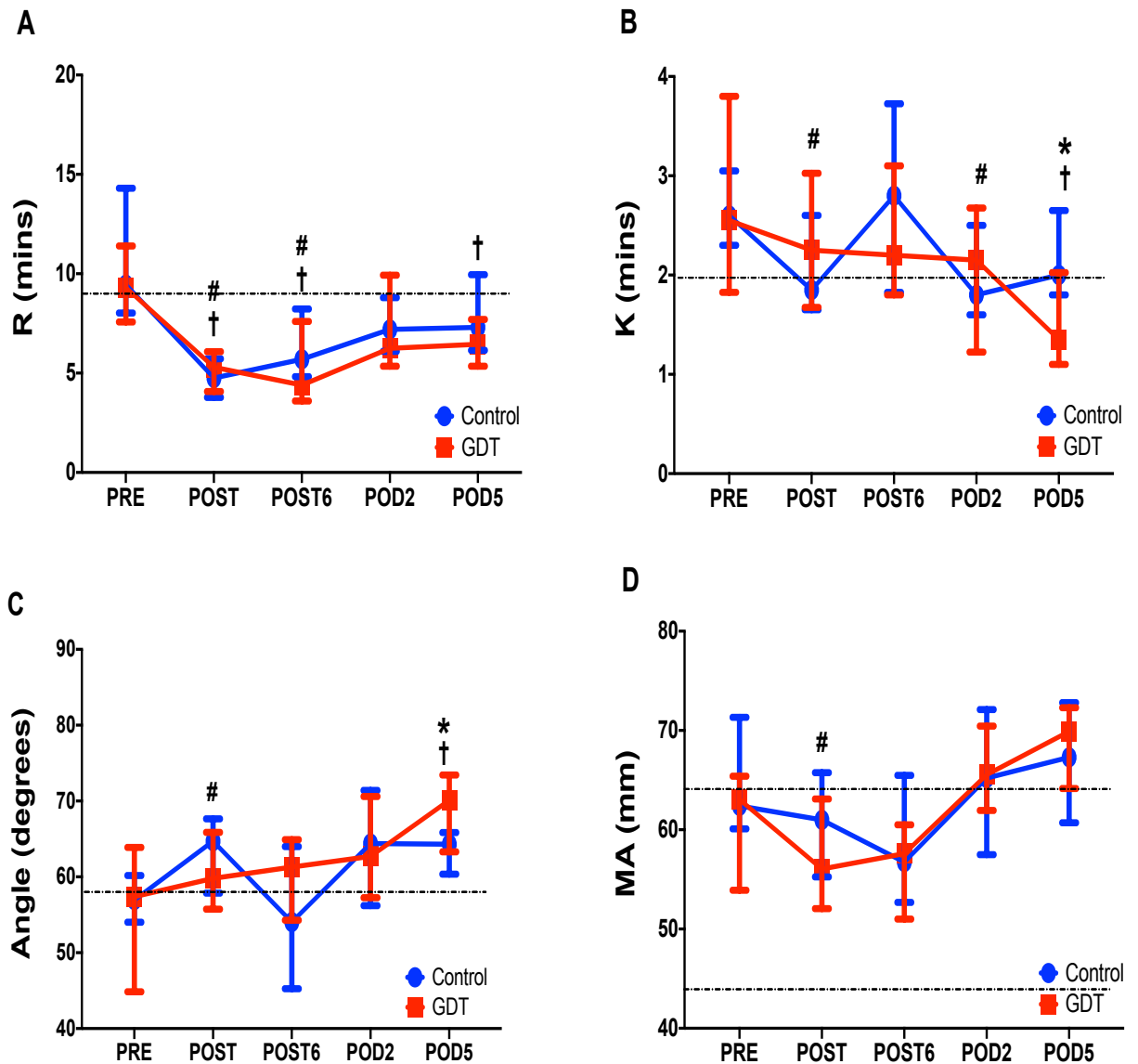


Figure 3.1 Longitudinal changes in TEG parameters following major intra-abdominal surgery in different haemodynamic intervention arms

Samples from both treatment arms, Control (n=26) and GDT (n=24), were collected at 5 Perioperative time points: PRE (immediately preoperatively); POST (immediately postoperatively); POST6 (following 6hr randomised postoperative haemodynamic treatment intervention); POD2 (postoperative day 2); POD5 (postoperative day 5).

Citrated native thromboelastography (TEG) parameters are shown: A. R-time. B. K-time C. Angle. D. Maximum amplitude (MA).

Dashed line demonstrates upper/lower limits of reference range for each TEG parameter (shown in text). Data are median (IQR).

p < 0.05 postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).

† p < 0.05 postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).

* p < 0.05 between Control and GDT treatment groups each perioperative time point (Mann Whitney test)

Table 3.4 TEG defined net effect on coagulation profile at each perioperative time point in control and goal directed therapy groups

	Control					GDT				
	R	K	Angle	MA		R	K	Angle	MA	
PRE	9.5 (8.0-14.3)	2.6 (2.3-3.1)	57.0 (54.0-60.2)	62.4 (60.1-71.3)	<i>Normo</i>	9.3 (7.6-11.4)	2.6 (1.8-3.8)	57.4 (44.9-63.9)	63.1 (53.9-65.4)	<i>Normo</i>
POST	4.8 (3.8-5.7)	1.9 (1.7-2.6)	64.7 (57.9-67.7)	61 (55.1-65.8)	<i>HyperE</i>	5.3 (4.1-6.1)	2.3 (1.7-3.0)	59.8 (55.7-65.9)	56.1 (52.1-63.1)	<i>HyperE</i>
POST6	5.7 (4.8-8.0)	2.8 (1.8-3.7)	54.1 (45.3-64.0)	56.75 (52.7-65.5)	<i>HyperE</i>	4.4 (3.6-7.6)	2.2 (1.8-3.1)	61.3 (54.3-64.9)	57.6 (51.0-60.5)	<i>HyperE</i>
POD2	7.2 (6.1-8.0)	1.8 (1.6-2.5)	64.4 (56.2-71.4)	65.2 (57.5-72.1)	<i>HyperEP</i>	6.3 (5.4-9.9)	2.2 (1.2-3.7)	62.7 (57.3-70.6)	65.6 (62.0-70.5)	<i>HyperEP</i>
POD5	7.3 (6.1-10.0)	2.0 (1.3-2.7)	64.3 (60.4-65.9)	67.3 (60.7-72.8)	<i>HyperEP</i>	6.5 (5.4-7.7)	1.4 (1.1-2.0)	70.1 (63.3-73.4)	69.9 (64.2-72.3)	<i>HyperEP</i>

Data are median (IQR). 5 perioperative time points: PRE (immediately preoperatively); POST (immediately postoperatively); POST6 (following 6hr randomised postoperative haemodynamic treatment intervention); POD2 (postoperative day 2); POD5 (postoperative day 5). Citrated native TEG parameters with normal ranges: R-time, 9-27 mins; K-time, 2-9 mins; Angle, 22-58 degrees; MA, 44-64 mm. Normo/HyperE/HyperP/HyperEP denotes net effect on coagulation profile: either normocoagulable or hypercoagulable based on TEG criteria for enzymatic (E), platelet (P) or combined enzymatic/platelet (EP) hypercoagulability

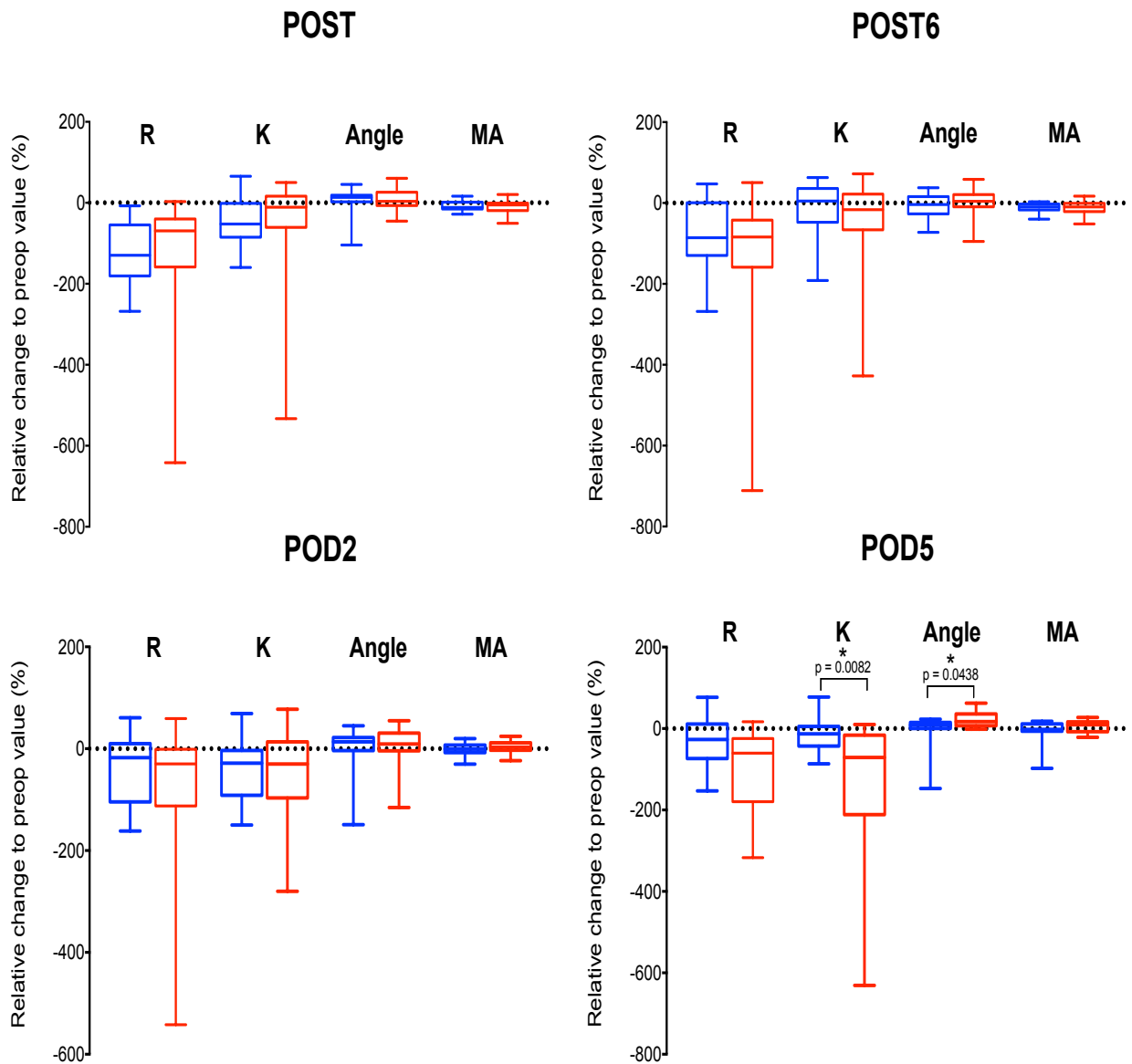


Figure 3.2 Relative percentage change in TEG parameters at each postoperative time point compared with baseline preoperative values

The relative percentage change at each postoperative time point in each treatment arm was compared to immediately preoperatively (PRE) for TEG parameters in each patient. POST (immediately postoperatively); POST6 (following 6hr randomised postoperative haemodynamic treatment intervention); POD2 (postoperative day 2); POD5 (postoperative day 5). Treatment arms were: Control (n=26, blue) or GDT (n=24, red). Citrated native thromboelastography (TEG) parameters are shown: R-time; K-time; Angle; Maximum amplitude (MA). Data are median (IQR and range shown).

*p < 0.05 (with p-values reported, by Mann-Whitney test, between Control and GDT treatment groups).

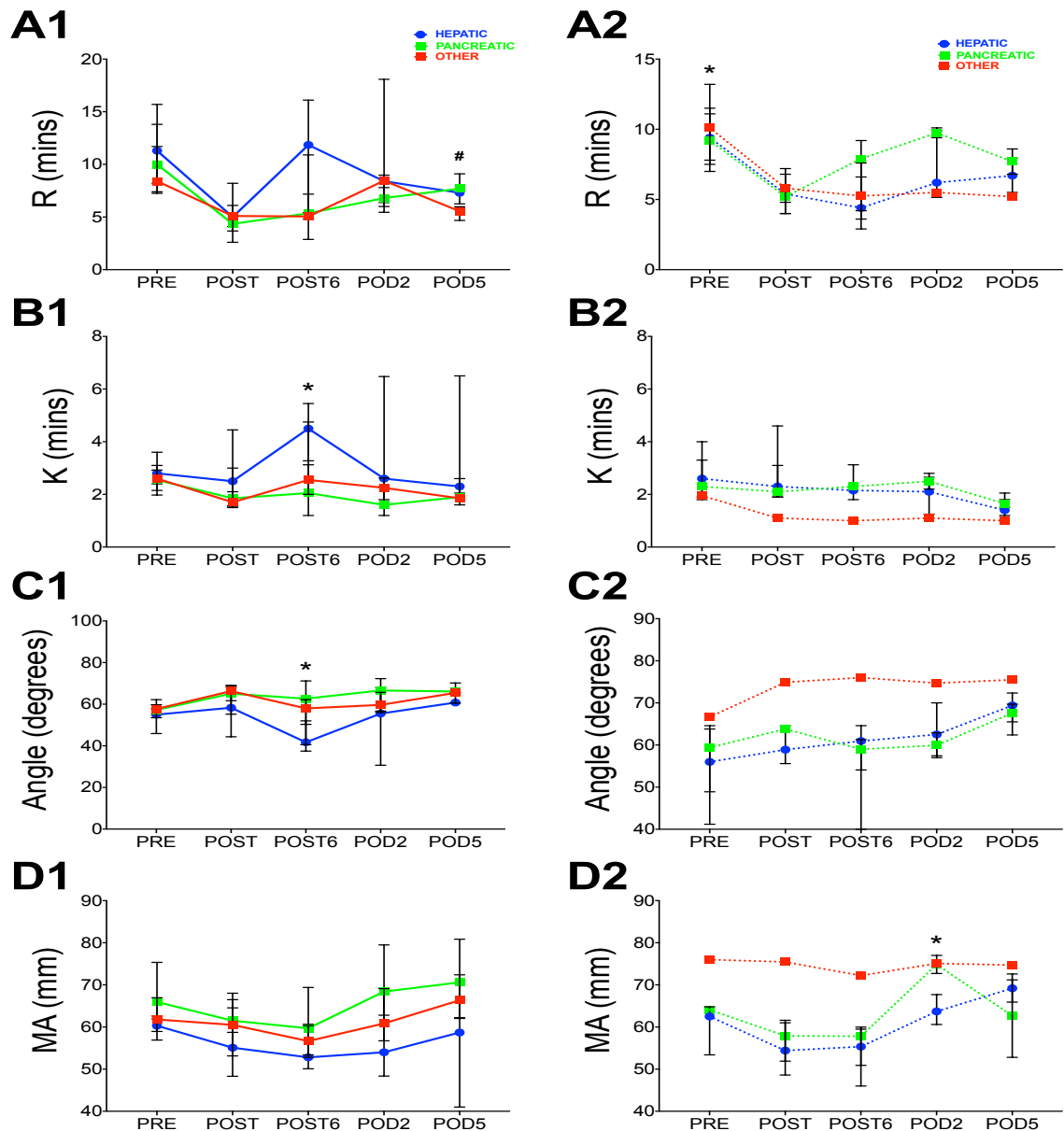


Figure 3.3 Longitudinal changes in TEG parameters following major intra-abdominal surgery in different surgical subgroups in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. Citrated native thromboelastography (TEG) parameters are shown: A1. R-time (Control). A2. R-time (GDT). B1. K-time (Control). B2. K-time (GDT). C1. Angle (Control). C2. Angle (GDT). D1. MA (Control). D2. MA (GDT)

Data are median (IQR, not presented for other surgical subgroup in GDT group).

Treatment arms: Control [n=26, solid lines with subgroups: hepatic (n=7); pancreatic (n=14); other (n=5)]; GDT [n=24, dotted lines with subgroups: hepatic (n=19); pancreatic (n=3); other (n=2)].

* p < 0.05 between hepatic and pancreatic surgical subgroup at given perioperative time point (Kruskal Wallis test with Dunn's multiple comparisons test)

p < 0.05 between pancreatic and other surgical subgroup at given perioperative time point (Kruskal Wallis test with Dunn's multiple comparisons test).

Table 3.5 Longitudinal changes in native and heparinase TEG parameters following major intra-abdominal surgery in different haemodynamic intervention arms

		Control (n=17)			GDT (n=14)		
		Native	Heparinase	p<0.05	Native	Heparinase	p<0.05
R	PRE	9.7 (6.2-16.8)	9.8 (6.20.5)	0.008	9.1 (5.9-28.2)	10.6 (4.8-26.6)	
	POST	4.9 (2.5-10.3)	5.5 (3.1-9.1)	0.01	4.8 (3.3-7.3)	6.3 (3.6-8.2)	0.001
	POST6	7.1 (2.5-21.8)	6.1 (2.5-16.6)		4.3 (2.3-7.8)	5.0 (2.6-8.6)	0.02
	POD2	7.2 (4.5-20.9)	7.4 (4.9-13.1)		5.8 (3.1-10.1)	6.7 (3.6-11.7)	0.02
	POD5	6.2 (4.4-8.6)	7.4 (4.9-8.6)	0.02	5.6 (4.6-8.6)	6.0 (5.1-9.4)	0.02
K	PRE	2.6 (1.9-5.4)	2.7 (1.9-5.0)		3.1 (1.1-7.2)	3.8 (1.3-9.6)	
	POST	1.8 (1.1-5.6)	2.0 (1.1-5.8)		2.3 (1.0-4.0)	1.8 (0.9-3.4)	
	POST6	3.4 (1.1-6.2)	2.2 (1.2-5.8)		2.1(0.8-9.3)	2.2 (0.8-10.2)	0.05
	POD2	1.7 (1.2-7.7)	1.8 (1.1-3.3)		2.0 (1.0-2.7)	2.2 (1.4-7.2)	0.02
	POD5	1.9 (1.5-10.0)	1.8 (1.6-7.5)		1.3 (1.0-2.4)	1.4 (0.9-2.8)	
Angle	PRE	57.6 (31.9-64.1)	56.4 (35.5-64.2)		53.0 (27.5-75.6)	47.4 (23.5-71.1)	
	POST	64.4 (37.4-74.7)	61.9 (37.2-77.0)		59.8 (45.2-77.1)	65.2 (49.6-76.0)	
	POST6	49.7 (34.3-75.0)	61.9 (39.6-71.2)	0.02	62.2 (32.1-79.0)	61.0 (30.9-77.4)	0.03*
	POD2	66.6 (23.2-75.5)	63.9 (47.8-76.0)		62.2 (32.1-79.0)	61.0 (29.8-70.1)	0.02*
	POD5	64.6 (47.5-68.6)	63.9 (20.3-68.1)		70.8 (59.5-75.5)	70.0 (55.1-76.0)	
MA	PRE	62.2 (51.0-81.4)	61.3 (48.8-82.8)		61.2 (49.6-82.4)	58.3 (45.1-78.5)	
	POST	68.5 (48.3-74.2)	58.3 (38.5-71.2)	0.04*	60.5 (45.1-79.6)	56.3 (47.7-79.8)	
	POST6	55.8 (43.1-76.1)	56.1 (38.2-73.1)		64.5 (49.4-77.0)	63.6 (50.3-73.6)	
	POD2	62.8 (46.7-80.5)	64.4 (50.3-80.3)		64.5 (49.4-77.0)	63.6 (50.3-73.6)	
	POD5	65.4 (30.5-74.1)	65.8 (34.8-76.1)		69.1 (59.8-74.7)	68.7 (57.1-72.4)	

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. Treatment arms were: Control (n=17) or GDT (n=14).

Data are median (range).

* p <0.05 between native and heparinase TEG parameters at each perioperative time point within each treatment arm (Wilcoxon matched pairs signed rank test): figures in bold* represent clinically relevant difference.

3.3.5 Longitudinal changes in conventional haematological variables and the effect of goal directed therapy

Haemoglobin concentration and haematocrit declined postoperatively and Remained significantly less than baseline up to and including POD5 (Figure 3.4), in both treatment groups. There were no differences between the control and GDT groups at any perioperative time point to suggest more haemodilution in the GDT group.

Platelet count was reduced in both treatment arms post-intervention and on POD2 in the GDT group but had returned to baseline by POD5 for the GDT group and by POD2 for the control group. Prothrombin time was elevated in both groups at every postoperative time point, except POD5 for the GDT group. There were no significant alterations in APTT except for a reduction seen immediately postoperatively in the control group only. Fibrinogen concentration was significantly reduced, in both treatment arms, until POD2. There was a difference between the control and GDT groups at both preoperative and postoperative time points until POD2.

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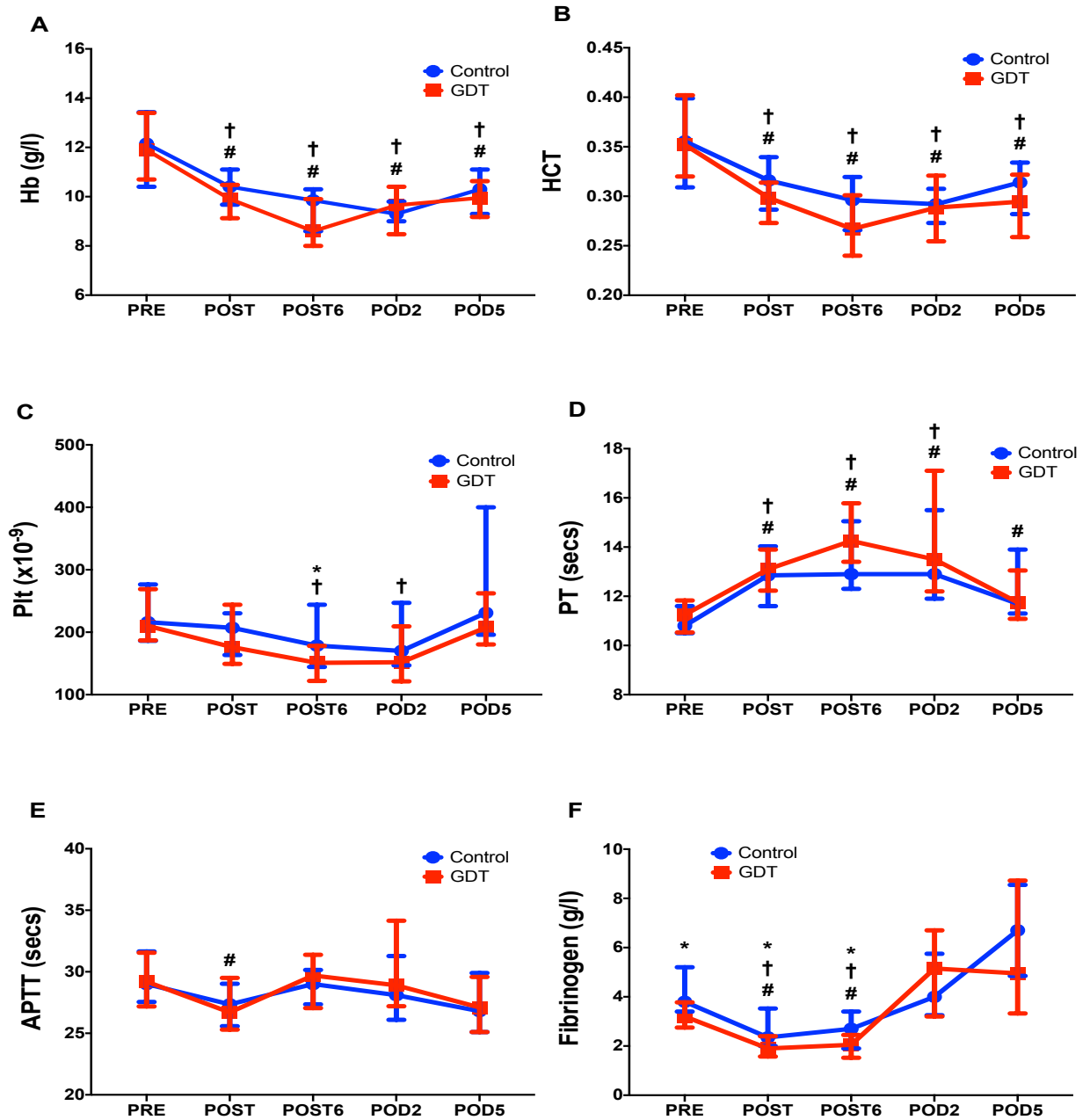


Figure 3.4 Longitudinal changes in routine haematological parameters following major intra-abdominal surgery in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. A. Haemoglobin (Hb). B. Haematocrit (HCT). C. Platelet count (Plt). D. Prothrombin time (PT). E. Activated thromboplastin time (APTT). F. Fibrinogen. Data are median (IQR).

$p < 0.05$ postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).

† $p < 0.05$ postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).

* $p < 0.05$ between Control and GDT treatment groups each perioperative time point (Mann Whitney test)

4.3.6 Longitudinal changes in specific coagulation biomarkers and individual clotting factors and the effect of goal directed therapy

Additional blood samples from 15 patients within the POM-O trial cohort had more detailed haematological assessment in a dedicated haemophilia research laboratory. Sample size was limited by funding for the additional laboratory expenses involved. This subset comprised patients randomised to postoperative interventions of both control (n=7) and GDT (n=8) treatment arms. However, it should be noted that within this subset, the control group consisted of 6 patients undergoing pancreatic resection (86%), whereas the GDT group had 7 patients having liver resection (87.5%), so the groups were not well matched in terms of surgical procedure.

D-dimer levels were increased from POD1 to POD5 in both treatment groups, with no inter-group difference detected (Figure 3.5). vWF antigen (vWF:Ag) was unchanged throughout the perioperative period in both groups.

Reductions in coagulation factors II, VII, X, XI, and XII, in comparison to baseline values, were demonstrated on POD1 in both groups, before returning to baseline (Figure 3.6). In the GDT group, reduced levels of factors II and X were shown immediately postoperatively to POD2 and immediately postoperatively for factor XII. In both groups, factor XIII:A was significantly reduced from POD1 to POD 5 in relation to preoperative levels. No significant difference between control and GDT groups was demonstrated for any coagulation factor at any time point.

Thrombin generation testing did not show any variation, in either LT (lag time i.e time needed for thrombin concentration to reach 1/6th of the peak concentration), endogenous thrombin potential (ETP) or slope (velocity index between end of lag time and peak thrombin generation), at any postoperative time point when compared to baseline preoperative values (Figure 3.7).

Furthermore, there was no difference in any parameter between the

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postoperative intervention groups.

The perioperative profile of the natural anticoagulants is shown in Figure 3.8. Antithrombin levels declined immediately postoperatively in both groups and remained reduced in the GDT group until POD5. Protein C reduced until POD2 and POD5 in the control and GDT groups respectively. There were similar reductions in Protein S with lower concentrations postoperatively until POD2 in both groups. There were no significant differences detected between treatment arms at each time point for any anticoagulant biomarker.

Selected biomarkers for endothelial injury were measured and presented in Figure 3.9. Syndecan-1 was significantly increased following the postoperative intervention in the GDT group with respect to both the control group and the baseline value within the GDT group. No differences were detected either between perioperative time points or between groups for soluble P-selectin, TFPI or thrombomodulin concentrations. Soluble tissue factor was reduced in the GDT groups on POD1 and POD2 but there was no demonstrable difference between the treatment arms at any time point

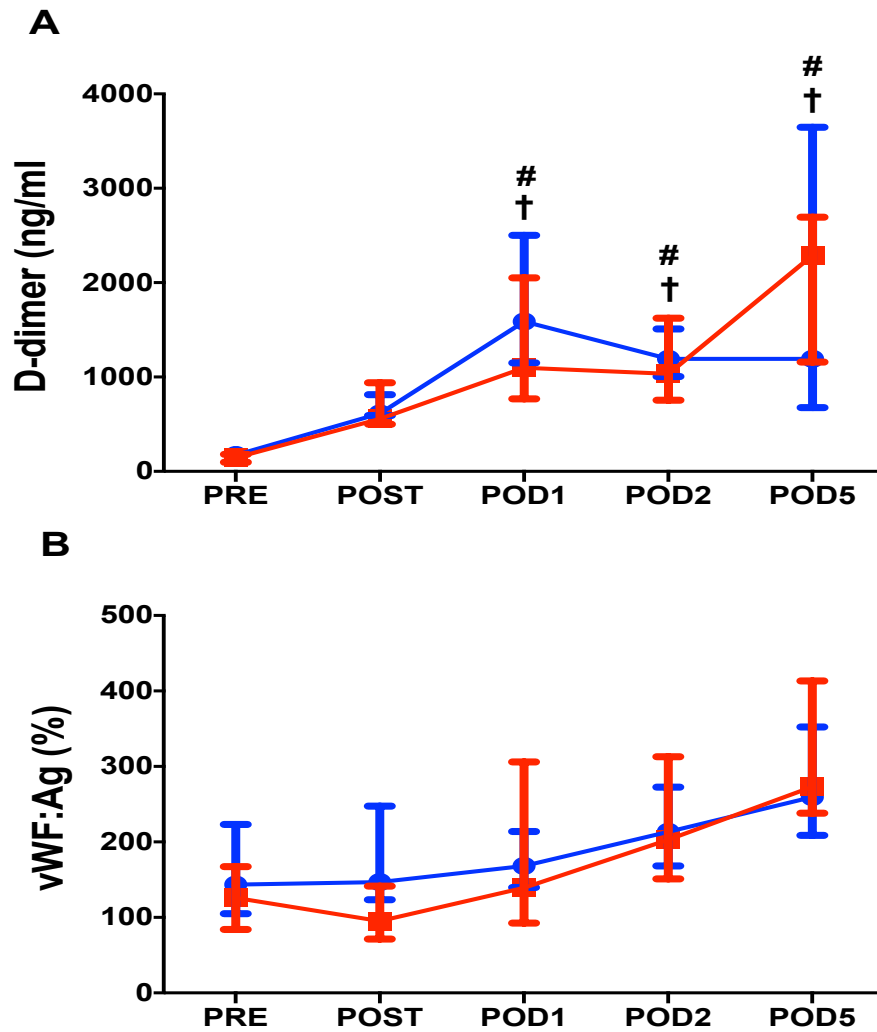


Figure 3.5 Longitudinal changes in D-dimer and von Willebrand factor antigen following major intra-abdominal surgery in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. A. D-dimer. B. von Willebrand factor antigen (vWF:Ag). Data are median (IQR).

$p < 0.05$ postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).

† $p < 0.05$ postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).

* $p < 0.05$ between Control and GDT treatment groups each perioperative time point (Mann Whitney test)

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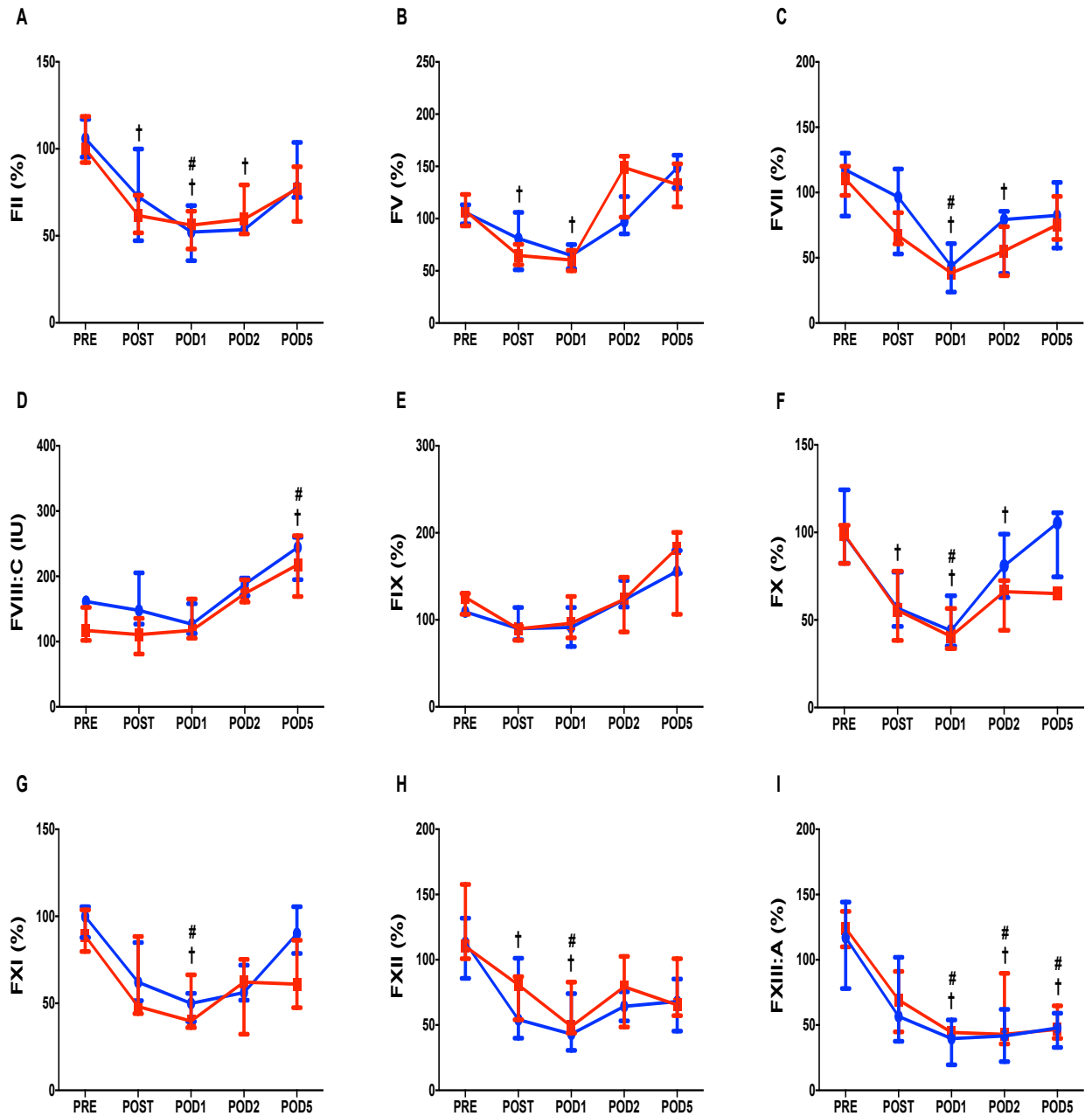


Figure 3.6 Longitudinal changes in individual clotting factors following major intra-abdominal surgery in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. A. Factor II (FII). B. FV. C. FVII. D. FVIII:C. E. FIX. F. FX. G. FXI. H. FXII. I. FXIII:A. Data are median (IQR).

p < 0.05 postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).

† p < 0.05 postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).

* p < 0.05 between Control and GDT treatment groups each perioperative time point (Mann Whitney test)

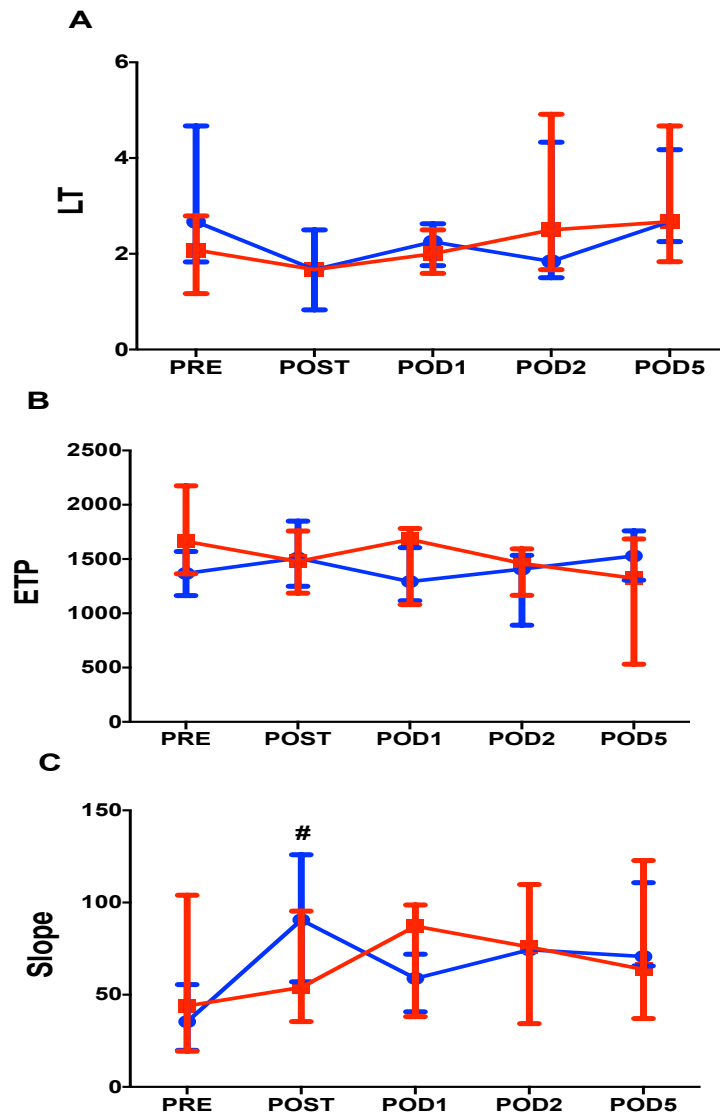


Figure 3.7 Longitudinal changes in thrombin generation assay variables following major intra-abdominal surgery in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. A. Lag time (LT). B. Endogenous thrombin potential (ETP). C. Slope. Data are median (IQR).

$p < 0.05$ postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).

† $p < 0.05$ postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).

* $p < 0.05$ between Control and GDT treatment groups each perioperative time point (Mann Whitney test).

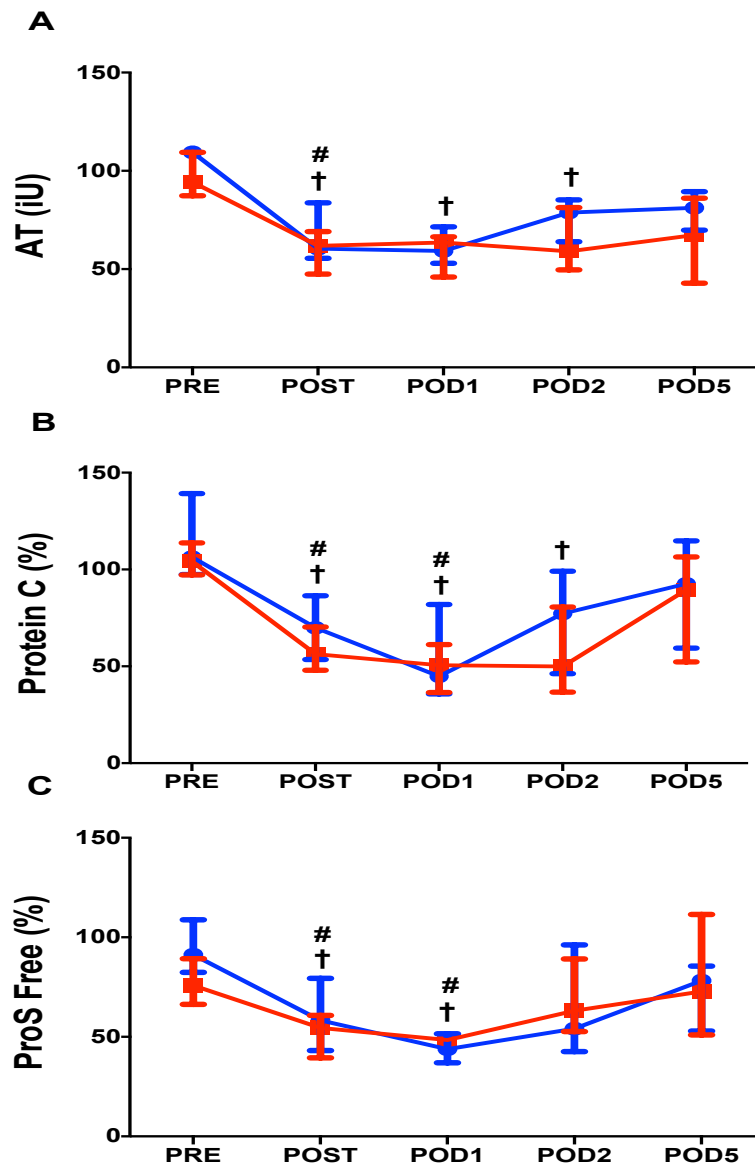


Figure 3.8 Longitudinal changes in natural anticoagulants following major intra-abdominal surgery in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. A. Antithrombin. B. Protein C. C. Protein S Free. Data are median (IQR).

$p < 0.05$ postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).

† $p < 0.05$ postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).

* $p < 0.05$ between Control and GDT treatment groups each perioperative time point (Mann Whitney test)

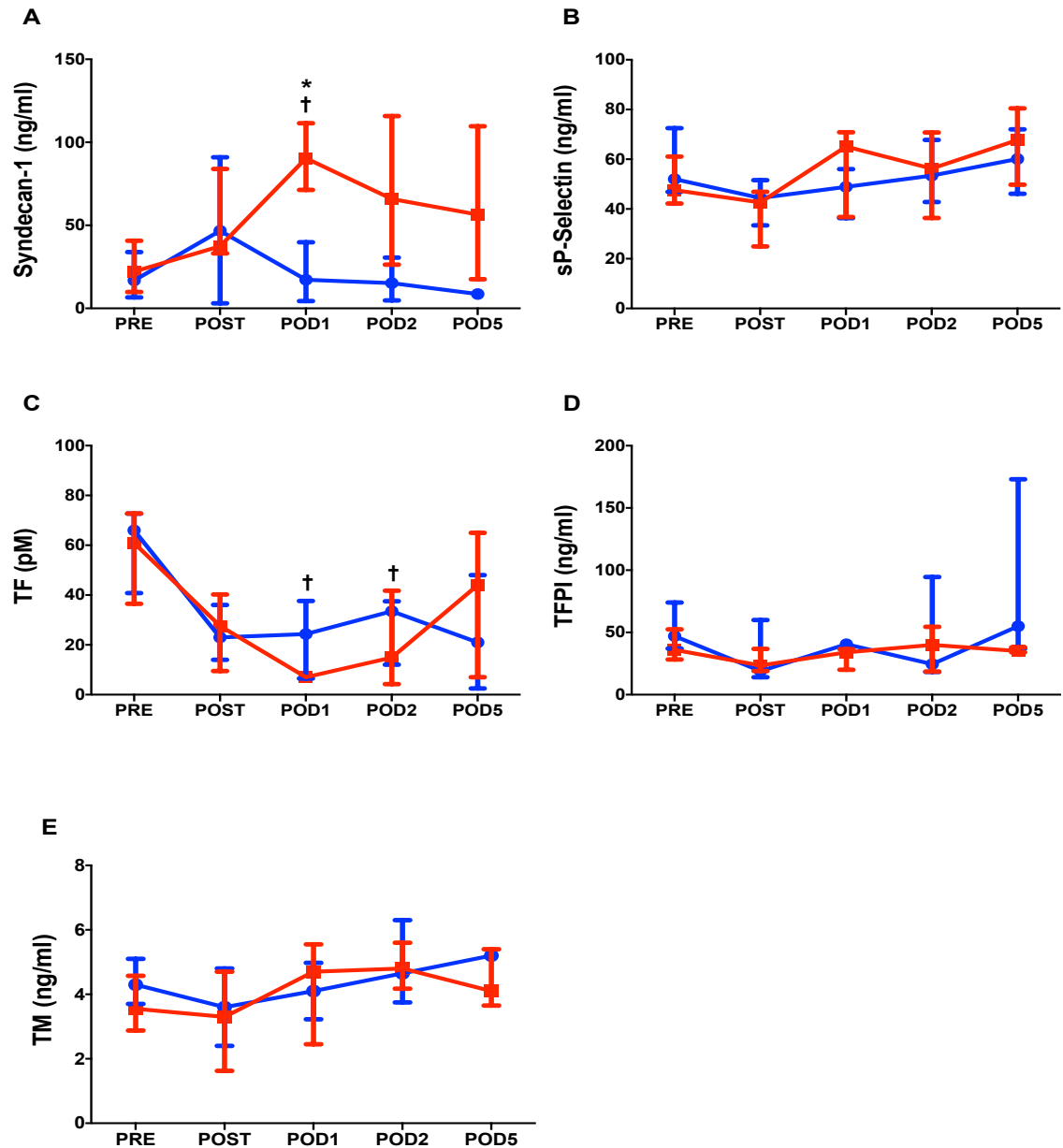


Figure 3.9 Longitudinal changes in selected biomarkers of endothelial injury following major intra-abdominal surgery in different haemodynamic intervention arms

Samples were collected at 5 perioperative time points: PRE; POST; POST6; POD2; POD5. A. Antithrombin. B. Protein C. C. Protein S Free. Data are median (IQR).
 # $p < 0.05$ postoperative time point vs. baseline (PRE) in Control group (Kruskal Wallis test with Dunn's multiple comparisons test).
 † $p < 0.05$ postoperative time point vs. baseline (PRE) in GDT group (Kruskal Wallis test with Dunn's multiple comparisons test).
 * $p < 0.05$ between Control and GDT treatment groups each perioperative time point (Mann Whitney test)

3.4 Discussion

3.4.1 Key findings

1. *Postoperative goal directed therapy was associated with administration of larger volumes of intravenous fluid in the immediate postoperative period.*

In the GDT treatment arm, the haemodynamic treatment algorithm used gelatin based colloid solution to optimise pre-defined haemodynamic variables. The excessive fluid administration shown was solely due to colloid administration, with no inter-group differences in the volume of crystalloid solution, blood or blood products. However, no difference between the control and GDT groups was demonstrated for haemoglobin or haematocrit to suggest significantly more haemodilution as a consequence of increased fluid administration.

2. *Thromboelastographic profiles seen postoperatively following major intra-abdominal surgery were similar for both control and goal directed therapy groups.*

Postoperative hypercoagulability was consistently demonstrated as per TEG criteria, with significant differences compared to baseline in the early postoperative period. GDT did not significantly affect either the absolute values of each TEG parameter or the relative change compared to control.

3. *In the hepatic surgical subgroup there was a trend towards return to baseline coagulation status immediately post treatment intervention in the control treatment arm.*

In determining the effect of surgical subtype on the alteration of coagulation status, the effect of GDT compared to control treatment was different in hepatic surgery subgroup as demonstrated by a transient relative return to baseline immediately post treatment intervention in K-time and angle in the control group, whereas in the GDT group, the corresponding values

GDT & perioperative coagulation following major surgery remained in the hypercoagulable range throughout the postoperative period.

4. *No consistent heparinoid effect is demonstrable at any perioperative time point.*

There was no clinically relevant difference between the native and heparinase thromboelastography variables in either treatment arm. Alteration of TEG angle postoperatively in the GDT group alone without corresponding changes in MA to signify modulation of hypercoagulability is difficult to consider as significant. There was a marked difference in the subset characteristics between the treatment groups in this subset so any effect potentially attributable to hepatic surgery, in terms of endogenous heparin and heparan sulfate proteoglycans, was not demonstrated in other TEG parameters. This subset comparison was not designed to assess the adequacy of thromboprophylaxis or indeed if low molecular weight heparin had been administered.

5. *Conventional coagulation tests (PT and APTT) failed to demonstrate postoperative hypercoagulability.*

Prothrombin time was actually prolonged postoperatively, in both treatment groups, until POD5, thereby masking the underlying hypercoagulable state. However, D-dimer was significantly elevated in both treatment groups from POD1 to POD5.

6. *Alterations in individual coagulation factors were consistent with similar previous studies.*

Factors II, VII, X, XI and XII levels were reduced, as reported in other surgical cohorts, in the immediate postoperative period only. Reductions in factor XIII:A were more prolonged, until POD5 in both treatment groups. The effect of goal directed therapy, not specifically examined in previous studies, was subtle with the GDT treatment arm associated with slightly prolonged reductions in factors II, VII and X, together with reductions in

GDT & perioperative coagulation following major surgery factor V, immediately postoperatively. However, no significant differences between treatment groups were demonstrated at the postoperative time points.

7. *Thrombin generation testing did not demonstrate postoperative hypercoagulability*

Thrombin generation variables were not significantly altered, in either treatment group, throughout the perioperative period. Thrombin generation testing was, however, performed in the absence of soluble thrombomodulin (TM) and not thrombomodulin-modified as in more recent studies.

8. *Goal directed therapy was associated with a higher degree of endothelial injury in the immediate post-intervention period*

Syndecan-1 levels were significantly higher in the GDT group following the postoperative intervention period, compared to the control group, indicating a relative increase of glycocalyx damage.

3.4.2 Importance of findings

This is the first study to fully characterise the effect of a goal directed therapy regimen on the coagulation profile in patients undergoing major surgery. The principal differences between the haemodynamic treatment algorithms were the volume of fluid, specifically colloid, administered and the use of dobutamine as vasopressor therapy in around 40% of the GDT group. The recent OPTIMISE trial (Pearse et al., 2014) similarly demonstrated extra colloid administration during a postoperative goal directed therapy intervention but other studies have produced conflicting results. This highlights the heterogeneity of goal directed therapy studies in terms of the haemodynamic treatment protocol used and the choice of fluid administered. POM-O used an individualised oxygen delivery target during the intervention period whereas other published studies have relied on population based values to determine

GDT & perioperative coagulation following major surgery
fluid responsiveness.

Both treatment groups were associated with a hypercoagulable profile in the postoperative period, as defined by thromboelastographic parameters outside the reference range, until at least postoperative day 5. The hypercoagulable values observed were reflective of both enzymatic and platelet hypercoagulability but the predominant mechanism was enzymatic. There were no consistent differences between the control and GDT groups in absolute TEG parameters to suggest that postoperative GDT significantly altered the pro-thrombotic postoperative profile. However, GDT was associated with an exacerbated hypercoagulable phenotype, with respect to relative changes compared to baseline, at postoperative day 5, in terms of TEG K-time and angle. This result should be interpreted with caution in the absence of further inter-group differences in TEG parameters.

A substantial finding from our results is that the goal directed therapy protocol used in this study was associated with promotion of endothelial damage. Syndecan-1, a transmembrane endothelial proteoglycan and constituent of the endothelial glycocalyx, was significantly elevated following the haemodynamic treatment intervention compared to the control group, indicating glycocalyx shedding as a consequence of this therapy.

The glycocalyx forms the endothelial surface layer, consisting of a variety of endothelial membrane-bound molecules, including glycoproteins and proteoglycans, that form a negatively charged barrier to circulating cells and macromolecules. It plays a key role in microvascular and endothelial physiology, by regulating microvascular tone and endothelial permeability, maintaining an oncotic gradient across the endothelial barrier, regulating adhesion/migration of leukocytes, and inhibiting intravascular thrombosis (Becker et al., 2010).

Destruction of the glycocalyx can lead to capillary leak, oedema, accelerated

GDT & perioperative coagulation following major surgery inflammation, platelet aggregation, hypercoagulability and loss of vascular responsiveness. Triggers of glycocalyx shedding include inflammation, trauma or hypervolaemia. Degradation occurs experimentally with pro-inflammatory mediators, such as $\text{TNF}\alpha$ (Chappell et al., 2009) and lipopolysaccharide (Marechal et al., 2008). High circulating syndecan-1 levels have been demonstrated in trauma patients, in correlation with elevated pro-inflammatory cytokines and biomarkers of coagulation activation, with associated increased mortality (Johansson et al., 2011). Hypervolaemia promotes glycocalyx shedding via the release of atrial natriuretic peptide (ANP) secondary to atrial stretch. Animal studies have demonstrated both elevated syndecan levels and increased extravasation of colloid following administration of ANP.

Our results demonstrate elevated syndecan-1 levels, with respect to both preoperative baseline and the control treatment group, immediately following goal directed therapy. The underlying mechanism cannot be confirmed but may be attributable to either relative excess fluid administration or a more pro-inflammatory phenotype in this treatment group.

In terms of defining the perioperative coagulation profile, postoperative hypercoagulability was not identified in either group by conventional coagulation testing or thrombin generation testing. Despite clearly defined hypercoagulability by TEG criteria in the postoperative phase, the changes seen in the laboratory parameters were confusing and often falsely interpreted by clinicians as being indicative of normo- or hypocoagulability. For example, using PT-INR values, in isolation, in the immediate postoperative period to determine whether pharmacological thromboprophylaxis can be administered risks failure to identify a hypercoagulable state. Thus if low molecular weight heparin is withheld on the basis of routine coagulation tests, a crucial window of opportunity for prevention of thromboembolic complications is being missed. Previous studies have highlighted the failure of routine laboratory parameters to detect the hypercoagulable state following major surgery (Lison et al. 2011), with our results confirming this finding. Particularly in the context

GDT & perioperative coagulation following major surgery of major hepatobiliary surgery, prothrombin time and activated partial thromboplastin time are shown to evaluate narrow aspects of haemostasis, rather than the overall haemostatic status.

Thrombin generation assays have been previously used to demonstrate normal or enhanced thrombin generation in the presence of prolonged PT and APTT, as seen in liver disease (Lisman et al., 2010). The total amount of thrombin generated during *in vitro* coagulation is measured in a global test, taking plasma concentrations of pro- and anticoagulants into account. However recent work has shown that the thrombomodulin modification of thrombin generation assays is required to identify the resultant hypercoagulability following liver surgery (Potze et al., 2015). Standard thrombin generation assays, as performed in our study, in the absence of thrombomodulin, do not detect hypercoagulability demonstrated by a combination of TEG parameters and alterations in individual pro- and anticoagulants.

The importance of identifying postoperative hypercoagulability has been highlighted by previous surgical cohort studies demonstrating an association between TEG-defined hypercoagulability in the perioperative phase and higher risk postoperative thromboembolic complications, including DVT/PTE, MI and ischaemic stroke (Kashuk et al., 2009; McCrath et al., 2005). Similarly, in a cardiac surgical cohort, hypercoagulability detected by similar criteria in patients undergoing coronary artery bypass grafting, has been shown to be associated with an adverse 30 day combined event rate of MI, stroke and mortality (Rafiq et al., 2012). The presence of hypercoagulability identified by TEG in these diverse surgical cohorts has therefore been shown to be predictive of postoperative thromboembolic events.

A multitude of similar studies have demonstrated the presence of hypercoagulability in the postoperative cohort, without demonstrating this association with adverse outcome, although largely in highly specific surgical

GDT & perioperative coagulation following major surgery subgroups (Bell et al., 1996; Bezeaud et al., 2007; Cerutti et al., 2004; Gibbs et al., 1992; Goobie et al.; Lindberg et al., 2000; Wilson et al., 2001).

Our study cohort reflects a high-risk surgical population having major intra-abdominal surgery. Similar studies have investigated hepatic surgery cohorts in isolation whereas we examined the coagulation changes in patients undergoing hepatic, pancreatic and other intra-abdominal oncological surgery together.

In comparing our study with cohorts linking postoperative hypercoagulability with adverse outcome, there are differences in how hypercoagulability has been defined. McGrath et al. used MA values alone and defined a dichotomous cut-off point between control and hypercoagulable groups as MA > 68mm. Kashuk et al. defined hypercoagulability as clot strength (G) >12.4 dynes/cm². The rationale for using the derived parameter G was suggested as a better reflection from a single parameter of the enzymatic and platelet components of haemostasis. Our study examined the differences seen in each standard measured TEG parameter to allow determination of the type of hypercoagulability, whether enzymatic, platelet or combined, to more accurately understand the pathogenesis of the resultant prothrombotic phenotype.

3.4.3 Strengths and limitations

This study is the first to specifically examine the effect of goal directed therapy on the coagulation profile rather than composite postoperative outcome measurements. Concurrent measurement of routine haematological parameters, thromboelastography, individual coagulation factors, natural anticoagulants and specific biomarkers of endothelial injury facilitated the most comprehensive evaluation of perioperative coagulation to date. In addition to confirming postoperative hypercoagulability, as per multiple previous studies, in both treatment groups, we have demonstrated that goal

directed therapy does not significantly modify the hypercoagulable state but may have potentiate endothelial injury, with increased glycocalyx damage.

If resources had not been limited, more detailed assessment of the inflammatory changes seen, with measurement of pro- and anti-inflammatory cytokines would have been useful. Furthermore, we were unable to measure microparticles in this sub-study, which would potentially have provided more mechanistic information.

The surgical cohort was largely comprised of hepatobiliary surgical patients with pre-existing cancer. Therefore, the confounding effect of underlying malignancy on the perioperative coagulation profile needs to be considered. Despite robust randomisation procedures in the main trial, our TEG study treatment groups were skewed with regard to the surgery undertaken, such that the goal directed therapy group was comprised of mostly liver resections whereas the control group have a higher proportion of pancreatic surgery. This difference was more pronounced in the subset undergoing more detailed haematological assessment. The effect of surgical subtype has been addressed in the previous chapter, but should be accounted for when interpreting these results.

Our study compared favourably in terms of cohort size to previously published studies on hypercoagulability in postoperative patients. The heterogeneity of the surgical cohort was also more noticeable than other similar studies, which largely concentrated on liver resection (Bezeaud et al. 2007, Cerutti et al. 2004). However, although the study used a comparatively large cohort, the sample size was determined pragmatically, by the number of patients recruited into the parent POM-O trial, with further sub-studies limited in number by restricted funding.

Our results are presented both as a total surgical population as well as within different surgical subgroups to examine potential differences between

changes seen in hepatic and non-hepatic surgical patients. Haemostatic balance after both liver resection and pancreatic surgery is unpredictable and so it is reasonable to consider these subgroups separately. During liver resection, in particular, conflicting influences exist between the removal of hepatic mass, impaired hepatic synthesis of clotting factors, vascular clamping and massive haemorrhage promoting hypocoagulability, whereas hypercoagulability can result from impaired hepatic synthesis of anticoagulants, extensive tissue trauma and haemodilution. Differential alteration in TEG parameters in liver surgery, compared to other major intra-abdominal surgery, was only demonstrated transiently in the control arm immediately after the haemodynamic intervention period. However, caution is needed in interpreting the surgical subgroup results given the strong preponderance of hepatic surgery patients in the GDT groups, making meaningful comparison within this treatment arm difficult.

TEG parameters were measured preoperatively, thus providing a baseline to quantify any changes seen postoperatively and also detect any presence of preoperative hypercoagulability. McGrath et al. did not measure preoperative values in their outcome study so it was not possible to guarantee that the postoperative hypercoagulable state was due to surgery itself or a pre-existing prothrombotic condition.

The length of postoperative TEG monitoring is one feasible limitation of our study. In detecting hypercoagulability up to postoperative day 5, we were unable to identify the time point at which there was a return to normocoagulability (or baseline value) in all surgical subgroups. Previous studies have detected postoperative hypercoagulability up to 7 days postoperatively following major intra-abdominal surgery (Mahla et al., 2001) and up to 6 weeks postoperatively following surgery for proximal femoral fracture (Wilson et al., 2001). Our study was not designed to follow up subjects beyond hospital discharge so TEG monitoring was therefore limited to within the short-term postoperative period.

Similarly, the objective of our study was not to investigate postoperative outcome, in terms of any association between postoperative hypercoagulability and thromboembolic complications in the short- and long-term postoperative phase. We limited our follow up to the time of hospital discharge and so data on any subsequent thromboembolic event was not collected.

3.4.4 Conclusion

Thromboelastography allowed the detection of the hypercoagulable state postoperatively, in the absence of any consistent changes in routine haematological parameters.

Postoperative hypercoagulability is not demonstrably modified by a goal directed haemodynamic therapy protocol although changes in individual components were seen. Endothelial injury is promoted by goal directed therapy, in the context of excessive fluid administration, in the immediate post-intervention period. Further work is required to assess the effect of different fluid regimens on both biomarkers of coagulation activation and endothelial injury, accounting for different underlying pathology and the type of surgery undertaken. This will allow more individualised assessment of thrombotic potential in patients undergoing major surgery and mitigate risk of thromboembolic complications.

Further work is required to delineate the association between TEG-defined postoperative hypercoagulability and incidence of postoperative thromboembolic complications such DVT/PTE, MI and ischaemic stroke. The duration of postoperative hypercoagulability following major intra-abdominal surgery needs to be further defined to allow for improvement of thromboprophylaxis and reduction of postoperative morbidity and mortality.

Chapter 4

Commonly used perioperative therapies and modulation of coagulation

4.1 Introduction

Postoperative hypercoagulability may manifest as thromboembolic complications, such as myocardial infarction, deep vein thrombosis and pulmonary thromboembolism, which are critical pathological events that increase morbidity in the perioperative period (Mantilla et al., 2002). Reducing the incidence of both venous and arterial thromboses are key goals of improving perioperative outcomes. However, preventing thrombosis, whilst mitigating the risk of bleeding, is a frequent perioperative conundrum (Acedillo et al., 2013; Korte et al., 2011). Although widespread introduction of pharmacological thromboprophylaxis regimens has significantly reduced the rates of thromboembolic complications in high-risk surgical populations (Agnelli, 2004), concern remains over bleeding complications in the context of both perioperative anticoagulant and anti-platelet administration. Low molecular weight heparins remain the commonest pharmacological thromboprophylaxis strategy used in surgical patients but may be associated with higher rates of postoperative bleeding complications in certain surgical specialties (Freedman et al., 2000). Similarly, perioperative administration of aspirin has been demonstrated to increase risk of major bleeding but has no significant effect on the rate of a composite of death or non-fatal myocardial infarction (Devereaux et al., 2014a). Therefore, challenges remain in reducing thrombotic risk, without exaggerating perceived bleeding risk, with existing pharmacological strategies.

Numerous perioperative therapies including statins, sympatholytics (β blockers and α_2 agonists), steroids, antioxidants and local anaesthetics have attracted attention, with postulated roles in reducing risk of arterial and venous thrombosis postoperatively. Inflammation-induced hypercoagulability may be modulated by these commonly administered drugs through off-target effects, although the precise mechanisms remain ill-defined.

Statin therapy has been recommended in high-risk surgical patients before

surgery, as preoperative therapy is associated with improved postoperative outcome (Dunkelgrun et al., 2009; Durazzo et al., 2004; Schouten et al., 2009; Warltier et al., 2006). More recently, attention has focused beyond the lipid-lowering effects of statins, with more understood about anti-inflammatory and anti-thrombotic properties. The 2009 JUPITER trial highlighted a potential anti-thrombotic effect, by demonstrating a significant reduction in deep vein thrombosis in normolipidaemic patients, with elevated CRP levels, treated with rosuvastatin (Glynn et al., 2009; Ridker et al., 2008). Furthermore, there is increasing evidence of an anti-inflammatory action, with inhibition of cytokine formation and adhesion molecule expression (Albert et al., 2005; Rezaie-Majd et al., 2002). Simvastatin has a powerful inhibitory effect on neutrophil recruitment, mediated by CXC chemokines (Zhang et al., 2011a). In experimental endotoxaemia, simvastatin has been shown to suppress the inflammatory response and attenuate both monocyte tissue factor expression and thrombin generation (Ferro et al., 2000; Steiner et al., 2005).

Perioperative β -blocker regimens have demonstrated a reduction of myocardial events in the perioperative period, although serious harmful effects, such as increased ischaemic stroke and non-cardiac mortality, have prevented widespread perioperative use in major non-cardiac surgery (POISE Study Group et al., 2008). The mechanism for preventing non-fatal myocardial infarctions is poorly understood. In addition to cardioprotection, β -blockers also restrain organ-specific inflammation during sepsis (Ackland et al., 2010). One plausible mechanism for modulation of hypercoagulability centres on catecholamines that are endogenously synthesized by immune cells, independently of the autonomic nervous system. Phagocyte and lymphocyte-derived catecholamines act in an autocrine/paracrine fashion, modulating lymphocyte trafficking, cell proliferation, cytokine production and the functional activity of leukocytes, independently of endothelial interactions (Flierl et al. 2007). Thus, activation of the adrenergic system during an inflammatory response greatly enhances the local inflammatory response and hence potentially contributes to a prothrombotic, hypercoagulable state.

Central sympatholytics such as clonidine, an α_2 agonist used commonly perioperatively, have been demonstrated to suppress acute inflammation with reduction of proinflammatory cytokines seen in both experimental models (Hofer et al., 2009) and in clinical cohorts (Kim and Hahn, 2000). Although smaller initial trials suggested clonidine reduces perioperative MI (Wallace and Wallace, 2006), the larger more recent POISE-2 trial (Devereaux et al. 2014) had conflicting results. Although it has been postulated that clonidine may modify perioperative coagulation, attenuation of the hypercoagulable response to the surgical stimulus has hitherto not been demonstrated (Ganter et al., 2005).

Beyond the well-established anti-inflammatory properties of steroid use, the relationship between steroids and perioperative coagulation remains controversial. Inhibition of platelet aggregation by dexamethasone has been demonstrated in animal models (van Giezen et al., 1994), while in human studies, glucocorticosteroids were historically found to significantly decrease the activated partial thromboplastin time, fibrinogen, and plasminogen concentrations, even after 2 days of treatment (Jørgensen et al., 1982). Administration of high dose dexamethasone in healthy volunteers has been shown to increase the concentration of von Willebrand factor (vWF) and soluble P-selectin, thus promoting coagulation (Jilma et al., 2005). Prolonged preoperative steroid therapy is not associated with either an increased bleeding tendency or an altered risk of thromboembolic complications to suggest manifested changes in coagulation status (Turan et al., 2010).

N-acetylcysteine (NAC) is a thiol-containing free radical scavenger, with antioxidant, anti-inflammatory and microcirculatory effects (Aitio, 2006). NAC administration is commonplace in the treatment of paracetamol overdose, prevention of acute kidney injury following contrast exposure (Birck et al., 2003) and major cardiovascular surgery (Macedo et al., 2006) and as a hepatoprotective agent, in improving graft function in orthotopic liver

transplantation (Hilmi et al., 2010; Thies et al., 1998), with equivocal results. Furthermore, it has been extensively investigated in sepsis, both clinically and in experimental models, with conflicting results (Emet et al. 2004; Parmentier et al. 2000). Previous studies on the effect of NAC on haemostatic parameters in healthy subjects have demonstrated reduction of both vitamin K dependent coagulant and anticoagulant proteins *in vivo* (Pizon et al. 2011), and elevation of prothrombin time *in vitro* (Knudsen et al., 2005). In surgical patients undergoing abdominal aortic aneurysm surgery, NAC administration was associated with a decreased PT value, prolonged coagulation time on thromboelastometry and attenuated platelet aggregation, with no significant effect on blood loss (Niemi et al., 2006). However, the exact mechanism by which NAC interferes with haemostasis remains undefined.

Local anaesthetics have similarly been reported to have anti-inflammatory and anti-thrombotic properties. Lidocaine has been shown to reduce inflammation following experimental sepsis (Gallos et al., 2004; Schmidt et al., 1997). Furthermore, infusion of lidocaine perioperatively reduces systemic cytokine levels (Herroeder et al., 2007). Bupivocaine has been demonstrated to reduce clot strength, as assessed by maximum amplitude on thromboelastography, at clinically relevant concentrations (Kohrs et al., 1999; Leonard et al., 2000).

Magnesium sulphate (MgSO_4) has been demonstrated to prevent arterial thrombus formation in a rat model (Mussoni et al., 2001), in addition to attenuating haemodilution-induced hypercoagulability in human healthy volunteers (Ruttmann et al. 2007). Recently, intraoperative MgSO_4 infusion to maintain Mg levels at the upper limit of normal range, has been shown to attenuate postoperative hypercoagulability in gynaecological patients undergoing pelviscopic surgery, as assessed by rotational thromboelastometry (Na et al., 2012).

To investigate the effect of these commonly used perioperative drugs on

coagulation, an experimental model was used to allow any effect of pre-treatment on human whole blood, in a relative hypercoagulable state, to be quantified *ex vivo*. Lipopolysaccharide (LPS) is used commonly as a laboratory model for inflammation and sepsis *in vivo* and *in vitro*, reproducing the inflammatory stimulus of a septic, or in the case of this study, surgical insult. LPS induced activation of coagulation has been robustly demonstrated, with thromboelastography shown to be a suitable monitoring tool (Koch et al., 2009b; Spiel et al., 2006; Zacharowski et al., 2006). This experimental protocol has been described in detail in Chapter 2. In summary, the effect of pre-treatment with selected therapies, with postulated thrombomodulatory potential, prior to stimulation or ‘spiking’ of whole blood with LPS *ex vivo*, was assessed by quantifying any modifying effect on TEG parameters following a pre-determined incubation period.

4.1.1 Hypothesis

Lipopolysaccharide administered to whole blood results in activation of coagulation producing hypercoagulability, as assessed by thromboelastography.

Selected perioperative therapies with putative anti-thrombotic activity attenuate the promotion of hypercoagulability by LPS.

4.1.2 Aims

The hypothesis was addressed by characterising the coagulation changes in whole blood, taken from both healthy volunteers and preoperative surgical patients, following the addition of LPS (model for inflammatory stimulus) and selected perioperative therapies.

Primary outcome

TEG (citrated native TEG) parameters following treatment with selected perioperative therapies, compared to control, in whole blood incubated with either LPS or endotoxin-free saline.

4.2 Methods

4.2.1. Study population

Following ethics committee approval and informed consent, venous blood samples were taken from healthy, drug-naïve volunteers at the Royal Free Hospital. Further blood samples were taken from surgical patients recruited to the POM-O (Post Operative Morbidity Oxygen) trial. Both healthy volunteers and preoperative surgical patients were used in the initial stages to assess the whole blood TEG response to LPS (100ng/ml dose only), to maximise our sample size. However, in determining the thrombomodulatory potential of selected drugs, only healthy volunteers were included in this experimental paradigm, as including both volunteers and surgical patients in the same experimental group was not considered appropriate. Recruitment and exclusion criteria have been described in detail in the Methods section.

4.2.2 Study protocol

Blood samples were obtained in a standardised manner from both healthy volunteers and preoperative surgical patients, as previously outlined. Following collection into Vacutainer tubes containing sodium citrate 3.8% (Becton Dickinson, Plymouth, UK), samples were gently inverted to ensure mixing of sample and then transferred in 1ml aliquots to polypropylene tubes. In LPS treated samples, 1ml blood was incubated at 37°C with 100ng/ml LPS (*Salmonella typhus*) or endotoxin-free sterile 0.9% NaCl for 4 hours. In drug treated samples, the following drugs were added to volunteer whole blood 20 mins prior to incubation with LPS or saline: dexamethasone (40ng/ml), metoprolol (100ng/ml), atenolol (500ng/ml), carvedilol (100ng/ml), clonidine (75ng/ml), simvastatin (10ng/ml), N-acetylcysteine (500ng/ml), lidocaine (5ng/ml) or magnesium sulphate (2mM). Different sample sizes were produced with different candidate drugs due mainly to drug availability and resources. In determining the effect of pre-treatment with β -blockers,

metoprolol was used as a β_1 adrenoceptor antagonist control within the other β -blocker studies, to account for inter-individual genetic variation in adrenergic receptor signalling, hence producing different sized treatment groups.

4.2.3 Simvastatin sub-study

To further elucidate the proposed mechanism of action by simvastatin, an additional drug was studied, namely the CXCR2 antagonist, SB265610 (1 μ M). This experimental drug was added in the same manner as the selected perioperative drugs prior to incubation with LPS or saline. The aim of this sub-study was to investigate if any anti-thrombotic effect seen by simvastatin on TEG could be recapitulated by blocking the CXC chemokine pathway.

Simvastatin reduces neutrophil infiltration most likely through reduced release of CXC chemokines, in animal models (Zhang et al. 2006, Zhang et al. 2010). Neutrophil depletion reverses *in vivo* experimental thrombosis (von Bruhl et al. 2012).

4.2.4 Thromboelastography

Thromboelastographic assays were performed immediately after the incubation period and following recalcification of incubated citrated blood, as described in Chapter 2. Citrated native TEG parameters were measured: R-time (reaction time, reference range 2-27 min); K-time (reference range 2-9 min); angle (α , reference range 22-58 degrees); maximum amplitude (MA, reference range 44-64 mm). Each TEG parameter and the terminology of hypercoagulability have been previously defined in the Methods section.

4.2.5 Statistical analysis

Data are expressed as means (with SD) or medians (with interquartile ranges) as appropriate. In data sets fewer than 5 subjects, individual raw data points

are presented. The normality of continuous data was assessed using the D'Agostino & Pearson normality test. Differences between paired treatment groups were compared using a 2-tailed paired t-test or Wilcoxon matched-pairs signed rank test for paired groups for parametric and non-parametric data respectively. Differences between unmatched multiple treatment groups were compared using ordinary one-way ANOVA (with Dunnett's multiple comparison test) or Kruskal Wallis test (with Dunn's multiple comparison test) for parametric and non-parametric data sets, as appropriate. Statistical significance was set at $p < 0.05$. GraphPad Prism 8 (San Diego, CA, USA) was used for statistical analysis.

Sample size calculations

We powered the primary outcome (TEG R-time) on the basis that potential anti-thrombotic therapies would reverse the LPS-induced decrease in R-time by 50%. Previous work described ~60% reduction in clotting time CT (R-time equivalent) following LPS incubation (Koch et al. 2009). Thus, for selected drugs to attenuate pre-defined LPS-induced hypercoagulability, at least 6 subjects per experimental comparison would be required to detect this R-time difference at the $\alpha=0.05$ level (80% power).

4.3 Results

4.3.1 Effect of lipopolysaccharide on whole blood coagulation

Lipopolysaccharide at test concentration 100ng/ml produced hypercoagulable changes in all TEG parameters (Figure 4.1). The relative change seen in each TEG parameter following LPS 100ng/ml was calculated with respect to control, with healthy volunteer (n=61) and surgical patient (n=16) samples producing similar responses, in terms of magnitude of LPS stimulated hypercoagulability (Figure 4.2).

Similar effects were evoked with reduced concentrations of LPS of 10ng/ml, except for the maximum amplitude which was not significantly increased at the lower concentration (Figure 4.3).

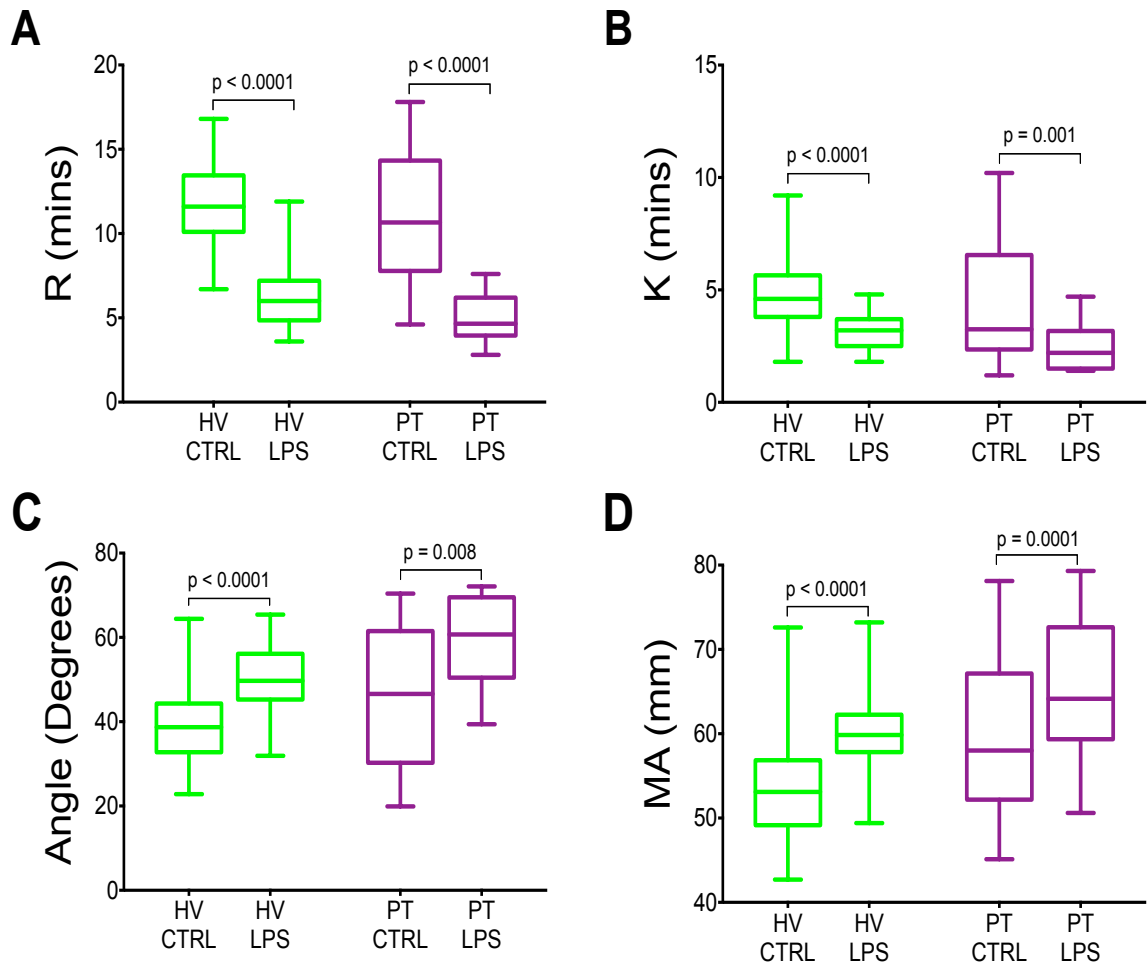


Figure 4.1 TEG parameters in whole blood from healthy volunteers and preoperative surgical patients incubated with LPS (100ng/ml) for 4 hrs

Samples from both healthy volunteers (HV; n=61) and preoperative surgical patients (PT; n=16) were incubated with either LPS (100ng/ml) or sterile saline (CTRL) to produce a 2 paired experimental groups. Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). p-values reported, by Wilcoxon matched-pairs signed rank test, CTRL vs. LPS.

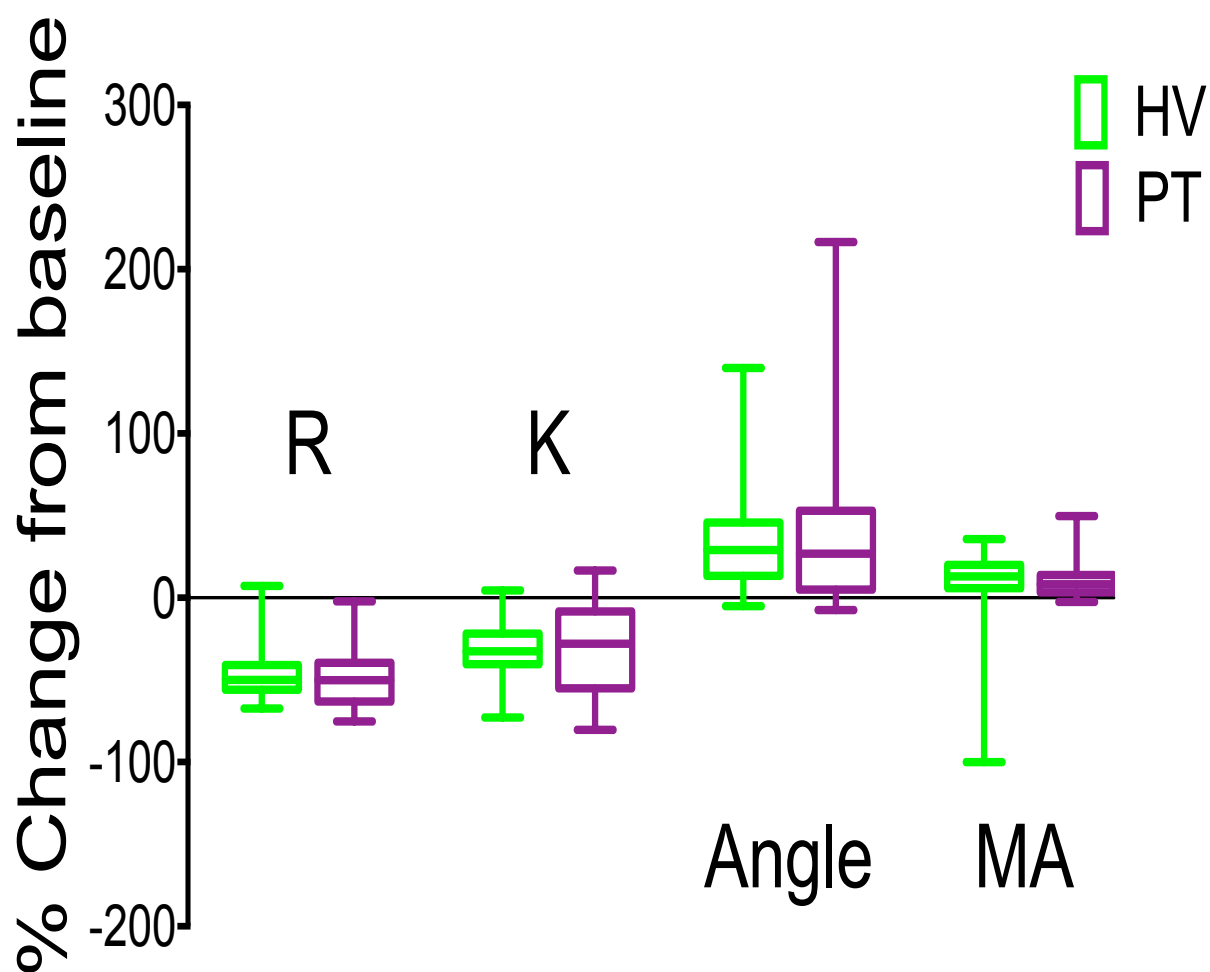


Figure 4.2 Comparison of LPS-induced TEG changes in healthy volunteers and preoperative surgical patients

Relative (%) LPS-induced changes of TEG parameters with respect to control were calculated for both healthy volunteers (HV; n=61) and preoperative surgical patients (PT; n=16), following incubation with LPS 100ng/ml. Data are median (IQR and range shown). * $p < 0.05$ HV vs. PT (Mann-Whitney test).

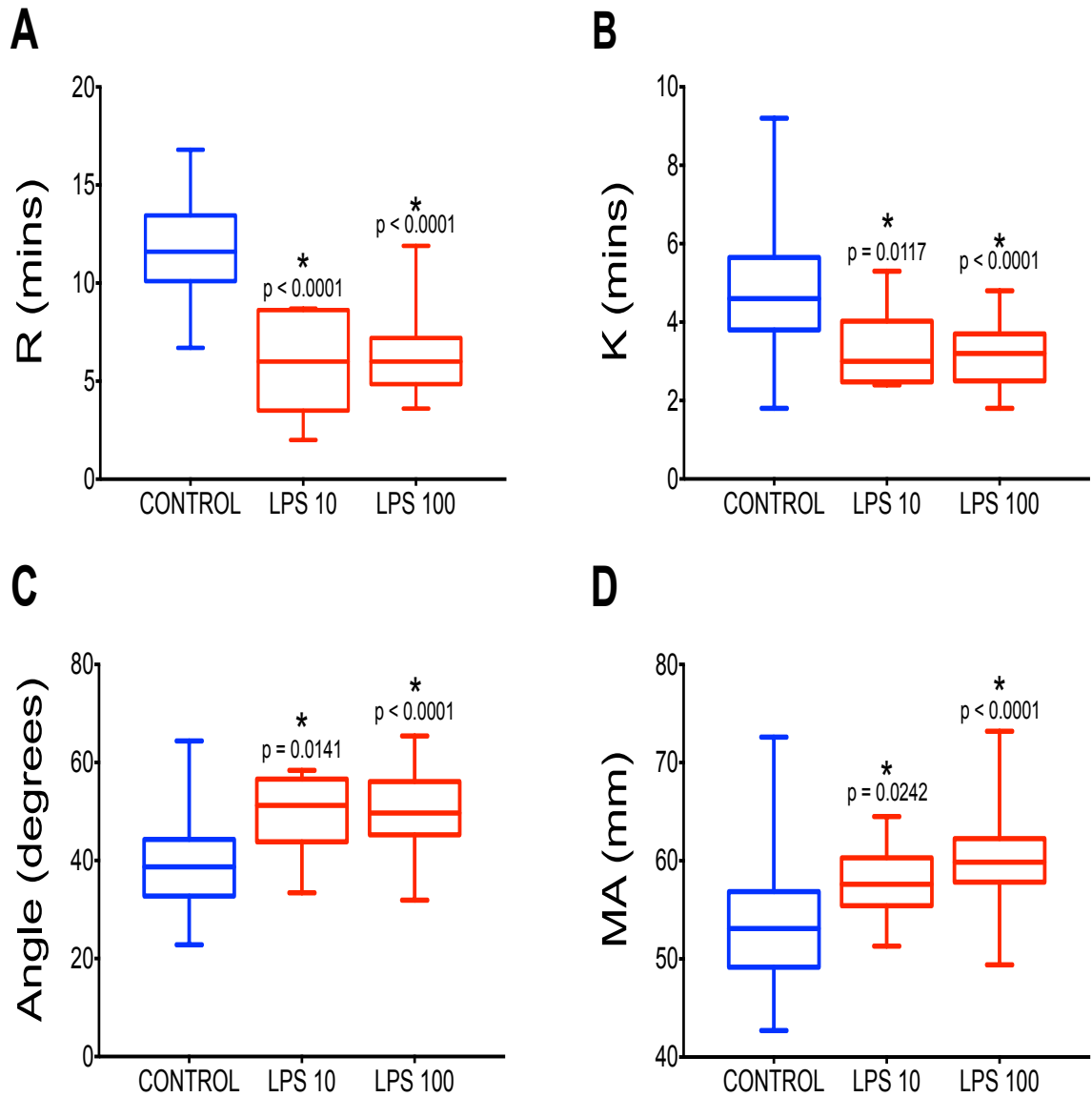


Figure 4.3 TEG parameters in whole blood incubated with different concentrations of LPS for 4 hrs

Samples from healthy volunteers were incubated with LPS 10ng/ml (n=6), LPS 100ng/ml (n=61) or sterile saline (CONTROL, n=77). Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). * p < 0.05 CONTROL vs. LPS at given concentration (Mann-Whitney test).

4.3.2 Effect of simvastatin on LPS-induced hypercoagulability

Pre-treatment with simvastatin (10ng/ml) attenuated the hypercoagulable effect following LPS incubation with relative increases in R-time and K-time, and relative reduction in angle, with respect to LPS alone (Figure 4.4). This effect related to enzymatic hypercoagulability rather than platelet hypercoagulability, as there was no effect on MA relative to LPS alone. There was no attenuation of LPS-induced hypercoagulability in any TEG parameter with simvastatin pre-treatment at higher concentration [25ng/ml] (Figure 4.5).

The effect of post-treatment with simvastatin (10ng/ml), 20 minutes after addition of LPS, was also investigated, to compare with the effect of pre-treatment. However, post-treatment did not attenuate LPS-induced hypercoagulability and conversely produced reduction in K-time and increase in MA, with respect to LPS alone (Figure 4.5).

In the simvastatin sub-study, pre-treatment with the CXCR2 antagonist SB265610 produced a similar effect to simvastatin on enzymatic hypercoagulability, with attenuation of the LPS-stimulated reduction in R-time while other TEG parameters were unaffected (Figure 4.6).

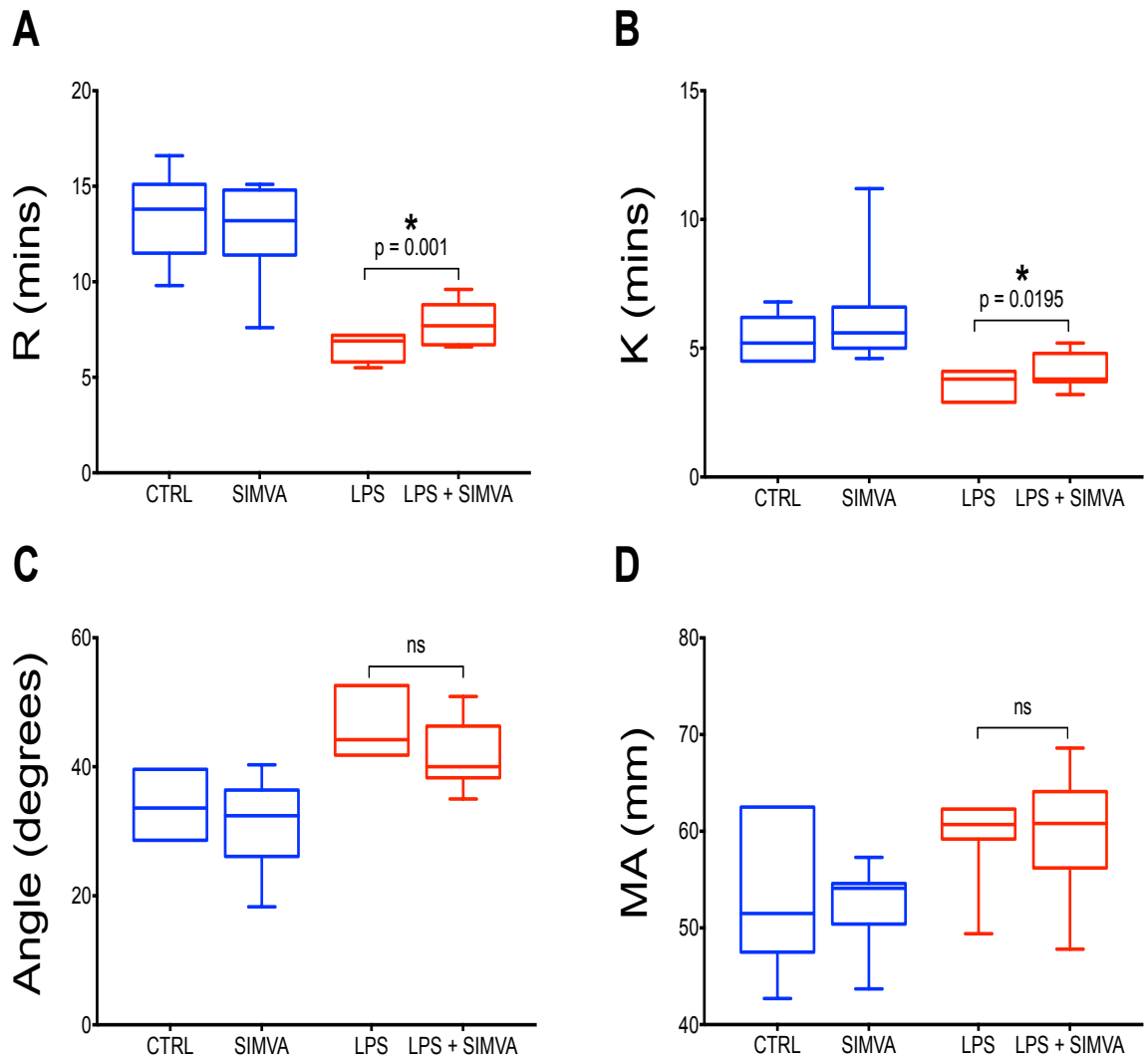


Figure 4.4 TEG parameters in whole blood pre-treated with simvastatin (10ng/ml) prior to incubation with LPS

Samples from healthy volunteers were pre-treated with simvastatin (10ng/ml) prior to incubation with LPS (100ng/ml) for 4hr (n=11). Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). p-values reported, by Wilcoxon matched-pairs signed rank test, LPS vs. LPS + simvastatin (SIMVA).

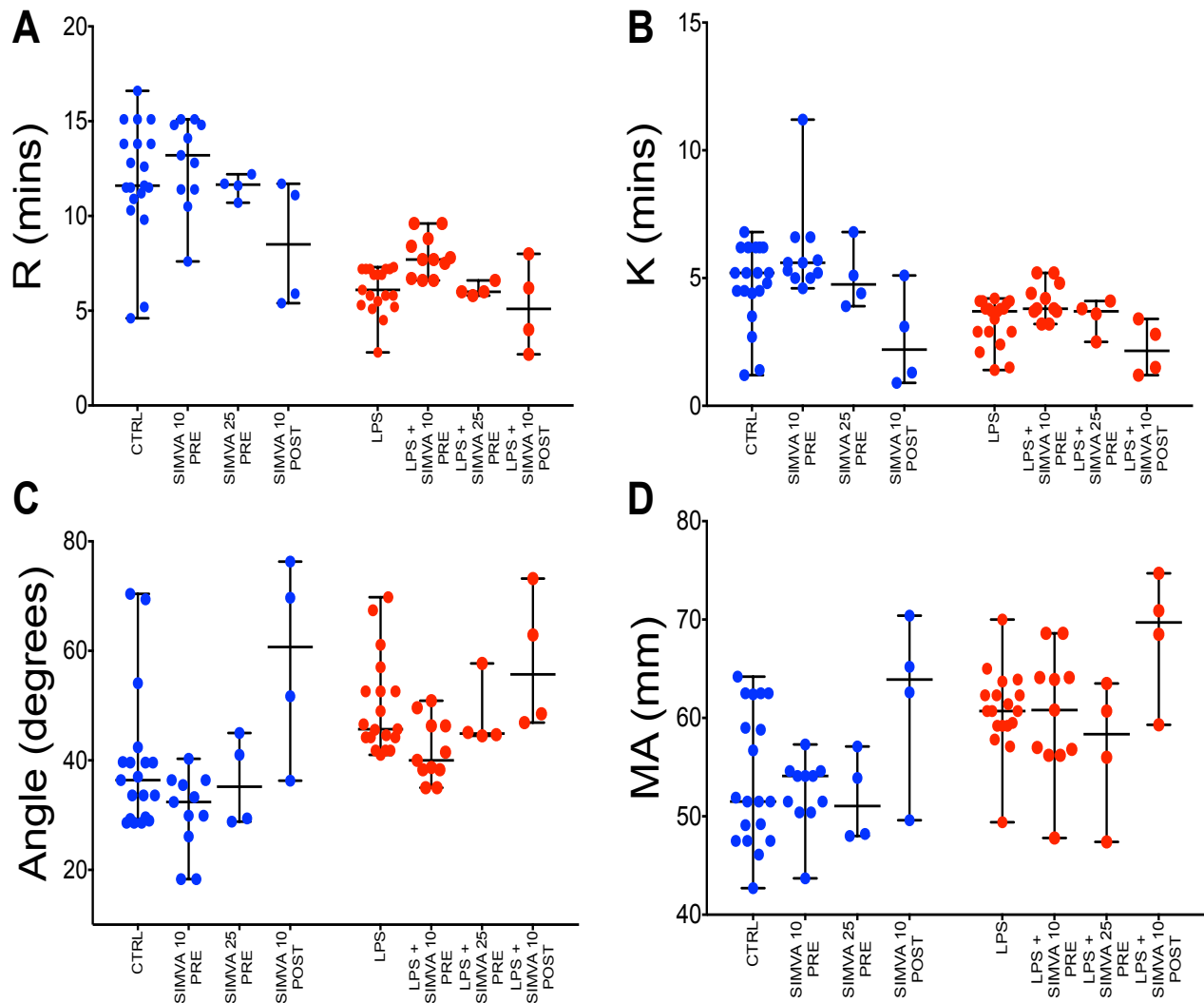


Figure 4.5 TEG parameters in whole blood either pre- or post-treated with simvastatin in relation the addition of LPS

Samples from healthy volunteers were either pre-treated with sterile saline (CTRL, n=19), simvastatin 10ng/ml (n=11); pre-treated with simvastatin 25ng/ml (n=4); post-treated with simvastatin 10ng/ml (n=4), in relation to addition of LPS followed by incubation for 4hr. Data are presented as individual raw data points with median and IQR shown. Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). * $p < 0.05$ LPS vs. LPS + simvastatin (SIMVA) at given concentration (Mann-Whitney test).

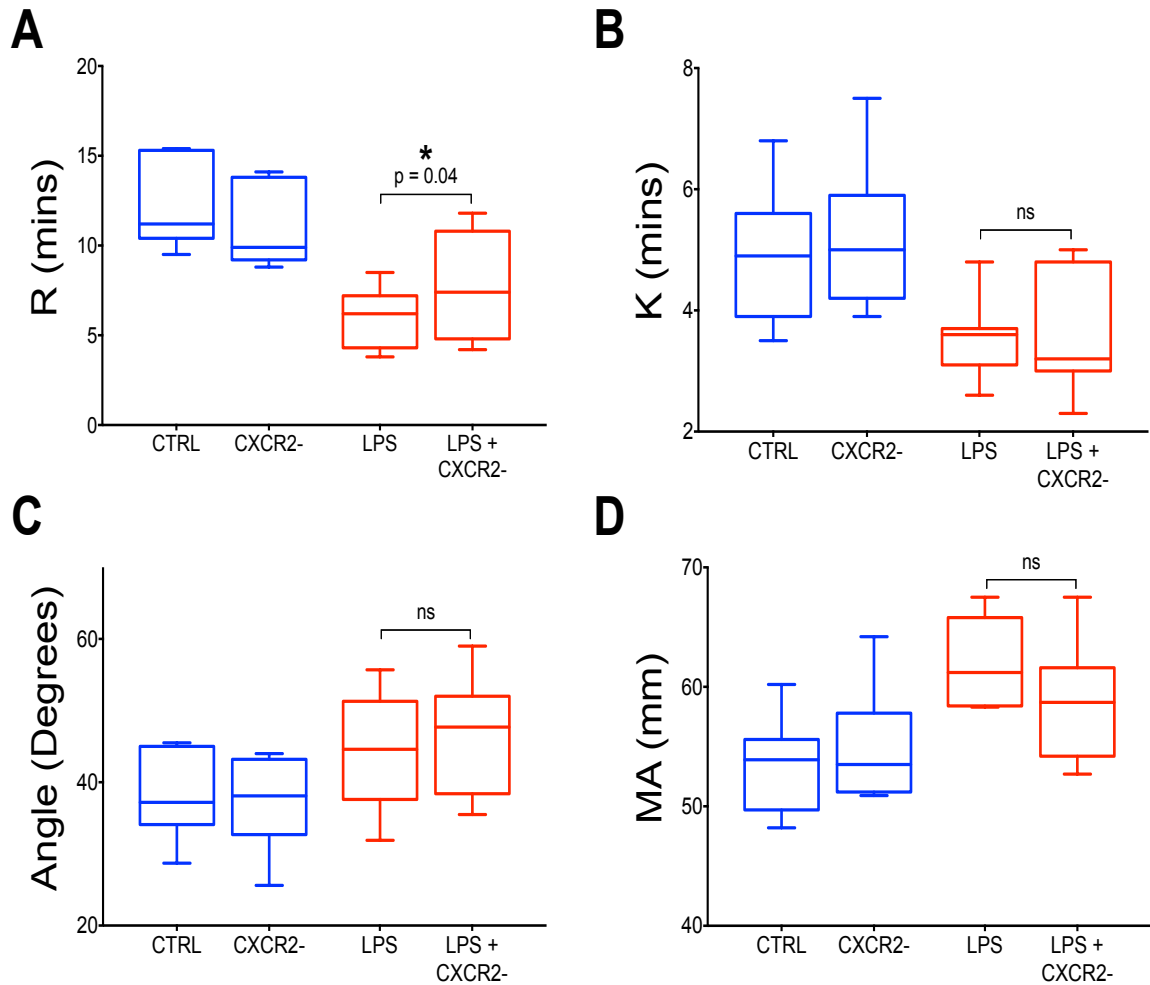


Figure 4.6 TEG parameters in whole blood pre-treated with CXCR2 antagonist, SB265610, prior to incubation with LPS

Samples from healthy volunteers were pre-treated with SB265610 (1 μ M) prior to incubation with LPS (100ng/ml) for 4hr (n=7). Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). p-values reported, by Wilcoxon matched-pairs signed rank test, LPS vs. LPS + SB265610 (CXCR2-).

4.3.3 Effect of sympatholytics on LPS-induced hypercoagulability

Pre-treatment with each selected β blocker did not attenuate the hypercoagulable effect of LPS with no significant difference seen for any TEG parameter (Figure 4.7).

Pre-treatment with clonidine also did not significantly affect LPS-induced hypercoagulability, at both normal (5ng/ml) and supramaximal (75ng/ml) dose, with no differences seen for any TEG parameter (Figure 4.8).

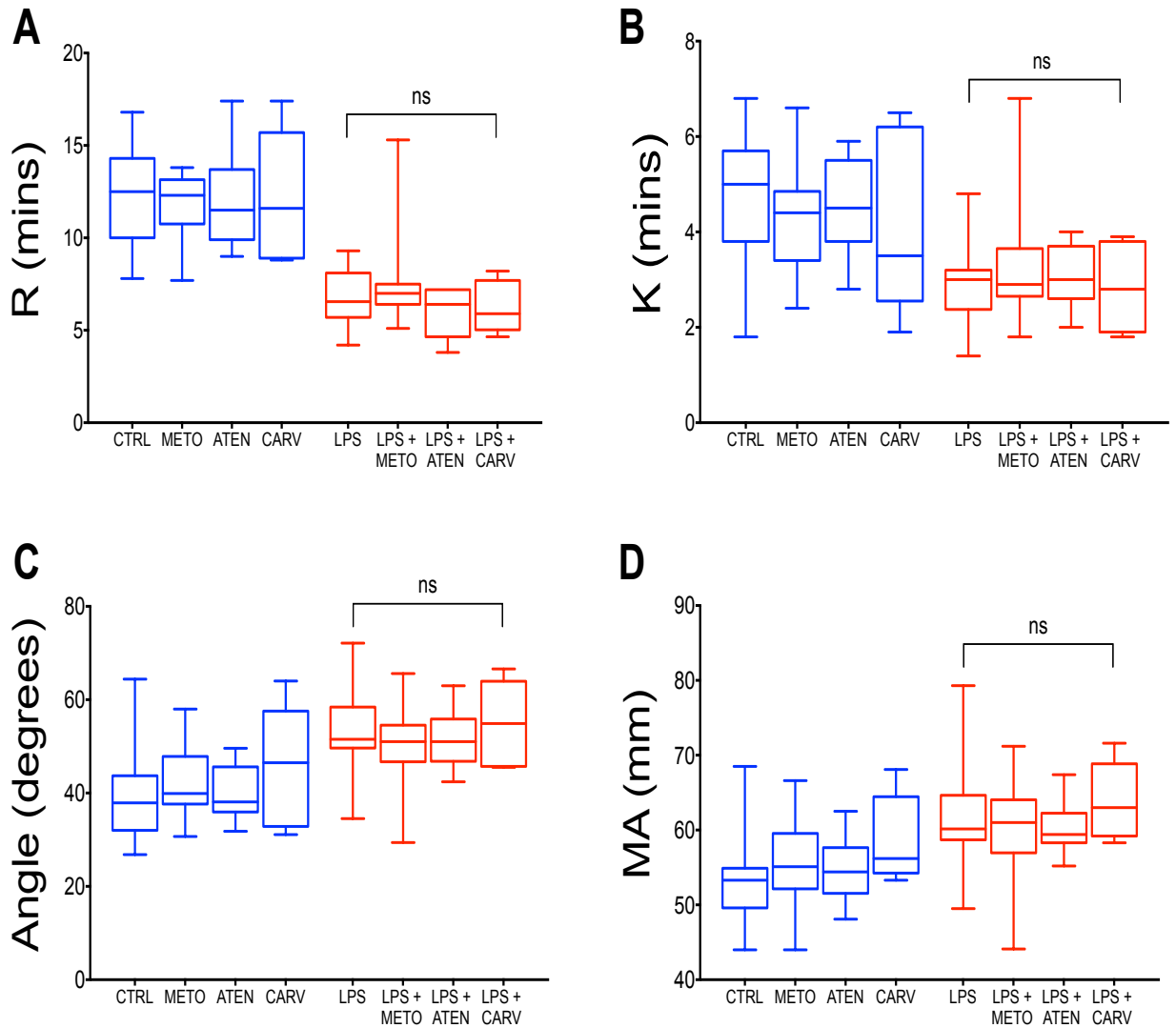


Figure 4.7 TEG parameters in whole blood pre-treated with selected β -blockers prior to incubation with LPS

Samples from healthy volunteers were pre-treated with the following β -blockers prior to incubation with LPS (100ng/ml) for 4 hrs: metoprolol, 100ng/ml (n=13); atenolol, 500ng/ml (n=9); carvedilol, 100ng/ml (n=5). Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). * $p < 0.05$ LPS vs. LPS + given β -blocker (Mann-Whitney test).

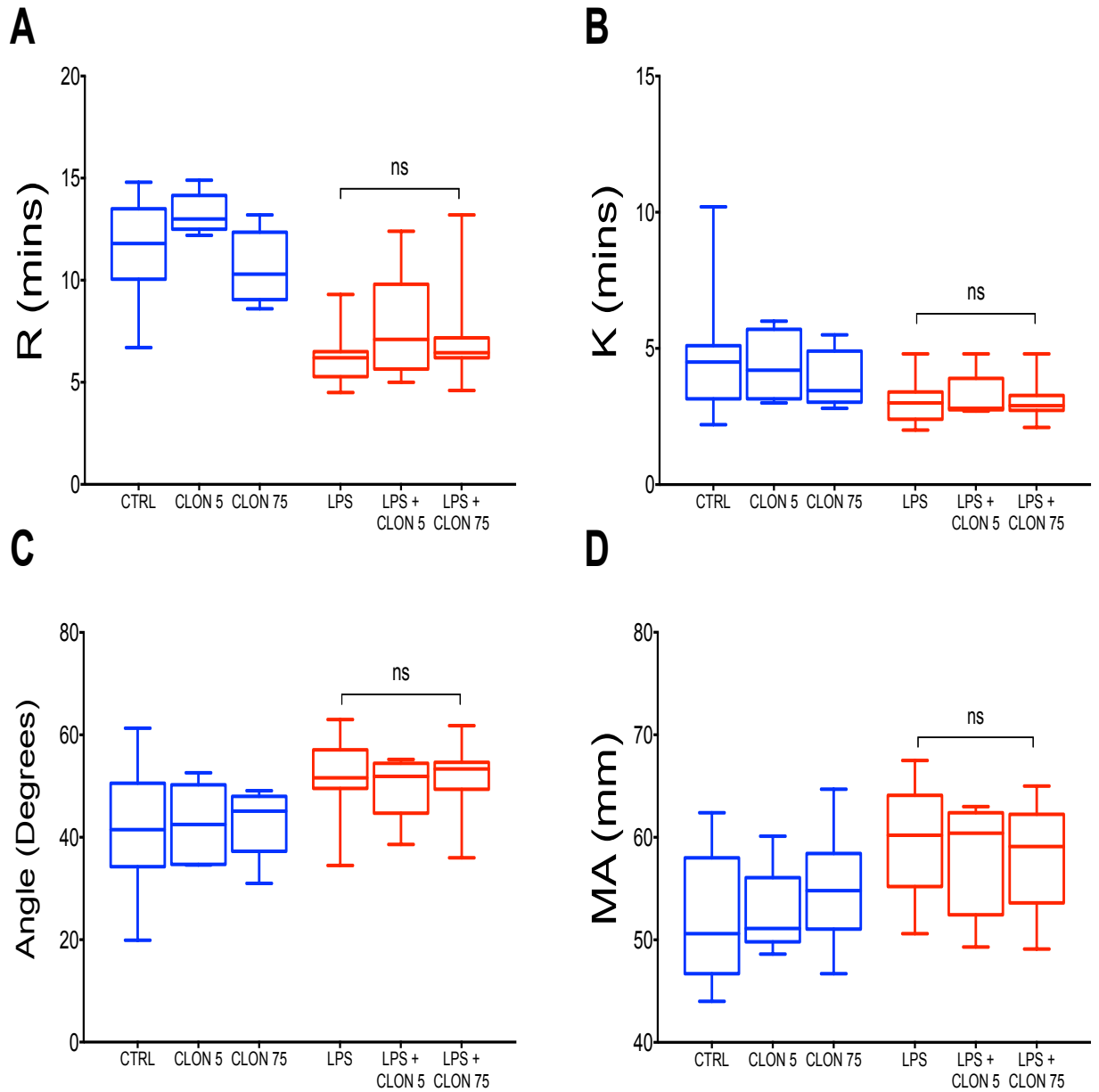


Figure 4.8 TEG parameters in whole blood pre-treated with clonidine prior to incubation with LPS

Samples from healthy volunteers were either pre-treated with either sterile saline (CTRL, n=17); clonidine, 5ng/ml (CLON 5, n=8) or 75ng/ml (CLON 75, n=9) prior to incubation with LPS for 4hrs. Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). * p <0.05 LPS vs. LPS + clonidine (CLON) at given concentration (Mann-Whitney test).

4.3.4 Effect of dexamethasone on LPS-induced hypercoagulability

Pre-treatment with dexamethasone attenuated the hypercoagulable effect following LPS incubation with a relative increase in R-time and reduction of MA, with respect to LPS alone (Figure 4.9). Therefore, this effect reflects modulation of both enzymatic and platelet hypercoagulability.

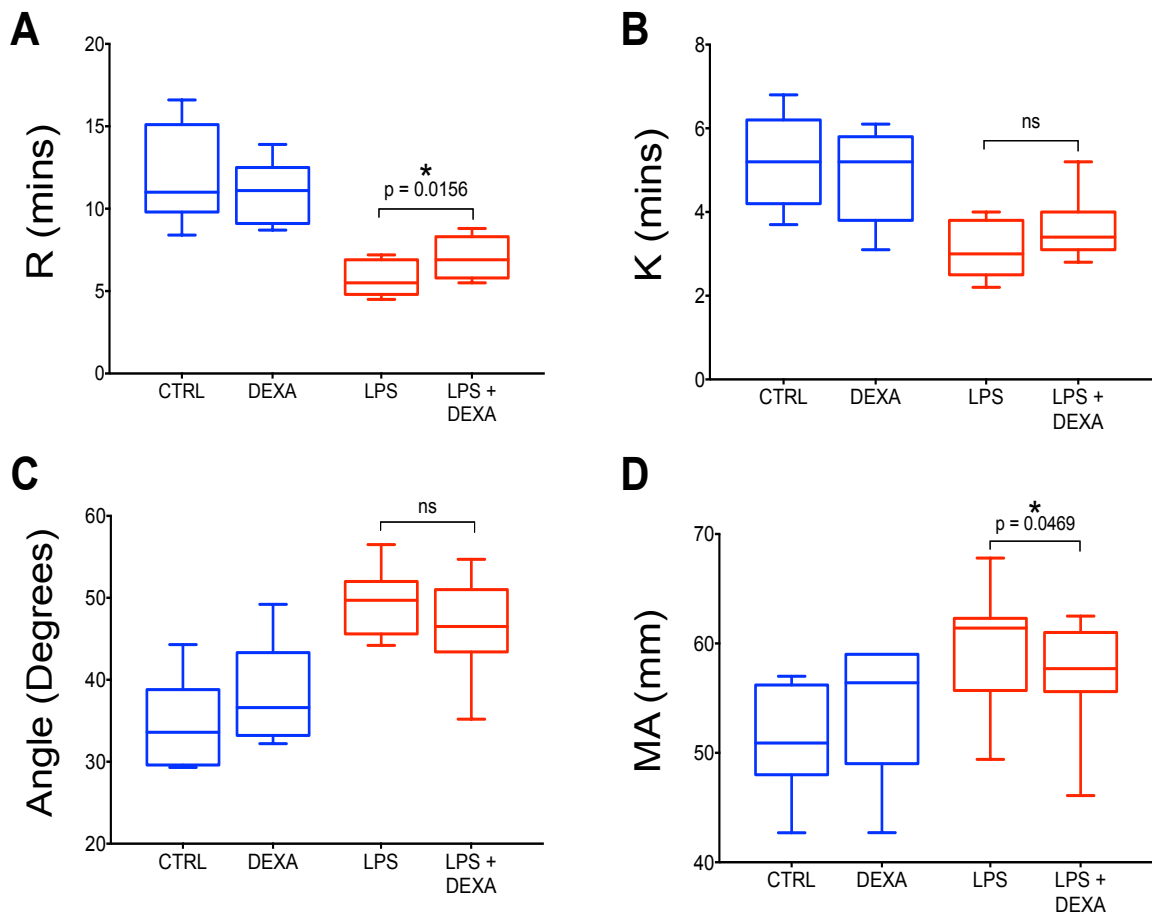


Figure 4.9 TEG parameters in whole blood pre-treated with dexamethasone prior to incubation with LPS

Samples from healthy volunteers were pre-treated with dexamethasone (40ng/ml) prior to incubation with LPS (100ng/ml) for 4hr (n=7). Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). p-values reported, by Wilcoxon matched-pairs signed rank test, LPS vs. LPS + dexamethasone (DEXA)

4.3.5 Effect of lidocaine on LPS-induced hypercoagulability

Pre-treatment with the selected local anaesthetic, lidocaine, did not attenuate the hypercoagulable effect of LPS with no significant difference seen for any TEG parameter (Figure 4.10).

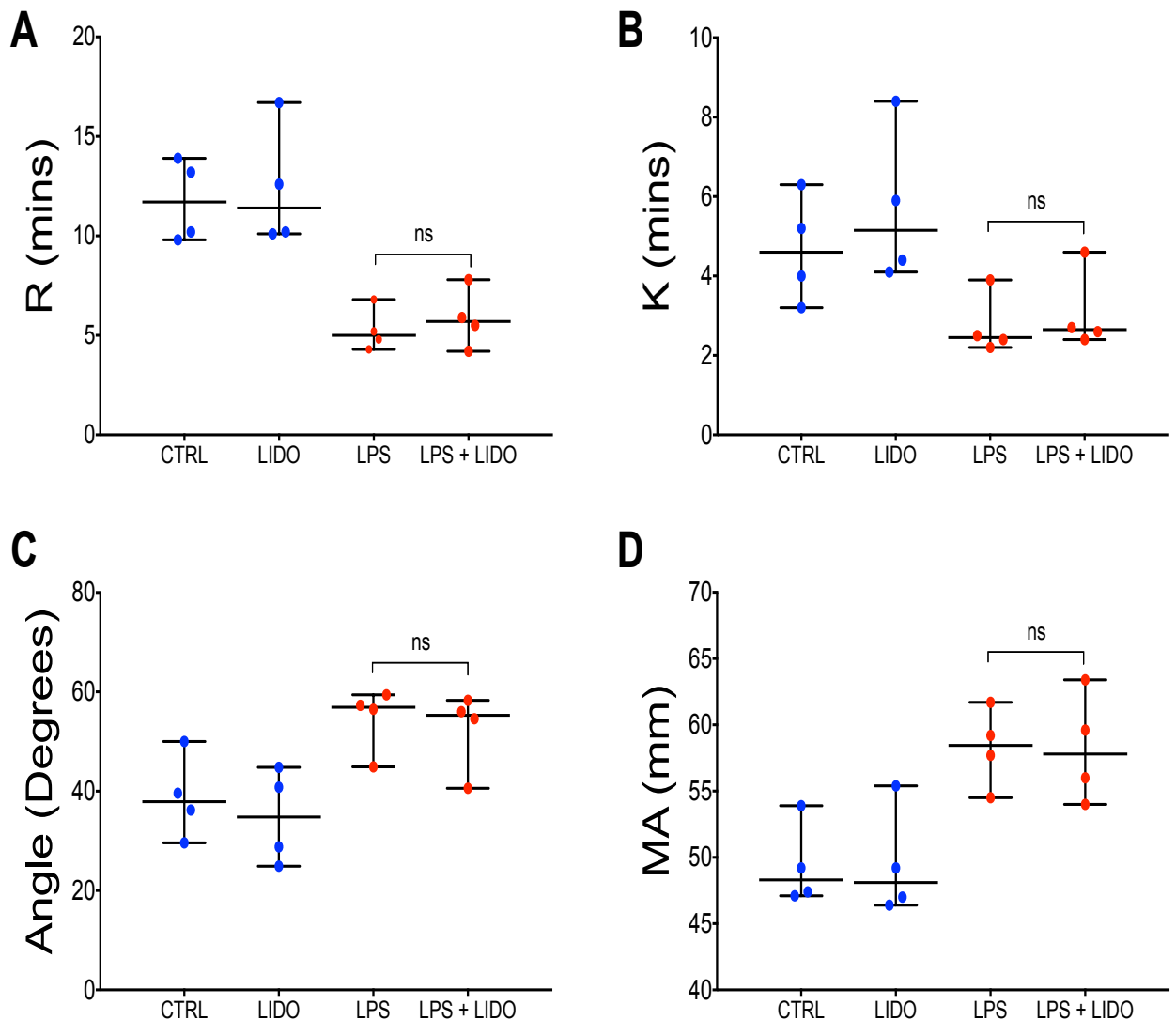


Figure 4.10 TEG parameters in whole blood pre-treated with lidocaine prior to incubation with LPS

Samples from healthy volunteers were pre-treated with lidocaine (5ng/ml) prior to incubation with LPS (100ng/ml) for 4hr (n=4). Data are presented as individual raw data points with median and IQR shown. Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). * p <0.05 LPS vs. LPS + lidocaine (LIDO), Wilcoxon matched-pairs signed rank test.

4.3.6 Effect of N-acetylcysteine (NAC) on LPS-induced hypercoagulability

Pre-treatment with the selected antioxidant, NAC, exacerbated the hypercoagulable effect of LPS, with further reduction in both R-time and K-time and relative increase in both angle and MA, compared to LPS alone (Figure 4.11). This represents a distinct promotion of both enzymatic and platelet TEG hypercoagulability.

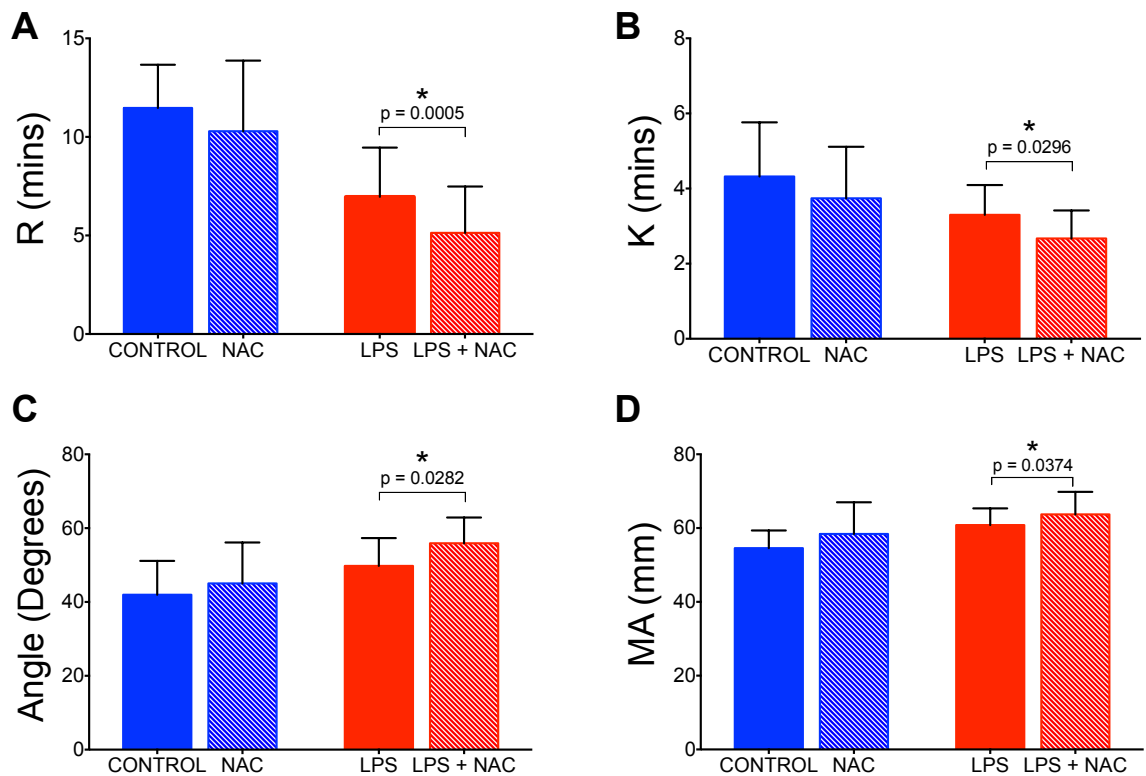


Figure 4.11 TEG parameters in whole blood pre-treated with NAC prior to incubation with LPS

Samples were pre-treated with N-acetylcysteine (500ng/ml) prior to incubation with LPS (100ng/ml) for 4hr (n=10). Data are mean (SD). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). p-values reported, by paired t-test, LPS vs. LPS + N-acetylcysteine (NAC).

4.3.7 Effect of MgSO₄ on LPS-induced hypercoagulability

Pre-treatment with MgSO₄ did not attenuate the hypercoagulable effect of LPS, with no significant difference seen for any TEG parameter (Figure 4.12).

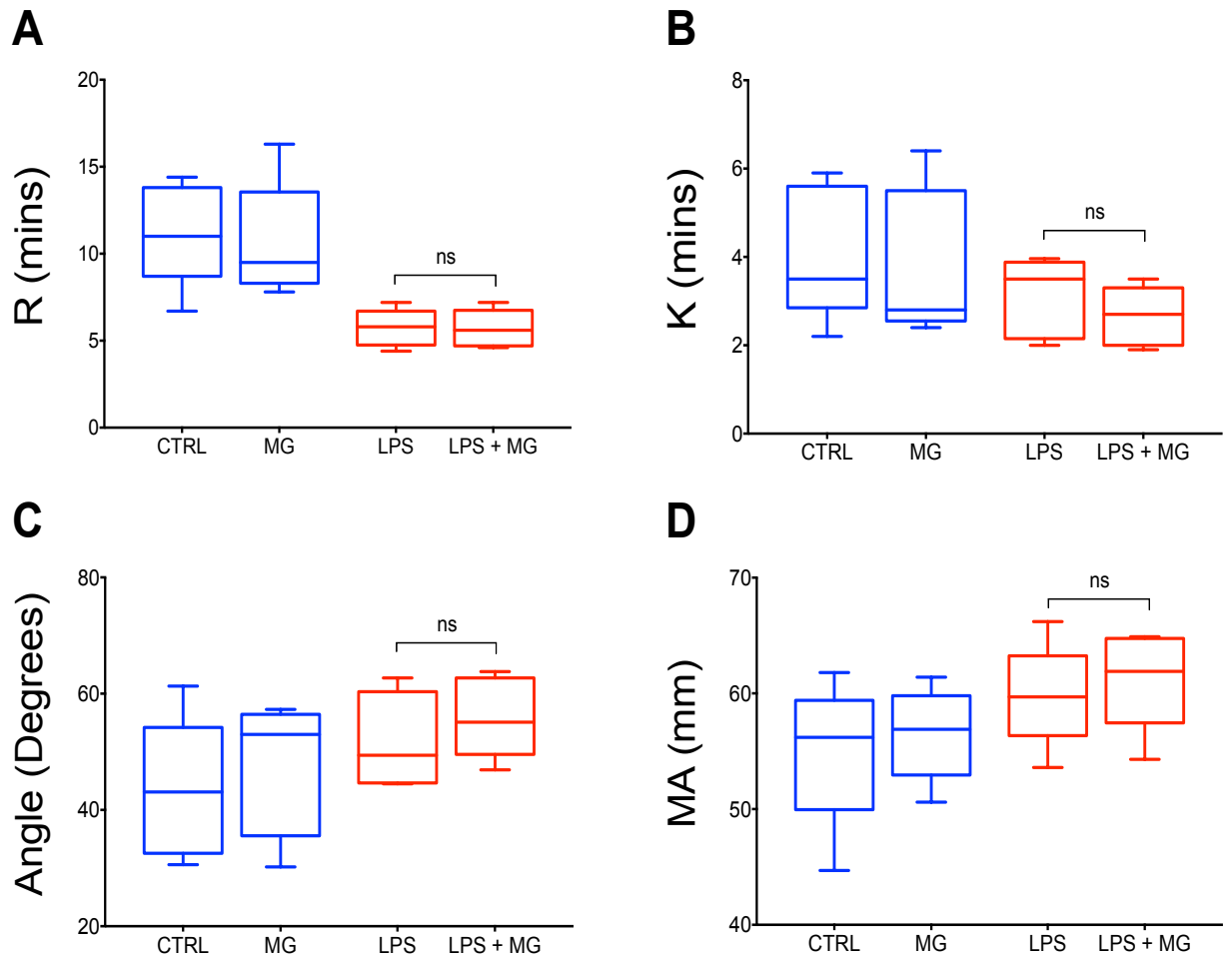


Figure 4.12 TEG parameters in whole blood pre-treated with MgSO₄ prior to incubation with LPS

Samples were pre-treated with MgSO₄ (2mM) prior to incubation with LPS (100ng/ml) for 4hr (n=5). Data are median (IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown with reference ranges in brackets: A. R-time (9-27 mins). B. K-time (2-9 mins) C. Angle. (22-58 degrees) D. Maximum amplitude (MA, 44-64mm). * $p < 0.05$ LPS vs. LPS + MgSO₄ (MG), Wilcoxon matched-pairs signed rank test.

4.4 Discussion

4.4.1 Key findings

1. Lipopolysaccharide produces hypercoagulability in both healthy volunteers and preoperative surgical patients

The principal finding is that LPS activates coagulation in whole blood *ex vivo*, as demonstrated by consistent significant changes in all TEG parameters, as reported in previous thromboelastographic studies. We observed significant changes in all TEG parameters, demonstrating both enzymatic and platelet hypercoagulability in the presence of LPS. This trend was seen at both concentrations of LPS tested, although the TEG maximum amplitude was not significantly increased at the lower LPS concentration. The magnitude of the changes seen with LPS were very similar to the hypercoagulable values seen postoperatively in our clinical study group (Chapter 3). Therefore, both our hypothesis that LPS induces TEG-hypercoagulability and the subsequent use of our *ex vivo* incubation model to further investigate potential therapeutic modulation have been vindicated.

2. Simvastatin attenuates the prothrombotic effect of LPS, with the CXCR2 antagonist SB225022 producing a similar effect

Pre-treatment with simvastatin attenuated but did not completely reverse LPS-induced enzymatic hypercoagulability. However, neither higher concentrations nor post-treatment produced the same effect. The specific CXCR2 antagonist SB225022 similarly attenuated LPS-induced enzymatic hypercoagulability, mirroring the effect seen with simvastatin.

3. Dexamethasone attenuated LPS-induced hypercoagulability by both enzymatic and platelet hypercoagulability criteria

No previous studies have demonstrated any effect on coagulation parameters in whole blood in this manner.

4. *N-acetylcysteine exacerbates hypercoagulability in the presence of LPS*

Surprisingly we found that NAC pre-treatment consistently promoted both enzymatic and platelet hypercoagulability with changes seen in all TEG parameters. This result was unexpected as previous *in vitro* studies of NAC pre-treatment in endotoxaemia have shown reduction of pro-inflammatory cytokines under mild oxidative conditions (Parmentier et al., 2000).

5. *Other commonly used perioperative drugs tested had no modifying effect on inflammation-induced hypercoagulability*

Pre-treatment with β -blockers, clonidine, magnesium sulphate and lidocaine had no significant effect on LPS-induced hypercoagulability.

4.4.2 Importance of findings

Our study confirmed that LPS produced a hypercoagulable phenotype, in whole blood *ex vivo*, in both healthy volunteers and preoperative surgical patients. In addition to demonstrating reductions in R-time following LPS incubation, as reported by previous thromboelastography studies (Koch et al., 2009b; Spiel et al., 2006; Zacharowski et al., 2006), we observed consistently significant changes in TEG angle and maximum amplitude, reflecting both enzymatic and platelet hypercoagulability.

The principal finding from this study was that inflammation-induced hypercoagulability was attenuated by pre-treatment with dexamethasone and simvastatin. Our results clearly showed an incomplete reversal of LPS-induced hypercoagulability which could suggest a potential role in modulating the pro-thrombotic perioperative phenotype. For dexamethasone this attenuation was in both enzymatic and platelet components of hypercoagulability whereas for simvastatin, changes related to enzymatic hypercoagulability only.

By demonstrating the modification by simvastatin of the procoagulant response to LPS in this *ex vivo* model, this supports *in vivo* data obtained from healthy subjects that reported inhibition of LPS-induced elevations of plasma high sensitivity C-reactive protein, monocyte chemoattractant protein (MCP)-1 and monocyte tissue factor expression by high dose simvastatin (Steiner et al., 2005). Furthermore, it may provide useful insight into the potential mechanism by which statin therapy has been shown to reduce thrombotic events in certain clinical cohorts (Glynn et al., 2009; Ridker et al., 2008).

In investigating a potential mechanism for an anti-inflammatory, anti-thrombotic action of simvastatin, we demonstrated that the specific CXCR2 antagonist SB225022 similarly attenuated LPS-induced enzymatic hypercoagulability, mirroring the effect seen with simvastatin. Although we cannot confirm a direct effect of simvastatin on the chemokine receptor from our results, as previously demonstrated (Zhang et al. 2011), we have extended these studies by demonstrating that CXCR2 antagonism is associated with partial reversal of a prothrombotic phenotype.

The results for dexamethasone suggest a more marked effect on LPS-stimulated hypercoagulability. However, clinical data have so far failed to demonstrate any reduction in major adverse events with perioperative steroids that are potentially attributable to hypercoagulability (Dieleman et al., 2012). Both *in vitro* and *in vivo* studies have produced conflicting results with regard to the overall effect on coagulation and the assessment of outcome in clinical studies has relied on composite outcome measures rather than specific coagulation parameters. This is the first study to demonstrate attenuation of combined pattern hypercoagulability following an *ex vivo* inflammatory stimulus. This should form the basis of more detailed investigation of the effect of steroid treatment on global coagulation indices *in vivo*.

In the LPS-TEG model, NAC pre-treatment generated an even more hypercoagulable phenotype, mostly in the presence of LPS. This was a surprising but consistent result with clearly demonstrated exacerbation of both enzymatic and platelet hypercoagulability. No previous studies had examined the effect of antioxidants on coagulation in this manner so further work is undoubtedly required to investigate this finding, both in terms of studying alternative antioxidants and examining the specific effect of NAC on coagulation parameters, including TEG, *in vivo*. However, our data may partly explain the failure of the experimental promise of NAC to translate to convincing improvements in clinical outcome.

Furthermore, our results did not support a thrombomodulatory role of magnesium in the presence of LPS. Intraoperative MgSO₄ infusion has been reported to reduce clotting times and maximal clot firmness in separate surgical cohorts by the same group, but alterations were inconsistent and arguably did not constitute hypercoagulability (Na et al., 2012, 2014)

4.4.3 Strengths and limitations

This study has more than 3-fold the subjects compared with previous LPS based reports and reflects changes in both healthy subjects and patients immediately prior to major surgery. Although previous studies have examined modification of LPS-stimulated blood by individual drug classes *in vivo* (Pernerstorfer et al., 1999), this study is unique in examining a comprehensive range of perioperative therapies in an *ex vivo* model.

However, there were several limitations to the study. This *ex vivo* model of LPS induced activation of coagulation was tested using thromboelastography as a surrogate for assessing perioperative thrombotic risk. Although we identified specific drug classes that attenuate inflammation-induced hypercoagulability, exploring the precise underlying mechanism involved was

beyond the scope of this study. It must be acknowledged that activation of coagulation *in vivo* is a complex process and our whole blood *ex vivo* model represented a much simpler paradigm which, in particular, did not examine the contribution of endothelium to coagulation activation. Although our model did not account for the effect of endothelial components such as endogenous anticoagulants, thrombomodulin and Protein C, whole blood analysis did allow examination of the cellular contribution to coagulation activation, especially the role of neutrophils. To account for any potential effect of haemodilution on hypercoagulability by the addition of both selected drugs and LPS, volumes added to whole blood samples were minimised in our experimental protocol.

Despite demonstrating LPS induced hypercoagulability at two different LPS concentrations, a comprehensive dose response assessment was not undertaken. The choice of dose of LPS was therefore based on both similar published experimental protocols (Koch et al. 2009; Zacharowski et al. 2006) and by initial experiments demonstrating that 100ng/ml consistently produced both enzymatic and platelet hypercoagulability, which was less convincingly seen at the lower dose of 10ng/ml.

In comparing the whole blood response to LPS in healthy volunteers and preoperative surgical patients, it was necessary to consider these as two separate groups. All volunteer blood sampling used venous blood whereas arterial blood was used in the patient group. Previous studies have demonstrated different TEG variables in corresponding arterial and venous samples taken during cardiac surgery, with a relatively hypercoagulability in arterial blood (Manspeizer et al., 2001). Therefore, any discrepancy between groups, in terms of sample collection and subject characteristics, was accounted for by excluding surgical patients from the drug pretreatment phase of this study.

Thromboelastography was used as our default monitoring tool as it allowed a global assessment of whole blood coagulation. This modality has been

previously validated, with alterations in TEG parameters corresponding to other markers of coagulation activation (Spiel et al., 2006), in particular TEG R-time with tissue factor expression (Lakshmanan et al., 2016). Clinically, numerous surgical cohort studies have demonstrated an association between TEG-defined hypercoagulability in the perioperative phase and higher risk of postoperative thromboembolic complications (Kashuk et al., 2009; McCrath et al., 2005), with no indication given by conventional haematological parameters such as APTT or INR (Hincker et al. 2014). A multitude of similar studies have demonstrated the presence of hypercoagulability in the postoperative cohort, without demonstrating this association with adverse outcome, although largely in highly specific surgical subgroups. Therefore, existing evidence suggests that TEG is a superior modality to conventional haematological parameters in assessing hypercoagulability in this model, but it is accepted that further work is warranted beyond our *ex vivo* model to fully characterise indirect effects of potential thrombomodulatory drugs on whole blood.

4.4.4 Conclusion

In assessing the thrombomodulatory potential of perioperative drugs, both steroids and statins attenuated while NAC exacerbated hypercoagulability in the presence of inflammation. However, further investigation in the clinical context, together with quantification of postoperative thrombotic events, is crucial to fully evaluate the significant potential clinical relevance of these findings.

Chapter 5

Modulation of autonomic regulation of coagulation

5.1 Introduction

The postoperative phase following major surgery is characterized by the concurrent activation of both inflammatory and coagulation systems. A significant proportion of postoperative morbidity, including major cardiovascular events, acute lung injury and gastrointestinal dysfunction, may be manifestations of an exaggerated pro-inflammatory pro-thrombotic response observed following surgical insult, contributing to end organ dysfunction and death.

Whereas the controlled production of pro-inflammatory cytokines, such as interleukins (IL)-1 β , IL-6, IL-8; tumour necrosis factor (TNF)- α and high mobility group box protein B1 (HMGB1) triggers beneficial inflammatory responses to promote local coagulation and contain infection and tissue damage, excessive or persistent cytokine production and subsequent systemic release leads to the systemic inflammatory response syndrome (Tracey, 2007). Increased plasma concentrations of pro-inflammatory cytokines have been demonstrated after major surgery and the magnitude of cytokine mediated inflammatory response is related to the extent of the surgical insult (Desborough, 2000). Furthermore, raised levels of pro-inflammatory cytokines have been shown to correlate with negative outcome measures including postoperative mortality (Sato et al., 2002; Welborn et al., 2000).

The inflammatory response is balanced by anti-inflammatory factors including cytokines IL-10 and IL-4, IL-1 receptor antagonist and transforming growth factor (TGF) β , produced through a normal immune response to inhibit release of TNF α and other pro-inflammatory cytokines. Neutralising the effects of excessive pro-inflammatory cytokine production by inhibition of single cytokines (such as monoclonal anti-TNF α therapy) has been successful in certain inflammatory conditions, such as rheumatoid arthritis. However, such targeted therapies have not been successful in models of SIRS and severe

sepsis (Abraham, 1999).

Recent research has highlighted the role of the autonomic nervous system in regulating the immune system and adjusting the inflammatory response by modulating the production of multiple inflammatory cytokines (Wang et al., 2003; Wittebole et al., 2006). In particular the cholinergic nervous system has been implicated, forming an 'inflammatory reflex' with both immunosuppressing and immunosensing roles. These effects are rapid, localised and integrated compared to the slower and more diffuse humoral anti-inflammatory mechanisms.

The afferent vagal nerve performs an immunosensing function with sensory vagal afferents shown to detect low tissue concentrations of cytokines and inform the brain of inflammation in the periphery via ascending fibres (Figure 5.1). Central integration of afferent and efferent components of this 'cholinergic anti-inflammatory pathway' occurs in the medullary nucleus tractus solitaries (NTS), which is the major site for termination of vagal afferents. NTS neurones then project to the dorsal motor nucleus of the vagus (DMN) where most of the efferent vagal preganglionic fibres originate (Pavlov et al., 2003).

Evidence for immunomodulation by the vagus nerve is now available for models of sepsis, haemorrhagic shock (Guarini et al., 2003), ischaemia-reperfusion injury (Saeed et al., 2005), localised inflammation (Golparvar et al., 2014) and pancreatitis (van Westerloo et al., 2006a).

Acetylcholine (ACh) is the principal neurotransmitter of the parasympathetic nervous system. Macrophages and other cytokine producing cells prominently express ACh receptors and, when exposed to ACh, are inactivated. ACh post-transcriptionally suppresses TNF synthesis and attenuating the release of pro-inflammatory cytokines, IL-1 β , IL-6 and IL-8 in experimental sepsis (Tracey et al., 2000).

The $\alpha 7$ nicotinic receptor ($\alpha 7$ nAChR) has been identified as the specific receptor responsible for mediating these immunomodulatory effects (Jonge and Ulloa, 2009; Wang et al., 2003). Selective $\alpha 7$ nAChR agonists have proven effective in reducing macrophage cytokine production and inflammation in animal models (van Westerloo et al., 2006b). Furthermore, nicotine has been shown to suppress LPS-induced inflammatory changes *in vivo*, together with increased circulating IL-10 levels (Wittebole et al., 2006).

nAChRs are traditionally described as ligand-gated ion channels which allow calcium ions to enter the cells. However, the intracellular signalling pathway which follows is complex and incompletely defined in non-neuronal cell types. In models of neuroprotection, the $\alpha 7$ nAChR has been shown to be closely associated with the activated phosphatidylinositol 3-kinase (PI3K) cascade, with nicotine exerting a neuroprotective effect by activating PI3K, which subsequently activates Akt and upregulates the anti-apoptotic protein Bcl-2 (Kihara et al., 2001). Studies in macrophages indicate that the anti-inflammatory potential of $\alpha 7$ nAChR is mediated by the inhibition of the transcription factor, nuclear factor kappa B (NF- κ B). During infection or trauma, bacterial components (LPS) or intracellular mediators (HMGB1) trigger pro-inflammatory pathways that converge on the activation of NF- κ B. Candidate intracellular signalling mechanisms between $\alpha 7$ nAChR activation and NF- κ B inhibition include the PI3K and Jak2-STAT3 pathways. Studies have shown that the PI3K pathway regulates LPS signalling in monocytes (Guha and Mackman, 2002), whereas $\alpha 7$ nAChR activation by nicotine has been linked to activation of transcription factor STAT3 via phosphorylation of tyrosine kinase Jak2 in macrophages (de Jonge et al., 2005). Therefore, in examining the anti-inflammatory potential of the $\alpha 7$ nAChR, consideration of the candidate intracellular pathways is required to fully understand how the immunomodulatory effect is mediated at a cellular level.

Despite rapidly emerging evidence for the 'cholinergic anti-inflammatory

pathway', the effect on coagulation of this previously unknown neural circuit requires further elucidation. Previous work has been confined to animal studies, with electrical vagal nerve stimulation shown to attenuate lipopolysaccharide (LPS)-induced activation of coagulation, as assessed by plasma concentrations of thrombin-antithrombin complexes and D-dimers (van Westerloo et al. 2006). PI3K pathway inhibition has been shown to suppress both LPS-induced TNF- α and tissue factor expression in both human monocytic cells and an animal model of endotoxaemia (Schabbauer et al., 2004). However, further demonstration of cholinergic modulation of coagulation with human or *in vivo* studies is not available. Given our previous observation that LPS activates coagulation in whole blood, as demonstrated by thromboelastography, the effect of nicotine and PI3K inhibition was investigated using the same LPS TEG experimental protocol.

5.1.1 Hypothesis

Lipopolysaccharide administered to whole blood results in activation of coagulation and produces hypercoagulability, as assessed by thromboelastography. Nicotinic ACh receptor activation, with nicotine, attenuates the promotion of hypercoagulability by LPS.

5.1.2 Aims

The hypothesis was addressed by characterising the coagulation changes in whole blood, taken from both healthy volunteers and preoperative surgical patients, following the addition of LPS (model for inflammatory stimulus) after pre-treatment with either nicotine or a PI3K inhibitor, at clinically relevant concentrations.

Primary outcome

TEG (citrated native TEG) parameters following pre-treatment with nicotine or the selective PI3K inhibitors, LY294002 and ZSTK474, compared to control in whole blood incubated with either LPS or endotoxin-free saline.

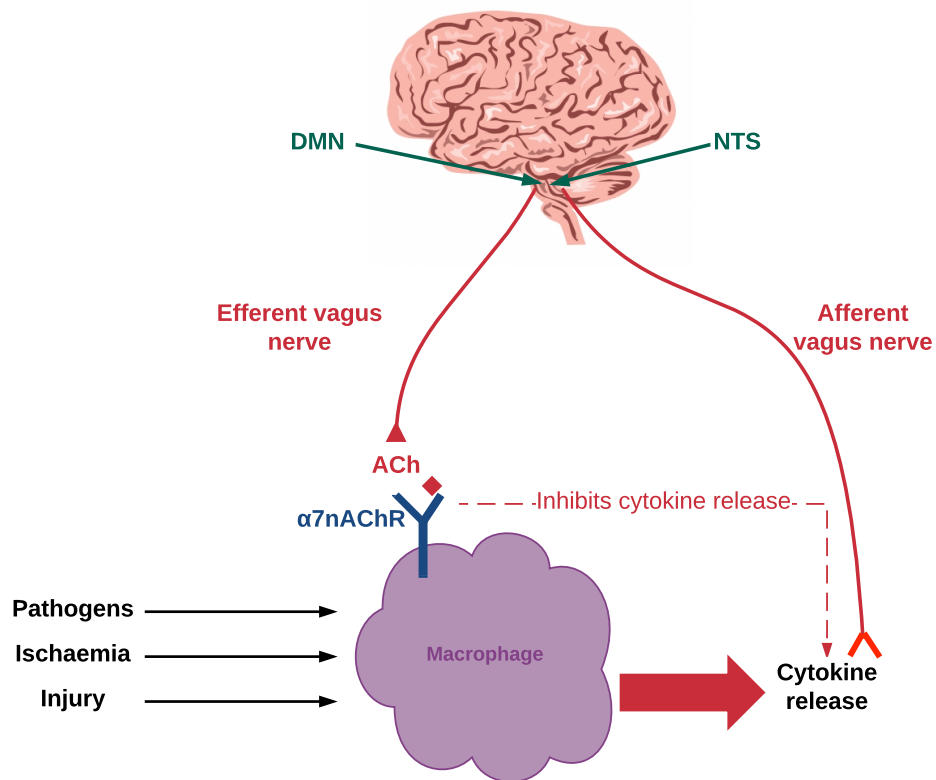


Figure 5.1 Cholinergic anti-inflammatory pathway

Tissue injury results in cytokine release from macrophages. Cytokines are sensed by the afferent vagus nerve and information relayed to nucleus tractus solaritus (NTS) and then to the dorsal motor nucleus. This results in the activation of the efferent vagus nerve with subsequent inhibition of cytokine production via activation of the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) located on macrophages and other immune cells. Afferent vagal signals that activate an efferent response to inhibit cytokine production is termed the *inflammatory reflex*

5.2 Methods

5.2.1 Study population

Following ethics committee approval and informed consent, venous blood samples were taken from healthy, drug-naive volunteers at the Royal Free Hospital. Recruitment and exclusion criteria have been described in detail in the Methods section.

5.2.2 Study protocol

Venous blood samples were obtained in a standardised manner from healthy volunteers, as previously outlined. Following collection into Vacutainer tubes containing sodium citrate 3.8% (Becton Dickinson, Plymouth, UK), samples were gently inverted to ensure mixing of sample and then transferred in 1ml aliquots to polypropylene tubes. Pre-treatment with 10 μ l of either sterile saline (control), or nicotine (100nM) was undertaken after 20 minutes. Following a further 20 minutes, each pre-treated whole blood sample had LPS (100ng/ml) added, prior to incubation at 37°C for 4 hours.

5.2.3 PI3K sub-study

To further elucidate the proposed mechanism of action by nicotine, additional drugs were studied, namely the selective PI3K inhibitors, LY294002 and ZSTK474. These experimental drugs were added in the same manner as the nicotine prior to incubation with LPS or saline. The aim of this sub-study was to investigate if any anti-thrombotic effect seen by nicotine on TEG could be recapitulated by pharmacological blockade of the PI3K pathway. Sample sizes in the PI3K inhibitor treatment groups were limited by the amount of drug solution available, resulting different sized drug pre-treatment groups.

5.2.4 Thromboelastography (TEG)

Thromboelastographic assays were performed immediately after the incubation period and following recalcification of incubated citrated blood, as described in Chapter 2. Citrated native TEG parameters were measured: R-time (reference range 2-27 min); K-time (reference range 2-9 min); angle (reference range 22-58 degrees); maximum amplitude (MA, reference range 44-64 mm). Each TEG parameter and the terminology of hypercoagulability have been previously defined in the Methods section.

5.2.5 Statistical analysis

Data are expressed as means (with SD) or medians (with interquartile ranges) as appropriate. The normality of continuous data was assessed using the D'Agostino & Pearson normality test. Differences between treatment groups were compared using a 2-tailed paired t-test for parametric data sets of 2 matched groups or a Kruskal Wallis test (with Dunn's multiple comparison test) or non-parametric data sets of 3 groups with different sample sizes. Statistical significance was set at $p < 0.05$. GraphPad Prism 8 (San Diego, CA, USA) was used for statistical analysis.

Sample size calculations

We powered the primary outcome (TEG R-time) on the basis that potential anti-thrombotic therapies would reverse the LPS-induced decrease in R-time by 50%. Previous work described ~60% reduction in clotting time CT (R-time equivalent) following LPS incubation (Koch et al. 2009). Thus, for selected drugs to attenuate pre-defined LPS-induced hypercoagulability, at least 6 subjects per experimental comparison would be required to detect this R-time difference at the $\alpha=0.05$ level (80% power).

5.3 Results

5.3.1 Effect of nicotine on LPS-induced hypercoagulability

Pre-treatment with nicotine (100nM) attenuated the prothrombotic effect of LPS incubation with a relative increase in R-time and reduction in angle, with respect to LPS alone (Figure 5.2). This effect related primarily to enzymatic hypercoagulability.

5.3.2 Effect of selective PI3K inhibitors on LPS-induced hypercoagulability

Pre-treatment with both selective PI3K inhibitors, LY294002 (20nM) and ZSTK474 (1 μ M), did not attenuate the hypercoagulable effect of LPS, with no significant difference seen for any TEG parameter (Figure 5.3).

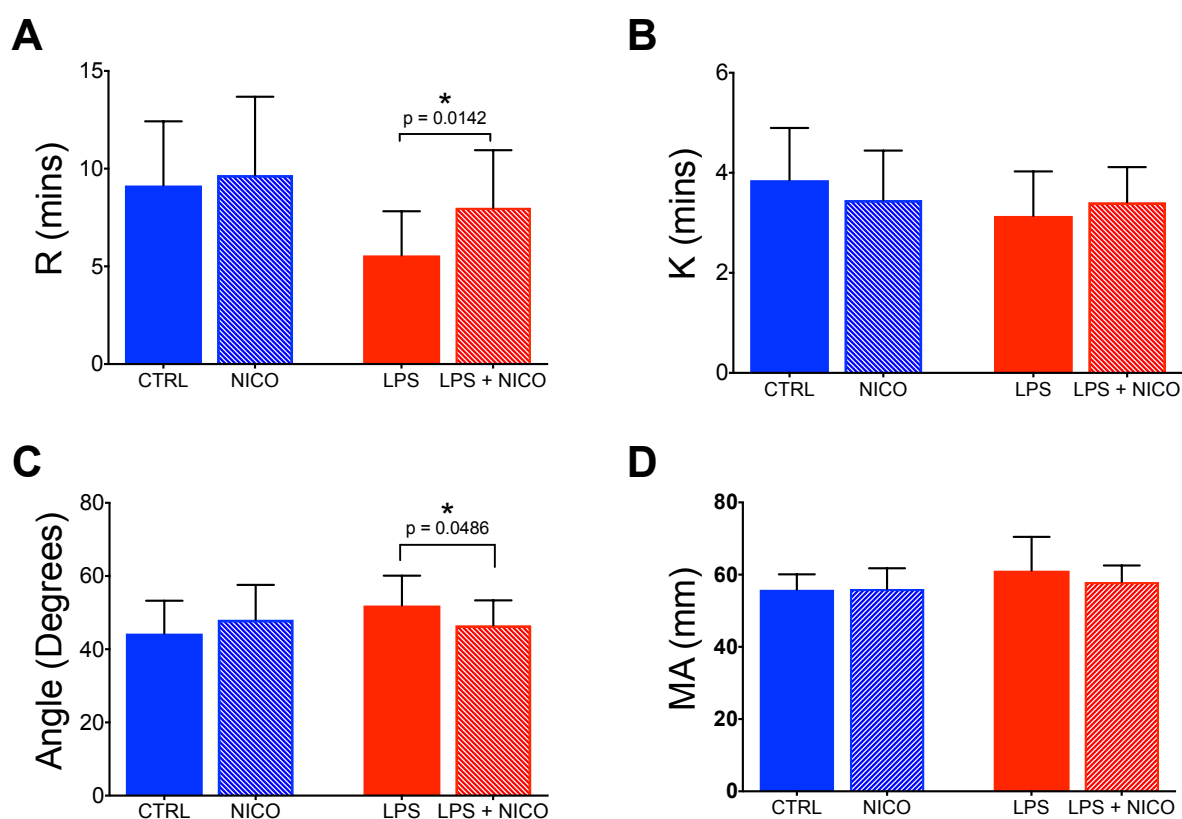


Figure 5.2 TEG parameters in whole blood pre-treated with nicotine prior to incubation with LPS

Samples were pre-treated with nicotine (n=8) or sterile saline prior to incubation with LPS (100ng/ml) for 4 hours to produce 4 experimental groups, control (CTRL), nicotine (NICO), LPS and LPS + nicotine (LPS + NICO). Data are mean (SD). Citrated native thromboelastography (TEG) parameters are shown: A. R-time. B. K-time C. Angle. D. Maximum amplitude (MA). * $p < 0.05$ LPS vs. LPS + Nicotine (2 tailed paired t-test)

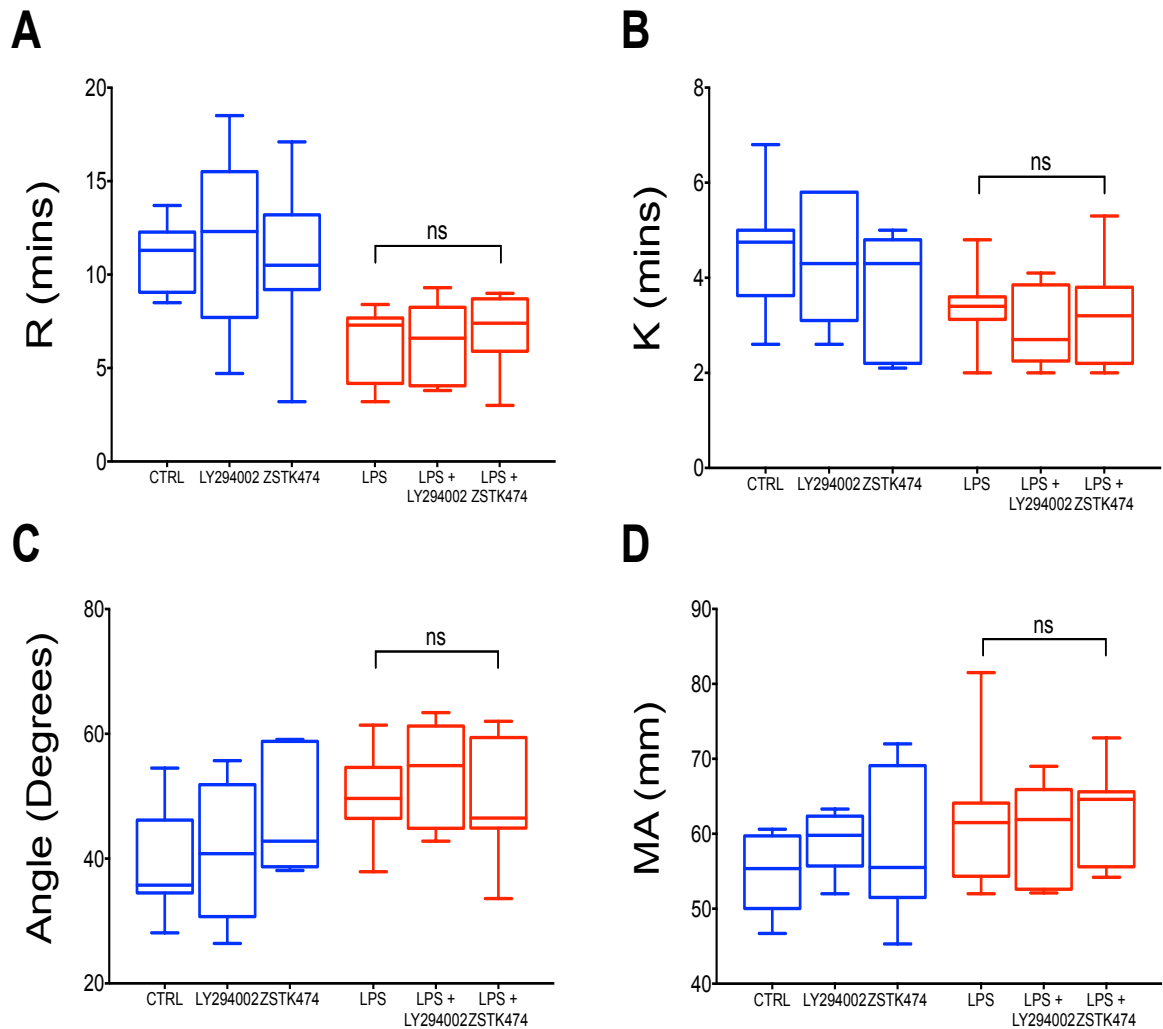


Figure 5.3 TEG parameters in whole blood pre-treated with selected PI3K inhibitors prior to incubation with LPS

Samples were pre-treated with PI3K inhibitor, LY294002 (n=5) or ZSTK474 (n=7), or sterile saline prior to incubation with LPS (100ng/ml) for 4 hours to produce experimental groups. Data are median (with IQR and range shown). Citrated native thromboelastography (TEG) parameters are shown: A. R-time. B. K-time C. Angle. D. Maximum amplitude (MA). * $p < 0.05$ LPS vs. LPS + PI3K inhibitor (Kruskal Wallis test). ns represents $p > 0.05$

5.4 Discussion

5.4.1 Key findings

1. Nicotine attenuates the prothrombotic effect of LPS

Pre-treatment with nicotine attenuated but did not completely reverse LPS-induced enzymatic hypercoagulability. This result corroborated previous work that vagal nerve stimulation inhibits activation of coagulation (van Westerloo et al. 2006), but is the first study to report pharmacological manipulation of the cholinergic anti-inflammatory pathway in whole human blood.

2. PI3K inhibitors have no effect on LPS-induced hypercoagulability.

The anti-thrombotic effect seen with nicotine was not recapitulated with either selective PI3K inhibitor tested. These results suggest that the attenuation of the pro-thrombotic effect of LPS by nicotine is not mediated by the PI3K intracellular pathway.

5.4.2 Importance of findings

This study supports the concept of the ‘cholinergic anti-inflammatory pathway’ having a further role in suppressing the activation of coagulation, by demonstrating the attenuation of inflammation induced hypercoagulability in human whole blood.

Autonomic dysregulation is an early and common feature following major surgery (Amar et al., 1998). Preservation of parasympathetic function confers cellular and anti-inflammatory protection across diverse organs (Andersson and Tracey, 2012). Experimental data have identified that vagal nerve activity is crucial for cardioprotection during ischaemia (Mastitskaya et al., 2012), an increasingly recognised source of perioperative morbidity. Furthermore, the parasympathetic neurotransmitter acetylcholine dampens inflammation by

nicotinic receptors in tissue macrophages, thus limiting multi-organ dysfunction (Andersson and Tracey, 2012). Therefore, these results draw attention to the additional role of the parasympathetic nervous system in regulation of coagulation, such that autonomic dysfunction may yield thromboembolic consequences, outside the end-organ sequelae already reported.

5.4.3 Strengths and limitations

This is the first study to specifically examine the effect of nicotine on inflammation-induced hypercoagulability, and in so doing confirms a role of the cholinergic pathway in suppressing the activation of coagulation. This non-specific nicotinic acetylcholine receptor agonist attenuated enzymatic hypercoagulability and highlights further potential targets for modulation of the pro-thrombotic phenotype. The LPS TEG experimental model used allowed a global assessment of haemostasis in human whole blood and thus supports previous work measuring individual components of coagulation activation, following vagal nerve stimulation, in animal studies (van Westerloo et al. 2006).

The strengths and limitations of this *ex vivo* model of LPS induced activation of coagulation have been discussed in Chapter 4. If resources had not been limited, the study would have benefitted from larger sample sizes and assessing the effect other nAChR agonists, particularly selective $\alpha 7$ nAChR agonists, in this same experimental model. The PI3K sub-study aimed to determine whether the effect seen with nicotine could be recapitulated by pharmacological blockade of a candidate intracellular signalling pathway. A similar pattern was not seen but that does not conclusively exclude the role of the PI3K pathway in autonomic regulation of coagulation. More comprehensive investigation should include determining whether Jak2 STAT3 pathway inhibition produces attenuation of inflammation-induced hypercoagulability in the same LPS model. However, the experimental

paradigm used in this study was not designed to ascertain the precise mechanism of nicotinic receptor activation in relation to coagulation activation at a cellular level so alternative models would be better placed to examine. Previous studies have demonstrated a reciprocal role between procoagulant activity and the PI3K signalling pathway but crucially this related to tissue factor expression on endothelial cells (Blum et al., 2001), which were absent from our experimental model.

5.4.4 Conclusion

In summary, this study demonstrated that inflammation-induced hypercoagulability is attenuated by non-specific nicotinic acetylcholine receptor activation when assessed by thromboelastography. This identifies a role for the parasympathetic nervous system in the modulation of coagulation changes following major surgery, such that preservation of parasympathetic function may confer favourable postoperative outcomes, with reduction of thrombotic risk being a potential component of improving morbidity. Further work is undoubtedly required to study the effect of specific nicotinic receptor ($\alpha 7$ nAChR) activation on biomarkers of coagulation activation and the effect of parasympathomimetics on *in vivo* models of coagulation with an intact endothelial component.

Chapter 6

Discussion

6.1 Summary of key findings

Data presented in this thesis demonstrate that postoperative hypercoagulability is seen in patients undergoing major intra-abdominal surgery up to at least postoperative day 5. This pro-thrombotic phenotype was diagnosed by pre-defined thromboelastography criteria to detect both enzymatic and platelet hypercoagulability. The results confirm that conventional coagulation tests are incapable of determining the hypercoagulable state in this population and are not accurate global assessments of haemostasis. Different surgical subtypes produce a broadly similar effect on coagulation, with subtle differences in pre-existing hypercoagulability relating to the underlying pathology. The postoperative goal directed fluid regimen studied was associated with excessive fluid administration but no adverse effect in terms of haemodilution-induced TEG-defined hypercoagulability was demonstrated. However, patients receiving goal directed therapy and the associated increased colloid fluid volume showed evidence of a higher degree of endothelial injury and specifically glycocalyx damage.

Ex vivo interrogation of inflammation-induced hypercoagulability using an experimental model demonstrated that the procoagulant response to an inflammatory insult such as major surgery is potentially modifiable by pre-existing perioperative therapies through off-target effects. Statin and steroid therapy attenuated hypercoagulability produced *in vitro* with the anti-oxidant N-acetylcysteine exacerbating the prothrombotic state both in the presence of inflammation. The results also support the concept of cholinergic autonomic modulation of coagulation with nicotine suppressing the hypercoagulable response to inflammation in the same experimental model.

6.2 Interpretation of results

The hypothesis that major surgery is associated with postoperative hypercoagulability has been robustly tested. The aim of the clinical TEG study was to elucidate the specific pattern of hypercoagulability in surgical cohorts and that has been achieved. This data supports previous work on specific surgical cohorts reporting procoagulant profiles postoperatively either by viscoelastic parameters or other biomarkers of coagulation activation. However, the identification of the hypercoagulable state cannot be regarded as confirmation of an increased risk of thromboembolic complications. Thus, it should be acknowledged that this study was not designed to determine the true predictive value of thromboelastography on postoperative thrombotic events.

Dai et al. published a systematic review of the literature to assess the accuracy of thromboelastography in predicting thromboembolic complications in different cohorts of surgical patients (Dai et al. 2008). The majority of published studies demonstrate a diagnostic and predictive value for TEG but the quality assessment of the data showed wide variation, in terms of verification of TEG data with other haematological parameters, blinding and insufficient details of TEG sampling and reference testing. The definition of hypercoagulability was inconsistent with different studies using a variety of single absolute or derived parameters, most commonly maximum amplitude or maximum clot firmness, to define the procoagulant subgroup. Therefore, these studies were assessing the predictive value of platelet hypercoagulability rather than both the enzymatic and platelet components of clot formation measurements. The results in this thesis demonstrate a combined pattern of hypercoagulability, with subtle differences seen depending on the underlying pathology and the nature of the surgery. It is important to examine the precise pattern of hypercoagulability not only in establishing potential mechanisms to modulate this response to surgery but

also to determine whether enzymatic or platelet hypercoagulability is more predictive of unfavourable outcome.

The clinical utility of viscoelastic testing in reliably determining the hypercoagulable state in the perioperative period is reiterated in this study. Conventional coagulation tests provided no consistent information on the global assessment of coagulation with interpretation of routine variables suggesting normo- or hypocoagulability despite clear evidence of hypercoagulability on TEG. Therefore, reliance on conventional coagulation tests, in current practice, to determine risk of thromboembolic complications or guide adequacy of thromboprophylaxis is fundamentally flawed. Failure to identify the prothrombotic phenotype in this manner may be associated with patient harm by not recognising the risk of thrombosis or appropriately preventing potential clinical sequelae. In particular, prothrombin time (PT/INR) is widely clinically used to determine suitability for administration perioperative low molecular weight heparin, with elevated INR deemed reflective of an 'auto-anticoagulated' state and an increased risk of postoperative bleeding, thus precluding the use of thromboprophylaxis. However, the correlation between PT and the clot initiation parameters, R-time (TEG) and CT (ROTEM), is poor (Coakley et al., 2006). The viscoelastic variables better reflect the overall haemostatic balance between pro- and anticoagulant factors in clot formation, rather than PT which quantifies specific procoagulant proteins traditionally considered to comprise the extrinsic system of coagulation.

In assessing the impact of major surgery on the coagulation profile, it is important to evaluate the relative contribution of each modifiable surgical and patient factor. This thesis aimed to investigate the effect of postoperative goal directed therapy on the coagulation profile postoperatively to determine whether different haemodynamic management strategies adversely affected the postoperative coagulation status. In the regimen studied, the main difference between treatment groups was the volume of colloid fluid

administered in the postoperative period. Although there was no demonstrable alteration of goal directed therapy on overall coagulation status as per thromboelastography criteria, there was an association with the degree of endothelial injury. This study did not determine how glycocalyx damage is potentiated in this therapy group but shear stress from relative hypervolaemia is one plausible mechanism. However, relative excess of fluid administered was not manifested in terms of laboratory evidence of significant haemodilution so alternative hypotheses should be considered. A key finding from the parent POM-O trial was a reduction of parasympathetic activity in the goal directed therapy, as detected by alterations in heart rate variability parameters (Ackland et al. 2015). Given the results from the *ex vivo* LPS TEG model, with nicotine attenuating inflammation-induced hypercoagulability, it is conceivable that the modulation of autonomic regulation of coagulation activation may be altered by components of goal directed therapy, such that suppression of the cholinergic anti-inflammatory reflex may be associated with a propensity to a hypercoagulable state.

Whereas the role of glycocalyx damage in sepsis and trauma is increasingly understood, the aetiology of endothelial injury and the resultant effect on the coagulation status in the perioperative setting undoubtedly requires further elucidation. Sepsis leads to ubiquitous degradation of the glycocalyx and altered endothelial permeability, with hypovolemia, hypoalbuminaemia, and oedema (Chelazzi et al., 2015). Inflammatory-mediated injury to glycocalyx may be responsible for numerous clinical sequelae of sepsis, including acute kidney injury, respiratory failure, and hepatic dysfunction. Biomarkers of glycocalyx degradation, such as circulating levels of syndecan or selectins, may be used as to indicate the severity of endothelial dysfunction in sepsis. In severely injured trauma patients, glycocalyx shedding is associated with reduced plasma colloid oncotic pressure and impaired thrombin generation (Rahbar et al., 2015).

However, the net effect of shedding glycocalyx components on the global

haemostatic picture following surgical trauma is poorly characterised. Elevated syndecan-1 and heparan sulphate levels have been demonstrated following major surgery (Rehm et al., 2007). Given heparan sulphate is a major component of the glycocalyx layer, the predicted effect of endothelial injury may be one of heparin-like activity with reduction in thrombin generation and a less hypercoagulable phenotype. However, endothelial injury also leads to adhesion of platelets, leukocytes and other neutrophil-endothelial cell interactions that promote coagulation activation. Trauma patients who had high levels of shed syndecan-1 demonstrated higher levels of pro-inflammatory cytokine IL-10 with a correlation with changes in endothelial permeability (Haywood-Watson et al., 2011). Preservation or restoration of the glycocalyx, assessed by both endothelial permeability and syndecan-1 levels, have been demonstrated with steroid treatment, antithrombin and fresh frozen plasma or whole blood resuscitation in different *in vitro* models (Chappell et al., 2007, 2009).

The results in this thesis suggest that glycocalyx damage is promoted by goal directed therapy in the immediate postoperative period but the net effect on global haemostasis is neutral. The lack of demonstrable haemodilution following an intervention associated with excessive fluid administration could potentially be attributable to increased endothelial permeability secondary to the glycocalyx damage seen. No significant endogenous heparin effect was demonstrated in the clinical TEG study to suggest an attributable rise in shed heparan sulphate. The aetiology of endothelial injury seen in surgical patients and the overall effect of coagulation is uncertain. Furthermore, the effect of limiting glycocalyx damage on perioperative coagulation, by potential therapeutic strategies to preserve or restore glycocalyx, requires specific investigation.

The inflammation-induced coagulation activation model was chosen to test the potential modification of the procoagulant effect of a standardised inflammatory stimulus by selected widely used perioperative therapies. This

facilitated *ex vivo* assessment of a wide range of medication with putative anti-inflammatory anti-thrombotic properties, via off-target effects. This model had the advantage of not exposing subjects directly to the inflammatory stimulus and allowing LPS treated samples to be pre-treated with multiple medications simultaneously. However, although this experimental paradigm demonstrated modulation of inflammation-induced hypercoagulability by simvastatin, dexamethasone and N-acetylcysteine, the results need to be interpreted cautiously due to the limitations of the experimental model. Activation of coagulation *in vivo* is much more complex than that seen in this model which, in particular, does not take into account the contribution by the endothelium and components such as endogenous anticoagulants, thrombomodulin and Protein C. The disadvantages of *ex vivo* investigation are somewhat offset by the use of whole blood analysis so the cellular contribution of particularly leucocyte mediated tissue factor production is accounted for.

In comparing the alteration in TEG parameters in the clinical study to the *ex vivo* experimental model, the pattern of predominantly enzymatic hypercoagulability and the magnitude of the changes demonstrated were similar. Therefore, although this simplified artificial model has limitations, it does provide a sound basis for further investigation of positive results. The *in vivo* effect of pre-treatment with statins, steroids and antioxidants should now be further assessed by testing if modification of TEG parameters is seen following systemic administration of LPS in either an animal model or human subjects. This *in vivo* endotoxaemia model has been widely used in the assessment of modulation of LPS-induced pro-inflammatory cytokine production and coagulation activation. Whereas primary outcome has been previously measured in this model in terms of individual biomarkers of activation of coagulation (Pernerstorfer et al., 1999; Steiner et al., 2005), thromboelastography is the ideal modality to evaluate the global effect on haemostasis, as a surrogate for thrombotic risk, in the same manner as the *ex vivo* paradigm.

The partial reversal of LPS-stimulated hypercoagulability by simvastatin and nicotine prompted sub-study analysis to investigate potential mechanisms for further anti-thrombotic properties of each therapy. Proposed actions for simvastatin have focussed on the inhibition of platelet-dependent neutrophil recruitment, mediated by CD40L, secreted from activated platelets, and CXC chemokines respectively (Zhang et al., 2011a). The aim of investigating the effect of a CXCR2 antagonist in the LPS TEG study was to demonstrate if the attenuation seen with simvastatin could be recapitulated but it did not provide specific clarification of the mechanism of action. Although the pattern of modulation of coagulation activation was similar, detailed mechanistic studies are necessary to confirm any proposed anti-thrombotic action. Flow cytometry studies to elucidate whether simvastatin alters CXCR2 expression on neutrophils would be beneficial in the same whole blood model, given that simvastatin reduces neutrophil recruitment via reduced CXC chemokine release in murine models (Zhang et al. 2011). Providing a biological explanation for the effect seen with simvastatin on inflammation-induced hypercoagulability would more robustly confirm the effect seen in this TEG model but also identify further potential therapeutic targets to modulate coagulation activation.

6.3 Importance of findings and clinical relevance

Thromboembolic complications are the leading cause of preventable postoperative morbidity and mortality. Of the 312 million surgical procedures performed worldwide each year (Weiser et al., 2016), around 8% are complicated by an arterial or venous thrombotic event (Donze et al., 2014). Postoperative myocardial infarction, ischaemic stroke, deep vein thrombosis and pulmonary thromboembolism (PTE) continue to represent a substantial healthcare burden, despite routine thromboprophylaxis and significant improvements in perioperative care. The scale of the problem is set to

increase with the expanding volume of surgery performed worldwide and the rising proportion of surgical patients with risk factors for perioperative hypercoagulability, such as advancing age and an underlying cancer diagnosis.

Although the data presented in this thesis did not include the rate of thromboembolic complications, the results demonstrate the significance of the perioperative shift to the prothrombotic phenotype. Key factors in improving postoperative outcomes will be more reliable identification of patients at increased risk of thromboembolic complications and appropriate risk stratification to enable more effective thromboprophylactic regimens to be implemented. Current strategies rely on generic patient and surgical factors to assess overall risk throughout the perioperative period. More dynamic assessment is required to reflect the differential risk throughout the perioperative period with easy and accurate identification of underlying perioperative hypercoagulability. Quantification of both preoperative risk, based on risk factors such as cancer or age, and postoperative risk, based on the response to surgical trauma and haemodynamic therapy, would allow more accurate evaluation of both the need for and duration of pharmacological thromboprophylaxis.

The impact of goal directed therapy on composite measures of postoperative outcome is well documented. The results from this thesis did not show any consistent exaggeration of the procoagulant response to surgery but did demonstrate potential harm attributable to endothelial damage. Despite the lack of net effect on global haemostasis, this finding highlights the importance of perioperative fluid regimens and the impact on endothelial function. Further exploration is required to elucidate the precise effect of goal directed therapy strategies on the glycocalyx, in combination with specific outcome measures of postoperative morbidity.

The results from the *ex vivo* incubation model demonstrate the potential of

certain commonly used perioperative therapies in the modulation of coagulation activation. Additional treatment benefit may be attributable to off-target effects, with anti-inflammatory anti-thrombotic properties, beyond their established mechanism of action. The prospect of utilising existing well-established medications in the perioperative phase to further reduce a component of perioperative risk is attractive and should be further investigated. Pre-treatment with appropriate doses of steroid or statin therapy could be evaluated in a corresponding *in vivo* LPS model to demonstrate if similar patterns of amelioration of inflammation-induced hypercoagulability are produced. Further mechanistic studies are needed to investigate the potential anti-thrombotic action and to identify additional therapeutic targets for modulation of this procoagulant response.

The experimental model employed was designed to reflect the inflammatory stimulus of surgical trauma. Although the results have been interpreted in the context of the perioperative coagulation status, the paradigm used is also applicable to the investigation of both the prothrombotic and pro-inflammatory state seen in sepsis and malignancy. Statin therapy, for example, has postulated benefit in the terms of cancer-related mortality (Nielsen et al. 2012). Further examination of the relationship between coagulation status, complication rate and mortality in this cohort may elucidate the mechanism by which statins confer this overall benefit. Moreover, the demonstrated alteration in coagulation status should be evaluated in parallel with biomarkers of inflammation to identify agents with potential to modulate other end-organ effects of the inflammatory response to surgery.

6.4 Further work

The results presented in this thesis highlight the considerable promise of thromboelastography in the reliable identification of perioperative hypercoagulability. Existing literature either demonstrates the presence of

hypercoagulability in specific postoperative surgical cohorts or, less commonly, the increased incidence of thromboembolic complications in TEG-defined hypercoagulable patients. It is vital that more high quality research is done to elucidate the efficacy and cost-effectiveness of thromboelastography in predicting and/or reducing thromboembolic complications. Alterations to the coagulation status should be examined in large cohorts of surgical patients into the extended postoperative phase with investigation of the incidence of both manifested and subclinical thrombotic events.

Consequently, the time taken to return to a normocoagulable state following different types of major surgery could be more reliably demonstrated and surgery-specific thromboprophylaxis regimens could be tested. The association between the incidence of thromboembolic events and both TEG-defined composite hypercoagulability and individual TEG parameters requires further more detailed investigation. There remains no consensus on how best to define hypercoagulability by TEG criteria and more comprehensive investigation of the impact of different aspects or parameters of global haemostasis on thrombotic risk is warranted.

Ultimately the predictive value of viscoelastic testing and its clinical utility in diagnosing perioperative hypercoagulability and identifying patients at increased thrombotic risk would be best assessed by a randomised controlled trial to compare rates of postoperative thromboembolic complications in subjects prescribed generic thromboprophylaxis regimens or more individualised protocols based on viscoelastic test criteria. The prospect of routine use of thromboelastography to assess hypercoagulability in the surgical patient would entirely depend on a demonstrable outcome benefit rather than detection of the hypercoagulable state.

The use of thromboelastography is well-established in the management of haemorrhage and clotting disorders associated with major trauma, liver transplantation and cardiac surgery in specialist centres (Hunt et al., 2015; Wikkelsø et al., 2016). Although no improvement in mortality has been

demonstrated, coagulation monitoring with TEG/ROTEM can reduce overall transfusion requirements as empirical therapy is eliminated and specific management of coagulation defects is instituted at an early stage. Clinical provision of viscoelastic testing has hitherto been limited by the moderate complexity of performing TEG and ROTEM analysis, such that the personnel running the tests need to be adequately trained, equipment required is subject to quality control procedures and existing modalities suffer from a unique set of pre-analytic and analytic variables that impact test reliability and reproducibility. However, modern adaptations of this technology, such as the TEG 6 machine, are significantly more self-automated, with ease of use provided by cartridge technology and machine portability and will represent true point of care tests. Therefore, access to viscoelastic tests and interpretation of TEG-derived parameters should become more readily available enabling more widespread adoption. Thus, real time dynamic characterisation to alterations in coagulation seen in both bleeding scenarios and in the context of perioperative hypercoagulability should become more routine practice in the future.

The *ex vivo* interrogation of inflammation-induced hypercoagulability has produced some interesting results and formed the basis for future investigation to refine the experimental model to more accurately reflect the perioperative scenario. Further studies should concentrate on whole blood and defining the *in vivo* effect of potential thrombomodulatory agents in modulating the procoagulant response to inflammation. Global haemostatic assessment with thromboelastography is superior to both conventional coagulation testing and individual biomarkers of coagulation activation and allows dynamic evaluation of the interaction between platelets, plasma and leucocytes. Crucially, *in vivo* investigation will demonstrate the contribution of the endothelium on the response to both an inflammatory stimulus and pre-treatment with selected perioperative therapies.

6.5 Conclusion

Hypercoagulability and increased risk of thromboembolic complications are commonly encountered in the perioperative phase of major surgery.

Thromboelastography is superior to conventional coagulation testing in demonstrating this prothrombotic phenotype and may have a role in guiding more adequate individualised thromboprophylaxis regimens. In mitigating this increased perioperative thrombotic risk, it is crucial to evaluate the impact of individual components of perioperative management, particularly intravenous fluid therapy and co-existing cancer diagnosis, in order to minimise the hypercoagulable response to the same surgical insult. Through further exploration of the relationship between inflammation and coagulation seen with surgical trauma, it may be possible to discover novel thrombomodulatory agents with minimal bleeding risk which can modify the risk of perioperative thromboembolic complications and improve postoperative outcome.

List of Associated Publications and Presentations

Individualised oxygen delivery targeted haemodynamic therapy in high-risk surgical patients: a multicentre, randomised, double-blind, controlled, mechanistic trial.

- Ackland GL, Iqbal S, Paredes LG, Toner A, Lyness C, Jenkins N, Bodger P, Karmali S, Whittle J, Reyes A, Singer M, Hamilton M, Cecconi M, Pearse RM, Mallett SV, Omar RZ; POM-O (PostOperative Morbidity-Oxygen delivery) study group.
- *Lancet Respiratory Medicine* 2015 Jan;3(1):33-41.

Baroreflex impairment and morbidity after major surgery.

- Toner A, Jenkins N, Ackland GL; POM-O Study Investigators.
- *British Journal of Anaesthesia* 2016 Sep;117(3):324-31.

Postoperative goal-directed therapy and development of acute kidney injury following major elective noncardiac surgery: post-hoc analysis of POM-O randomized controlled trial

- Patel A, Prowle JR, Ackland GL, POM-O Study Investigators
- *Clinical Kidney Journal* 2017 Jun; 10(3): 348–356.

Magnesium and rotational thromboelastometry (ROTEM®)

- Lyness C, Mallett SV.
- *Anaesthesia*. 2015 Mar;70(3):362-3

Thromboelastography screening of perioperative anti-thrombotic drugs reveals that simvastatin reverses hypercoagulability through a CXC chemokine/CXCR2 receptor mechanism

- Lyness C, Mallett SV, Ackland GL.
- *Poster presentation, Perioperative Research Trainee Forum (PORTL) Meeting, London, November 2012*

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