Predicting excess bleeding due to haemostatic failure following cardiac surgery requiring cardiopulmonary bypass

Charles Llewellyn Percy

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Summary

Bleeding following cardiac surgery requiring cardiopulmonary bypass (CPB) is associated with increased morbidity. Identification of patients at increased risk of bleeding might allow intervention to prevent bleeding developing.

In this thesis, clotting factors, anticoagulants and calibrated automated thrombin generation were investigated as potential methods for identifying such patients. Post-CPB FXIII, fibrinogen and platelet count were significantly lower in those who bleed more than 2 mL/kg/hr for two consecutive hours and in those who bleed in excess of 1 litre at 24 hours. ROC analysis demonstrated these had modest predictive value. Calibrated automated thrombography was unable to identify patients at risk of bleeding.

Calibrated automated thrombography was also used to investigate the effects of haemostatic treatment (FFP, rFVIIa, PCC and TFPI inhibition) on thrombin generation *in vitro*. Blocking the effect of TFPI produced the greatest improvement in thrombin generation.

The effect of CPB on platelet phospholipids was investigated using mass spectrometry. Post-CPB the ability to externalise phosphatidylethanolamine and phosphatidylserine was impaired. The ability to externalise and synthesise 12-HETE-PC and 12-HETE-PE in response to both thrombin and collagen post-CPB was also reduced. The effect of these phospholipids on thrombin generation and the ability to identify patients at risk of bleeding was then investigated. Thrombin generation using liposomes containing 12-HETE-PC or 12-HETE was lower in patients who required haemostatic treatment for post-CPB bleeding compared to those who did not. This suggests there are variations between individuals in the way their coagulation factors interact with oxidised phospholipids and that this may influence bleeding.

Finally a cell based model of thrombin generation was developed using monocytes as a source of tissue factor and incorporating the observed changes in phospholipids, clotting factors and anticoagulants. This model provides a basis to further investigate the influence of different TF expressing cells on thrombin generation which may affect bleeding.

Publications

Papers

Percy CL, Hartmann R, Jones RM, Balachandran S, Mehta D, Dockal M, Scheiflinger F, O'Donnell VB, Hall JE, Collins PW. Correcting thrombin generation ex vivo using different haemostatic agents following cardiac surgery requiring the use of cardiopulmonary bypass. Blood Coagulation and Fibrinolysis, 2015; 26(4): 357-367.

Posters

Percy CL, Jones R, Mehta D, Balachandran S, Hall JE, O'Donnell VB, Collins PW. Thrombin generation and post-operative bleeding in patients undergoing surgery requiring cardiopulmonary bypass. Oral poster presentation at: 24th Congress of the International Society on Thrombosis and Haemostasis, 29th June to 4th July 2013, Amsterdam, Netherlands.

Percy CL, Hartmann R, Kolm A, Panholzer E, Mehta D, Balachandran S, Hall JE, Dockal M, Scheiflinger F, Collins PW. The effect of Tissue Factor Pathway Inhibitor (TFPI) on thrombin generation and post-operative bleeding in patients undergoing surgery requiring cardiopulmonary bypass. Oral poster presentation at: 24th Congress of the International Society on Thrombosis and Haemostasis, 29th June to 4th July 2013, Amsterdam, Netherlands.

Abbreviations

ACT	Activated Clotting Time
APC	Activated Protein C
APTT	Activated Partial Thromboplastin Time
AT	Antithrombin
CABG	Coronary Artery Bypass Grafting
CAT	Calibrated Automated Thrombography
CPB	Cardio-pulmonary bypass
CV	Coefficient of Variation
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DMPE	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine
DMPS	1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine
ELISA	Enzyme Linked Immunosorbent Assay
ETP	Endogenous Thrombin Potential
FII	Prothrombin
FIIa	Thrombin
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FIX	Factor IX
FIXa	Activated factor IX
FX	Factor X
FXa	Activated factor X
FXI	Factor XI

FXIa	Activated factor XI
FXII	Factor XII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
FFP	Fresh Frozen Plasma
HETE	Hydroxyeicosatetraenoic acid
12-LOX	12-Lipoxygenase
OR	Odds Ratio
nM	nanomole/L
NPV	Negative Predictive Value
PC	Phosphatidylcholine
PCC	Prothrombin Complex Concentrate
PE	Phosphatidylethanolamine
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
pM	picomole/L
PPP	Platelet Poor Plasma
PPV	Positive Predictive Value
PRP	Platelet Rich Plasma
PS	Phosphatidylserine
PT	Prothrombin Time
rFVIIa	Recombinant activated factor VII
SAPC	1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
SAPE	1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine
SAPS	1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine
SD	Standard Deviation
TAFI	Thrombin Activated Fibrinolysis Inhibitor
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TRAP	Thrombin Receptor Activating Peptide

TXA_2	Thromboxane A_2
UK	United Kingdom of Great Britain and Northern Ireland
USA	United States of America
VWF	Von Willebrand Factor
x *g*	multiples of gravity

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Chapter 1 General Introduction

1.1 Cardiac Surgery requiring the use the cardiopulmonary bypass

Coronary artery bypass grafting (CABG) for atherosclerosis remains the most frequent indication for surgery requiring the use of cardiopulmonary bypass (CPB) (1). However, the clinical need for valve replacement or repair is steadily increasing and in the UK, operations for aortic and mitral valve disease doubled in the period 2003-2008 (1).

The purpose of CPB is to allow a blood-less field and the heart to be stopped, thus providing the cardiac surgeon with the possibility of anastomosing arterial or venous grafts to the coronary arteries, bypassing any atherosclerotic stenosis and thus improving the supply of oxygenated blood to the myocardium. The other purpose is to allow the myocardium to be entered and repair or replacement of the cardiac valves to be undertaken. It can also allow for the replacement of the ascending thoracic aorta. A schematic diagram of a CPB circuit is shown in Figure 1.1. Whilst for revascularisation percutaneous coronary intervention has supplemented surgery and in some cases replaced it (2), and in selected cases CABG can be undertaken without the use of CPB (3), at present cardiopulmonary bypass remains an essential procedure for the majority of cardiac surgery.

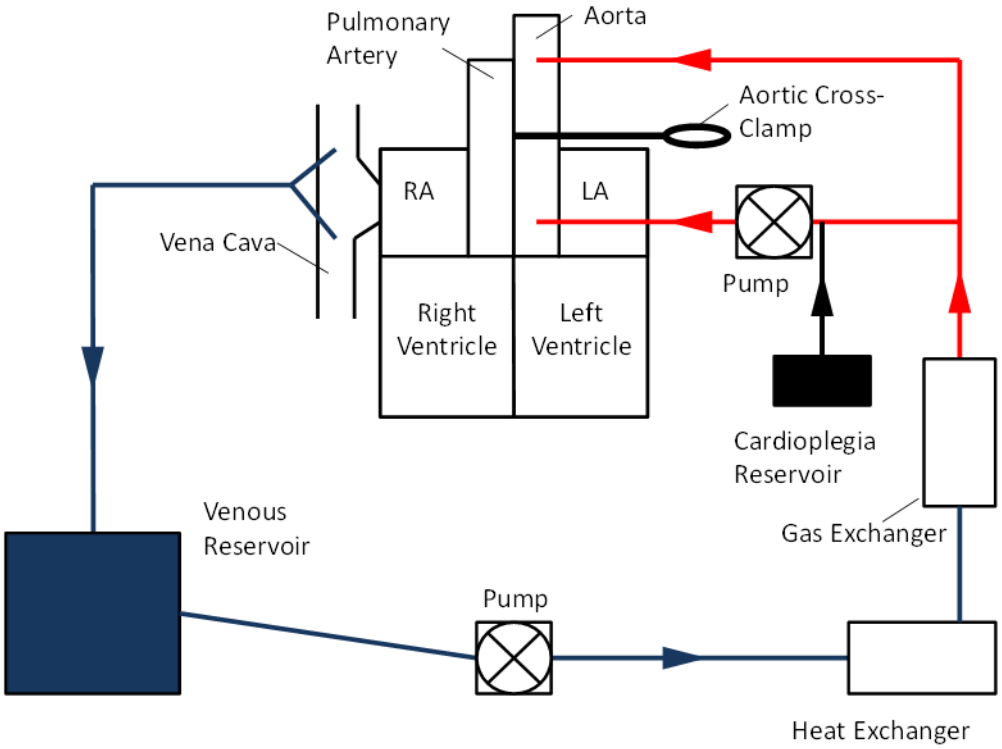


Figure 1.1 Schematic representation of a cardiopulmonary bypass circuit. Filling of the heart is prevented by diverting blood flow via cannulae in the inferior and superior vena cava (blue) and directing this to the venous reservoir. Oxygenated blood (red) is returned to the systemic circulation via a cannula in the aorta, distal to the position of aortic cross-clamp. Cardioplegia solution (black) is mixed with oxygenated blood and perfused via the coronary arteries to maintain the heart in asystole (RA = right atrium; LA = left atrium).

1.2 Complications of surgery requiring CPB

Outcomes from cardiac surgery have continued to steadily improve and this has enabled increasingly complex surgery to be performed (1). However, as with any intervention, complications are experienced by some patients and these include stroke, cardiac arrhythmia, renal failure, myocardial infarction, sepsis and bleeding (4-6). All these complications can result in increased morbidity, a prolonged period of invasive ventilation and intensive (level 3) or high dependency (level 2) care.

The focus of this thesis is on the prediction of bleeding. Bleeding following surgery requiring cardiopulmonary bypass is associated with increased morbidity and mortality (7). It accounts for around 4% to 15% of red blood cells transfused in the UK and USA, and approaches 20% globally (7, 8). A similar pattern has been observed for platelet and plasma transfusions. A survey from the UK in 2012 found cardiac surgery accounted for 10% of the units of platelets transfused (9), whilst another study reported that cardiac surgery was responsible for 12.7% of the plasma transfused (10). Despite this comparatively heavy usage, 70-80% of the blood products transfused are given to only 12-20% of patients (7, 8). This suggests that better identification of patients at risk of bleeding may allow a reduction in blood product use by allowing preventative measures to be taken before excessive bleeding develops.

Definitions of bleeding following cardiac surgery have varied between studies and have included: blood loss in excess of 1 litre at 24 hours (11); blood loss exceeding 200 mL/kg per hour (12); blood loss exceeding 2 mL/kg per hour for two or more consecutive hours (12); the need for haemostatic treatment (13); return to theatre for re-exploration for bleeding (1, 13).

1.2.1 Morbidity associated with bleeding and red cell transfusion

In a large retrospective study of 1,188 patients undergoing cardiac surgery, Christensen et al reported an incidence of excess post-operative bleeding of 6.4%. This was associated with a near three-fold higher 30-day mortality (odds ratio [OR] = 2.9, $p < 0.001$), an increased incidence of stroke (OR = 3.3, $p = 0.0033$), an unsurprising increased risk of needing to return to theatre for re-exploration (OR = 103.655, $p = 0.0001$), and an increased likelihood of intensive care unit stay longer than 72 hours (OR = 1.3, $p < 0.0001$) and mechanical ventilation longer than 24 hours (OR = 3.4, $p = 0.0002$) (12).

Even if major bleeding does not develop, transfusion of red cells is common, with 50-80% of patients in one study being reported to have received at least one unit of red cells within a day of surgery (14). Transfusion of blood products has in itself been associated with post-CPB complications. A large retrospective study of 10,425 patients reported red cell transfusion was an independent risk factor for early mortality after CABG, and the risk increased with increasing numbers of red cell units transfused (15). Renal failure has also been reported more frequently in those receiving red cell transfusions, although whether this is causal or an indicator of bleeding leading to reduced renal perfusion is uncertain (16, 17). Finally, red cell transfusion has also been associated with an increased risk of postoperative infection. In an observational study of 5,158 patients who underwent cardiac surgery, overall 48% were transfused at least one unit of red cells, with 63-65% of those undergoing dual CABG and valve replacement or surgery on the thoracic aorta requiring transfusion of a median of 3-4 units (18). Whilst the overall incidence of infection was only 5.8%, for each unit transfused there was a 23% increase in the risk of infection in the first 65 post-operative days. Platelets and fresh frozen plasma (FFP) appear to confer no increase in risk (19).

The exact mechanism for these observations is unclear. These increased risks may in part be associated with the duration of blood product storage prior to transfusion (20). Storage of red cells leads to a variety of changes including a reduction in deformability, a reduction in 2,3-diphosphoglycerate levels which increases the affinity of haemoglobin for oxygen, thus reducing oxygen delivery to the tissues, and reductions in the concentration of adenosine triphosphate (ATP) and nitric oxide (21-24). In addition during storage of blood products, elastase, histamine, soluble-HLA, soluble Fas-ligand, TGF-β1 and the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 have all been reported to increase (25).

Regardless of the exact mechanism by which red cell transfusion increases morbidity, given that bleeding is a reason for transfusion, accurate identification of those at risk of bleeding and early intervention might improve patient outcomes.

1.3 Current concepts of normal haemostasis

1.3.1 Primary haemostasis

After breach of a vessel wall, a variety of components are responsible for the initial prevention of bleeding. These include platelets, Von Willebrand factor, collagen and laminin.

Platelets are formed from megakaryocytes through a complex system of actin and tubule formation. They contain alpha and delta (or dense granules), mitochondria and a tubular system which allows a change of shape following activation. Platelet activation serves two important purposes. Firstly it provides a phospholipid surface upon which procoagulant and anticoagulant proteins can assemble and interact. Secondly, aggregation leads to the development of a stable thrombus to plug the physical defect that has led to bleeding. Platelets are initially brought to the site of an injury by interaction between GPIbα and Von Willebrand factor (VWF).

VWF is synthesised by endothelial cells, where it is stored in Weibel-Palade bodies and in megakaryocytes where it ultimately ends up in platelet α-granules. VWF is also present in the sub-endothelium where it is associated with collagen and laminins (26). VWF binds to GPIbα on platelets via its A1 domain, a process which is influenced by shear rate, and to collagen via its A3 domain. Thus VWF is able to bring platelets into proximity with collagen to allow their activation via GPVI/FCRγ (27). Binding of collagen to the GPVI/FCRγ complex leads to activation

of downstream signalling pathways and release of intracellular calcium, which in turn leads to release of ADP and ATP from delta granules. Platelet activation also occurs through the interaction of laminin and the platelet surface integrin α6β1 (26).

VWF also influences platelet aggregation. Its C1 domain contains a binding site for the platelet integrin αIIbβ3. The latter becomes activated on the platelet surface following activation via collagen binding. VWF and fibrinogen can both act to cross-link between αIIbβ3 on adjacent platelets leading to aggregation.

1.3.2 Platelet Activation

Adhesion and aggregation via integrin interaction is mediated by tyrosine kinase and G-protein linked pathways. Ligand binding and clustering of integrins leads to actin polymerisation and activation of phospholipase C (PLC). After initial activation via integrin signalling, a wide variety of secondary activation pathways are triggered (28, 29). Triggers include ADP released from dense granules which activates the platelet membrane receptors $P2Y_1$ and $P2Y_{12}$, and thrombin generated as part of secondary haemostasis which activates PAR1 and PAR4. Platelet activation can also be mediated by thromboxane A_2 which is a product of arachidonic acid metabolism. Arachidonic acid can be generated from membrane phospholipids through the action of phospholipase A_2 (PLA_2) activated by calcium released from platelet dense granules (30). Arachidonic acid is then then further metabolised by cyclooxygenase-1 (COX-1) to prostaglandins G_2 and then H_2. Prostaglandins H_2 then serves as the substrate for thromboxane synthase to produce thromboxane A_2.

1.3.3 Secondary haemostasis

The current theory underlying haemostasis has evolved considerably over the last 50 years. The original coagulation cascade model, whilst useful to assist in the identification of the causes of abnormalities in the commonly used coagulation tests of the prothrombin time (PT) and activated partial thromboplastin time (APTT), has now been superseded by the cell based model (31).

Tissue Factor (TF) expression on cells is the first step in the initiation of coagulation. There has been a degree of controversy as to which cells in the blood actually express TF(32). Monocytes have long been recognised as being able to express TF in response to stimuli such as lipopolysaccharide (LPS), IL-1α and TNF-β (33, 34). In addition TF can be expressed on macrophages in response to CD40L, vascular smooth muscle cells in response to platelet derived growth factor , eosinophils in response to platelet activating factor (PAF) and granulocyte monocyte-colony stimulating factor (GM-CSF), endothelial cells by LPS, IL-1α and TNF-β, and astrocytes (35-39). In addition, there have been reports of TF being identified in platelets (40, 41). However, there is an increasing body of evidence to show that platelets do not express TF themselves (42, 43), but instead their membranes are able to incorporate TF expressing microparticles derived from other cell types (44, 45).

Tissue factor can be expressed in active and inactive conformations, also referred to as decrypted and encrypted forms. Decryption has been suggested to require the formation of disulphide bond between cysteine residues at position 186 and 209 and the co-expression of phosphatidylserine (46). The presence of phosphatidylserine (PS) alone may not be only determinant of tissue factor activity on the cell membrane, as lower thrombin generation has been reported when using tissue factor isolated from a monocyte cell line and then relipidated

with PS compared to tissue factor presented in intact cell membranes (47). Distinct lipid rafts containing cholesterol may also play a role in regulating TF activity (48).

Factor VII (FVII) activation takes place via a variety of mechanisms. *In vitro* experiments have demonstrated that incubation of plasma at cold temperatures results in a significant increase in activated factor VII (FVIIa) (49, 50). Factors IXa, Xa, XIIa and thrombin have also been demonstrated to able to convert FVII to FVIIa and that the conversion of FVII to FVIIa is increased in the presence of TF (51-53). FVII has also been reported to undergo auto-activation by FVIIa (54).

TF in conjunction with FVIIa proceeds to cleave factor X (FX) to Xa (FXa) and factor IX (FIX) to IXa (FIXa). This leads to the amplification phase where a small amount of thrombin is generated which is then able to activate factor XI (FXI) along with the factors VIII (FVIII) and V (FV). In the propagation phase, activated FVIII (FVIIIa) acts as a cofactor for FIXa which increases its ability to activate FX. Activated FV (FVa) acts as a cofactor to FXa to significantly increase the production of thrombin, which in turn cleaves fibrinogen to fibrin and allows the formation of a definitive clot. These reactions require the presence of a phospholipid surface and ionic calcium. Ionised calcium plays a key role in acting as a cofactor to allow the gla-domain containing coagulation factors, II, VII, IX and X, to interact with phospholipid surfaces (55). Thrombin also activates factor XIII (FXIII). Activated FXIII (FXIIIa) catalyses the cross-linking between the D moieties of fibrin molecules, resulting in enhanced clot strength and a reduction in susceptibility to fibrinolysis (56).

An alternative pathway for activation of coagulation involves contact activation. This pathway has generally been thought to be of importance *in vitro* where, amongst others, glass, silica

and anionic polymers have been recognised activators. More recent studies have found that polyphosphates appear to be the initiator of this pathway *in vivo* (57). Long-chain polyphosphates, such as those found in Salmonella, are the most potent (58). However, platelet dense granules also contain short-chain polyphosphates which can also mediate contact activation (59), albeit less potently than long-chain polyphosphates. Contact activation results in proteolysis of high molecular weight kininogen and prekallikrein. These activate factor XII which in turn cleaves FXI. This rapidly results in the formation of the tenase complex consisting of FIX and its cofactor FVIII, a complex that has significantly higher affinity for FX compared to TF-FVII. This pathway may also be important in cardiopulmonary bypass where the material of the bypass circuit may initiate contact activation.

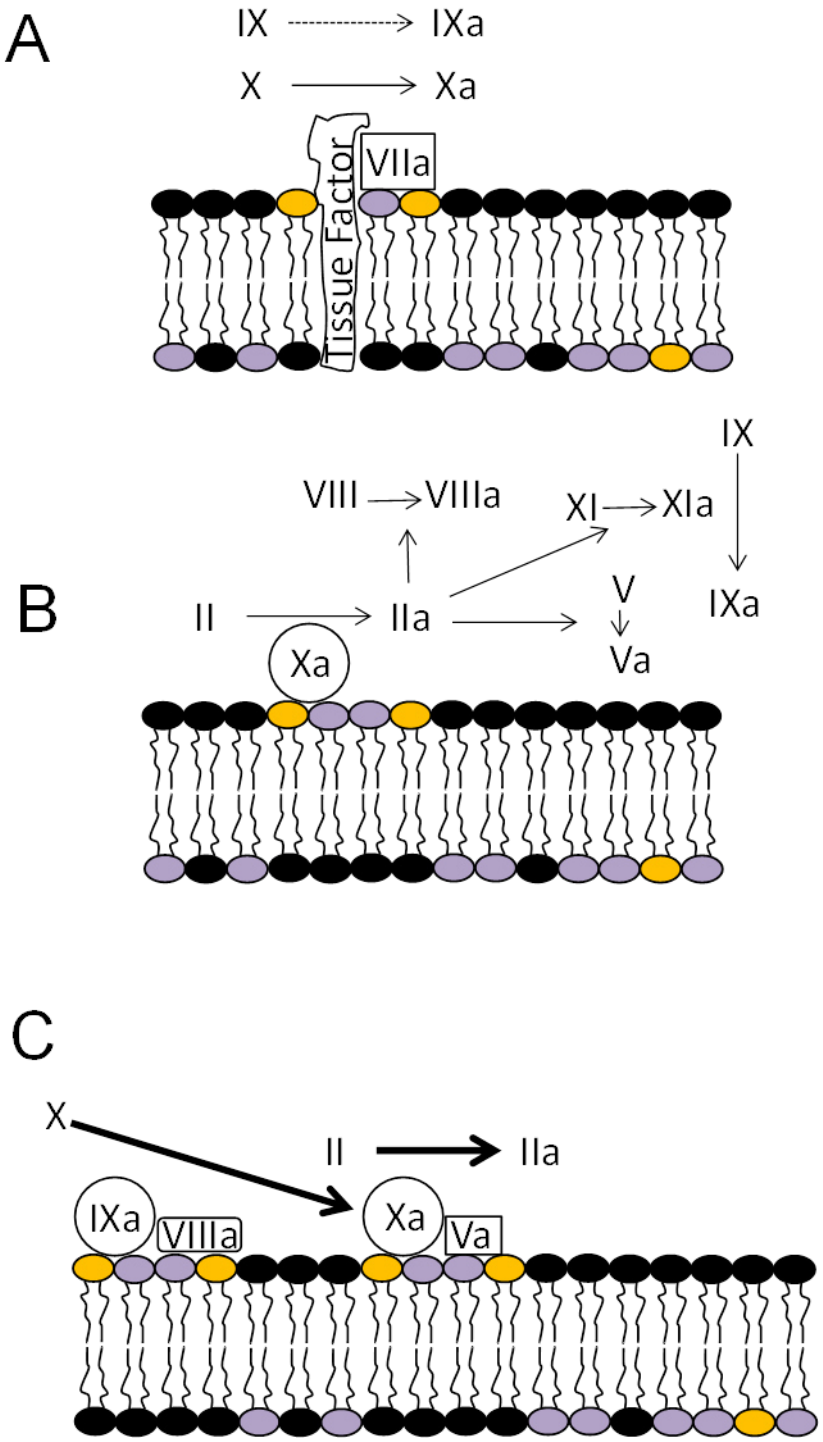


Figure 1.2.The current concept of coagulation begins with an initiation phase (Panel A), where TF-FVIIa activates FX and FIX. In the amplication phase (Panel B), FXa cleaves prothrombin (II) to thrombin (IIa), which cleaves FVIII to FVIIIa, FV to FVa and FXI to FXIa. This results in the propagation phase (Panel C) where the the tenase complex (FIXa and FVIIIa) cleaves large amounts of FX to FXa. This forms the prothrombinase complex with FVa which leads to the generation of a burst of thrombin.

To tightly regulate this process there are inhibitors at each stage. Tissue Factor pathway inhibitor (TFPI) is a key inhibitor of the initiation phase of the coagulation cascade and the majority is associated with the endothelium bound to glycosaminoglycans (60, 61). It is a protease inhibitor which binds to FVIIa and FXa (62). Its importance can be inferred from the finding that unstimulated monocytes express TFPI in excess of TF, presumably as a means of preventing inadvertent triggering of coagulation that might otherwise occur (63). It has three kunitz-type domains. The first of these binds to FVIIa while the second binds FXa. The third domain binds to protein S which potentiates the anti-FXa activity of TFPI. TFPI is released from the endothelium by heparin (64). TFPI is cleared via hepatocytes, mediated by LDL receptor related peptide (65), and also via the renal tract (66, 67).

The amplification phase is regulated by protein C which is activated by thrombin in the presence of thrombomodulin. Activated protein C and its cofactor protein S inactivate factors Va and VIIIa by proteolysis. Antithrombin, synthesised in the liver, forms a complex with thrombin and to a lesser extent FXa, FIXa and FXIa. These reactions are potentiated by naturally occurring heparins and other glycosaminoglycans. Alpha-2-macroglobulin is an additional inhibitor of thrombin which forms a one-to-one covalently bound complex with it, preventing interaction between thrombin and its substrates by steric hindrance.

1.3.4 Fibrinolysis

Once a clot has been formed, it will ultimately need to be degraded once the underlying lesion has been repaired. Once fibrin is generated it binds tissue plasminogen activator, generated by the endothelium, which in turn cleaves plasminogen at Arg561-Val 562. The product, glu-plasmin, then undergoes autocatalytic cleavage of its N-terminal region to form lys-plasmin, which has an exposed lysine binding site that enhances its ability to cleave fibrin and

fibrinogen. PAI-1 and PAI-2 inhibit plasminogen whilst thrombin-activatable fibrinolysis inhibitor (TAFI) and α_2-antiplasmin regulate plasmin activity.

TAFI is predominantly synthesised in megakaryocytes and the liver. Thrombin is its primary activator. Thrombin in conjunction with thrombomodulin is able to activate TAFI with over a thousand folder greater efficiency than on its own (68). TAFI activation involves cleavage at the arginine residue at position 92 which allows substrates to access its active site (69). Plasmin is also able to activate TAFI, a process that may be physiologically important for TAFI activation away from the endothelium and thrombomodulin bound thrombin (70-72). TAFI regulates plasmin activity by cleaving C-terminal lysine residues from fibrin, thus removing the binding site for plasmin. This process appears to act on a threshold based mechanism, whereby fibrinolysis is only inhibited when TAFI reaches or exceeds a certain concentration (73).

1.4 Phospholipids

1.4.1 Role in haemostasis

The role of phospholipids in coagulation has long been recognised as essential, indeed their inclusion in routine laboratory assays APTT and PT is required in order for clotting to take place. What has become increasingly clear is that different phospholipids have different functions and effects. For example, whilst phosphatidylcholine has generally been considered to be inert, the importance of the amount in a given membrane is demonstrated by the observation that below a certain amount thrombin generation may be impaired (74, 75).

Membrane asymmetry is important to prevent coagulation. In 1977 Zwaal et al demonstrated that in un-activated platelets the inner membrane was able to shorten clotting times whilst the external membrane in comparison did not (76). Thrombin stimulation of platelets was shown in 1976 to result in exposure of phosphatidylethanolamine (PE) (77). Total PE and PC in the platelet membranes were shown to be reduced 20% and 9% respectively by thrombin stimulation with the arachidonate content of PC reducing (78), indicating hydrolysis by PLA_2.

Under resting conditions, the external platelet membrane is composed predominantly of the inert phospholipid PC. On activation of the platelet, the negatively charged phospholipids PE (77, 79) and phosphatidylserine (PS) (80) move to the external membrane to support coagulation. Phospholipid exposure on cell membranes is considered to be mediated by three types of transporter as shown in Figure 1.3. Flippases are aminophospholipid translocases that mediate the movement of PE and PS from the external membrane to the internal membrane. A number of membrane proteins have been described with this activity, with those in platelets being ATP dependent (81). Floppase activity, where predominantly PC is moved passively to the external membrane thus maintaining membrane asymmetry, has been associated with the ABC class of transmembrane transporter proteins (82), although the precise identity of these transporter proteins remains uncertain. Finally scramblase activity is calcium dependent, and on activation of platelets allows the rapid exposure of the negatively charged phospholipids PE and PS on the external membrane (83). Four different proteins have been reported to have scramblase activity, with each appearing to function in specific situations: TMEM16F plays a role in platelets, XKR8 and PLSCR1 in response to apoptotic stimuli and PLSCR3 specifically in mitochondrial degradation (84). The clinical importance of exposure of platelet membrane phospholipids is demonstrated by Scott's Syndrome, a disorder whereby platelets are unable to externalise aminophospholipids (85), leading to a reduction in their ability to support thrombin generation.

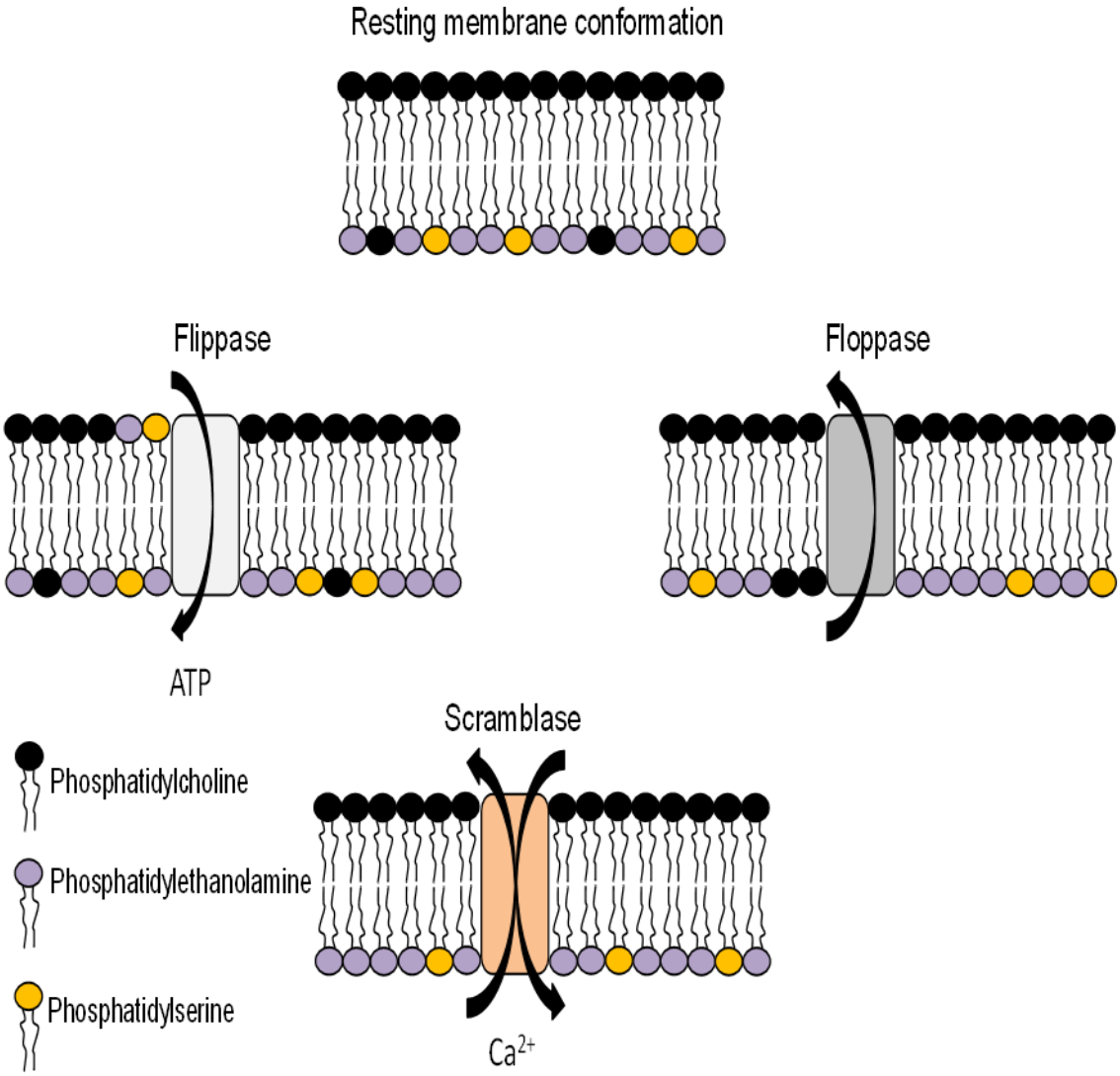


Figure 1.3 There are three types of transporter considered to be involved on the movement of phospholipids between the external and internal membrane of cells. Flippase activity is energy dependent and requires ATP to maintain membrane asymmetry; floppase activity appears to be a passive process, whilst scramblase activity is calcium dependent.

The existing routinely available coagulation assays have been shown to be affected by the phospholipid composition of the reagents used. Changing the composition of thromboplastin reagents by varying the proportions of PE, PC and PS has been shown to alter the sensitivity of the PT for deficiencies of FII, FV, FVII and FX (86).

Binding of FXa to membranes composed of PC and PS induces a conformational change in the protease which increases its affinity for interaction with FVa as assessed by fluorescence anisotropy (87). Evidence also exists suggesting that PS containing phospholipid membranes may alter the substrate specificity of FXa to favour the production of thrombin via its intermediate cleavage to meizothrombin rather than prothrombin 2 (88).

1.4.2 Platelet phospholipid structure and metabolism

Glycero-phospholipids have a characteristic structure composed of a head group, such as choline, serine or ethanolamine, a phosphate group, a glycerol backbone and two fatty acids. The glycerol backbone consists of three carbon atoms, termed sn1 to sn3. The fatty acid chains attach at the sn1 and sn2 positions whilst the phosphate group attaches at the sn3 position (89).

These different sections of the structure each confer different physical properties. The head group is hydrophilic and charged leading to the typical orientation in a lipid bilayer of head groups on the outer surface and fatty acid chains orientated towards the centre.

The fatty acids can either be unsaturated (one or more carbon double bonds present) or saturated (no carbon double bonds present). Increasing fatty acid saturation been shown to

influence coagulation (90). The fatty acids at the sn1 position can be attached by an acyl, or a vinyl-ether bond in the case of plasmalogens (91, 92), whilst those at the sn2 position are attached via an acyl bond only. Studies using X-band electron paramagnetic resonance (EPR) spectroscopy, whereby the EPR spectrum is measured of a magnetically anisotropic probe embedded in the lipid membrane, have shown that the fluidity of the membrane is influenced by the fatty acid composition, with the gel-liquid transition temperature being determined at least in part by the fatty acid composition (93).

Plasmalogen and acyl phospholipids possess distinct physical properties. For example, the phase transition point for plasmalogens from gel to liquid occurs at a lower temperature compared to acyl phospholipids (94). In addition liposomes composed of plasmalogens fuse more readily than those composed of acyl forms (95). These properties would therefore influence membrane structure and composition which would be of relevance to coagulation and this is further supported by the finding that majority of PE in platelets is the plasmalogen form (96). In addition plasmalogens have been reported to play a role in cell signalling and prevention of oxidative damage, and it may be these functions which explain why decreased levels have been reported to associated with neurodegenerative conditions and inborn errors of metabolism (89).

The fatty acid composition of platelet glycerophospholipids has been reported to predominantly consist of, in decreasing order of abundance, arachidonic acid, palmitic acid, stearic acid, oleic acid and linoleic acid (96). The phospholipid molecule can be cleaved or modified at different positions by a variety of enzymes (97). Phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2) hydrolyse phospholipids at the sn1 and sn2 positions respectively. Phospholipase C (PLC) can cleave at the phosphate group from the glycerol back bone, whilst

phospholipase D (PLD) cleaves the head group from the phosphate group. This is summarised in Figure 1.4.

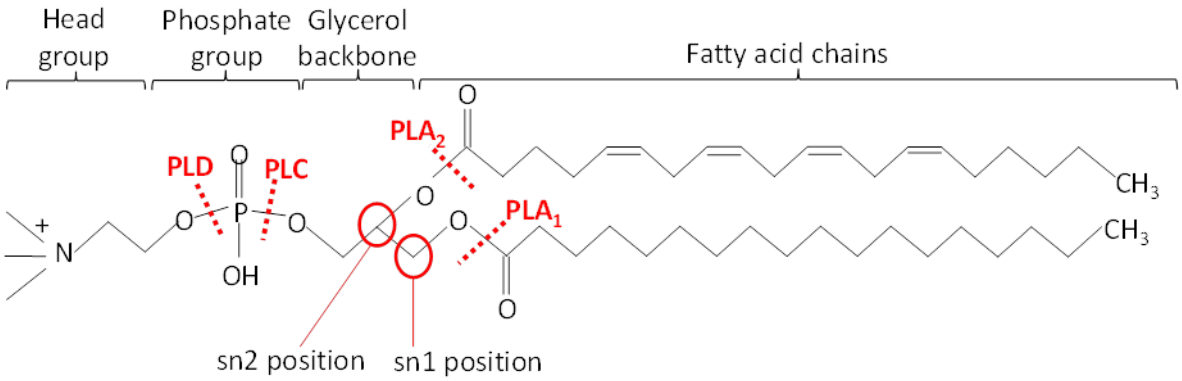


Figure 1.4 Example structure of a typical glycerophospholipid (1- stearoyl-sn2-arachidonyl-phosphatidylcholine). The different components of the structure are annotated, along with enzymatic cleavage points for phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD). Adapted from O'Donnell et al (97).

Following hydrolysis by PLA_2, liberated arachidonic acid can undergo oxidation by lipoxygenase (LOX) followed by reduction by glutathione peroxidase to from hydroxyeicosatetraenoic acid (HETE). Alternatively the action of cyclooxygenase (COX) can generate prostaglandins. In platelets, 12-LOX is the only isoform identified and oxidises the 12th carbon atom, whereas in macrophages it is 15-LOX and 5-LOX in eosinophils. The oxidation occurs in three steps: 1) hydrogen is abstracted from the arachidonic acid molecule; 2) radical rearrangement; 3) oxygen is inserted onto the arachidonic acid molecule, the position depending on the LOX isoform (98). The hydroperoxide product is unstable and is reduced to the corresponding hydroxide form by glutathione peroxidase. A novel family of oxidised phospholipids, 12-HETE-PE and 12-HETE-PC, has recently been described that are rapidly generated by 12-LOX in platelets after stimulation with thrombin or collagen (99). The 12-hydroxyeicosatetraenoic acid produced is re-esterified by fatty acyl co-A ligase to form the oxidised phospholipids. This process is summarised in Figure 1.5 and examples of the structures of arachidonate containing

PE, PS and PC species are shown in Figure 1.6. These HETE-phospholipids have been shown to increase thrombin generation measured by calibrated automated thrombography (99).

They are likely to play an important role *in vivo*, as a number of recent studies have demonstrated that platelet 12-LOX activity can be stimulated by thrombin via PAR4, collagen via GPVI, and also immunoglobulin via the Fcγ Receptor IIa (100, 101). This suggests a potential role in secondary haemostasis. Oxidisation of fatty acids can result in differences in the polarity and hydrophilicity of the parent phospholipid compared to their un-oxidised forms, which may result in changes to the properties of the lipid membrane such as fluidity and orientation of other phospholipid molecules.

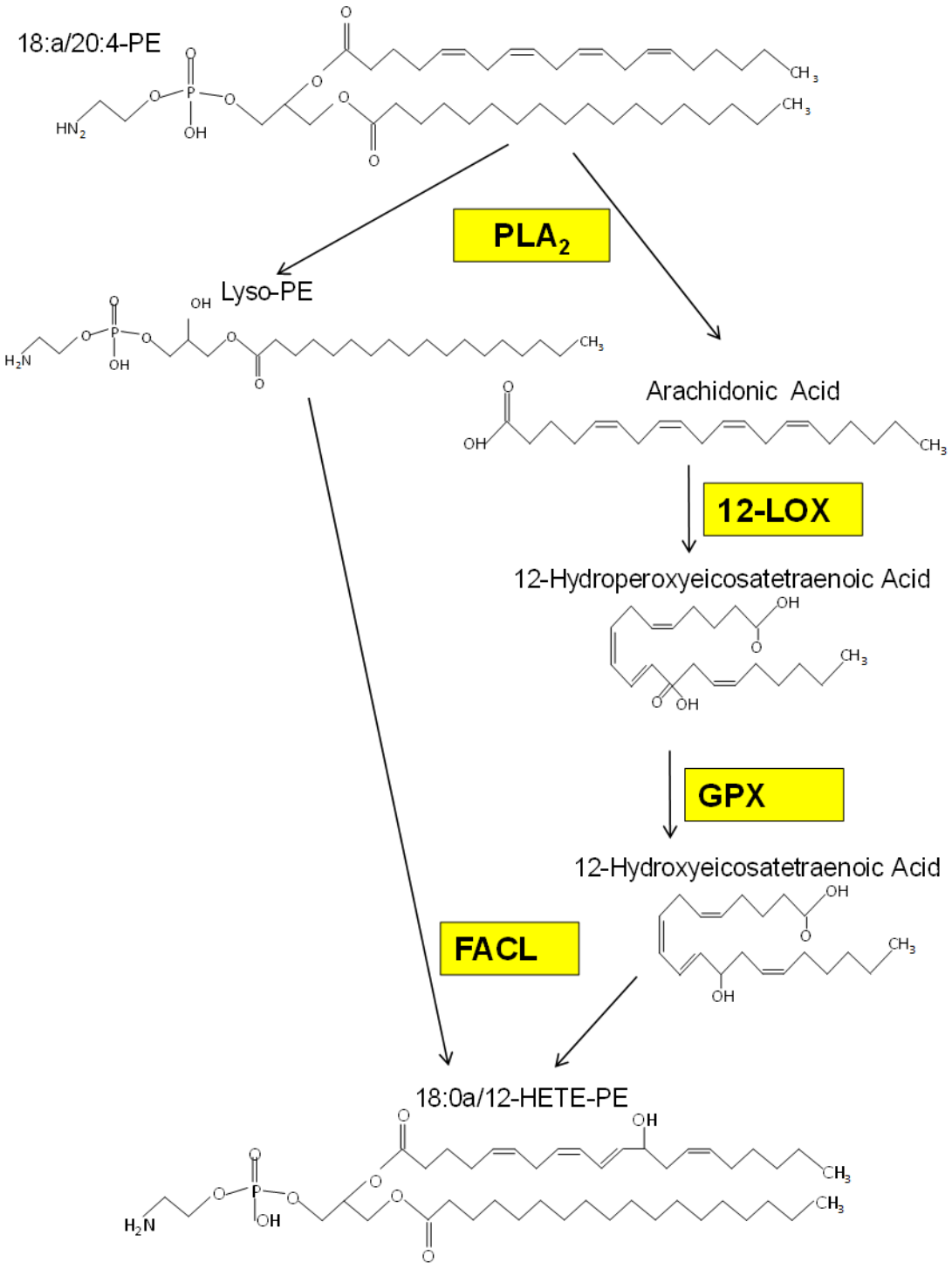


Figure 1.5 The enzymatic degradation of the phosphatidylethanolamine species 18:0a/20:4-PE. PLA_2 cleaves 18:0a/20:4-PE into lyso-PE and arachidonic acid. 12-lipoxygenase (12-LOX) may then oxidise arachidonic acid to 12-hydroperxoxyeicosatetraenoic acid, an unstable product which is then reduced by glutathione peroxidase (GPX) to produce 12-hydroyeicosatetraenoic acid (12-HETE). This can then be reincorporated into lyso-PE by Fatty Acyl-CoA Ligase (FACL) to form 12-hydroxyeicosatetra-phophatidylethanolamine (12-HETE-PE). Adapted from O'Donnell et al (97).

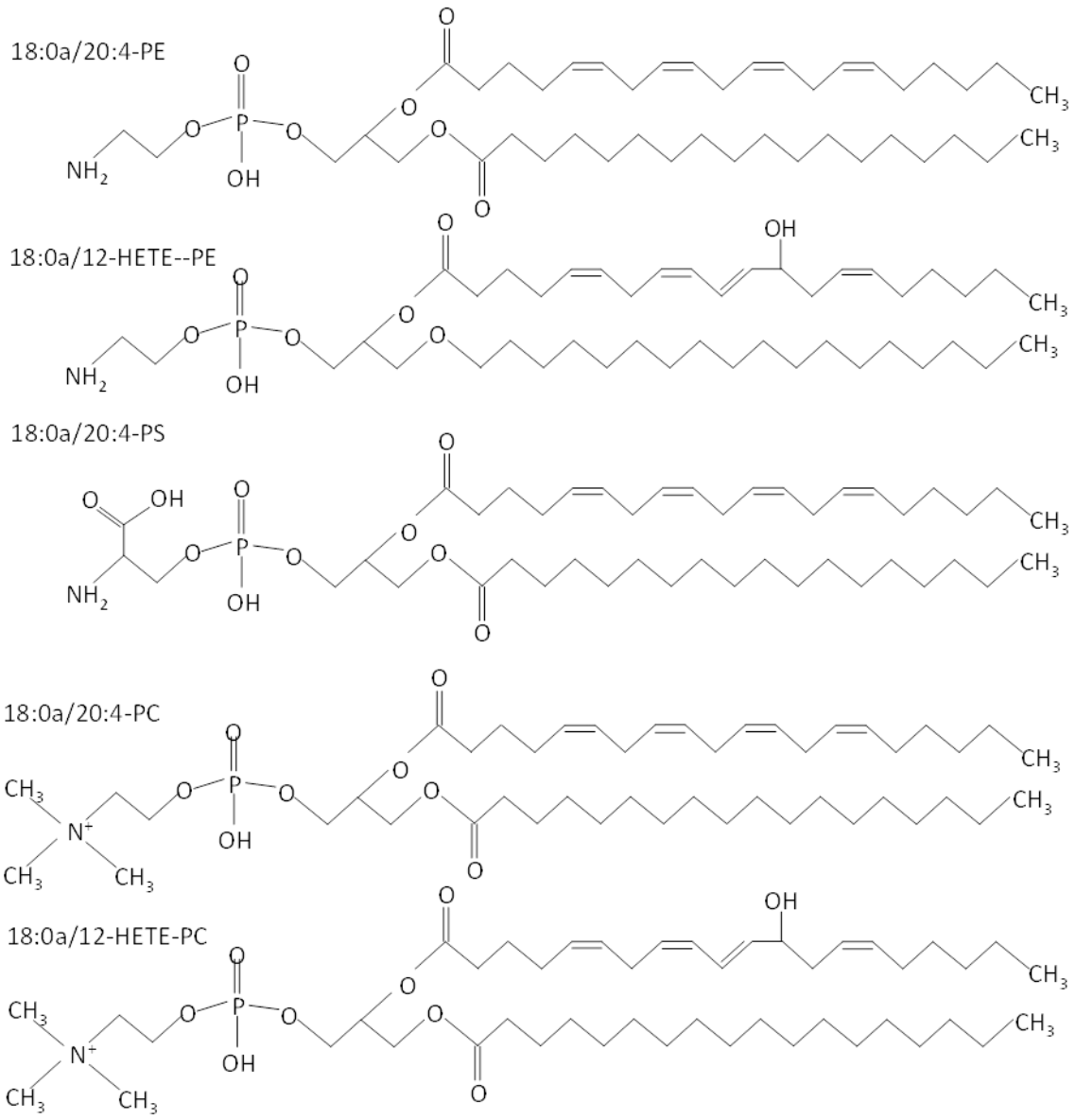


Figure 1.6 Examples of the structures arachidonic acid containing species of phosphatidylethanolamine (18:0a/20:4-PE), phosphatidylserine (18:0a/20:4-PS) and phosphatidylcholine (18:0a/20:4-PC). The structure of the corresponding 12-hydroxyeicosatetrenoate derivatives of phosphatidylethanolamine (18:0a/12-HETE-PE) and phosphatidylcholine (18:0a/12-HETE-PC) are shown for comparison.

1.5 Effect of CPB on haemostasis and associations with bleeding

Bleeding following surgery requiring the use of cardio-pulmonary bypass can have various causes. These include dilution and consumptive of clotting factors, anticoagulation,

thrombocytopenia and or platelet dysfunction (102) and surgical relating to breach of the vasculature.

During cardiopulmonary bypass, intravascular volume needs to be maintained in order for the bypass machine to continue to function optimally. These fluids consist of a mixture of colloids and crystalloids. A greater proportion of the former tend to remain in the intravascular space whilst only around 20% of the latter remains. Colloids may cause impairment of coagulation factor function through adsorption and steric hindrance as well as dilution. Crystalloids tend mainly to cause dilution, however excess sodium ions may interfere with coagulation by increasing thrombin's cleavage of substrate.

Prothrombin fragment 1.2 has been used in the past as a marker of thrombin generation. In general, it has been reported to increase post-CPB, along with an increase in thrombin-antithrombin complexes. Low levels pre-CPB have been reported to be associated with increasing blood loss post-CPB (103), suggesting that reduced thrombin generation is a risk factor for post-CPB bleeding.

Kininogen levels increase during-CPB indicating cleavage of high molecular weight kininogen and activation of the contact pathway of coagulation (104). In general studies have reported a decrease in prothrombin, FV and FX, FVII and XI to be unchanged or reduced, and FVIII and IX to be increased or unchanged post-CPB (105, 106). The differences in the reported effect of CPB are likely to relate to differences in the timing of post-CPB samples.

Postoperative blood loss has been reported to be weakly inversely correlated with fibrinogen concentration measured 2 hours post-CPB ($r= -0.33, p = 0.019$) and FXIII activity measured pre and 2-hrs post ($r= -0.34$, $p=0.0009$ and $r=-0.41$, $p=0.003$, respectively), suggesting that defective clot strength may have been the cause of blood loss (105). Pre-operative fibrinogen concentration has been reported to be an independent predictor of post-CPB blood loss with a moderate inverse correlation ($r= -0.53$, $p<0.0001$), but in the absence of a clear cut-off level this was of limited clinical utility (107). Post-operative bleeding has been shown to inversely correlate with fibrinogen level at the end of CPB (108) and those with a level <2.0 g/L have been reported to be significantly more likely to require in excess of 5 red cell units transfused in the first 24 hours post-CPB than those with a level ≥ 2.0 g/L ($p<0.0001$) (109). FXIII has been shown to be reduced post-CPB (105). The post-operative FXIII level has been shown to have variable effects on bleeding. Some studies have reported no association (108), whilst others have reported an association between FXIII, clot strength determined by thromboelastography and bleeding (105, 110).

CPB has also been shown to affect the concentrations of anticoagulants. Total protein C levels fall post cardiopulmonary bypass, but activated protein C increases (111, 112). In addition full length and total TFPI have been reported to increase post-CPB (113). TFPI may play a role in bleeding post-CPB because heparin, the standard anticoagulant used during cardiopulmonary bypass, releases TFPI from the endothelium (64).

1.6 Effect of CPB on platelets

Following CPB, numerical (11, 114) and functional defects of platelets have been reported. Platelet aggregation is reduced in response to ADP (115-118). P-selectin, a marker of platelet activation, has generally been reported to be either unchanged (119) or increased (120, 121).

Studies measuring Annexin V binding suggest there may also be changes in the phospholipids expressed on the external membrane of platelets post CPB (120, 122). Annexin V binds to negatively charged phospholipids and therefore has been used as an indicator for the presence of these lipids. In addition a reduction in mean platelet volume post-CPB has been reported, suggesting a loss of phospholipid membrane may occur (123).

1.7 Haemostatic agents for management of bleeding post-CPB

At present there are comparatively few methods to correct haemostatic failure. These consist of platelet transfusions, fresh frozen plasma (FFP), cryoprecipitate, or the unlicensed use of recombinant factor VIIa (rFVIIa), prothrombin complex concentrate (PCC), or fibrinogen concentrate. Inhibition of fibrinolysis by tranexamic acid (a synthetic lysine analogue which reduces fibrinolysis by competing for plasmin binding to lysine residues on fibrin), and previously aprotinin (a broad spectrum serine protease inhibitor), is also routinely used.

FFP has variable clinical efficacy and it has been reported that volumes of 30 mL/kg are needed to obtain a clinically relevant increase in clotting factors in critically ill patients (124). In the setting of cardiac surgery, a large volume load such as this may have deleterious effects on cardiac function. Studies where lower volumes of FFP were used to treat bleeding showed no difference in mortality whether FFP was given or not (125). Furthermore FFP has risks associated with transfusion such as transfusion related acute lung injury (126).

rFVIIa has been used outside of its licensed indication to manage bleeding post-CPB, typically where other measures have been ineffective (127). However, no randomised controlled trials exist and two recent meta-analyses were unable to demonstrate a reduction in mortality but did find an increased incidence of thrombotic events (128, 129).

PCCs either contain FII, FIX and FX or FII, FVII, FIX and FX depending on the manufacturer and the licensing jurisdiction, along with other vitamin K dependent factors including protein C and S. There are also variations in the amount of heparin present depending on the manufacturer (130). Successful off label use of PCCs to manage excess bleeding following cardiac surgery has been reported in a number of small studies which were either case series or retrospective analyses (131-133). As with rFVIIa, no randomised trials have been reported thus far, making the dose to administer and the selection or patients a matter of clinical judgement.

Fibrinogen concentrate has been used in a number of small prospective studies which have suggested a reduction in transfusion requirements and bleeding (134-136). However larger studies are required to provide better data on overall efficacy and safety in the setting of CPB.

Finally, inhibition of fibrinolysis has been a longstanding part of management of patients undergoing CPB. Tranexamic acid and aprotinin both reduce post-operative bleeding and transfusion requirements (137, 138). However, aprotinin was withdrawn from the market in 2007 due to safety concerns, and although since reintroduced in Europe and Canada, its use has remained limited (139).

1.8 Assays for monitoring haemostasis

1.8.1 Routine laboratory coagulation assays

Current routinely available assays of coagulation consist of the prothrombin time (PT) and activated partial thromboplastin time (APTT). The end point of both these assays is the earliest formation of fibrin, and modern automated coagulometer detect this as a change in light

absorbance. However, this end point is reached early in the overall process of producing a definitive clot; therefore these assays have limited value to assessing overall haemostatic function. In the setting of cardiac surgery requiring CPB there is no strong evidence to suggest they can predict bleeding.

Their main role lies in identifying individual clotting factor deficiencies. These are FVII in the case of the PT; FVIII, FIX, FXI, FXII, Prekallikrein and High Molecular Weight Kininogen in the case of the APTT; FII, FV, FX where both are prolonged. They can also be readily used to monitor many of the anticoagulants. The PT is routinely used to monitor warfarin, whilst the APTT is particularly sensitive to unfractionated heparin, an anticoagulant used clinically in various settings, including cardiac surgery. Unfractionated heparin consists of molecules of heparin sulphate of varying size. These bind to antithrombin via aspartate residues and potentiate its ability to inactivate FIIa, FXa and FIXa.

1.8.2 Activated Clotting Time (ACT)

During cardiac surgery requiring CPB a rapid and simple test is required to adjust the heparin dose. It is of critical importance to maintain adequate anticoagulation to prevent blood clotting in the bypass apparatus. The activated clotting time (ACT) is used for this purpose. Whole blood is added to a test tube containing silica, or another contact activator, along with a magnetic stir bar. The device is then able to record at which point the stir bar is no longer able to move which provides the end point for the test. During CPB, the heparin dose is adjusted to maintain the ACT in excess of 400 seconds. However, the ACT will also be prolonged by thrombocytopenia, platelet dysfunction, coagulation factor deficiencies, defective fibrin polymerisation and hypothermia. It is not used to assess haemostasis or guide haemostatic therapy

1.8.3 Thromboelastographic methods

TEG and ROTEM are methods that can be used to monitor global haemostasis, and both lend themselves to near patient testing. Although the precise nomenclature is different, they both give similar results: a reaction time during which the initial activation of haemostasis is taking place; a maximum speed of formation and firmness of any clot formed; an indication of the activity of fibrinolysis.

The TEG apparatus uses a system where the cuvette rotates. The reaction cuvettes usually contain a substance to hasten clot formation. They can also contain hepzyme to remove the effects of heparin, and inhibitors of platelet aggregation to increase sensitivity to fibrin formation. A pin attached to a pressure transducer is inserted and as the clot forms the change in pressure is recorded to provide a curve, differing components of which correspond to different stages of clot formation and fibrinolysis. ROTEM follows similar principles to TEG, the main difference being that the pin rotates whilst the cuvette remains static. By using different substances in the cuvette, the test can be made more sensitive to the contribution of fibrinogen (FIBTEM) or remove the effect of heparin (HEPTEM).

There have been conflicting reports of the role of TEG and ROTEM in predicting bleeding post-CPB. Only one has found any association between pre-CPB thromboelastography parameters and post-CPB bleeding, and those correlations were weak (140).

On arrival in the intensive care unit post-CPB the TEG parameters including maximum amplitude (MA), reaction time (k), and alpha-angle have been reported to identify patients who went on to bleed excessively or needed haemostatic treatment (141, 142). However the

positive predictive value was at best 62% and the studies were small, making clinical intervention prior to bleeding developing more difficult to judge.

Using ROTEM, a number of studies have reported an association between a variety of parameters and post-operative blood loss when measured either at the end of CPB or on admission to the intensive care unit (143, 144). However, in a study of 58 patients Davidson et al reported that at least one ROTEM parameter was abnormal in 94% of patients who did not go on to bleed excessively. Overall they found the positive predictive value (PPV) of ROTEM to identify those who bled >200mL/hr was only 14.8%, although the negative predictive value (NPV) was better at 100% (145). One study has even reported that ROTEM and TEG had less clinical utility in predicting blood loss than standard coagulation screens with lower ROC values (146).

Overall there is little evidence to show that thromboelastography measured pre-CPB can predict bleeding post-CPB. In the post-CPB setting thromboelastography can help guide treatment in a patient who is bleeding. However, using the results to guide preventative treatment prior to bleeding developing may result in a quarter to a third of patients receiving unnecessary intervention.

1.8.4 Platelet aggregometry

More recently, studies have investigated the use of whole blood impedance aggregometry for monitoring platelet dysfunction. The ADP lesion post-CPB is well described, and using reduced aggregation in response to stimulation by ADP has been shown to have moderate predictive value for post-CPB bleeding (>800 mL at 12 hours) with a sensitivity of 70%, specificity of 66%,

NPV of 92% and PPV of 29% (147). Using the need for transfusion as a surrogate for bleeding, the sensitivity was 88% and specificity 59%, whilst the PPV was 65% and NPV 85% (148).

1.8.5 Calibrated Automated Thrombography (CAT)

Thrombin generation has been assessed using various methods. Many studies have assessed thrombin generation by measuring thrombin-antithrombin (TAT) complexes by ELISA, however, this does not give any real time data on how much thrombin can be generated at a given time point. Early methods described to measure thrombin generation in real time were reliant on subsampling from a cuvette or test tube and then measuring the clotting time of that sample, a method that firstly given the time taken to perform such assays, limited the number of time points that could be measured and secondly lacked sensitivity to measure very small amounts of thrombin (149).

Subsequently the 1980s saw attempts to measure thrombin in real time using chromogenic assays. Initially these still relied on sub-sampling from a cuvette or test tube in which the thrombin generation reaction was taking place. A thrombin substrate was then added to the sub-sample and the colour change measured. This was then compared with a standard curve generated from solutions with a known concentration of thrombin and then the thrombin concentration of the test sample was calculated (150). However, these methods remained unsatisfactory due to their time consuming nature and potential for error.

In 1993 an improved method allowing continuous measurement of thrombin generation was described (151). In this method the optical density of the test plasma was measured every two seconds and the velocity of change plotted against time. The concentration of thrombin was then calculated based on the formula for velocity of change in optical density at given time (vt)

=Kcat x [Thrombin] x [Substrate]/(Km + [Substrate]). By rearranging this formula, the concentration of thrombin could be calculated using the equation, [Thrombin] = vt(Km + [Substrate])/Kcat x [Substrate].

However, because the light absorbance increased as fibrin was formed this resulted in a potential for erroneous calculation of the amount of thrombin generated. It was also realised that it was necessary to compensate for the effect of thrombin bound to α_2-macroglobulin. α_2-macroglobulin is an *in vivo* inhibitor of thrombin with a normal plasma concentration of 2.5 to 4 µmol/L. *In vivo* when in complex with thrombin, it prevents the binding of thrombin substrates through steric hindrance. However, small peptides, such as chromogenic or fluorogenic substrates used in thrombin generation assays, are still able to access the active site of thrombin, thus leading to a change in colour or fluorescence (152, 153). Physiologically this signal would be irrelevant as *in vivo* thrombin would be unable to bind to its usual substrates.

Consequently, Hemker and colleagues developed a method to account for this by using a calibrator substance, essentially thrombin-α_2-macroglobulin, and assigning this an activity based on measurement of a known amount of thrombin. Their algorithm then compared measurements from the test well with measurements from the calibrator well and then subtracting the difference to calculate the actual thrombin generation in the test well at any given point. However, chromogenic methods remained hindered by the need to defibrinate the plasma, as otherwise the developing fibrin clot would impair the detection of the chromogenic signal.

In 2003, Hemker and colleagues described a method for continuous measurement of thrombin generation using a fluorogenic substrate (154). The benefit of this technique is that a large number of experiments can be performed simultaneously, coupled with reduced variability due to the automation of the initiation of the reaction and recording of the fluorescent signal and the absence of the need for subsampling. Thrombin generation can be measured in either platelet poor plasma (PPP) or platelet rich plasma (PRP). The reaction is triggered using a solution containing phospholipids and tissue factor (for PPP), or tissue factor only (PRP), which is then added to the test plasma before addition of the fluorogenic substrate and calcium chloride.

The parameters generated using this technique are the lag time, peak thrombin concentration, time to peak thrombin concentration and endogenous thrombin potential. The latter is derived by integrating the first derivative of the fluorescence curve obtained and is indicative of the total amount of thrombin that might be generated over a given period. A typical thrombin generation curve is shown in Figure 1.6.

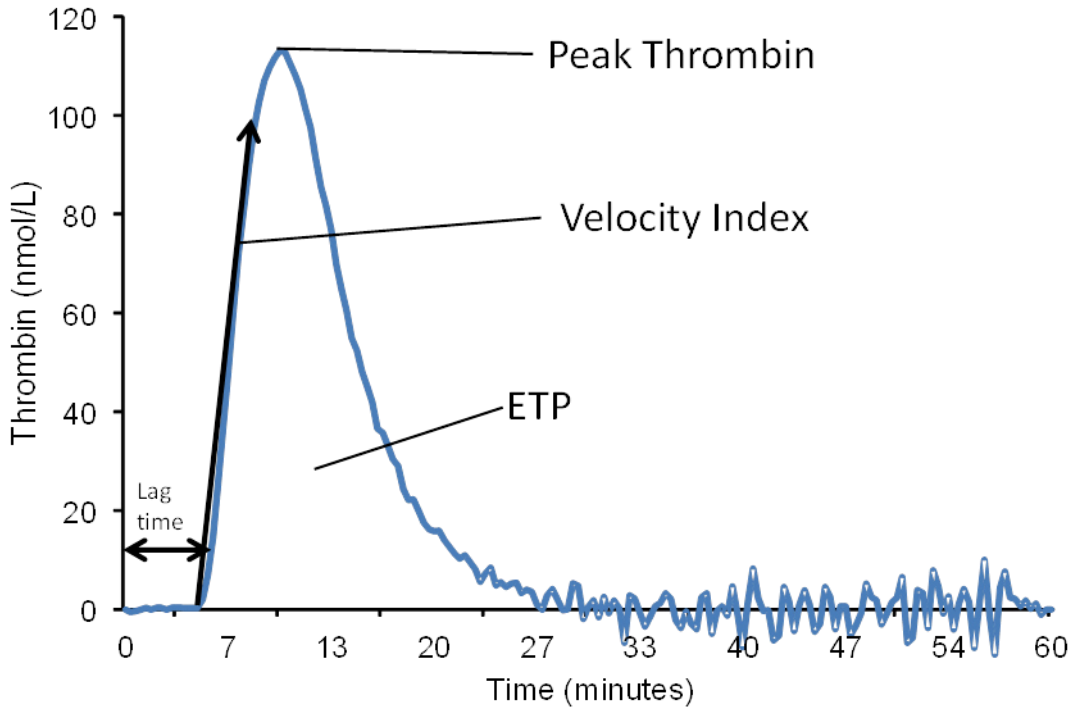


Figure 1.7 Example of thrombin generation curve obtained using calibrated automated thrombography, showing the parameters of lag time, velocity index, peak thrombin and ETP. The lag time is the time for the first 10 nmol/L of thrombin to be generated. The endogenous thrombin potential (ETP) is the area under the curve.

The Thrombinoscope software subtracts substrate conversion attributable to the α2-macroglobulin-thrombin complex from the total leaving only conversion that can be attributed to thrombin alone. The fluorogenic substrate used is Z-Gly-Gly-Arg-AMC. Cleavage by thrombin results in release of fluorescent 7-amino-4-methylcoumarin. Different plasmas will quench fluorescence to varying degrees, often referred to as the inner filter effect. The initial rate of change in fluorescence is proportional to α2-macroglobulin-thrombin activity, whilst deviation from the initial velocity is dependent on the level of fluorescence. Every measured fluorescence level requires a conversion factor to convert the change in fluorescence into a thrombin concentration. Failure to compensate for the inner filter effect and α2-macroglobulin-thrombin activity can result in inaccurate estimation of the ETP and peak thrombin (155).

There has been increasing evidence that the conditions under which CAT is performed can be adjusted to investigate different aspects of coagulation. Collection of blood samples into bottles containing corn trypsin inhibitor (CTI) prevents contact activation. This has been shown to increase the sensitivity of the assay to changes in the propagation and amplification phases of coagulation when coupled with the use of concentrations of tissue factor below 1 pmol/L (156, 157). Meanwhile addition of thrombomodulin increases the sensitivity of the assay to protein C (158). The temperature at which the assay is performed also has been shown to impact on the results. The relationship has been contrary to what might have been anticipated, with thrombin generation parameters being lower when measured at 37 ^{o}C compared to when measured at lower temperatures (159). This is in contrast to other studies where thrombin generation has been assessed using a cell based assay or measurement of thrombin-antithrombin complexes, which have shown a decrease with falling temperatures (160, 161). One possible explanation that has been suggested is that at higher temperatures the speed of diffusion becomes more important in determining thrombin generation, because the stoichiometric interaction of thrombin with its inhibitors increases more rapidly compared to the activity of proteases involved in coagulation (162). Recently, a number of studies have shown different phospholipids can alter the results of CAT, either by being present as microparticles, or through altering the composition of the phospholipids used in the trigger solution (99, 163, 164). CAT is also sensitive to TFPI, with *in vitro* studies showing TFPI reduces thrombin generation in a concentration dependent manner (165).

Clinical relevance of CAT has been demonstrated in the setting of haemophilia A where it has been shown to be potentially useful in tailoring treatment (166, 167), in liver disease for assessing haemostasis (158) and in monitoring anticoagulation (158, 168). Recently, two studies have reported that CAT can identify patients at risk of bleeding following CPB, even when samples where measured preoperatively (11, 169). This raises the possibility that

patients at risk of bleeding might be identified preoperatively and appropriate action taken to reduce that risk. This would improve patient outcome by avoiding or minimising the complications of bleeding and transfusion post-CPB described earlier.

1.9 Mass Spectrometry to investigate platelet phospholipids

The different head groups of phospholipids also allow for derivatisation to aid in identification. Recently a method has been described where PE and PS, which have a primary amine group, can be derivatised using a cell impermeable and cell permeable biotin reagent and then quantified using mass spectrometry (99). For the first time this allows a means of quantifying these lipids on the external cell membrane and also measuring changes in response to stimulation with agonists, such as thrombin and collagen in the case of platelets. Biotinylation works by the non-enzymatic reaction of a biotinylated linker protein with an active group on the molecule of interest. In the case of PE and PS, N-hydroxysuccinimidobiotin (NHS-Biotin) reacts spontaneously with the primary amine present on the phospholipid head group, resulting in the formation of a biotinylated phospholipid.

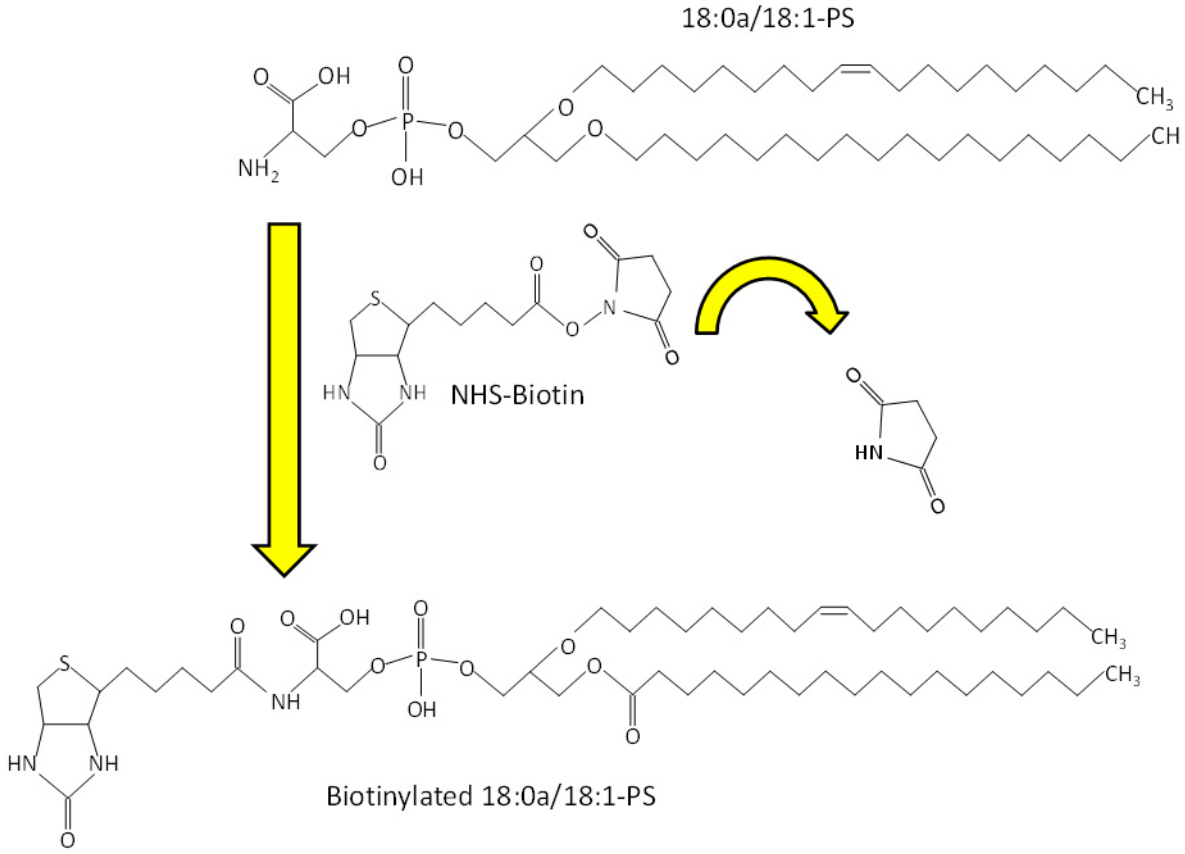


Figure 1.8 Mixing N-hydroxysuccinimidobiotin (NHS-Biotin) with phosphatidylserine at room temperature results in derivisation of the serine head-group through the addition of biotin to the amine group. This reaction is non-enzymatic and is favoured when the pH is above 7.

Modern methods of mass spectrometry allow detailed investigation of the structure of molecules. One such method is tandem mass spectrometry, which combined with high performance liquid chromatography (HPLC) can allow the identification of lipids based on their mass, charge and retention time (170). The mass spectrometer used to identify and quantify the phospholipids described in this thesis was a Q-trap 4000 manufactured by AB SCIEX (Framingham, MA, USA).

The Q-trap 4000 contains three quadropoles (Q1-Q3). A quadropole consists of four paired rods, across which electrical currents of differing voltages can be applied. These contrasting

currents will cause ions that pass between the quadropoles to oscillate. The degree of oscillation is determined by the mass to charge ratio (m/z) of the ion with the result that the ions are separated based on the speed with which they pass through the quadropole. This separation then allows their detection. The lipid molecules undergo initial separation based on their molecular weight and polarity using HPLC, which leads to different retention times. They then enter the ionisation chamber of the Q-trap where they are bombarded by electrons resulting in the molecules taking on a charge. The ions then enter Q1 where, having set the specific m/z ratio of the ions of interest, those ions are separated. Only ions with the m/z selected are allowed to enter the collision chamber, Q2. In Q2 they are fragmented by being bombarded with an inert gas, such as nitrogen, in a process termed collision activated dissociation. These fragmented, or daughter ions, then pass into the third quadrupole Q3, where they undergo further separation based on their m/z ratio and then pass to the detection chamber which provides a mass spectrum for the original precursor or parent ion that originated from Q1. This allows each ion, and ultimately each molecule to be identified based on the daughter ions generated.

1.10 Hypothesis and aims

If it were possible to identify patients at increased risk of bleeding prior to undergoing CPB, it might be feasible to intervene to prevent bleeding developing. This would reduce blood product transfusion, which would improve patient outcomes and reduce the demand on the blood supply chain. Calibrated automated thrombin generation may be a suitable test to inform this strategy, and it may also provide a means of monitoring the effect of haemostatic treatment if this is required. In addition, new methods of measuring platelet phospholipids provide an opportunity to develop coagulation assays which use phospholipids which better reflect the situation *in vivo*. Based on the available evidence the following hypothesis and aims were generated which provided the basis for this thesis.

1.10.1 Hypothesis

Thrombin generation assays are useful for identifying patients at increased risk of excess bleeding following CPB and can be used in clinical practice to inform blood product usage.

1.10.2 Primary aim

To evaluate whether abnormalities in thrombin generation can identify patients at increased risk of bleeding post-CPB.

1.10.3 Secondary aims

- To investigate whether calibrated automated thrombin generation is useful in identifying patients at increased risk of excess bleeding post CPB as defined by bleeding up to 24 hours, rate of blood loss, need for re-exploration or need for infusion of haemostatic products.
- Investigate whether calibrated automated thrombin generation can be used in routine clinical practice as a near patient test.
- Refine the calibrated automated thrombin generation assay to optimise its sensitivity to the haemostatic defect associated with CPB by varying the concentration and source of tissue factor, composition of phospholipids vesicles and presence or absence of corn trypsin inhibitor.
- To investigate the effect of different haemostatic products to correct thrombin generation *in vitro.*
- Investigate the effect of CPB on the externalisation of 12-HETE-PE, PE, PS and the synthesis of 12-HETE-PE and 12-HETE-PC in unstimulated and stimulated platelets.

Chapter 2 Materials and Methods

Patients were recruited who were undergoing cardiac surgery requiring the use of CPB. Informed consent was obtained and the study received approval from the South West Wales local research ethics committee (reference 11/WA/0215). Research and Development approval was obtained from Cardiff and Vale University Health Board.

2.1 Criteria for Study Participation

Inclusion criteria were age 18 years or older, elective and urgent cardiac surgical procedures involving valve procedures, re-sternotomy, operations on the aorta or coronary artery bypass surgery combined with another procedure such as value replacement. Patients on warfarin were included after routine treatment to reverse the international normalised ratio (INR), along with patients with any abnormality of pre-operative coagulation tests or liver impairment. Exclusion criteria were routine coronary artery bypass grafting as the sole procedure, administration of antiplatelet agents other than aspirin within the preceding 5 days, a platelet count below $120x10^9$/L, haemoglobin less than 100 g/L, or emergency procedures.

2.2 Complications Recorded

Final follow up was at discharge from the hospital or 30 days post-surgery, whichever was sooner. At that time the following was recorded:

- The length of time ventilated
- Duration of level 3 and level 2 care
- Time from operation until discharge

- Major complications of cardiac surgery: myocardial infarction (defined as an elevated cardiac troponin), significant haemodynamic instability (defined as use of two or more inotropic drugs or intra-aortic balloon pump), occurrence of atrial fibrillation lasting for more than 24 hours, reoperation for bleeding for any cause, prolonged ventilation (with or without tracheostomy defined as requirement for positive pressure ventilation for > 48 hours), clinically presenting cardiovascular accident, sepsis and renal impairment requiring renal replacement therapy

2.3 Definition of bleeding

Excess bleeding was defined as a composite of any one of the following: > 1 L in 24 hours; > 200 mL per hour for 2 consecutive hours; > 2 mL/kg per hour for 2 consecutive hours in the first 6 hours post-CPB; return to theatre for reoperation due to bleeding; requirement for haemostatic therapy.

2.4 Demographic data

The following demographic data were recorded: age, weight, gender, anticoagulant and antiplatelet medication history, type of operation, duration of aortic cross-clamping, duration of cardiopulmonary bypass, time and dose of heparin administration, time and dose of protamine given, volume and time of intravenous crystalloid, colloid and blood products administered once pre-operative blood samples had been taken and for 24hrs after the end of surgery. The volume of cell salvage blood and the hourly and total output from surgical drains were also recorded for the first 24 hours post-operatively. A copy of the data collection proforma is included in Appendix 1.

2.5 Anaesthetic and Surgical technique

Cardiac surgery and CPB were initiated in a standard way as per the institutional protocols of the University Hospital of Wales. The cardiopulmonary bypass circuits and priming fluids were the same in all cases. The cardiopulmonary bypass circuits were manufactured by Chalice Medical (Worksop, Nottinghamshire, UK) and Sorin Group Inc (Arvada, CO, USA). Peripheral intravenous cannulae were inserted as was an arterial cannula, through which blood samples for the study were drawn (see later). Anaesthesia consisted of standard induction with propofol, midazolam, fentanyl and the muscle paralysing agent rocuronium, before switching to isoflurane for maintenance of anaesthesia. Following induction of anaesthesia a central venous catheter was inserted. Unfractionated heparin was used as an anticoagulant to maintain the activated clotting time (ACT) >400 seconds. Protamine at a dose of 1 mg per 100 units of heparin was given after the end of the cardiopulmonary bypass prior to the removal of the arterial and venous cannulae. Heparin reversal was defined as the ACT returning to within 10% of baseline. The ACT was monitored using a Helena Actalyke MINI II (Helena Laboratories, Beaumont, Texas, USA).

2.6 Sample Collection

Whole blood samples were taken into vacutainer bottles containing 3.2% trisodium citrate (Greiner Bio-One, Stonehouse, UK) at a ratio of 9 parts whole blood to 1 part 3.2% trisodium citrate.

1.2mg /mL Corn Trypsin Inhibitor (CTI) was purchased from Cambridge Biosciences (Bar Hill, Cambridge, United Kingdom). This was diluted to a concentration of 1 mg/mL in tris-buffered saline (TBS) pH 7.4. TBS was composed of 20 mmol/L tris(hydroxymethyl)aminomethane and 136 mmol/L sodium chloride dissolved in distilled water with the pH adjusted to 7.4 using hydrochloric acid.

A solution of 4% trisodium citrate was made by dissolving 4g of trisodium citrate in 100 mL distilled water. Blood collection tubes were then prepared by adding 400 µL of 4% trisodium citrate to 100 µL 1 mg/mL CTI solution in a 5 mL screw-cap vial. Whole blood when collected into this tube was at a ratio of 1 part anticoagulant solution to 9 parts whole blood (i.e. 0.5 mL anticoagulant to 4.5 mL whole blood).

Blood samples were taken from an arterial line before heparin administration and 30 minutes after reversal of heparin by protamine sulphate.

PPP was prepared by centrifuging samples at 1570 x *g* for 15 minutes. The plasma was then removed and centrifuged a second time at 1570 x *g* for 15 minutes, after which the plasma was divided into 1 mL aliquots and frozen at -80 ^{o}C for testing later.

Samples for Full Blood Count recording where collected into vacutainers containing EDTA (BD, Oxford, UK).

2.7 Full Blood Count Measurement

Full blood counts were performed on an ABX Pentra DX 120 automated analyser (Horiba Medical, Northampton, UK).

2.8 Coagulation factor and anticoagulant assays

2.8.1 Assays of coagulation factor II, V, VII, VIII, IX, X, fibrinogen and protein C, free protein S, antithrombin and heparin

The prothrombin time (PT), activated partial thromboplastin time (APTT), Clauss fibrinogen and factors II, V, VII, VIII, IX, X, XI, antithrombin, protein C, free protein S and post-operative

anti-Xa activity were measured on an ACL 500 Top (Instrumentation Laboratory, Cheshire, UK) automated coagulometer using standard manufacturer protocols and reagents.

For measurement of the PT, FII, FV, FVII and FX, HemosIL™ RecombiPlasTin 2G was used. This was supplied as a vial of lyophilised powder and a vial of diluent. The diluent was allowed to equilibrate to room temperature for 15 minutes before adding the contents to a vial of lyophilised reagent. This was then left to stand for 30 minutes at room temperature, swirling occasionally, before use. Lyophilised factor II, V, VII and X deficient plasmas, calibration plasma, normal control and Special Test Control Level 2 plasmas were reconstituted with distilled water and allowed to stand for 30 minutes at room temperature, swirling occasionally, before use.

For measurement of the APTT, FVIII, FIX and FXI, HemosIL™ $CaCl_2$ (0.02 mol/L) and HemosIL™ SynthASil APTT reagents were used. A vial of reagent was allowed to equilibrate to room temperature for 15 minutes before use. Lyophilised factor VIII, IX and XI deficient plasmas, calibration plasma, normal control and Special Test Control Level 2 plasmas were reconstituted with distilled water and allowed to stand for 30 minutes at room temperature, swirling occasionally, before use.

Fibrinogen was measured using the Clauss method. A vial of lyophilised QFA thrombin reagent was reconstituted with distilled water and allowed to stand for 30 minutes at room temperature, swirling occasionally, before use. Lyophilised calibration plasma, normal control and Special Test Control Level 2 plasmas were reconstituted with distilled water and allowed to stand for 30 minutes at room temperature, swirling occasionally, before use.

Antithrombin was measured using a kit supplied by IL consisting of vials of FXa reagent (containing bovine Xa and heparin) and Chromogenic Xa substrate (N-α-Z-D-Arg-Gly-Arg-pNA2HCl). Each vial was swirled gently prior to use to ensure mixing of the contents. Heparin was measured by anti-Xa activity using the similar Liquid Heparin kit, the difference being that the FXa reagent did not contain heparin.

Free protein S was measured using a kit consisting of vials of lyophilised C4b-binding protein attached to latex beads, a vial of buffer to use for reconstitution of the former, and a vial of anti-protein S monoclonal antibody.

Protein C was quantified using a kit containing a vial of concentrated diluent, a vial of lyophilised protein C activator (venom of Agkistrodon contortrix contortrix) and a vial of protein C chromogenic substrate (pyroGlu-Pro-Arg-pNA.HCL). The diluent vial was diluted 1 part to 10 parts distilled water; the vials lyophilised reagents were reconstituted with distilled water and allowed to stand at room temperature for 30 minutes before use, with occasional gentle swirling to mix the contents.

2.8.2 TFPI ELISA

Full-length and total TFPI were measured by an ELISA technique. All chemicals were purchased from VWR International Ltd, unless otherwise stated. Distilled, laboratory grade water was used for all buffers.

The following buffers and reagents were assembled:

1. Hydrochloric acid 25% (v/v)
2. 1 mol/L Sodium Hydroxide

3. 10% Tween 20 (w/v)
4. Tris-buffered Saline (TBS): 25 mmol/L Tris(hydroxymethyl)-aminomethane; 150 mmol/L sodium chloride, pH 7.4
5. TBS -Tween 20: 25 mmol/L Tris(hydroxymethyl)-aminomethane; 150 mmol/L sodium chloride; 0.1% v/v Tween 20
6. 0.5 mol/L EDTA, pH 8.0
7. Washing buffer: TBS/0.1% Tween 20 v/v
8. Blocking Buffer: TBS/2% (v/w) dry milk powder (BioRad Laboratories Ltd, Hemel Hempstead, UK)
9. Sample and antibody dilution buffer: 20 mmol/L EDTA in TBS-T/1% (v/w) dry milk powder
10. Substrate solution: Sure Blue TMB (1-component peroxidase substrate, Co KPL)
11. Stopping reagent: 1 mol/L Hydrochloric acid
12. Capture antibody (total TFPI): Mouse monoclonal antibody against human TFPI kunitz domain-2 (Sanquin, Amsterdam, Netherlands)
13. Capture antibody (full-length TFPI): Mouse monoclonal antibody against human TFPI C-terminus domain (Sanquin, Amsterdam, Netherlands)
14. Detection antibodies:
    a. Rabbit polyclonal antibody against human TFPI (American Diagnostica GmbH, Pfunstat, Germany)
    b. Anti-rabbit IgG peroxidase conjugate (Sigma-Alrdich, Dorset, UK)
15. Recombinant human full-length TFPI 1 µg/mL (Baxter Innovations GmbH, Vienna, Austria)

Method:

Detection antibodies were diluted in TBS to a concentration of 1 µg/mL. A 96 well plate (Nunc Maxisorp, eBioscience Ltd, Hatfield, UK) was coated by adding 100 µL of the detection

antibody to the relevant wells before covering and incubating at 4^{o}C overnight. Then the contents were discarded and the plate washed 3 times using the washing buffer described above. 250 µL of blocking buffer to each well and the plate was covered and placed on a plate shaker at room temperature for 60 minutes, before discarding the contents and washing the plate 3 times. Citrated patient samples and the TFPI standard were thawed by placing in a water bath for 5 minutes, with the temperature set at 37 ^{o}C. The standard was diluted in the sample dilution buffer from 1:1.25 to 1:0. Patient and control samples were diluted 1:10 and 1:20 for full-length TFPI and 1:25 and 1:50 for total TFPI using the same buffer. Diluted standard and patient samples were added to the plate in duplicate (100 µL per well) and 100 µL of buffer was added to two additional wells for blank correction. The plate was then covered, placed on a plate shaker and incubated at room temperature for 2 hours. The contents of the plate were discarded and the plate washed 5 times. The first detection antibody was diluted 0.5 µg/mL using the dilution buffer (see 9 above) and 100 µL added to each well. The plate was covered, placed on plate shaker and incubated at room temperature for 1 hour before discarding the contents and washing 5 times. The second detection antibody was diluted to 0.2 µg/mL with the dilution buffer and 100 µL added to each well. The plate was once again covered, placed on a plate shaker and incubated at room temperature for 1 hour. The contents of the plate were discarded and the plate washed 5 times. TMB was pre-warmed to 37 ^{o}C and 100 µL was added to each well. The plate was then incubated at room temperature for 7 minutes, protected from the light. The reaction was stopped by adding 50 µL of 1 mol/L hydrochloric acid to each well. Light absorbance at 450 nm was measured using a plate reader (Biotek, Winoosi, WT, USA) and data analysed using Gen 5.1 software.

2.8.3 Von Willebrand Factor ELISA

All chemicals were purchased from VWR International Ltd, unless otherwise stated. The following reagents were prepared:

1. Technoclone reference plasma reconstituted with sufficient distilled water to provide a VWF concentration of 100% (Technoclone, Vienna, Austria)
2. Cryocheck abnormal 1 and 2 control plasma (Precison Biologic Inc, Dartmouth, NS, USA)
3. Anti-rabbit VWF antibody (Dako, Ely, UK)
4. Sodium carbonate buffer (16 mmol/L anhydrous sodium bicarbonate, 34 mmol/L sodium hydrogen carbonate, pH 9.6)
5. Anti-VWF horse-radish peroxidase conjugate antibody (Dako, Ely, UK)
6. 2% polyvinylpyrrolidine (PVP) in sodium carbonate buffer
7. Phosphate buffered saline (PBS)/ 0.0005% (v/v) Tween 20, pH 7.2
8. PBS/0.0005% (v/v)Tween 20 with 0.1% (v/w) Bovine Serum Albumin (BSA)
9. Red-Stop reagent (Skybio Ltd, Wyboston, Befordshire, UK)

Procedure for the ELISA:

The Anti-rabbit VWF antibody was diluted 1 in 600 with sodium carbonate buffer and then 200 µL was added per well to a 96-well Dynex Immunlon 4HBX plate (Fisher Scientific, Loughborough, UK). The plate was covered and incubated over night at 4^{o}C. The contents of the wells were decanted and 200 µL of 2% PVP was added to each well. The plate was then covered and incubated for 30 minutes at room temperature. The contents were discarded and the plate was washed 3 times by adding 300 µL of PBS/Tween 20 to each well using a multi-channel pipette and the aspirating with a stylet attached to a vacuum pump. The reference plasma was diluted 1 in 40 with PBS/Tween 20/0.1 %BSA. The control plasmas were thawed for 5 minutes in a water bath set to 37 ^{o}C before being diluted. Cryocheck abnormal 1 control plasma was diluted 1 in 40 and Cryoheck abnormal 2 control plasma 1 in 20, both using PBS/Tween 20/0.1 %BSA. Citrated patient plasma was thawed for 5 minutes in a water bath set to 37 ^{o}C , vortex-mixed and then diluted 1 in 80 with PBS/Tween 20/0.1 %BSA. Then 100 µL of PBS/Tween 20/0.1% BSA was added to each well on the plate. Next, 100 µL of plasma

(reference, control or test) was added to one well and then serial dilutions were made on the plate (4 for control and test plasma; 8 for reference plasma). The plate was covered and incubated at room temperature for 2 hours, before decanting the contents and washing 3 times as described earlier. The anti-VWF horse-radish peroxidase conjugate antibody was diluted 1 in 1000 with PBS/Tween 20/0.1% BSA and 100 µL added to each well. The plate was again covered and incubated at room temperature for 1 hour. The contents were decanted and the plate washed 3 times as previously. 100 µL of TMB reagent was added to each well and the plate was incubated at room temperature for 7 minutes, protected from the light. The reaction was stopped by adding 50 µL of Red-Stop per well. Light absorbance at 650 nm was measured using a plate reader (Biotek, Winoosi, WT, USA) and data analysed using Gen 5.1 software.

2.8.4 Factor XIII Assay

Measurement of FXIII activity was performed using the Technochrom FXIII assay kit, purchased from Technoclone, Vienna, Austria. Following the instructions provided, a vial of lyophilised NADPH reagent was reconstituted with 3 mL of distilled water and left for 30 minutes at room temperature, swirling occasionally. The contents of this vial was then added to a vial to a vial of lyophilised FXIII activator reagent (composed of thrombin and calcium), which was then left to reconstitute for 30 minutes at room temperature, swirling occasionally. Meanwhile a vial of FXIII inhibitor reagent (composed of a tetrapeptide to prevent fibrin polymerisation) was mixed with 1 mL of FXIII stabiliser solution (containing glycine ether ester and PI(1-12) which is cross-linked by FXIIIa via a glutamate residue, releasing ammonia) and left for 30 minutes at room temperature, swirling occasionally.

To measure FXIII activity, calibration plasma (Technoclone, Vienna, Austria) was reconstituted to 100% FXIII activity using distilled water. Control plasma (Low Abnormal, FXIII activity as per product insert, Technoclone, Vienna, Austria) was also reconstituted with distilled water. Both

were allowed to stand at room temperature for 30 minutes with occasional agitation before use. Citrated patient samples were then thawed in a water bath at 37 ^{o}C for 5 minutes. On removal each aliquot was briefly mixed using a vortex-mixer and then used immediately. A volume of 10 µL of calibration plasma, control plasma and test samples was added in duplicate to separate wells of a 96 well plate (flat-bottom Immulon 2Hb 96-well plate, Diagnostica-Stago, Asnières sur Seine, France). Then 100 µL of either sample reagent or blank reagent was added to the respective wells. The plate was then placed in a plate reader (Biotek, Winnosi, VT, USA) preheated to 37 ^{o}C, programmed using Gen 5.0.1 software as follows:

a. Incubate for 4 minutes and 50 seconds
b. Shake for 10 seconds
c. Measure absorbance at 340 nm at 5, 7, 9 and 11 minutes
d. Subtract background absorbance using value for blank well
e. Calculate mean velocity of change (V)

A calibration factor (F) was then calculated using the following formula:

$$F=\text{Assigned FXIII calibration value}/(V_{(\text{calibration test wells})} - V_{(\text{calibration blank wells})})$$

The FXIII activity of patient and control plasma was then calculated as a percentage of the calibration plasma using the following formula:

$$\text{Factor XIII concentration} = F(V_{(\text{sample test wells})} - V_{(\text{sample blank wells})})$$

2.8.5 TAFI ELISA

TAFI was measured using an Imunclone kit from Sekisui Diagnostics (West Malling, UK). The vials supplied, consisting of lyophilised TAFI calibrator and high and low control plasmas, were each reconstituted with 0.5 mL distilled water and left at room temperature for 30 minutes. The TAFI calibrator was then diluted 1 in 50 with distilled water. From this final solution a

standard curve was constructed by preparing the following dilutions using distilled water: 1 in 1; 1 in 2; 1 in 4; 1 in 10; 1 in 20; 0 in 1. The control plasmas (high and low) were each diluted 1 in 50 using distilled water. Frozen citrated plasma from patients was thawed in a water bath set at 37 ^{o}C for 5 minutes. These were then diluted 1 in 100 and 1 in 200 using distilled water. 100 µL of samples, controls and calibrator solutions were added in duplicate to the well strips provided (wells were pre-coated with an anti-human-TAFI antibody). The wells were then covered and incubated at 37 ^{o}C for 2 hours. The contents of the wells were then discarded by inversion of the plate and violent tapping. The wells were then washed 5 times using the wash solution provided by adding 300 µL to each well using a multi-channel pipette and then aspirating with a stylet attached to a vacuum pump. 200 µL of anti-human TAFI horse-radish peroxidase antibody conjugate was then added to each well using a multi-channel pipette. The wells were again covered and incubated at 37 ^{o}C for 1 hour. At the end of the incubation period the contents were discarded and the plate washed as described earlier. 200 µL of TMB substrate provided was then added to each well using a multi-channel pipette. The wells were the covered and incubated in the dark for 5 minutes at room temperature. The reaction was then stopped by adding 50 µL of 0.45 mol/L sulphuric acid to each well. After a further ten minutes at room temperature, light absorbance at a wave length 450 nm was measured in a plate reader and data analysed using Gen 5.1 software (Biotek, Winoosi, WT, USA).

2.9 Thrombin generation assays

2.9.1 Calibrated automated thrombography

Thrombin generation was measured using the method described by Hemker and colleagues (154). Experiments were performed by mixing 20 µL of trigger solution (tissue factor mixed with phospholipid vesicles) with 80 µL of plasma in a U-bottom Immulon 2Hb 96-well plate (Diagnostica-Stago, Asnières sur Seine, France). The plate was maintained at 37 ^{o}C using a plate warmer while the samples were pipetted onto the plate. Thrombin generation was initiated

by adding Flu-Ca (2.5 nmol/L fluorogenic substrate Z-Gly-Gly-Arg from Bachem, UK and 0.1 mol/L calcium chloride, 20 mol/L HEPES, 0.02 % (w/v) sodium azide, 60 g/L bovine serum albumin, pH 7.35) to the trigger-plasma mixture and fluorescence was measured using a Fluoroscan reader (Thermo Scientific, UK) run using Thrombinoscope software (Synapse BV, Maastricht, Netherlands). The effect of the protein C pathway was measured by performing thrombin generation with a commercial trigger containing thrombomodulin (Diagnostica-Stago, Asnières sur Seine, France). Commercially available trigger solutions with 1 pmol/L TF (Stago PPP Low) and 5 pmol/L TF (Stago PPP) were also used. All commercial trigger reagents were a gift from Diagnostica-Stago (Asnières sur Seine, France).

2.9.2 Preparation of trigger solutions for thrombin generation assays

All un-oxidised phospholipids were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). The following buffers were prepared:

1. Buffer A consisting of 20 mmol/L HEPES, 140 mmol/L NaCl, pH 7.35 in distilled water.
2. Buffer B consisting of 0.5% w/v BSA dissolved in Buffer A.

2.9.2.1 Liposomes for investigation of the effect of different concentrations of tissue factor

Liposomes were prepared by mixing 120 µL of 25 mg/mL DOPS, 120 µL of 25 mg/mL DOPE and 458 µL 20 mg/mL DOPC at a ratio of 1:1:3 (mole: mole: mole) in a glass vial and then drying under an argon gas stream. Once dry, 4 mL buffer A was added and the mixture was vortexed before placing on a roller-mixer for 2 hours. The suspension was then passed 29 times through a mini-extruder device with membranes of 100 µm pore diameter (Avanti Polar Lipids, Alabaster, USA).

The contents of a vial of Innovin (Siemens, Germany) were reconstituted with 10 mL of distilled water to yield a solution assumed to have a TF concentration of 6 nmol/L, based on the product insert. This solution was further diluted in buffer B to give a TF concentration of 500 pmol/L. Trigger solutions were then prepared by diluting the phospholipid suspension and Innovin solution in buffer B to achieve a concentration of phospholipids of 24 μmol/L and concentrations of TF of 3 pmol/L, 6 pmol/L, 18 pmol/L, 30 pmol/L and 60 pmol/L. The final in-well TF concentration during the thrombin generation assay was 0.5 pmol/L, 1 pmol/L, 3 pmol/L, 5 pmol/L and 10 pmol/L respectively. These solutions were then aliquoted and frozen at -80 ^{o}C for use later. The TF activity if these trigger solutions was checked using a TF ELISA technique, the results of which are shown in Appendix 3.

2.9.2.2 Liposomes for investigation effects of 12-HETE-PE and 12-HETE-PC on thrombin generation

For experiments using 12-HETE-PC and 12-HETE-PE, liposomes were constructed as summarised in Table 2.1 using 65 % 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC), 5% 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine (SAPS), 30% 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (SAPE), 65% SAPC, 5% SAPS, 20% SAPE and 10% 12-HETE-PE or 55% SAPC, 5% SAPS, 30% SAPE and 10% 12-HETE-PC. All 12-HETE-PE and PC was provided by Dr Yoel Garcia (Department of Chemistry and the Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, TN, U.S.A). After drying under a stream of nitrogen, the phospholipids were re-suspended in buffer A. Recombinant full length tissue factor (rTF) (Haematologic Technologies Incorporated, Essex Junction, VT, USA) was diluted in water to a concentration of 30 nmol/L. This was then added to the lipid suspension to give a concentration of 300 pmol/L and the mixture subjected to 10 cycles of freeze-thawing in liquid nitrogen each complete cycle lasting 1 minute. The suspension was then passed through a mini-extruder with 100 μm pore filters to obtain the final liposome preparation. This was then

diluted 1 in 5 in buffer B to give a final concentration of 24 µmol/L phospholipids and 60 pmol/L rTF. The final concentration of TF available to trigger thrombin generation was not known because not all TF would have been incorporated into the liposomes and half would be expected to face internally. Thrombin generation assays were then performed in the same manner described earlier.

Table 2.1 Volumes of different phospholipids used to prepare a 0.5 mL suspension of each liposome.

Lipid	Stock Conc. (mmol/L)	Volume of stock sol. (µL) to make 0.5 mL of 100 µmol/L solution	0% HETE	10% HETE-PC	10% HETE-PE
SAPE	1.303	46	13.8	13.8	9.2
SAPS	1.200	50	2.5	2.5	2.5
SAPC	1.235	48.6	31.6	26.8	31.6
12-HETE-PE	1.276	47	0	0	1.7
12-HETE-PC	1.238	42	0	4.2	0

2.9.3 Thrombin generation assays to investigate effect of different haemostatic agents

Thrombin generation assays were performed as described in section 2.9.1, using the PPP low trigger reagent.

2.9.3.1 Calculation of concentrations of Anti-TFPI antibody

Thrombin generation was measured in the presence and absence of the anti-TFPI antibody AF2974 (R&D Systems, Abingdon, UK) at a concentration of 100 nmol/L, this concentration having previously been determined to inhibit concentrations of full length TFPI up to 6 nmol/L (personal communication from Erwin Panholzer, Baxter Biosciences, Vienna, Austria, titration curve included in Appendix 2).

2.9.3.2 Calculation of concentrations of FFP

FFP was obtained from the Welsh Blood Service. The post-operative plasma volume for each patient was estimated using the formula, 0.07 x weight x (1-haematocrit). The mean plasma volume was then calculated for the whole cohort. Mean weight was also calculated. These figures where then used to calculate the volume of FFP to add to each sample to be equivalent to 15mL/kg, 20mL/kg and 50mL/kg. This was 0.308 mL FFP, 0.411 mL FFP and 0.617mL FFP per mL patient plasma respectively. The activity of factors II, V, VII, VIII, IX, X, XI, antithrombin, protein C and S in the FFP were measured as described earlier. The results are shown in Table 2.2.

Table 2.2 Activity of coagulation factors and anticoagulants in FFP.

Factor	Activity
II	92.5 iu/dL
V	87.9 iu/dL
VII	81.5 iu/dL
VIII	92.2 iu/dL
IX	110.2 iu/dL
X	97.3 iu/dL
XI	113.6 iu/dL
Protein C	100 %
Free protein S	100 %
Antithrombin	113.9 iu/dL

2.9.3.3 Calculation of concentrations of rFVIIa, PCC

The mean weight of the overall cohort was used to calculate the amount of rFVIIa to add to plasma to be equivalent to doses of 45 µg/kg, 90 µg/kg and 180 µg/kg. Recombinant Factor VIIa was purchased from Novonordisk (NovoSeven, Novonordisk, Denmark). The final concentrations of rFVIIa in the spiked plasma were 0.93 µg/mL, 1.85 µg/mL and 3.7 µg/mL respectively.

The mean weight of the overall cohort was also used to calculate the amount of PCC to add to plasma equivalent to doses of 25 units/kg, 35 units/kg and 50 units/kg. PCC was a gift from CSL Behring (Beriplex, CSL Behring UK Ltd, Hayward Heath, UK). The final concentrations of PCC in the spiked plasma were 0.51 units/mL, 0.72 units/mL and 1.03 units/mL respectively.

2.10 Analysis of platelet phospholipids by flow cytometry and mass spectrometry

2.10.1 Preparation of washed platelets for phospholipid extraction and flow-cytometry

The following solutions were prepared:

1. Acidified citrate dextrose (ACD) (85 mmol/L trisodium citrate, 65 mmol/L citric acid, 100 mmol/L glucose, dissolved in distilled water and pH adjusted to 5.4)

2. Modified Tyrode's buffer (134 mmol/L sodium chloride, 12 mmol/L sodium bicarbonate, 2.9 mmol/L potassium chloride, 0.34 mmol/L disodium phosphate, 1 mmol/L magnesium chloride, 10 mmol/L HEPES, 5 mmol/L glucose, dissolved in distilled water and pH adjusted to 7.4)

Method:

Venous blood was drawn into a syringe containing ACD to produce final ratio of blood 8.1%: ACD 1.9% (v/v). For the purposes of the experiments described, 41.9mL of whole blood was drawn from a central venous cannula into a 50 mL syringe containing 8.1 mL of acidified citrate dextrose (ACD), before administration of heparin and 30 minutes after heparin reversal. In the laboratory the ambient air temperature was maintained at 21 ˚C by air conditioning. The contents of the syringe were split equally into two 50 mL falcon tube and centrifuged at 250 x

g for 10 minutes with no brake. The platelet rich plasma was removed using a Pasteur pipette into a fresh 50 mL falcon tube, taking care not to disturb the red cell – plasma interface and avoiding air bubbles. The red cells and leucocytes were discarded and the platelet rich plasma was centrifuged at 900 x *g* for 10 minutes with no brake. The resulting platelet poor plasma was discarded leaving a pellet of platelets at the bottom of the tube. This pellet was gently re-suspended in 2 mL of a solution of 1 part ACD to 9 parts modified Tyrode's buffer (v/v) using a Pasteur pipette. The volume was made up to 10 mL by adding a further 8 mL of the diluted ACD solution. The tube was then centrifuged at 800 x *g* for 10 minutes with no brake. The supernatant was discarded and the platelet pellet re-suspended in 2 mL modified Tyrode's buffer. A 0.1% (v/v) solution of trypan blue (Sigma-Aldrich, Dorset, UK) was prepared by dilution with modified Tyrode's buffer. An aliquot of the platelet suspension was then diluted 1 in 50 in the 0.1% Trypan blue solution; 10 µL of this suspension was then pipetted under a glass clover slip of an improved Neubauer Haemocytometer and the platelets were counted. A minimum of 100 cells were counted using the large 4 outer quadrant squares (each large outer square contains 16 squares and has a total area of 1 mm^2). The number of platelets in the original suspension was calculated as follows: platelets/mL = number of cells counted x (Dilution factor / Number of quadrants used to count) x 10^4 x 2(to take account of the volume of the original suspension being 2 mL). Further modified Tyrode's buffer was added to add the original suspension to obtain final platelet concentration of $2x10^8$/mL, the volume determined by dividing total number of platelets by the desired concentration.

2.10.2 Flow cytometry on whole blood and washed platelets for CD61 and Annexin V

All antibodies were purchased from Becton Dickinson. A 1 in 10 Dilution of the 10X Annexin V Buffer supplied in Annexin V kit was prepared. The following tubes were prepared:

1. Unstained
    a. 20 µL Whole blood or PRP

b. 80 µL 1 in 10 dilution of Annexin V buffer

2. CD61 PC7
    a. 10 µL CD61 PC7
    b. 20 µL Whole blood or PRP
    c. 70 µL 1 in 10 dilution of Annexin V buffer
3. CD61 PC7 / Annexin V FITC
    a. 10 µL CD 61 PC7
    b. 20 µL Whole blood or PRP
    c. 70 µL 1 in 10 dilution of Annexin V buffer
4. Fixed (3% paraformaldehyde) / Annexin V (positive control)
    a. 20 µL Whole blood or PRP
    b. 80 µL Annexin V buffer

The tubes were incubated for 15 minutes in the dark. Then 10 µL was transferred from tube 3 and added to a fresh tube containing 89 µL Annexin V Buffer and 1 µL Annexin V. From tube 4, 10 µL was transferred to a fresh tube containing 80 µL Annexin V buffer, 9 µL 30% paraformaldehyde and 1 µL Annexin V. All tubes were then incubated for 15 minutes. Then 10 µL was transferred from tubes 1 and 2 to fresh tubes. These four final tubes were then made up to a volume of 500 µL using modified Tyrode's buffer. These were then run on a Beckman Coulter Cyan ADP Flowcytometer and the data analysed using Summit software version 4.3 (Beckman Coulter, Fullerton, CA, USA). Platelets were first identified using forward and side scatter. Gating on this population then allowed identification of CD61 and Annexin V positive events. Each condition was run in triplicate.

2.10.3 Stimulation of washed platelets prior to phospholipid extraction

The following reagents were prepared:

1. A solution of 20 units/mL human thrombin (Sigma-Aldrich, Dorset, UK) was prepared by diluting to the desired concentration using distilled water.
2. A solution of 20 mmol/L calcium chloride by diluting a pre-prepared solution of 1 mol/L calcium chloride in modified Tyrode's buffer
3. A solution of 200µg/mL collagen (Mascia-Brunellli, Milan, Italy), using the diluent provided.
4. 5mg/mL EZ-Link Sulfo-NHS-Biotin (Thermo-Fisher Scientific, Cramlington, UK) in modified Tyrode's buffer
5. 0.0366g/mL Lysine (Sigma-Aldrich, Dorset, UK) in modified Tyrode's buffer
6. 6.8276mg/mL EZ-Link NHS-Biotin (Thermo-Fisher Scientific, Cramlington, UK) dissolved in DMSO

Table 2.3 Volumes of reagents and washed platelets used in platelet stimulation experiments.

Condition	Modified Tyrode's Buffer	Platelet Suspension	Calcium Chloride	Agonist
Untreated	330 µL	1000 µL	70 µL	Nil
Thrombin	316 µL	1000 µL	70 µL	14 µL
Collagen	260 µL	1000 µL	70 µL	70 µL

Plastic Eppendorf tubes, prepared as shown in Table 2.3 were incubated in a water bath at 37 ˚C for 10 minutes. They were then removed from water bath and 140 µL was removed to fresh Eppendorf tubes to use for biotinylation of total phospholipids as described later.

To the remaining suspension in each tube, 602 µL Sulfo-NHS-EZ linked Biotin was added and incubated for 10 minutes at room temperature. To bind any un-reacted biotin, 504 µL of lysine

solution was added to each tube and incubated for 10 minutes at room temperature. Finally 94 µL of modified Tyrode's buffer was added to each tube to make the volume up to 2600 µL.

For biotinylation of total phospholipids, 60 µL of modified Tyrode's solution and 20 µL of EZ-link NHS Biotin solution were added to each tube containing 140 µL of the platelet suspension. The tubes were then incubated at room temperature for 10 minutes. The volume of each tube was then made up to 400 µL by the addition of 180 µL modified Tyrode's buffer.

2.10.4 Extraction of externalised biotinylated phospholipids

Chloroform, methanol and HPLC grade water were purchased from Fisher Scientific (Loughborough, UK). Glass extraction tubes (Fisher Scientific, Loughborough, UK) were labelled and 9.75 mL of a mixture chloroform-methanol (1:2 v/v) was added to each tube. Next 10 µL of 1ng/µL biotinylated 1,2-dimyristoyl-sn-glycero-3-PS (DMPS-B) and biotinylated 1,2-dimyristoyl-sn-glycero-3-PE (DMPE-B) were added to each tube, followed by the platelet samples. The contents of each tube were then mixed thoroughly for 1 minute using a vortex mixer. Next 3.25 mL of chloroform was added to each tube before mixing for a further minute. A volume of 3.25 mL HPLC grade water was then added to each tube and the contents mixed on a vortex mixer for a further minute. The glass tubes were then centrifuged at 500 x *g* for 5 minutes in a refrigerated centrifuge cooled to 4˚C. The lower layer from each tube was then transferred to a fresh set of glass extraction tubes and the contents dried using a vacuum dryer for 30-60 minutes. Once dry, 100 µL of methanol was added to each tube and the contents re-suspended by vortex mixing for 1 minute. The contents of each tube was then transferred to a glass HPLC vial and frozen at -80 ^{o}C prior to analysis.

2.10.5 Phospholipid extraction for measurement of total phospholipids

Two sets of glass extraction tubes (Fisher Scientific) were labelled with the relevant sample numbers. A mixture of 1 part chloroform to 2 parts methanol (v/v) was prepared and 1.5 mL added to the first set of tubes. Then 10 µL of 1ng/µL internal standards consisting of DMPS-B, DMPE-B and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were added to each tube followed by the platelet samples. The contents of each tube were then thoroughly mixed for 1 minute using a vortex mixer. Next 0.5 mL of chloroform was added to each tube before mixing for a further minute, followed by 0.5 mL HPLC grade water and mixing for a final minute. The tubes were then centrifuged at 500 x *g* for 5 minutes at 4 ^{o}C using a refrigerated centrifuge. The lower layer from each tube was then transferred to a fresh set of glass extraction tubes and the contents dried using a vacuum dryer for 30-60 minutes. Once dry, 100 µL of methanol was added to each tube and the contents re-suspended by vortex mixing for 1 minute. The contents of each tube was then transferred to a HPLC vial and frozen at -80 ^{o}C prior to analysis.

2.10.6 Preparation of biotinylated phospholipid standards

Biotinylated 1,2-dimyristoyl-sn-glycero-3-PS (DMPS; 14:0/14:0-PS) and 1,2-dimyristoyl-sn-glycero-3-PE (DMPE; 14:0/14:0-PE) were prepared by pipetting 1 mg of each lipid into separate glass extraction vials and then drying under a stream of nitrogen gas. Each lipid was then re-suspended by adding 220 µl chloroform and 110 µl methanol to each vial and vortex mixing for 1 minute. Next 6.6 mg of NHS biotin and 3.3 µl tri-ethylamine were added each vial was incubated for 30 minutes at room temperature. To try to remove as much unreacted biotin as possible, the vials were then centrifuged at 500 x *g* for 5 minutes and upper three quarters of the solution removed to a fresh glass vial using a glass Pastuer pipette. The contents of each vial was then dried under a nitrogen gas stream before re-suspending by adding 0.5 mL methanol and vortex mixing for 1 minute. The lipid preparation was then purified by using

reverse phase HPLC on a Discovery C18 column (25 cm x 4.6 mm, 5µm, Sigma Aldrich, Dorset, UK) with the following conditions:

1. Flow rate of 1 mL/min.
2. Gradient elution profile of 50 % mobile phase B to 100 % mobile phase B (mobile phase A consisted of 5 mmol/L ammonium acetate in HPLC grade water; mobile phase B consisted of 5 mmol/L ammonium acetate in methanol) over 15 min. The gradient was then held at 100% B for 20 minutes before re-equilibrating to 50% B.
3. UV absorbance was measured at 205 nm.

On identification of the peak in UV absorbance, the fraction was collected. The process was repeated until all the original lipid preparation had been purified. The fractions were then pooled in a single glass vial and dried in a stream of nitrogen gas. The glass vial in which the sample was to be stored was weighed, with care being taken to ensure that the vial was clean, dry and gloves were worn at all times when handling. The purified lipid sample was then pipetted into the pre-weighed glass vial and the solvent evaporated using a stream of nitrogen. The glass vial was then reweighed and the difference in weight used to determine the weight of the biotinylated lipid present. The contents of the vial were then re-suspend in methanol added at a volume sufficient to produce a concentration of the lipid of 100 ng/µL and then stored under nitrogen gas at -80 ^{o}C for use later.

Biotinylated versions of the following lipids for use as internal standards were provided by Dr Stephen Clark and Dr Christopher Thomas: 1-stearoyl-2-oleoyl-sn-glycero-3-PS (SOPS; 18:0a/18:1-PS), 1,2-dioleoyl-sn-glycero-3-PS (DOPS; 18:1a/18:1-PS), 1-stearoyl-2-arachidonoyl-sn-glycero-PS (SAPS; 18:0a/20:4-PS), 1-(1Z-stearoyl)-2-arachidonoyl-sn-glycero-3-PE (SpAPE;

18:0p/20:4-PE), 1-stearoyl-2-arachidonoyl-sn-glycero-3-PE (SAPE; 18:0a/20:4-PE) and 18:0a/12-HETE-PE.

2.10.7 Mass-spectrometry to identify and quantify externalised and total PE, PS, 12-HETE-PE

Samples were run using HPLC/MS/MS using a Shimadzu DGU 14A degasser HPLC system with SIL-HTc Auto-sampler and LC-10AD *VP* µ Binary Pump System attached to an AB Sciex 4000 Q-Trap mass spectrometer. The methods used were those described by Thomas and Clark et al (171).

For the measurement of biotinylated PE, PS and 12-HETE-PE, reverse phase HPLC was performed using an Ascentis C18 column (5µm, 150 × 2.1 mm, Sigma Aldrich, Dorset, UK) with the following conditions at 22 °C:

1. Flow rate 400 µL/min.
2. Isocratic mobile phase consisting of 20 mmol/L ammonium acetate in methanol.

From each sample, 10µL was injected on to the HPLC column. The phospholipids were then detected using the multiple reaction monitoring (MRM) transitions listed in Table 2.4. PE species were monitored by formation of the sn2 carboxylate anion, whilst PS species were monitored by neutral loss of biotinylated serine. Biotinylated 12-HETE-PE species were monitored by the ion formed from the characteristic fragmentation of the oxidized sn2 lipid.

Table 2.4 MRM transitions used to monitor biotinylated PE, PS and 12-HETE-PE.

Lipid	Mass	Biotinylated Mass	m/z $[M-H]^-$	Biotinylated MRM transition
DMPE (14:0/14:0-PE)	635	861	860	860 →227
SpAPE (18:0p/20:4-PE)	751	977	976	976 →303
SAPE (18:0a/20:4-PE)	767	993	992	992→303
PpAPE (16:0p/20:4-PE)	723	949	948	948 →303
SOPE (18:0a/18:1-PE)	745	971	970	970 →281
OpAPE (18:1p/20:4-PE)	749	975	974	974 →303
DMPS (14:0/14:0-PS)	679	905	904	904 →591
SOPS (18:0a/18:1-PS)	789	1015	1014	1014→701
DOPS (18:1a/18:1-PS)	787	1013	1012	1012 →699
SAPS (18:0a/20:4-PS)	811	1037	1036	1036 →723
18:0a/12-HETE-PE	783	1010	1009	1009 → 179
18:0p/12-HETE-PE	767	994	993	993 → 179
18:1p/12-HETE-PE	765	992	991	991 → 179
16:0p/12-HETE-PE	739	966	965	965 → 179

2.10.8 Mass Spectrometry for 12-HETE-PC, SAPC and PAPC

Using the same HPLC system and mass spectrometer described earlier, reverse phase HPLC was performed using a Luna C18 (3 µm,150 × 2 mm) column (Sigma-Aldrich, Dorset, USA) and conditions at 22 ° C as follows:

Flow rate 0.2 mL/min Gradient elution profile - 50 % mobile phase A and 50% mobile phase B to 100 % mobile phase B (mobile phase A: methanol: acetonitrile: water, 60: 20: 20, 1 mmol/L ammonium acetate; mobile phase B: methanol, 1 mmol/L ammonium acetate) over 10 min

followed by 30 min at 100 % mobile phase B before re-equilibration to 50 % mobile phase A and 50% mobile phase B.

From each sample, 10µL was injected on to the HPLC column and the MRM transitions listed on Table 2.5 were monitored in negative mode. PC species were monitored by formation of the sn2 carboxylate ion, with 12-HETE-PC monitored by the ion resulting from characteristic fragmentation of the oxidized sn2 lipid.

Table 2.5 MRM transitions monitored for detection of PC and 12-HETE-PC.

Lipid	**Mass**	**m/z $[M\text{-}CH_3]^-$**	**MRM transition**
DMPC (14:0/14:0-PC)	678	663	663 →227
SAPC (18:0/20:4-PC)	810	795	795 →303
PpAPC (16:0/20:4-PC)	782	767	767 →303
16:0a/12-HETE-PC	798	783	783→ 179
18:0a/12-HETE-PC	826	811	811→179

2.10.10 Phosphatidylcholine Lipid Standard Curve

A solution containing the internal standard DMPC at concentration of 0.02 ng/µL was prepared by diluting DMPC in methanol. A solution of the analyte standards (C) was prepared by diluting SAPC and 16:0/12-HETE-PC together in methanol such that each had a concentration of 2 ng/µL. Then serial dilutions of the test standard mixture (see table below) was prepared as outlined in Table 2.6 and then adding 100 µL of each dilution to 100 µL of the internal standard solution.

Table 2.6 Preparation of standard curve for quantification of PC and 12-HETE-PC.

Dilution Number	Procedure	Concentration of each analyte standard	Final Concentration (after adding internal standard)	Amount on column (final)
1	Undiluted mixture C	2 ng/µL	1 ng/µL	10 ng
2	150 µL of (1) with 150 µL methanol	1 ng/µL	0.5 ng/µL	5 ng
3	60 µL of (2) with 240 µL methanol	0.2 ng/µL	0.1 ng/µL	1 ng
4	150 µL of (3) with 150 µL methanol	0.1 ng/µL	50 pg/µL	500 pg
5	60 µL of (4) with 240 µL methanol	20 pg/µL	10 pg/µL	100 pg
6	150 µL of (5) with 150 µL methanol	10 pg/µL	5 pg/µL	50 pg
7	60 µL of (6) with 240 µL methanol	2 pg/µL	1 pg/µL	10 pg
8	150 µL of (7) with 150 µL methanol	0.5 pg/µL	0.5 pg/µL	5 pg
9	60 µL of (8) with 240 µL methanol	0.2 pg/µL	0.1 pg/µL	1 pg

2.10.11 Biotinylated Lipid Standard Curve

A solution containing the internal standards DMPE-B and DMPS-B, each at a concentration of 0.02 ng/µL, was prepared by diluting in methanol. A solution of the analyte standards (F) was prepared by diluting SAPS-B, SOPS-B, DOPS-B, S(p)APE-B, SAPE-B and 18:0/12-HETE-PE-B together in methanol such that each had a concentration of 2 ng/µL. Then serial dilutions of the test standard mixture were prepared, as outlined in Table 2.7, before then adding 100 µL of each dilution to 100 µL of the internal standard solution to produce the final concentration.

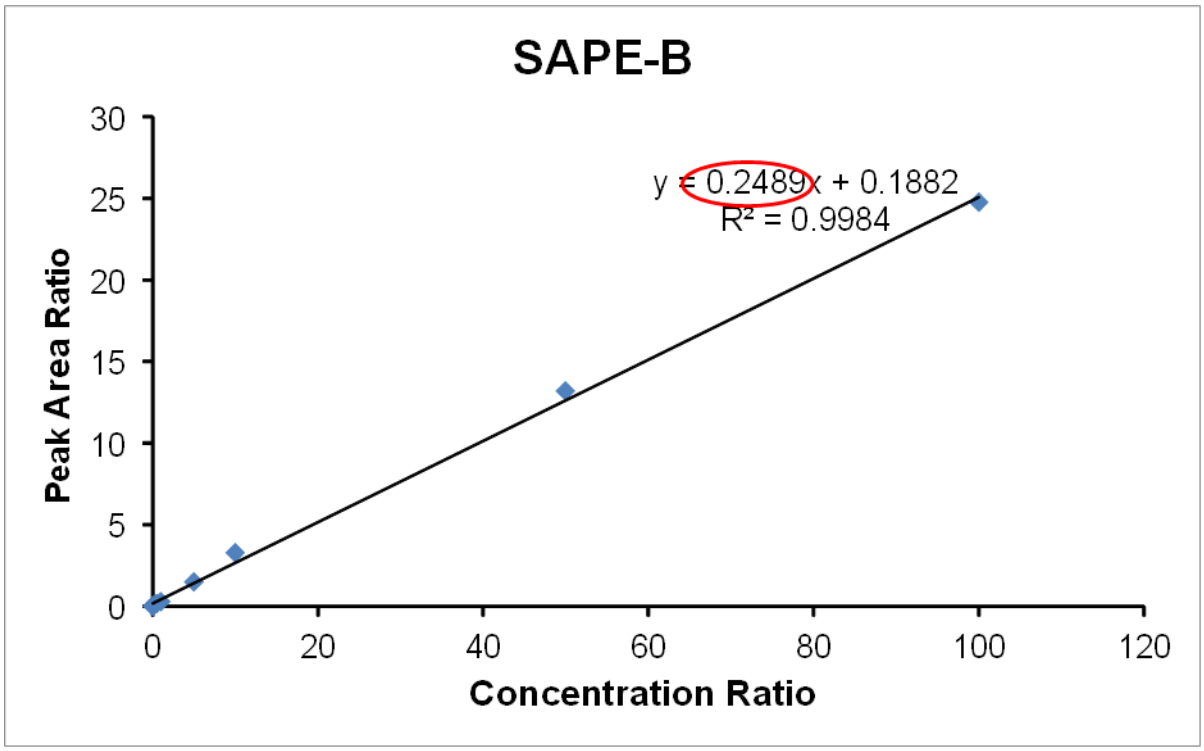
Table 2.7 Preparation of standard curve for quantification of biotinylated PE, PS and 12-HETE-PE.

Dilution Number	Procedure	Concentration of each analyte standard	Final Concentration (after adding internal standard)	Amount on column (final)
1	Undiluted mixture F	2 ng/µL	1 ng/µL	10 ng
2	150 µL of (1) with 150 µL methanol	1 ng/µL	0.5 ng/µL	5 ng
3	60 µL of (2) with 240 µL methanol	0.2 ng/µL	0.1 ng/µL	1 ng
4	150 µL of (3) with 150 µL methanol	0.1 ng/µL	50 pg/µL	500 pg
5	60 µL of (4) with 240 µL methanol	20 pg/µL	10 pg/µL	100 pg
6	150 µL of (5) with 150 µL methanol	10 pg/µL	5 pg/µL	50 pg
7	60 µL of (6) with 240 µL methanol	2 pg/µL	1 pg/µL	10 pg
8	150 µL of (7) with 150 µL methanol	0.5 pg/µL	0.5 pg/µL	5 pg
9	60 µL of (8) with 240 µL methanol	0.2 pg/µL	0.1 pg/µL	1 pg

2.10.12 Quantification of phospholipids

Each standard curve was run as described for the relevant samples (biotinylated and non-biotinylated). The peak area for each lipid was integrated. For each analyte standard, the ratio of analyte standard (AS) to internal standard (IS) for ng amounts of lipid in each standard vial was calculated followed by the ratio for AS to IS peak area in counts per second (cps). These two ratios were then plotted against each other as shown in Figure 2.1, and an equation generated to provide the slope of the line of best fit.

Figure 2.1 Example of plot generated for weight ratio against peak area ratio for SAPE-B showing equation including line slope. The slope is circled in red.



The amount of each lipid present in each patient sample was calculated by first integrating the peak area for the lipid and internal standard and then applying the following formula:

Amount of lipid present in ng = (Lipid peak area / internal standard peak area) x (amount of internal standard added [in all cases 10 ng]/ slope from standard curve)

The amount of lipid was then expressed as ng per $2x10^8$ platelets.

2.11 Monocyte Isolation for Cell-based coagulation model

2.11.1 *In vitro* Modelling

Monocytes were isolated in a Class 2 tissue culture hood from buffy coats purchased from the Welsh Blood Service. Sterile Phosphate Buffered Saline (PBS) suitable for cell culture was purchased from Life Technologies Ltd (Paisley, UK). The contents of the Buffy coat were diluted

at a ratio of 1 to 1 with PBS. This was then overlaid onto Lymphoprep (Alere Ltd, Stockport, UK) at a ratio of 2:1 in 50 mL falcon tubes. The tubes were centrifuged at 800 x *g* with no brake applied in a refrigerated centrifuge with the temperature set to 4 ^{o}C (Heraeus, Sweden) for 20 minutes. The interface layer was then carefully removed using a sterile pipette and diluted 1 to 1 with ice cold PBS containing 0.4% (w/v) trisodium citrate pH 7.4. The tube was then centrifuged at 400 x *g* with no brake applied, at 4 ^{o}C for 10 minutes. The supernatant was carefully removed and the pellet re-suspended in 45 mL of ice-cold PBS/0.4% Citrate. This process was repeated a further 5 times (centrifuging with the brake on) until the supernatant at the end of centrifuging was no longer cloudy. The pellet was then re-suspended in 2 mL RPMI 1640 media (Life Technologies Ltd Paisley, UK). An aliquot of the cell suspension was diluted 1 in 50 with a solution of 1% (v/v) trypan Blue. The number of cells present was counted using a modified Neubauer haemocytometer and calculated using the formula described in the section on preparation of washed platelets. The original suspension was then diluted further with RPMI fortified with 100 units/ mL penicillin and streptomycin with 2 mmol/L glutamine to yield a cell concentration as desired (see Chapter 6).

200 µL of this suspension was then placed in wells of a 96 well plate BD cell culture plate, the type determined by the experiment being performed (see Chapter 6). Some wells were left blank and some contained media only. The plate was then covered and incubated for 2 hours at 37 ^{o}C in a humidified cell culture incubator with carbon dioxide set to 5%. The media was removed and the wells were then washed with 300 µL RPMI to remove non-adherent cells. 200 µL of media was then added to each well, either containing RPMI alone or RPMI with varying concentrations of LPS. The plate was then covered and incubated under the same conditions for a further 18 hours. At the end of incubation, the media was removed and the wells washed with further RPMI. 20 µL of liposomes were then added (or calibrator) to the wells, followed by 80 µL of pooled PPP from a minimum of 4 normal donors (blood collected

into sodium citrate and CTI as described in section 2.6 above). Thrombin generation was then measured as described earlier.

2.11.2 Crystal Violet Staining for adherent cells in 96-well plate

The contents of the wells of the 96 well plate were removed and the cells were fixed by adding 200 µL ethanol per well. The plate was incubated at room temperature for 10 minutes. The ethanol was then removed and 200 µL of 0.1% (w/v) crystal violet solution was added, after which the plate was incubated at room temperature for 30 minutes. The crystal violet solution was then discarded and the plate washed 5 times with distilled water. Then 200 µL of 0.2% (v/v) Triton X was added to each well to lyse the cells and light absorbance was measured at 570 nm on Biotek plate reader and analysed using Gen 5.1 software (Biotek, Winoosi, VT, USA).

2.12 Cascade model using purified coagulation factors and anti-coagulants

Coagulation factors II, V, and X were purchased from Cambridge Biosciences Ltd (Cambridge, UK). Lyophilised factors VIII and IX were purchased from Tebu-Bio UK (Peterborough, UK). Lyophilised protein S and antithrombin were purchased from Enzyme Research Laboratories Ltd (Swansea, UK). Human full length TFPI was a gift from Dr Michael Dockal, Baxter Biosciences GmbH, Vienna, Austria. Lyophilised reagents were reconstituted with distilled water. All reagents were diluted to the desired concentration using distilled water and then frozen at -80 ^{o}C until further use.

The concentration of these coagulation factors was calculated so that the final in-well concentration was the same as the activity measured using the ACL500 Top. All coagulation and anti-coagulant factors were diluted in distilled water before adding to the respective wells of a 96 well plate. Thrombin generation assays were performed as described earlier. 20 µL of trigger solution or calibrator was added to each well, followed by 80 µL of the coagulation factor mix. Each condition was run in duplicate.

2.13 Statistical Analysis

Data was analysed using PASW Statistics version 18 software (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago, MI, USA). The Mann Whitney-U test was applied to examine differences between unrelated variables, whilst the Wilcoxon Rank test was used to examine differences between related samples. Analysis of multiple related samples was performed using Friedman's test. Spearman's correlation coefficients were calculated to investigate the relationship between full-length TFPI and thrombin generation parameters Analysis of multiple related samples was performed using Friedman's test. Comparison of means was performed using Student's t-test for paired variables and ANOVA for multiple related variables. A receiver operator characteristic (ROC) analysis was performed where factors where significantly different between patients that bleed excessively and those that did not.

Chapter 3 The effect of CPB on clinical parameters, coagulation factors and thrombin generation and their association with bleeding

3.1 Introduction

Surgery requiring cardiopulmonary bypass is associated with increased morbidity and mortality (172, 173) and accounts for around 4% to 15% of red blood cells transfused in the UK and USA (174, 175). Blood products are a limited resource and may become scarcer in the future due to the impact of an aging population; therefore, the need to target blood products to those with greatest need will increase.

Bleeding following cardiopulmonary bypass may be caused by dilution and consumptive of clotting factors, anticoagulation, thrombocytopenia and or platelet dysfunction (102) and surgical causes. Recent studies have reported that thrombin generation performed in both pre- and post-operative blood samples may identify patients at increased risk of excess bleeding (11, 169). This raises the possibility that intervention could be targeted at these patients before bleeding becomes established, thus improving patient outcome and reducing the use of blood products. Thrombin generation assays are potentially more useful than routine tests of coagulation because they integrate the effects of both pro and anticoagulant factors. Previous studies have shown that pro-coagulant factors (176) and protein C levels fall post cardiopulmonary bypass, but activated protein C increases (111, 112). Thrombin generation assays performed in the presence of thrombomodulin might therefore better describe global haemostasis. Tissue Factor Pathway Inhibitor (TFPI) may also play a role in

bleeding because heparin, the standard anticoagulant used during cardiopulmonary bypass, releases TFPI from the endothelium (64).

At present thrombin generation assays lack standardisation (177, 178). Some groups advocate the addition of corn trypsin inhibitor (CTI) to reduce contact activation and this has been shown to increase sensitivity to the propagation phase at low concentrations of tissue factor (156, 157). The optimal concentration of tissue factor to use in the assays is also uncertain. Lower concentrations may be more physiologically relevant and altering the concentration used may change the sensitivity of the assay to detect patients at risk of excess bleeding.

The composition and volume of intravenous fluids infused peri-operatively may also influence bleeding. Crystalloids have been shown to cause dilution of coagulation factors which may influence haemostasis (179). Colloids fluids are frequently used for resuscitation in the setting of intravascular volume depletion, especially where there was a desire to avoid total body fluid overload. However, colloids carry their own problems in that they have been reported to affect haemostasis (180).

The experiments detailed in this chapter sought to identify clinical and haemostatic parameters that might influence postoperative bleeding and to confirm whether thrombin generation assays were able to identify individuals at increased risk of bleeding. In addition the effect of changing the conditions of thrombin generation assay was explored.

3.2 Results

3.2.1 Demographic data and clinical parameters

A total of 102 patients were recruited to this section of the study. 10 patients were excluded from further analysis as measurement of heparin anti-Xa activity in post-operative samples revealed persistent contamination (>0.3 anti-Xa units/mL) despite the ACT after protamine administration having been within 10% of the starting value. A further 5 patients were found to have received one or more units of FFP in the interval between protamine being given and blood samples being taken and were therefore also excluded. This left 87 patients in the subsequent analysis.

Table 3.1 Type of operation by frequency of bleeding.

Type of Operation	Total undergoing procedure	No Bleeding	> 1L	> 200mL/hr	> 2mL/kg/hr	Need for haemostatic treatment	Re-operation	Excess bleeding by any end point
AVR	21	11	4	3	4	10	2	10
AVR + another procedure	35	21	6	5	7	14	1	14
CABG (Decision not to proceed with planned AVR)	1	0	1	1	1	1	1	1
LV Aneurysm-ectomy + CABG	1	0	1	0	1	1	0	1
MV Repair / Replacement alone	5	4	1	0	0	1	0	1
MV Repair + another procedure	12	9	0	0	0	3	0	3
Redo AVR	2	2	0	0	0	1	0	0
Redo AVR + another procedure	3	0	2	1	2	3	2	3
Replacement of portion of ascending aorta +/- another procedure	7	1	3	0	0	6	1	6
Total	87 (100%)	48 (55%)	18 (21%)	10 (11%)	15 (17%)	37 (43%)	7 (8%)	39 (45%)

AVR= aortic valve replacement, MV= mitral valve, MVR= Mitral Valve Replacement, TA= tricuspid annuloplasty, LV= Left Ventricle, CABG= Coronary Artery Bypass Graft.

Table 3.2 Clinical Parameters of patients recruited divided by end point.

End point	Whole Cohort	<1L	>1L	<200mL/hr	>200mL/hr	<2ml/kg/hr for 2 hours	>2ml/kg/hr for 2 hours	No Haemostatic treatment	Haemostatic treatment	No Re-op	Re-op
Age (years)	68 28-88 (62-76)	69 29-88 (62-76)	63 28-81 (58-71)	69 59-88 (61-76)	67 28-81 (65-75)	68 29-88 (61-76)	69 28-81 (67-75)	68 29-88 (62-76)	67 28-88 (61-76)	69 29-88 (62-76)	66 28-71 (46-69)
Weight (kg)	78 48-134 (67-90)	76 48-134 (66-89)	90 60-121 (72-97)	78 48-134 (67-90)	87 60-99 (71-94)	80 48-134 (66-90)	75 60-99 (68-90)	75 48-120 (66-88)	84 58-134 (69-96)	77 48-134 (67-90)	88 74-99 (81-94)
Sex (M/F)	60/27	46/23	14/4	25/25	8/2	50/22	10/5	31/16	29/11	55/25	5/2
Wafarin/Heparin pre-op (yes/no)	9/78	6/63	3/15	7/70	2/8	7/65	2/13	4/43	5/35	8/72	1/6
Aspirin pre-op (yes/no)	34/53	26/43	8/10	30/47	4/6	27/45	7/8	19/28	15/25	33/47	1/6
Time on bypass (minutes)	144 64-265 (115-178)	139 64-250 (114-163)	175* 79-270 (158-203)	145 67-270 (115-178)	144 79-203 (124-201)	143 64-270 (114-167)	156 79-203 (144-200)	132 64-230 (113-158)	162** 79-270 (133-203)	151 64-270 (115-178)	151 79-230 (111-217)
Aortic Cross-clamp time (minutes)	112 50-219 (95-145)	111 50-217 (91-139)	143 61-228 (125-167)	115 50-228 (95-143)	104 61-178 (92-160)	112 50-228 (91-141)	127 61-178 (100-157)	106 50-210 (91-131)	131** 61-228 (101-176)	123 50-228 (95-143)	118 61-199 (82-183)
Days ventilated	<1 <1-8 (<1-1)	<1 <1-8 (<1-1)	1 1-2 (<1-1)	<1 <1-8 (<1-1)	<1 <1-2 (<1-1)	<1 <1-8 (<1-1)	<1 <1-2 (<1-1)	<1 <1 <1	1 <1-8 (<1-1)	<1 <1-8 (<1-1)	1 1-6 (2-4)
Duration of Level 2/3 care (days)	3 1-20 (3-5)	3 1-20 (2-5)	4 2-13 (3-6)	3 1-20 (2-5)	4 3-13 (2-6)	3 1-20 (2-5)	4 3-13 (3-6)	3 1-10 (3-5)	3 2-20 (3-5)	3 1-20 (3-5)	8*** 3-13 (5-10)
Days to discharge post-op	12 5-49 (8-16)	12 5-49 (8-17)	12 5-21 (7-14)	12 5-49 (8-16)	13 5-21 (8-14)	13 5-21 (8-16)	14 5-21 (8-14)	12 5-35 (9-15)	12 5-49 (7-18)	13 5-49 (8-16)	17*** 13-28 (14-21)
Complications (yes/no)	27/60	19/50	8/10	20/57	7/3	18/54	9/6	10/37	17/23	20/60	7/0

Data shown are the median and range (inter-quartile range). *Statistically significant difference between >1L and <1L ($p < 0.05$. **Statistically significant difference between no haemostatic treatment and need for haemostatic treatment ($p < 0.05$). ***statistically significant difference between reoperation and no reoperation ($p < 0.05$).

Table 3.3 Intravenous fluids and blood products administerted intra- and post-operatively divided by bleeding end point.

End point	Whole Cohort	<1L	>1L	<200mL/hr	>200mL/hr	<2ml/kg/hr for 2 hours	>2ml/kg/hr for 2 hours	No Haemostatic treatment	Haemostatic treatment	No Re-op	Re-op
Intraoperative volume crystalloid (mL/kg)	13 0-36 (8-20)	13 0-32 (8-18)	14 5-36 (8-22)	14 0-36 (8-20)	11 5-27 (8-16)	13 0-32 (8-20)	14 5-36 (9-16)	14 0-32 (8-21)	14 5-36 (8-17)	13 0-36 (8-20)	11 5-37 (5-22)
Intraoperative volume of colloid (mL/kg)	13 0-47 (8-18)	13 0-47 (8-18)	17 4-35 (10-19)	13 0-47 (8-17)	17 8-24 (16-21)	13 0-47 (8-17)	17*** 8-35 (16-24)	14 0-47 (7-17)	14 4-35 (9-19)	13 0-47 (8-18)	17 11-28 (16-26)
Post-operative volume crystalloid (mL/kg)	13 0-30 (10-16)	13 0-28 (11-16)	11 0-30 (0-17)	13 0-29 (10-16)	13 0-30 (11-23)	13 0-28 (10-16)	14 0-30 (12-23)	13 0-23 (10-15)	13 0-30 (10-17)	13 0-30 (10-16)	11 0-30 (6-22)
Post-operative volume of colloid (mL/kg)	23 0-52 (18-29)	23 0-52 (19-30)	20* 0-34 (10-23)	23 0-52 (19-30)	11** 0-34 (4-22)	23 0-52 (19-30)	18*** 0-34 (9-22)	26 9-52 (21-30)	19‡ 0-40 (11-23)	23 0-52 (17-28)	28 20-34 (22-34)
Cell Salvage Volume (mL/kg)	9 3-52 (7-12)	10 3-52 (7-12)	9 6-24 (8-11)	10 3-52 (7-12)	9 6-13 (8-11)	9 3-52 (7-12)	9 6-13 (8-11)	10 3-16 (7-12)	9 5-52 (7-11)	9 3-52 (7-12)	13 11-29 (12-21)
Number of red cell units transfused	1 0-12 (0-3)	1 0-8 (0-2)	4* 0-12 (1-7)	1 0-12 (0-2)	5** 0-9 (3-8)	1 0-8 (0-2)	5*** 0-12 (3-8)	1 0-5 (0-2)	3‡ 0-12 (0-5)	1 0-12 (0-3)	6‡‡ 0-8 (6-8)
Number of platelet units transfused	0 0-5 (0-1)	0 0-5 (0-1)	1* 0-4 (1-3)	0 0-5 (0-1)	3** 1-3 (1-3)	0 0-5 (0-1)	2*** 1-4 (1-3)	0 0-1 (0-0)	1‡ 0-5 (1-2)	0 0-4 (0-1)	3‡‡ 1-5 (3-4)
Number of FFP units transfused	0 0-12 (0-4)	0 0-12 (0-4)	8* 0-12 (4-9)	0 0-12 (0-4)	9** 4-12 (6-11)	0 0-12 (0-4)	8*** 4-12 (4-10)	0 - -	4‡ 2-12 (5-7)	0 0-12 (0-4)	10‡‡ 8-12 (10-12)

Data shown are the median and range (inter-quartile range). *Statistically significant difference between >1L and <1L ($p< 0.05$). **Statistically significant difference between >200mL/hr and <200mL/hr ($p< 0.05$) . ***Statistically significant difference between >2mL/kg/hr and <2mL/kg/hr ($p< 0.05$). ‡ Statistically significant difference between no haemostatic treatment and haemostatic treatment ($p< 0.05$). ‡‡ Statistically significant difference between no re-operation and reoperation ($p< 0.05$).

The majority of patients (90%) underwent heart valve surgery with or without an additional procedure. Replacement of the aortic valve, with or without another procedure, accounted for 64% of all operations. There was no significant difference between the type of operation and the frequency of bleeding (Table 3.1). Patients who bled excessively received significantly more red cells and platelet transfusions regardless of bleeding endpoint ($p<0.01$) (Table 3.3). Patients who received haemostatic treatment with FFP, cryoprecipitate and fibrinogen had a significantly longer time on bypass (162 versus 132 minutes, $p=0.005$) and longer aortic cross-clamp times (131 versus 106 minutes, $p=0.003$) (Table 3.2). Patients who bled in excess of 1L at 24 hours also spent a longer on bypass than those who did not (175 versus 139 minutes, $p=0.04$). Post-operative colloid administration was lower in those who bled compared to those who did not, apart from those needing to return the theatre where there was no difference (>1L 20mL/kg versus 23 mL/kg, $p=0.02$; >200mL/hr 11 versus 23, $p=0.04$; >2mL/kg/hr 18 versus 23, $p=0.007$; need for haemostatic treatment 19 versus 26, $p<0.0001$). This finding is most likely due to these patients receiving blood products rather than colloids or crystalloids. There was no significant difference between those who bled and those who did not in the frequency of preoperative aspirin administration, other anticoagulant use, or in the residual anti-Xa activity in post-operative samples.

3.2.2. Clotting factors, inhibitors and platelet count

The results for this section are summarised in tables 3.4 to 3.9. Overall the median concentration of clotting factors was significantly lower post cardiopulmonary bypass compared to before. In contrast the overall median concentrations of VWF increased. The anticoagulants antithrombin, protein C and S were also lower post-cardiopulmonary bypass. Total TFPI increased significantly while full length TFPI decreased.

Comparing the levels between the different end-points for bleeding, there were no significant differences in pre-operative samples. Post-operatively, prothrombin (49 iu/dL versus 53 iu/dL, p=0.028), Factor V (42 iu/dL versus 50 iu/dL, p=0.038) and X (42 iu/dL versus 47 iu/dL, p=0.034), Factor XIII (46% versus 59%, p=0.013), Fibrinogen (1.4 g versus 1.7g, p=0.031) and platelet count ($101x10^9$/L versus 110 $x10^9$/L, p=0.042) were significantly lower in those who went on to bleed more than 2 mL/kg/hr for two consecutive hours in the first six hours after the end of surgery. ROC analysis showed these had modest value in predicting bleeding.

Prothrombin (50 iu/dL versus 53 iu/dL, p=0.045), Factor XIII (46% versus 58%, p=0.012), Fibrinogen (1.4 g/L versus 1.7 g/L, p=0.006) and platelet count (104 $x10^9$//L versus 118 $x10^9$//L, p=0.048) were also lower in those who went on to bleed in excess of 1 litre at 24 hours. ROC analysis suggested fibrinogen had modest predictive value.

For those bleeding at >200mL/hr for 2 consecutive hours, Factor XIII was significantly lower (45% versus 58%, p=0.048), as was Factor VII (56 iu/dL versus 70 iu/dL, p=0.039). ROC analysis demonstrated these had modest predictive value.

There were no significant differences in post-operative clotting factors, inhibitors or platelet counts for those who required haemostatic treatment. The same applied to those who needed to return to theatre for re-operation due to bleeding.

Table 3.4 Concentrations of clotting factors, inhibitors and platelet count before and after CPB for all patients.

End point	Whole Cohort Pre-CPB	Whole Cohort Post-CPB
APTT	29	31*
(sec)	23-38	22-46
	(28-31)	(30-35)
PT	11	15*
(sec)	10-16	12-22
	(10-12)	(14-17)
Fibrinogen	3.2	1.6*
(g/L)	1.7-7.9	1.1-3.8
	(2.6-4.0)	(1.4-1.9)
Factor II	95	52*
(iu/dL)	44-152	33-83
	(85-107)	(47-60)
Factor V	92	50*
(iu/dL)	51-135	17-93
	(76-109)	(40-58)
Factor VII	104	68*
(iu/dL)	48-192	25-146
	(80-118)	(58-80)
Factor VIII	129	92*
(iu/dL)	75-262	49-216
	(106-157)	(74-117)
Factor IX	133	104*
(iu/dL)	59-224	62-157
	(117-151)	(93-123)
Factor X	90	46*
(iu/dL)	27-148	20-57
	(78-104)	(38-54)
Factor XI	95	60*
(iu/dL)	42-183	35-137
	(82-109)	(50-72)
Full length TFPI	21	15*
(ng/mL)	7-39	4-35
	(16-24)	(11-22)
Total TFPI	56	113*
(ng/mL)	32-99	60-165
	(48-67)	(93-127)
VWF	145	172*
(iu/dL)	62-322	84-367
	(113-188)	(136-206)
Anti-Thrombin	92	51*
(%)	11-144	30-77
	(83-100)	(45-58)
Protein S	84	46*
(%)	43-125	30-85
	(72-98)	(39-55)
Protein C	106	61*
(%)	64-204	39-93
	(92-119)	(52-69)
Factor XIII	104	56*
(%)	30-213	21-107
	(90-117)	(45-67)
TAFI	101	69*
(%)	42-182	31-120
	(83-125)	(60-80)
Platelet Count	224	115*
($x10^9$/L)	124-670	42-258
	(188-258)	(98-137)
Anti-Xa activity	-	0.12
(iu/mL)		0.00-0.30
		(0.08-0.19)

Data shown are the median and range (inter-quartile range). * indicates significantly different results between pre and post samples ($p<0.05$).

Table 3.5 Concentrations of clotting factors, inhibitors and platelet count before and after CPB comparing those who bled less or more than 1L.

End point	<1L Pre-CPB	<1L Post-CPB	>1L Pre-CPB	>1L Post-CPB	ROC area post-CPB (95% CI)
APTT (sec)	30 23-38 (28-32)	31 22-46 (30-34)	28 25-32 (27-30)	34 27-46 (30-37)	-
PT (sec)	11 10-16 (10-12)	15 12-20 (14-17)	11 10-14 (11-12)	16 13-22 (15-18)	-
Fibrinogen (g/L)	3.2 1.7-7.9 (2.6-4)	1.7 1.1-3.8 (1.4-2)	3 2-4.6 (2.4-4)	1.4* 1.1-2.2 (1.3-1.7)	0.75 (0.6-0.9)
Factor II (iu/dL)	96 44-152 (82-110)	53 33-83 (47-63)	92 60-117 (87-100)	50* 39-58 (44-53)	0.66 (0.5-0.83)
Factor V (iu/dL)	87 51-135 (74-104)	50 17-93 (41-59)	98 60-121 (84-112)	46 26-59 (35-56)	-
Factor VII (iu/dL)	104 48-192 (79-122)	68 33-125 (59-80)	99 48-151 (80-107)	67 25-146 (56-76)	-
Factor VIII (iu/dL)	129 75-262 (104-156)	97 49-216 (78-119)	122 79-252 (112-169)	74 52-142 (67-109)	-
Factor IX (iu/dL)	130 59-224 (114-152)	107 65-157 (94-124)	134 94-183 (127-148)	100 79-144 (86-105)	-
Factor X (iu/dL)	90 27-148 (76-104)	47 20-77 (38-56)	92 48-114 (84-103)	45 24-57 (35-48)	-
Factor XI (iu/dL)	95 42-183 (82-107)	62 35-137 (51-73)	88 58-183 (76-125)	57 36-76 (45-61)	-
Full length TFPI (ng/mL)	21 7-39 (17-24)	16 4-35 (11-22)	20 10-38 (15-25)	13 5-33 (11-17)	-
Total TFPI (ng/mL)	57 32-99 (48-67)	115 60-165 (95-127)	53 36-79 (48-61)	100 65-160 (87-116)	-
VWF (iu/dL)	135 62-322 (109-180)	171 85-367 (136-207)	176 95-268 (144-217)	174 84-277 (132-203)	-
Anti-Thrombin (%)	93 61-144 (84-101)	52 30-77 (47-59)	92 11-109 (81-100)	45 35-60 (43-56)	-
Protein S (%)	80 43-112 (70-94)	46 30-85 (40-56)	94 58-125 (78-104)	45 35-63 (38-51)	-
Protein C (%)	106 64-204 (91-121)	62 39-93 (54-70)	104 76-149 (96-112)	58 42-74 (49-65)	-
Factor XIII (%)	105 59-213 (91-119)	58 21-107 (47-68)	98 30-131 (73-111)	46* 25-67 (43-55)	0.66 (0.5-0.83)
TAFI (%)	97 42-182 (82-121)	70 31-120 (60-80)	105 56-175 (97-136)	66 35-117 (61-88)	
Platelet Count ($x10^9$/L)	226 124-670 (188-267)	118 42-258 (101-139)	224 134-448 (202-239)	104* 62-173 (88-124)	0.6 (0.4-0.8)
Anti-Xa activity (iu/mL)	-	0.13 0.0-0.29 (0.08-0.19)	-	0.12 0.1-0.3 (0.07-0.19)	-

Data shown are the median and range (inter-quartile range). * indicates significantly different results between post-CPB samples in those bleeding <1L or >1L ($p<0.05$). CI= confidence interval.

Table 3.6 Concentrations of clotting factors, inhibitors and platelet count before and after CPB comparing those who bled at a rate of more or less than 200 mL/hr.

End point	<200mL/hr Pre-CPB	<200mL/hr Post-CPB	>200mL/hr Pre-CPB	>200mL/hr Post-CPB	ROC area post-CPB (95% CI)
APTT (sec)	30 23-38 (30-32)	31 22-46 (30-35)	28 26-31 (27-30)	34 27-46 (30-36)	-
PT (sec)	11 10-16 (10-12)	15 12-20 (14-17)	11 10-14 (11-12)	17 14-22 (15-18)	-
Fibrinogen (g/L)	3.2 1.7-7.9 (2.6-4)	1.7 1.1-3.8 (1.4-2)	3 2-4.6 (2.6-4)	1.4 1.2-2.2 (1.3-1.7)	-
Factor II (iu/dL)	96 44-152 (83-109)	53 33-83 (47-61)	90 60-107 (85-100)	50 39-57 (43-52)	-
Factor V (iu/dL)	87 51-135 (76-105)	50 17-93 (40-58)	101 60-117 (93-112)	46 26-59 (32-56)	-
Factor VII (iu/dL)	104 48-192 (79-120)	70 33-146 (60-81)	102 48-120 (80-110)	56* 25-82 (47-67)	0.71 (0.53-0.89)
Factor VIII (iu/dL)	128 75-162 (101-157)	94 49-216 (77-119)	144 106-208 (114-157)	73 52-135 (70-109)	-
Factor IX (iu/dL)	133 59-224 (115-152)	106 65-157 (94-124)	128 98-183 (121-146)	100 79-144 (84-104)	-
Factor X (iu/dL)	92 27-148 (77-104)	47 20-77 (38-56)	85 52-113 (81-97)	41 29-50 (32-47)	-
Factor XI (iu/dL)	95 42-183 (82-108)	60 35-137 (50-72)	88 63-132 (85-125)	60 37-76 (54-61)	-
Full length TFPI (ng/mL)	20 7-32 (16-24)	15 4-35 (11-22)	24 10-32 (20-26)	17 9-33 (11-18)	-
Total TFPI (ng/mL)	56 32-99 (48-67)	114 60-165 (94-127)	51 36-79 (48-61)	103 65-153 (82-129)	-
VWF (iu/dL)	143 62-352 (109-181)	171 85-367 (134-205)	188 113-260 (144-227)	174 84-277 (145-217)	-
Anti-Thrombin (%)	93 61-144 (84-101)	51 30-77 (81-95)	88 11-103 (45-59)	45 35-57 (43-52)	-
Protein S (%)	82 43-112 (71-95)	46 30-85 (40-56)	94 58-125 (74-104)	44 35-55 (38-50)	-
Protein C (%)	106 64-204 (92-121)	62 39-93 (54-70)	96 76-149 (94-108)	55 42-71 (46-62)	-
Factor XIII (%)	105 59-213 (90-118)	58 21-107 (45-68)	89 30-131 (72-98)	45* 25-64 (40-55)	0.70 (0.54-0.87)
TAFI (%)	100 42-182 (83-124)	70 31-120 (60-80)	105 56-159 (94-135)	64 35-117 (61-88)	-
Platelet Count ($x10^9$/L)	223 124-670 (188-265)	117 42-258 (98-138)	227 134-254 (214-239)	110 80-173 (90-129)	-
Anti-Xa activity (iu/mL)	-	0.13 0-0.3 (0.08-0.19)	-	0.11 0.1-0.26 (0.07-0.19)	-

Data shown are the median and range (inter-quartile range). * indicates significantly different results between post-CPB samples in those bleeding <200 mL/hr or >200 mL/hr($p<0.05$). CI= confidence interval.

Table 3.7 Concentrations of clotting factors, inhibitors and platelet count before and after CPB in those who bled more or less than 2 mL/kg/hr for 2 consecutive hours.

End point	<2mL/kg/hr for 2 hours Pre-CPB	<2mL/kg/hr for 2 hours Post-CPB	>2mL/kg/hr for 2 hours Pre-CPB	>2mL/kg/hr for 2 hours Post-CPB	ROC area post-CPB (95% CI)
APTT (sec)	30 23-38 (28-32)	31 22-46 (30-34)	38 26-32 (27-30)	34 27-46 (31-37)	-
PT (sec)	11 10-16 (10-12)	15 12-20 (14-17)	11 10-14 (10-12)	16 13-22 (15-18)	-
Fibrinogen (g/L)	3.2 1.7-7.9 (2.6-3.8)	1.7 1.1-3.8 (1.4-2)	3.3 2-4.6 (2.6-4.2)	1.4* 1.2-2.2 (1.3-1.7)	0.68 (0.53-0.83)
Factor II (iu/dL)	95 44-152 (82-120)	53 33-83 (48-62)	98 60-137 (87-107)	49* 39-60 (43-52)	0.68 (0.55-0.81)
Factor V (iu/dL)	87 51-135 (75-104)	50 17-93 (41-58)	100 60-121 (84-113)	42 26-59 (32-52)	0.68 (0.53-0.83)
Factor VII (iu/dL)	102 48-192 (78-117)	70 33-146 (60-80)	106 48-151 (88-120)	63 25-97 (54-75)	-
Factor VIII (iu/dL)	126 75-262 (101-154)	98 49-216 (79-121)	153 79-252 (114-173)	73 52-135 (67-95)	-
Factor IX (iu/dL)	133 59-224 (115-150)	107 65-157 (95-124)	134 94-183 (121-154)	100 79-144 (84-104)	-
Factor X (iu/dL)	90 27-148 (76-104)	47 20-77 (39-57)	94 52-113 (84-104)	42* 29-50 (35-47)	0.68 (0.55-0.80)
Factor XI (iu/dL)	94 42-183 (82-102)	60 35-137 (51-72)	107 58-183 (85-127)	59 36-76 (42-65)	-
Full length TFPI (ng/mL)	20 7-39 (17-24)	15 4-35 (11-22)	23 10-38 (16-27)	17 9-33 (11-22)	-
Total TFPI (ng/mL)	56 32-99 (48-67)	115 60-165 (95-128)	55 36-79 (48-67)	102 65-153 (87-112)	-
VWF (iu/dL)	136 62-322 (109-180)	174 85-367 (137-207)	182 95-268 (144-227)	154 84-277 (128-198)	-
Anti-Thrombin (%)	93 61-144 (83-101)	52 30-77 (47-59)	92 11-112 (84-100)	45 35-57 (41-52)	-
Protein S (%)	83 43-112 (72-95)	47 30-85 (41-56)	91 58-125 (74-104)	42 31-55 (37-48)	-
Protein C (%)	106 64-204 (91-120)	62 39-93 (54-70)	105 76-149 (95-112	55 42-71 (46-62)	-
Factor XIII (%)	104 59-154 (90-118)	59 21-107 (46-68)	98 30-131 (73-114)	46* 25-64 (42-55)	0.71 (0.59-0.84)
TAFI (%)	99 42- 182 (82-121)	70 31-120 (60-79)	105 56-175 (89-137)	65 35-117 (59-88)	
Platelet Count ($x10^9$/L)	222 124-670 (188-265)	119 42-258 (101-138)	230 134-448 (207-240)	101* 62-173 (86-129)	0.66 (0.50-0.83)
Anti-Xa activity (iu/mL)	-	0.13 0-0.3 (00.85-0.19)	-	0.1 0.1-0.26 (0.07-0.135)	-

Data shown are the median and range (inter-quartile range). * indicates significantly different results between post-CPB samples in those bleeding <2mL/kg/hr or >2 mL/kg/hr ($p<0.05$). CI= confidence interval.

Table 3.8 Concentrations of clotting factors, inhibitors and platelet count before and after CPB in those who did and did not require haemostatic treatment.

End point	No Haemostatic Treatment Pre-CPB	No Haemostatic Treatment Post-CPB	Haemostatic Treatment Pre-CPB	Haemostatic Treatment Post-CPB
APTT (sec)	29 26-38 (28-31)	31 22-46 (30-34)	30 23-38 (27-32)	32 24-46 (30-36)
PT (sec)	11 10-15 (10-12)	15 12-20 (14-17)	11 10-16 (10-12)	15 12-22 (14-17)
Fibrinogen (g/L)	3.4 1.7-7.9 (2.6-4)	1.7 1.2-3.8 (1.5-2)	3 2-5.9 (2.6-4)	1.5 1.1-3.3 (1.3-1.9)
Factor II (iu/dL)	95 44-142 (81-108)	52 33-83 (47-61)	98 49-152 (87-104)	53 37-78 (47-60)
Factor V (iu/dL)	87 55-135 (72-107)	50 29-93 (42-61)	94 51-121 (83-109)	47 17-64 (37-56)
Factor VII (iu/dL)	104 57-166 (78-122)	66 33-123 (58-78)	101 48-192 (82-115)	71 25-146 (59-80)
Factor VIII (iu/dL)	129 75-262 (106-154)	98 49-216 (79-121)	126 75-260 (106-169)	85 52-169 (70-113)
Factor IX (iu/dL)	134 87-224 (115-157)	107 69-157 (95-125)	130 59-183 (118-146)	103 65-144 (86-121)
Factor X (iu/dL)	87 27-145 (76-106)	46 20-67 (38-55)	96 51-148 (83-103)	46 30-77 (38-53)
Factor XI (iu/dL)	96 55-181 (82-108)	60 35-101 (52-73)	93 42-183 (82-109)	59 36-137 (49-72)
Full length TFPI (ng/mL)	21 7-39 (16-25)	15 4-35 (11-22)	20 10-38 (17-24)	16 8-33 (12-21)
Total TFPI (ng/mL)	57 32-99 (47-69)	115 62-155 (94-136)	55 36-84 (48-66)	104 60-165 (89-125)
VWF (iu/dL)	144 62-274 (105-181)	172 85-367 (140-204)	149 73-322 (117-188)	169 84-289 (132-208)
Anti-Thrombin (%)	93 69-117 (82-102)	51 30-75 (47-57)	92 11-144 (84-99)	50 34-77 (45-58)
Protein S (%)	80 43-112 (69-96)	46 30-85 (39-55)	88 58-125 (74-99)	46 31-71 (40-55)
Protein C (%)	107 64-164 (91-120)	61 39-87 (52-71)	104 76-204 (95-112)	61 42-93 (54-67)
Factor XIII (%)	104 59-198 (90-116)	58 21-107 (47-67)	103 30-213 (89-117)	52 25-85 (43-64)
TAFI (%)	99 61-182 (85-125)	69 39-120 (52-79)	103 42-175 (83-123)	71 31-117 (59-88)
Platelet Count ($x10^9$/L)	228 124-393 (184-265)	119 42-227 (99-142)	223 134-670 (189-247)	113 62-258 (94-133)
Anti-Xa activity (iu/mL)	- 	0.13 0.01-0.3 (0.09-0.18)	- 	0.11 0.0-0.29 (0.07-0.19)

Data shown are the median and range (inter-quartile range). * indicates significantly different results between post-CPB samples in those bleeding did not did not require haemostatic treatment ($p<0.05$).

Table 3.9 Concentrations of clotting factors, inhibitors and platelet count before and after CPB in those who did and did not undergo re-operation due to bleeding.

End point	No Re-op Pre	No Re-op Post	Re-op Pre	Re-op Post-
APTT	29	31	30	34
(sec)	25-38	22-46	23-31	30-46
	(28-32)	(30-35)	(28-31)	(30-40)
PT	11	15	11	17
(sec)	10-16	12-20	11-14	14-22
	(10-12)	(14-17)	(11-12)	(15-18)
Fibrinogen	3.1	1.6	3.5	1.4
(g/L)	1.7-7.9	1.1-3.8	2.3-4.5	1.2-2.2
	(2.6-4.0)	(1.4-1.9)	(2.6-4)	(1.3-1.7)
Factor II	95	53	94	47
(iu/dL)	44-152	33-83	60-101	37-57
	(84-108)	(48-61)	(85-98)	(39-52)
Factor V	92	50	90	30
(iu/dL)	51-135	24-93	60-111	17-59
	(79-109)	(41-58)	(76-101)	(26-56)
Factor VII	104	70	99	57
(iu/dL)	48-192	25-146	48-110	37-73
	(79-120)	(60-81)	(80-107)	(47-67)
Factor VIII	127	93	174	82
(iu/dL)	75-262	49-216	114-252	52-135
	(104-156)	(75-118)	(118-208)	(73-113)
Factor IX	133	105	129	84
(iu/dL)	59-224	65-157	98-173	79-105
	(115-151)	(95-124)	(127-144)	(81-104)
Factor X	90	46	97	39
(iu/dL)	27-148	20-77	52-106	29-48
	(77-104)	(38-55)	(81-103)	(30-47)
Factor XI	95	60	98	49
(iu/dL)	42-183	35-137	63-161	37-65
	(82-108)	(51-72)	(85-127)	(42-60)
Full length TFPI	20	15	22	17
(ng/mL)	7-39	4-35	10-31	8-33
	(16-24)	(11-22)	(20-26)	(10-22)
Total TFPI	56	114	52	107
(ng/mL)	32-99	60-165	42-79	65-153
	(48-67)	(94-127)	(44-61)	(87-129)
VWF	145	172	165	193
(iu/dL)	62-322	85-367	113-268	84-290
	(111-184)	(137-204)	(126-260)	(87-277)
Anti-Thrombin	92	51	92	46
(%)	11-144	30-77	87-100	34-57
	(83-101)	(45-58)	(88-96)	(35-56)
Protein S	84	47	83	40
(%)	43-125	30-85	63-104	32-50
	(72-96)	(40-56)	(74-100)	(35-44)
Protein C	106	62	103	50
(%)	64-204	39-93	76-112	45-62
	(92-121)	(54-70)	(95-112)	(46-59)
Factor XIII	104	58	95	49
(%)	59-213	21-107	30-131	25-64
	(89-117)	(45-67)	(90-114)	(40-55)
TAFI	99	71	127	69
(%)	42-182	31-120	64-175	46-117
	(83-123)	(60-80)	(104-159)	(61-103)
Platelet Count	224	116	232	108
($x10^9$/L)	124-670	42-258	207-448	62-173
	(188-258)	(98-138)	(214-349)	(94-129)
Anti-Xa activity	-	0.12	-	0.1
(iu/mL)		0.0-.03		0.01-0.2
		(0.08-0.19)		(0.05-0.13)

Data shown are the median and range (inter-quartile range). * indicates significantly different results between post-CPB samples in those bleeding did not did not require re-operation ($p<0.05$).

3.2.3 Relationship between intraoperative fluids and coagulation factor concentrations

Colloids and crystalloids had different effects on the concentration of coagulation factors and anticoagulants. The volume of intraoperative colloid received in mL per kilogram body weight of the patient, showed a weak inverse correlation with full length TFPI, antithrombin, protein C and free protein S (Figure 3.1). A similar relationship was seen for FII, FV, FX, FXIII and fibrinogen (Figures 3.2 and 3.3).

The volume of intravenous crystalloid was inversely correlated with free TFPI and fibrinogen. The effect of colloid is likely to be due to dilution in view of the mild inverse correlation with post-CPB haemoglobin concentration (Figure 3.4A). Less crystalloid appears to have remained in the intravascular space compared to colloid as the volume of crystalloid did not correlate with post-CPB haemoglobin (Figure 3.4B).

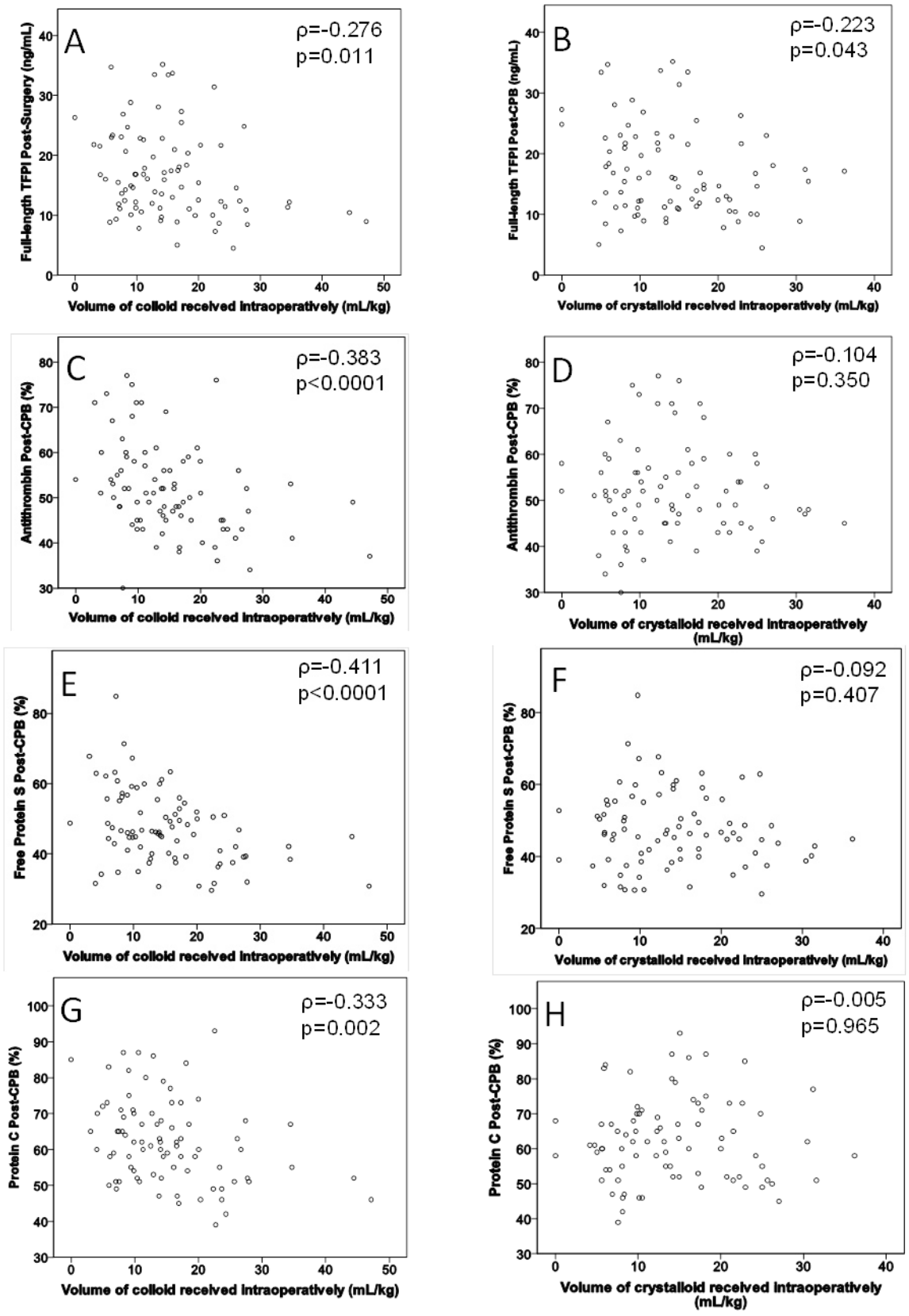


Figure 3.1 Correlation plots between intravenous fluids received intra-operatively and full-length TFPI (A,B), antithrombin (C,D), free protein S (E, F) and protein C (G,H). Spearman's correlation coefficients (ρ) are shown with p- values.

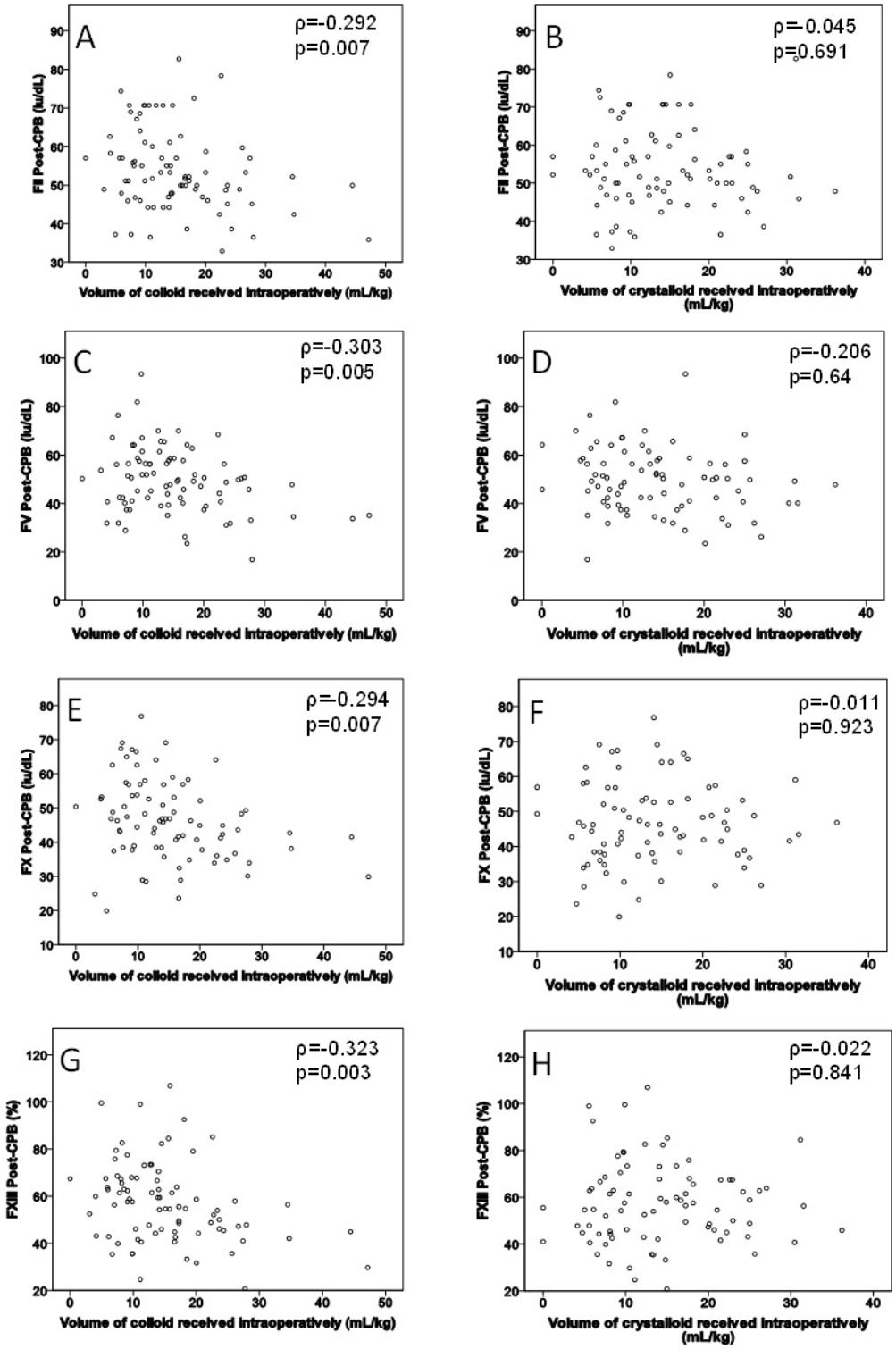


Figure 3.2 Correlation plots between intravenous fluids received intra-operatively and FII (A, B), FV (C, D), FX (E, F) and FXIII (G, H). Spearman's correlation coefficients (ρ) are shown with p-values.

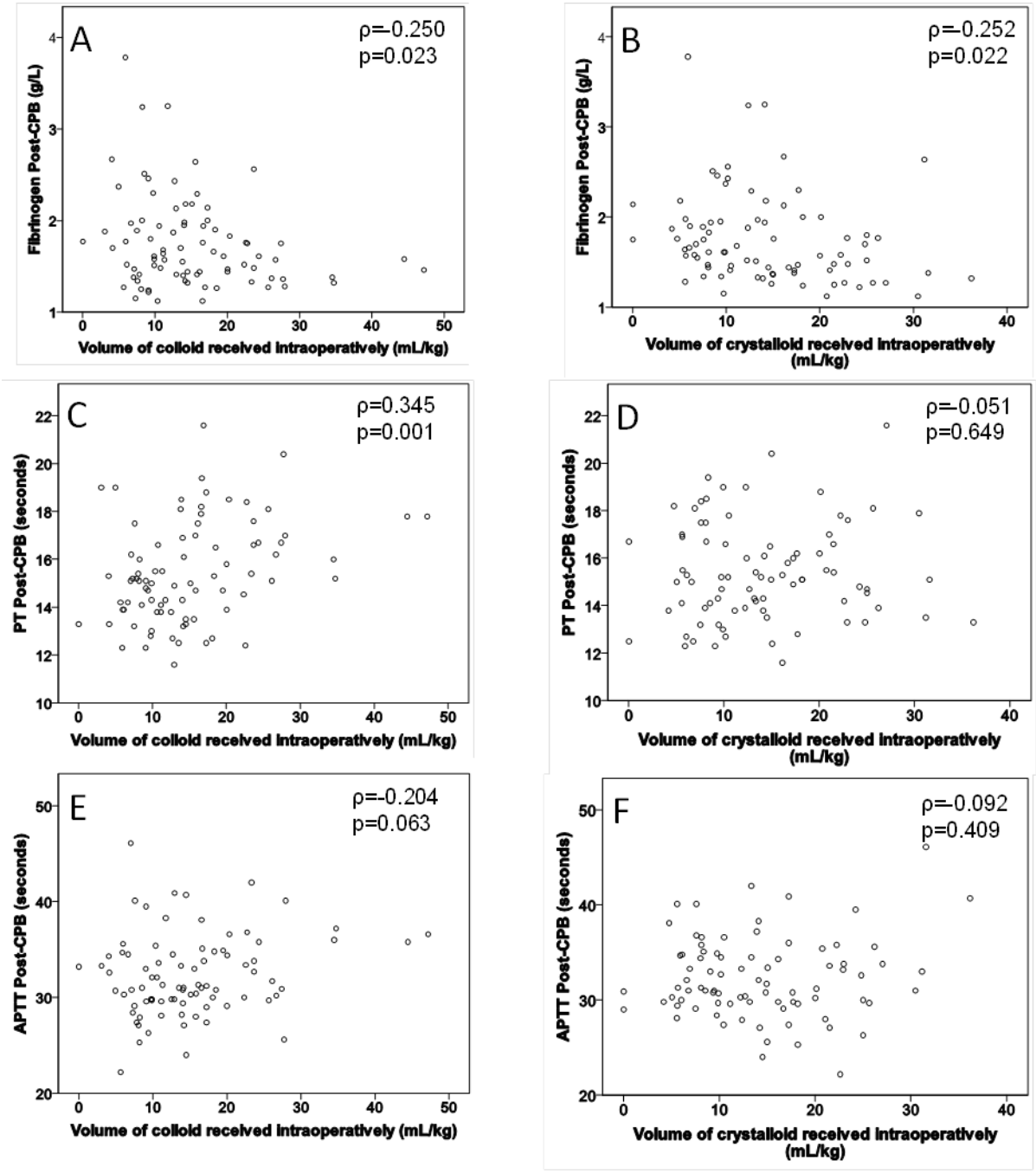


Figure 3.3 Correlation plots between intravenous fluids received intra-operatively and fibrinogen (A, B), PT (C, D) and APTT (E, F). Spearman's correlation coefficients (ρ) are shown with p- values.

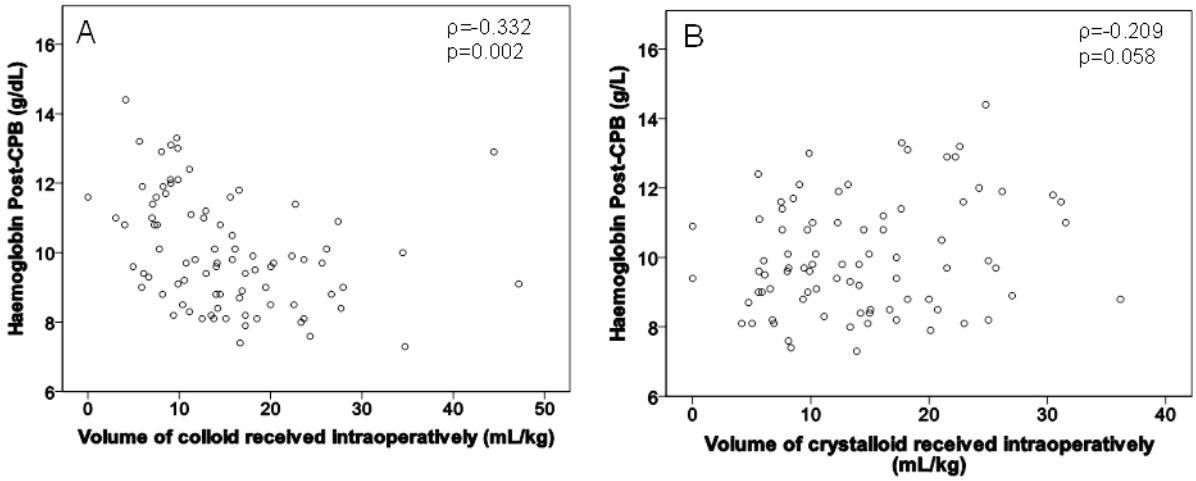


Figure 3.4 Correlation plots between the volume of intraoperative fluids infused and the haemoglobin concentration. Spearman's correlation coefficients (ρ) are shown with p- values.

3.2.4 Thrombin Generation Assays

There was a progressive concentration dependent increase in the ETP, peak thrombin, Velocity Index and concentration dependent shortening of the lag time as the concentration of tissue factor increased in both pre and post-CPB samples when collected into CTI with citrate (ETP summarised in Figure 3.5, peak thrombin Figure 3.6, lag time Figure 3.7 and Velocity Index Figure 3.8). In contrast in pre-CPB samples when using citrate alone these parameters were similar when using triggers using 0.5-5 pmol/L (pM) tissue factor, with a significant increase between 5 and 10 pM. Post-cardiopulmonary bypass there was a tissue factor concentration dependent increase mirroring that seen in CTI. At tissue factor concentrations below 5 pM, thrombin generation parameters were lower in samples anticoagulated with CTI compared to citrate.

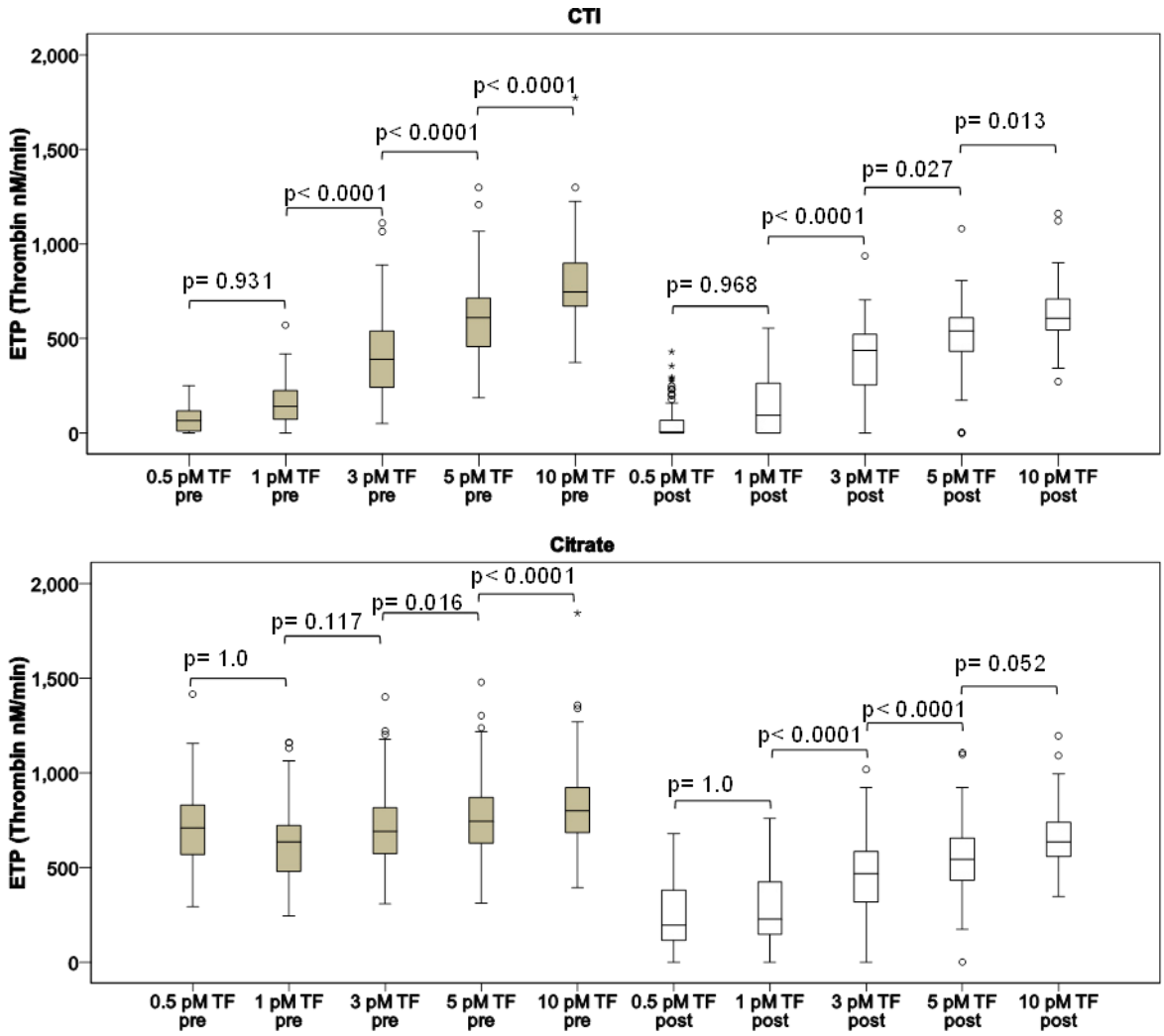


Figure 3.5 Changes for in Endogenous Thrombin Potential (ETP) between pre and post-cardiopulmonary bypass samples using triggers of varying tissue factor concentrations taken into CTI (upper panel)and citrate (lower panel). Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

The ETP was lower post-CPB regardless of anticoagulant used. Samples collected into CTI demonstrated an increase in ETP as the concentration of tissue factor in the trigger solution increased. In contrast, samples taken into citrate showed little difference in the ETP pre-CPB using trigger solutions containing 0.5 to 3 pM tissue factor, with a concentration dependent increase only being observed using tissue factor concentrations of 5 pM and higher. Post-CPB there was an increase in ETP as the concentration of tissue factor in the trigger solution increased as seen with CTI. The median peak thrombin concentration and velocity index,

obtained when using triggers with tissue factor concentrations between 0.5 and 5 pM, was paradoxically no different or slightly higher in post-CPB in samples collected into CTI compared to pre-CPB samples. This may be due to the reduced full-length TFPI concentrations allowing greater activation of the initiation phase of coagulation at low tissue factor concentrations compared to the preoperative samples.

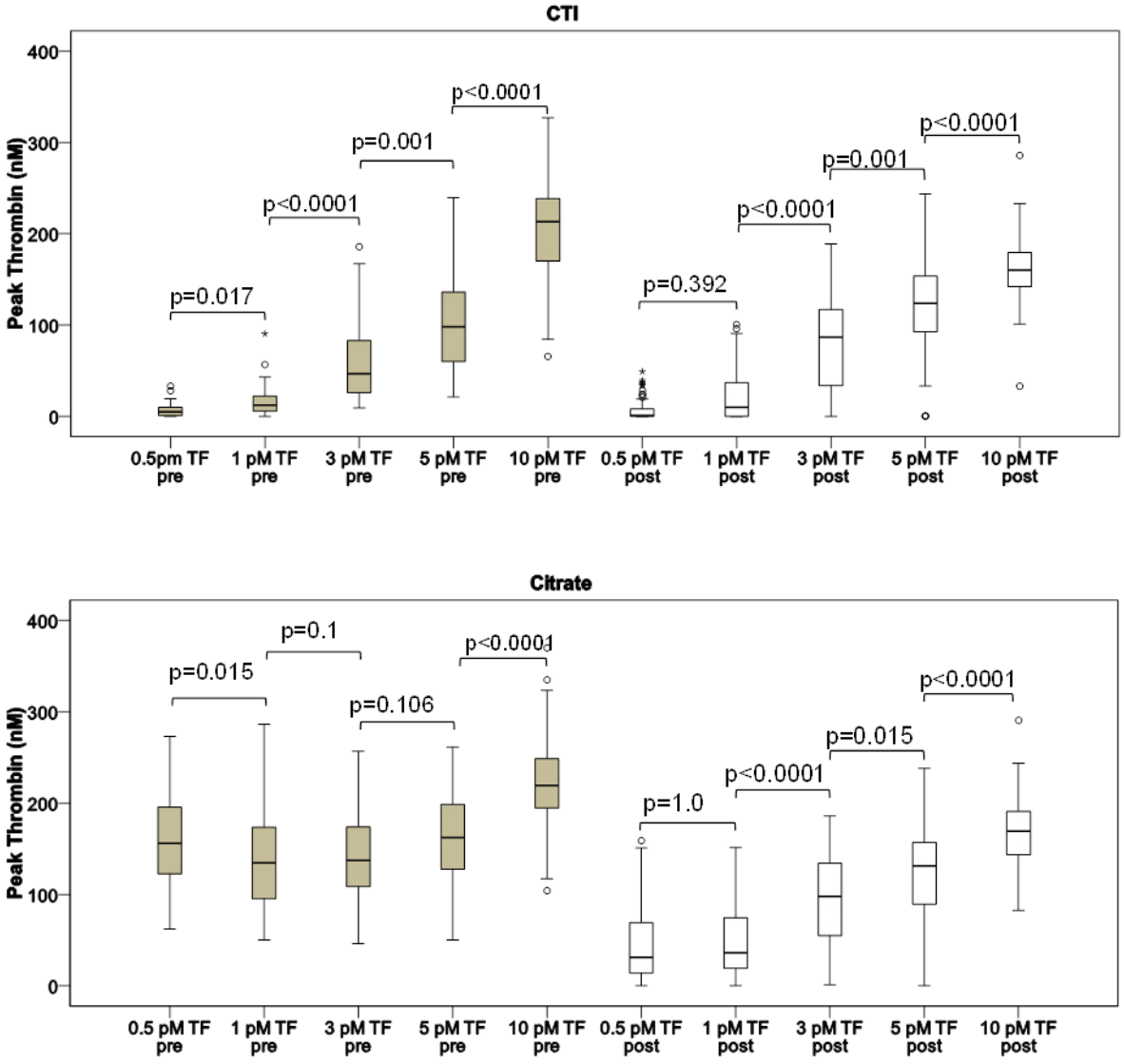


Figure 3.6 Changes for in peak thrombin between pre and post-cardiopulmonary bypass samples using triggers of varying tissue factor concentrations taken into CTI (upper panel) and citrate (lower panel). Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

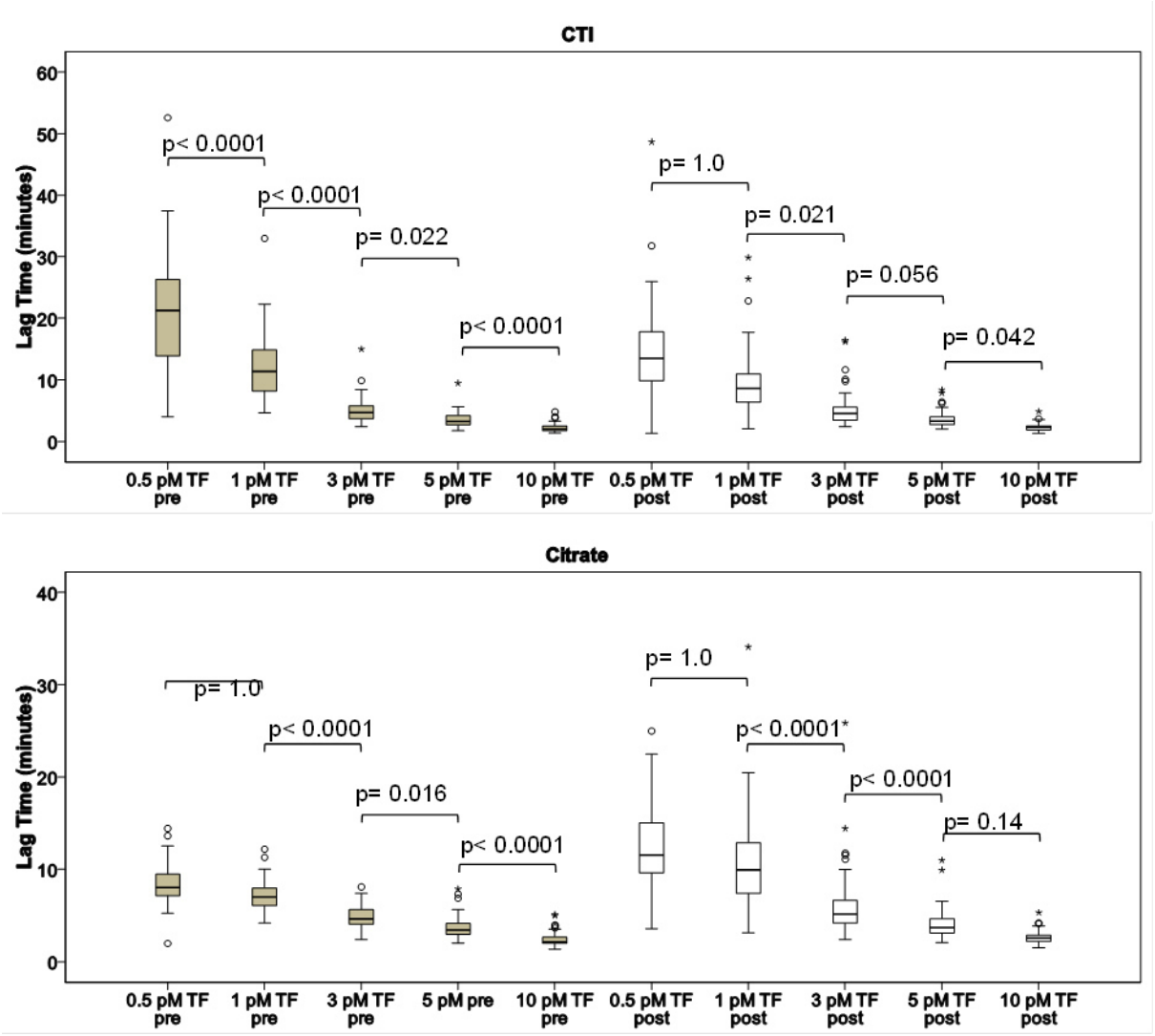


Figure 3.7 Changes for in lag time between pre and post-cardiopulmonary bypass samples using triggers of varying tissue factor concentrations taken into CTI (upper panel) and citrate (lower panel). Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

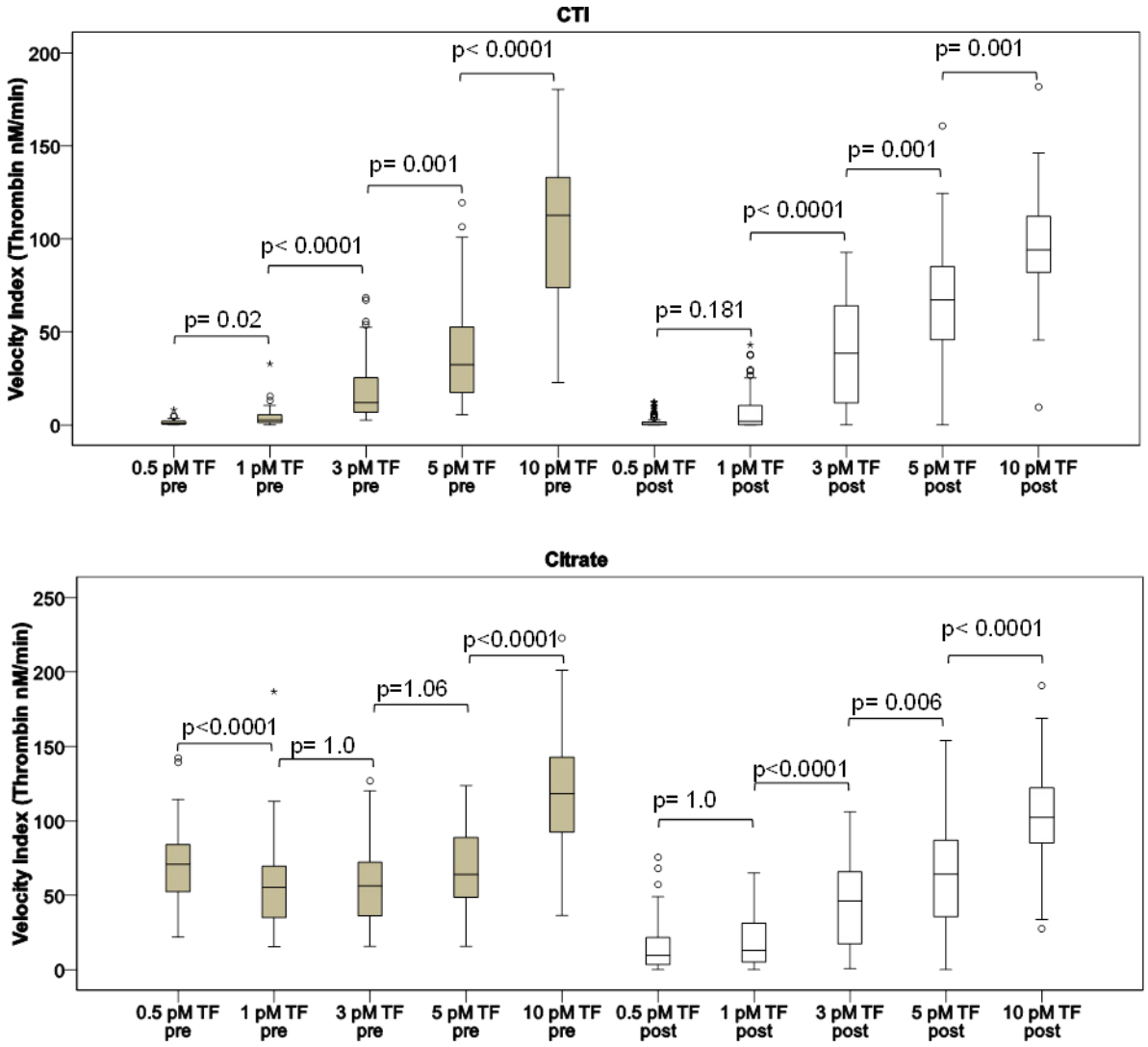


Figure 3.8 Changes for in velocity index between pre and post-cardiopulmonary bypass samples using triggers of varying tissue factor concentrations taken into CTI (upper panel) and citrate (lower panel). Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

3.2.5 Commercial thrombin generation reagents

Commercial thrombin generation trigger solutions consisted of Stago PPP Low and Stago PPP which according to the manufacturer have a tissue factor activity equivalent to 1 pM and 5 pM respectively. Using these reagents there was a significant reduction in the ETP and peak thrombin concentration post-CPB (Figures 3.9A and 3.9B). The lag time was significantly increased using the Stago PPP low reagent, but was only increased using the Stago PPP reagent when thrombin generation was measured in samples collected into CTI (Figure 3.9C). There was no difference in the velocity index using either of the reagents when thrombin generation was measured in samples collected into CTI, but was significantly reduced when measured in samples collected into citrate alone (Figure 3.9D).

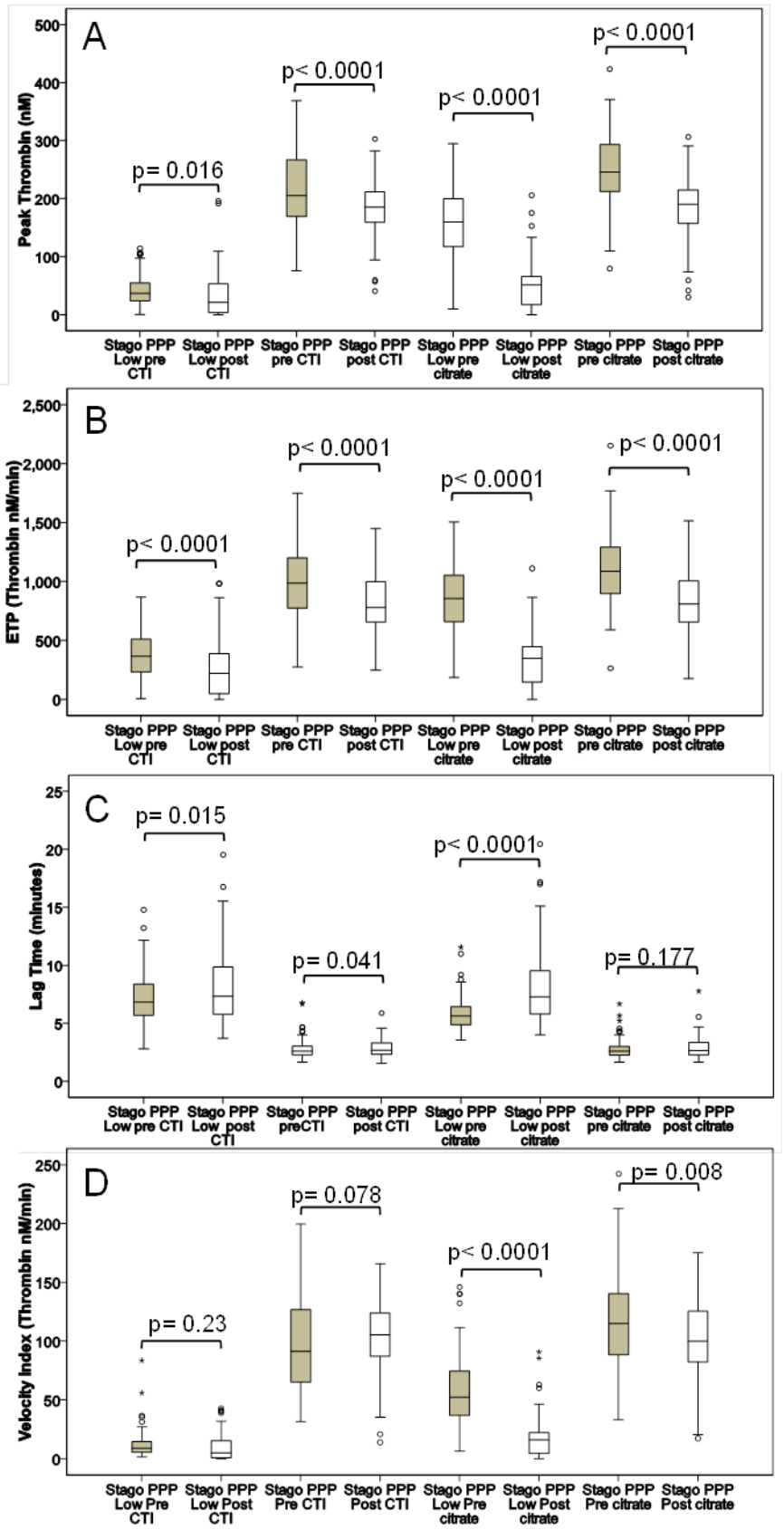


Figure 3.9 Peak thrombin (A), ETP (B), lag time (C) and velocity index (D) measured pre (shaded boxes) and post-CPB in samples taken into citrate containing CTI or citrate alone, using Stago PPP Low or PPP trigger solutions. Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

3.2.6 Near-patient testing

In order to reduce the delay in availability of laboratory results to clinical areas, near patient testing has been shown to be of value. In order to investigate the feasibility of performing calibrated automated thrombography close to the operating theatre, two members of staff were trained in how to perform the assay. A Fluoroscan Reader and desk-top computer with associated Thrombinoscope software were provided on loan by Diagnostica-Stago. A room was identified close to the main operating theatres. The equipment, along with a centrifuge was installed. Ten patients were included in this study. As is shown by Figure 3.10, whilst the ETP using the PPP Low reagent did show a moderate positive correlation, there was no correlation using the PPP reagent and no correlation with either reagent for peak thrombin. The poor correlation demonstrates that calibrated automated thrombography is too complex a technique to use in this setting.

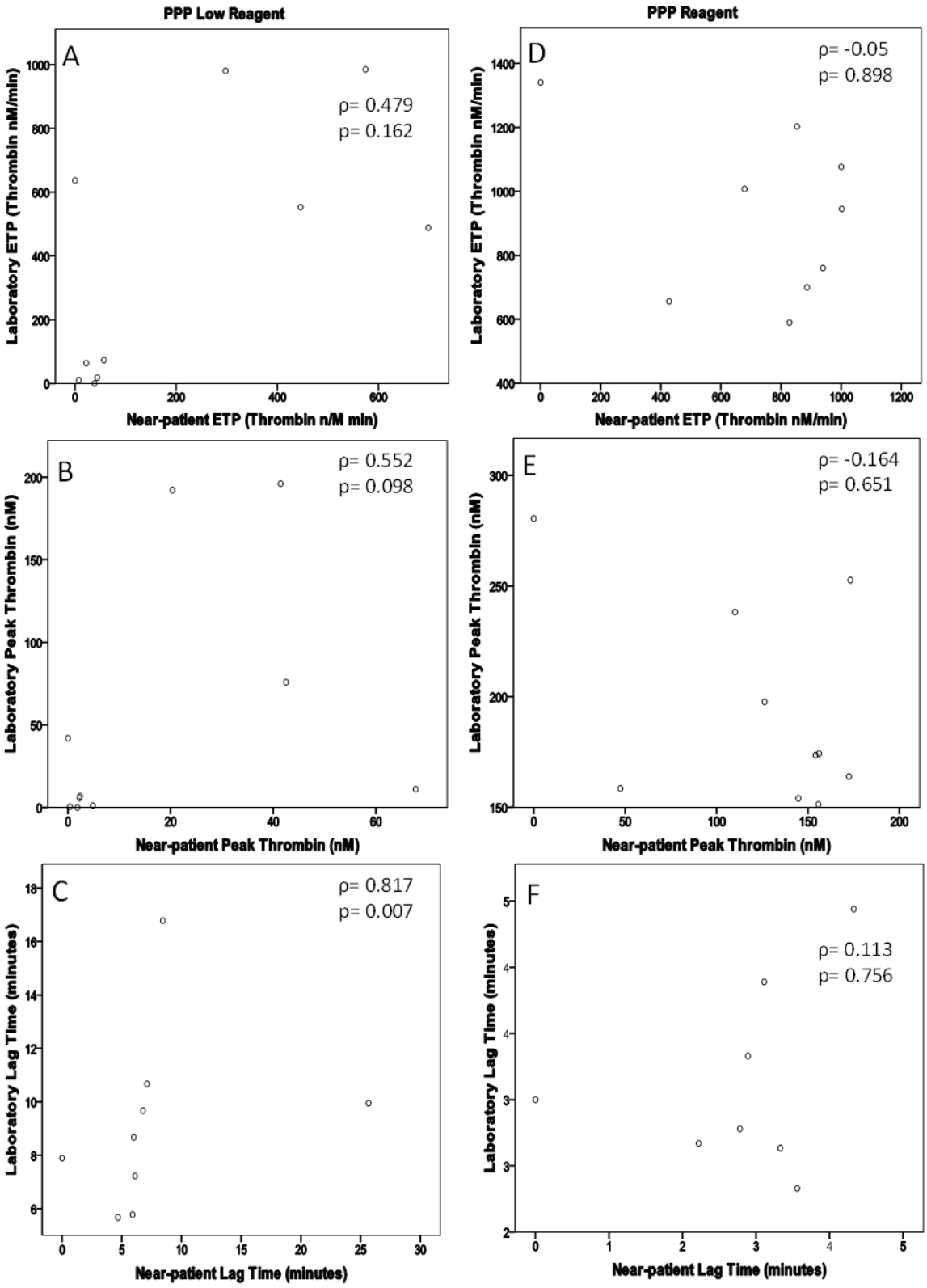


Figure 3.10 Scatter plots comparing thrombin generation parameters obtained from near patient testing with those obatined in the laboratory. Spearman's correlation coefficients (ρ) are shown with p- values.

3.2.7 Association of thrombin generation assays and bleeding end points

The lag time measured in samples taken into CTI in post-CPB samples was shorter in those who lost more than 1 L at 24 hours when using triggers containing 0.5 pM, 1 pM, 5 pM, 10 pM of tissue factor or the Stago PPP solution (Figure 3.11). This suggests the bleeding in these patients may be the result of a persistent low level activation of the coagulation cascade which over time may lead to consumption of coagulation factors leading to bleeding. A ROC analysis was undertaken for the post-CPB lag time. This indicated that there was no clinical utility in identifying patients who bled in excess of 1 L at 24 hours (Table 3.10).

Regardless of anticoagulant or trigger solution used, there was no significant difference in ETP, peak thrombin or velocity index comparing patient who did or did not bleed for any of the bleeding endpoints used in the study. This applied to both pre and post-CPB samples. The results are summarised in tables 3.11 3.15 for ETP, tables 3.16-3.20 for peak thrombin and tables 3.21-3.25 for the velocity index.

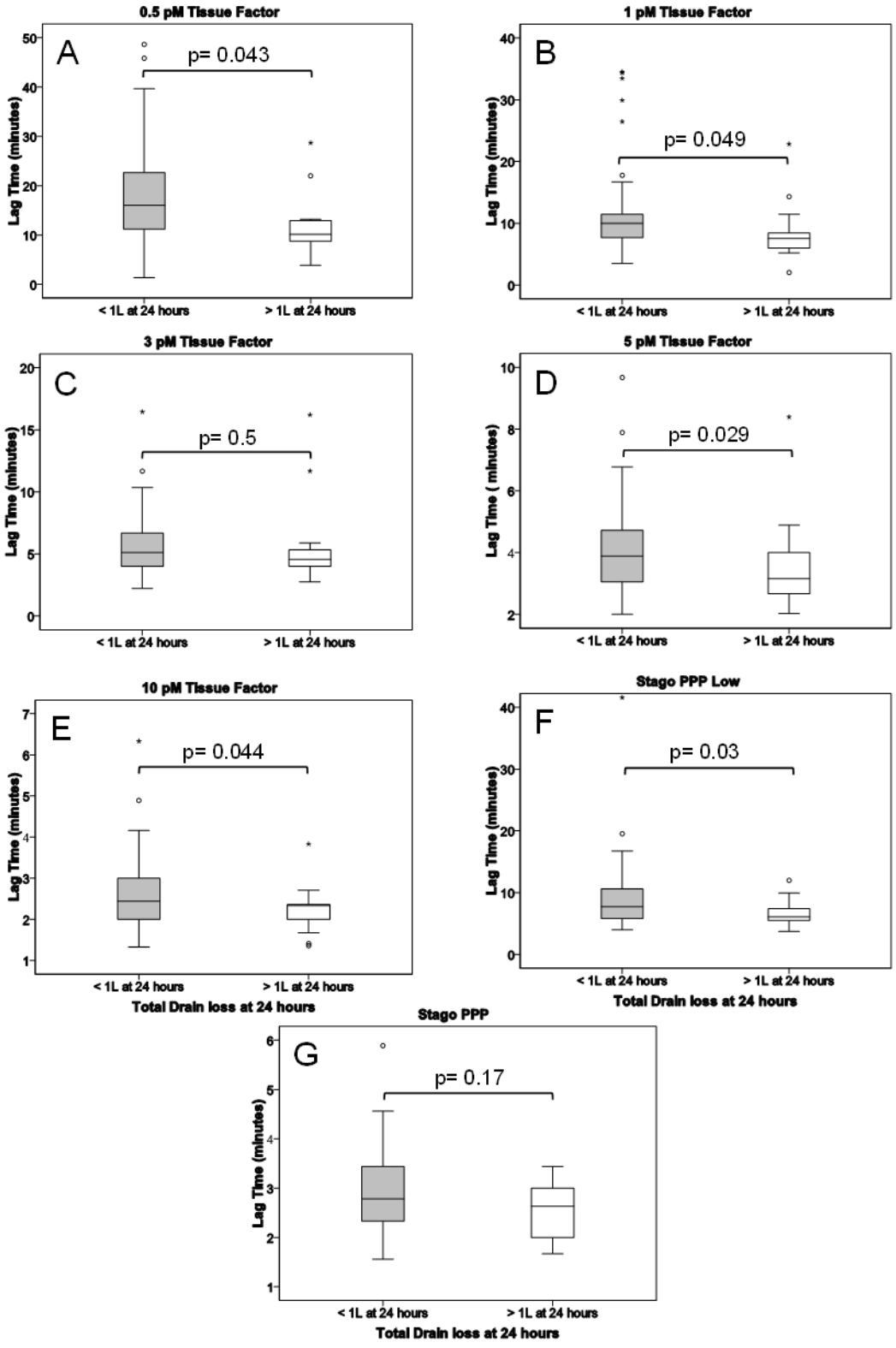


Figure 3.11 Lag time post-CPB in samples collected into CTI in those with a drain blood loss more or less than 1 litre at 24 hours. The lag time was shorter in those who bled more than 1 L (A, B D-G), although no different when a trigger containing 3 pM TF was used (C). Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

Table 3.10 ROC curve values for the post-CPB lag time using different trigger solutions in discriminating those who bled > 1L at 24 hours post-CPB.

Trigger Solution	ROC area post-CPB (95% CI)
0.5 pM	0.33 (0.14-0.52)
1 pM	0.24 (0.09-0.40)
3 pM	0.49 (0.29-0.68)
5 pM	0.33 (0.14-0.53)
10 pM	0.35 (0.17-0.53)
PPP Low	0.35 (0.19-0.52)
PPP	0.41 (0.24-0.58)

Table 3.11 ETP using different trigger solutions before and after cardiopulmonary bypass comparing those who bled less or more than 1L.

End Point	<1L Pre-CPB	<1L Post-CPB	>1L Pre-CPB	>1L Post-CPB
0.5 pM TF CTI	74 0-250 (18-127)	6 0-354 (0-64)	32 0-245 (3-109)	5 0-429 (0-95)
0.5 pM TF Citrate	716 207-1415 (563-853)	209 0-681 (104-388)	672 429-884 (570-754)	197 0-539 (147-291)
1 pM TF CTI	141 0-419 (71-221)	101 0-492 (0-266)	127 0-570 (58-226)	50 0-554 (0-291)
1 pM TF Citrate	642 160-1160 (496-728)	231 0-758 (140-424)	583 380-905 (434-693)	214 0-288 (151-424)
3 pM TF CTI	396 50-1110 (250-524)	409 0-936 (258-538)	359 129-730 (219-620)	471 54-620 (338-523)
3 pM TF Citrate	701 224-1402 (578-844)	454 0-1017 (321-578)	616 402-904 (486-748)	469 38-755 (287-563)
5 pM TF CTI	612 151-1298 (423-721)	530 0-1080 (429-628)	578 341-826 (525-714)	550 247-706 (445-587)
5 pM TF Citrate	755 220-1478 (625-891)	543 0-1108 (431-682)	723 439-915 (634-763)	539 174-765 (437-608)
10 pM TF CTI	746 211-1773 (670-912)	618 271-1160 (548-748)	733 461-930 (662-819)	592 342-764 (522-656)
10 pM TF Citrate	801 232-1843 (677-938)	652 348-1195 (565-763)	784 485-936 (429-829)	614 347-781 (503-651)
PPP Low CTI	637 10-835 (248-503)	162 0-985 (45-383)	264 126-867 (201-628)	221 0-863 (67-390)
PPP Low Citrate	858 184-1508 (716-1056)	346 0-1112 (129-459)	834 426-1217 (578-1011)	347 46-866 (175-415)
PPP CTI	988 276-1751 (806-1194)	799 248-1148 (685-1003)	892 523-1406 (689-1204)	718 410-1120 (626-801)
PPP Citrate	1088 265-2154 (912-1290)	814 177-1517 (663-976)	1026 591-1615 (802-1216)	725 429-1087 (608-943)
PPP+TM CTI	528 157-1282 (392-747)	598 47-1077 (477-796)	593 168-1158 (242-885)	605 271-1025 (492-706)
PPP+TM Citrate	534 0-1648 (434-749)	549 53-942 (408-688)	591 157-1185 (304-881)	537 256-946 (403-651)

Data shown are the median and range (inter-quartile range). Units for ETP are nM/min.

Table 3.12 ETP using different trigger solutions before and after cardiopulmonary bypass comparing those who bled at a rate of more or less than 200 mL/hr.

End point	<200mL/hr Pre-CPB	<200mL/hr Post-CPB	>200mL/hr Pre-CPB	>200mL/hr Post-CPB
0.5 pM TF CTI	74 0-250 (18-127)	6 0-429 (0-76)	18 0-133 (0-105)	2 0-198 (0-95)
0.5 pM TF Citrate	709 207-1415 (568-840)	202 0-681 (91-388)	725 429-879 (666-758)	192 140-464 (156-284)
1 pM TF CTI	142 0-570 (78-225)	101 0-554 (3-281)	67 0-304 (5-183)	22 0-467 (0-196)
1 pM TF Citrate	631 160-1160 (480-721)	231 0-758 (131-433)	641 384-781 (478-722)	214 151-473 (180-414)
3 pM TF CTI	384 50-1110 (249-512)	431 0-936 (258-522)	514 129-720 (207-625)	472 54-620 (430-573)
3 pM TF Citrate	692 224-1402 (573-830)	452 0-1017 (319-578)	684 429-857 (456-798)	473 113-755 (446-563)
5 pM TF CTI	605 151-1298 (445-716)	540 0-1080 (429-616)	672 341-792 (525-714)	546 351-706 (450-587)
5 pM TF Citrate	744 220-1478 (625-883)	543 0-1108 (429-659)	746 439-871 (691-813)	539 361-765 (468-608)
10 pM TF CTI	746 211-1773 (665-909)	614 271-1160 (548-729)	734 461-860 (701-819)	588 397-764 (522-663)
10 pM TF Citrate	801 232-1843 (677-930)	639 347-1195 (560-747)	777 485-865 (735-825)	608 394-781 (503-673)
PPP Low CTI	383 10-835 (241-513)	223 0-985 (54-388)	252 159-867 (201-476)	217 0-489 (10-307)
PPP Low Citrate	864 184-1508 (685-1048)	346 0-1112 (132-459)	823 556-1217 (632-941)	336 70-546 (242-415)
PPP CTI	1002 276-1751 (784-1204)	788 248-1448 (670-1003)	867 625-1406 (689-1026)	718 538-996 (656-801)
PPP Citrate	1088 265-2154 (905-1290)	814 177-1517 (655-976)	961 679-1468 (802-1130)	725 498-1062 (655-912)
PPP+TM CTI	530 157-1282 (386-764)	599 47-1077 (477-796)	490 168-1158 (242-869)	584 278-760 (497-706)
PPP+TM Citrate	535 0-1648 (408-686)	549 53-946 (655-976)	547 243-1185 (802-1130)	519 281-903 (655-912)

Data shown are the median and range (inter-quartile range). Units for ETP are nM/min.

Table 3.13 ETP using different trigger solutions before and after cardiopulmonary bypass in those who bled more or less than 2 mL/kg/hr for 2 consecutive hours.

End point	<2mL/kg/hr for 2 hours Pre-CPB	<2mL/kg/hr for 2 hours Post-CPB	>2mL/kg/hr for 2 hours Pre-CPB	>2mL/kg/hr for 2 hours Post-CPB
0.5 pM TF CTI	69 0-250 (17-116)	6 0-429 (0-67)	32 0-245 (4-124)	5 0-248 (0-90)
0.5 pM TF Citrate	706 207-1415 (559-851)	213 0-680 (115-395)	720 429-884 (660-775)	185 7-464 (143-272)
1 pM TF CTI	138 0-419 (71-217)	99 0-554 (0-278)	154 0-570 (54-283)	53 0-467 (4-277)
1 pM TF Citrate	628 160-1160 (481-724)	236 0-758 (148-453)	642 384-905 (468-710)	199 5-473 (154-338)
3 pM TF CTI	387 50-1110 (235-508)	420 0-936 (262-523)	728 129-730 (264-622)	468 54-625 (339-548)
3 pM TF Citrate	692 224-1402 (270-843)	466 0-1017 (322-585)	692 429-904 (579-793)	467 38-755 (219-535)
5 pM TF CTI	607 151-1298 (414-719)	543 0-1080 (431-623)	657 341-826 (527-694)	530 247-706 (448-581)
5 pM TF Citrate	745 220-1478 (619-890)	554 0-1108 (431-623)	728 439-915 (683-821)	519 174-765 (448-581)
10 pM TF CTI	745 211-1773 (658-911)	611 271-1160 (544-748)	748 461-930 (704-859)	595 342-764 (541-672)
10 pM TF Citrate	797 232-1843 (671-931)	635 348-1195 (561-750)	811 485-936 (754-844)	608 347-781 (524-675)
PPP Low CTI	366 10-835 (235-510)	225 0-985 (59-387)	267 159-867 (217-554)	215 0-624 (25-349)
PPP Low Citrate	861 184-1508 (660-1053)	350 0-1112 (147-469)	832 556-1217 (696-97)	313 0-805 (153-410)
PPP CTI	995 276-1751 (777-1204)	789 248-1448 (685-1005)	517 168-1158 (765-1113)	717 410-1066 (640-891)
PPP Citrate	1098 265-2154 (899-1293)	818 177-1517 (660-1013)	996 678-1468 (849-1167)	696 429-1062 (622-906)
PPP+TM CTI	529 157-1282 (381-762)	606 47-1077 (492-805)	517 168-1158 (320-877)	577 271-865 (407-840)
PPP+TM Citrate	535 0-1648 (426-758)	554 53-946 (413-687)	582 243-1185 (407-840)	503 198-903 (345-628)

Data shown are the median and range (inter-quartile range). Units for ETP are nM/min.

Table 3.14 ETP using different trigger solutions before and after cardiopulmonary bypass in those who did and did not require haemostatic treatment.

End point	No Haemostatic Treatment Pre-CPB	No Haemostatic Treatment Post-CPB	Haemostatic Treatment Pre-CPB	Haemostatic Treatment Post-CPB
0.5 pM TF CTI	74 0-250 (19-116)	6 0-429 (0-53)	64 0-245 (6-118)	5 0-286 (0-96)
0.5 pM TF Citrate	703 354-1415 (552-870)	195 0-676 (119-418)	715 207-1097 (614-784)	200 0-681 (110-319)
1 pM TF CTI	141 0-417 (94-213)	126 0-554 (0-250)	128 0-570 (61-234)	91 0-467 (0-296)
1 pM TF Citrate	647 252-1160 (479-745)	241 0-758 (168-454)	632 160-1129 (489-700)	213 0-575 (107-405)
3 pM TF CTI	389 50-1066 (228-483)	409 0-936 (205-510)	394 116-1110 (274-599)	455 17-704 (343-548)
3 pM TF Citrate	691 308-1402 (574-858)	448 0-924 (296-571)	693 224-1222 (567-789)	473 38-1017 (329-576)
5 pM TF CTI	607 186-1207 (456-696)	562 0-1080 (426-651)	590 151-1298 (467-717)	523 173-786 (444-584)
5 pM TF Citrate	745 322-1478 (619-913)	589 0-1108 (443-697)	737 220-1302 (644-841)	511 174-1097 (431-599)
10 pM TF CTI	781 384-1773 (654-911)	602 271-1160 (552-749)	737 211-1298 (681-875)	615 342-1121 (530-679)
10 pM TF Citrate	791 407-1843 (671-930)	609 353-1195 (558-747)	800 232-1339 (728-892)	639 347-1091 (557-691)
PPP Low CTI	637 21-835 (248-495)	192 0-985 (59-390)	352 10-867 (223-516)	221 0-624 (45-380)
PPP Low Citrate	864 275-1508 (722-1030)	346 0-1112 (133-469)	845 184-1334 (637-1048)	374 0-805 (150-432)
PPP CTI	988 610-1751 (768-1208)	817 248-1148 (685-1005)	927 276-1673 (796-1183)	758 410-1237 (658-930)
PPP Citrate	1159 720-2154 (899-1323)	824 177-1517 (657-1014)	1071 265-1729 (849-1206)	762 215-1190 (651-932)
PPP+TM CTI	530 157-1282 (401-762)	599 47-1077 (513-853)	515 168-1158 (356-781)	585 271-916 (461-711)
PPP+TM Citrate	537 0-1648 (462-817)	574 53-946 (413-710)	532 157-1185 (366-749)	514 198-903 (391-653)

Data shown are the median and range (inter-quartile range). Units for ETP are nM/min.

Table 3.15 ETP using different trigger solutions before and after cardiopulmonary bypass in those who did and did not undergo re-operation due to bleeding.

End point	No Re-op Pre-CPB	No Re-op Post-CPB	Re-op Pre-CPB	Re-op Post-CPB
0.5 pM TF CTI	75 0-250 (15-120)	6 0-429 (0-67)	22 0-245 (2-50)	5 0-198 (2-136)
0.5 pM TF Citrate	701 207-1415 (566-819)	195 0-681 (115-380)	796 429-884 (715-855)	242 15-464 (169-326)
1 pM TF CTI	142 0-419 (71-226)	99 0-554 (0-278)	72 0-570 (31-137)	18 0-467 (4-252)
1 pM TF Citrate	616 160-1160 (478-717)	231 0-758 (148-429)	711 384-905 (647-751)	228 5-473 (168-389)
3 pM TF CTI	393 50-1110 (242-531)	436 0-936 (262-523)	274 129-730 (232-672)	475 54-620 (277-557)
3 pM TF Citrate	691 224-1402 (573-798)	461 0-1017 (321-571)	778 452-904 (516-855)	475 38-755 (280-570)
5 pM TF CTI	611 151-1298 (456-714)	541 0-1080 (431-623)	547 341-826 (469-722)	524 323-706 (448-574)
5 pM TF Citrate	742 220-1478 (621-872)	544 0-1108 (429-656)	828 439-915 (701-861)	523 174-765 (453-591)
10 pM TF CTI	746 211-1773 (662-905)	604 271-1160 (544-721)	708 467-930 (686-839)	656 467-764 (567-686)
10 pM TF Citrate	797 232-1843 (673-924)	619 347-1195 (544-741)	800 498-936 (751-860)	660 503-858 (624-729)
PPP Low CTI	366 10-867 (235-516)	221 0-985 (49-379)	267 159-628 (189-449)	215 0-491 (50-440)
PPP Low Citrate	856 184-1508 (660-1053)	344 0-1112 (133-449)	836 561-1090 (732-976)	404 46-486 (300-437)
PPP CTI	987 276-1751 (777-1208)	789 248-1448 (659-1001)	916 626-1064 (779-1031)	700 507-996 (658-739)
PPP Citrate	1087 265-2154 (898-1294)	818 177-1517 (657-1007)	1076 782-1205 (874-1113)	721 498-1032 (632-827)
PPP+TM CTI	523 157-1282 (381-767)	598 47-1077 (492-788)	607 201-885 (380-811)	655 278-760 (401-711)
PPP+TM Citrate	533 0-1648 (426-758)	543 53-946 (409-685)	665 243-883 (469-811)	520 281-775 (345-670)

Data shown are the median and range (inter-quartile range). Units for ETP are nM/min.

Table 3.16 Peak thrombin concentration using different trigger solutions before and after cardiopulmonary bypass comparing those who bled less or more than 1L.

End point	<1L Pre-CPB	<1L Post-CPB	>1L Pre-CPB	>1L Post-CPB
0.5 pM TF CTI	5 0-19 (1-10)	0.5 0-39 (0-9)	2 0-33 (0-10)	0.5 0-49 (0-8)
0.5 pM TF Citrate	161 62-273 (121-198)	35 0-159 (11-70)	152 96-260 (139-188)	30 1-104 (20-50)
1 pM TF CTI	12 0-57 (6-22)	12 0-100 (0-36)	12 0-90 (4-22)	4 0-96 (0-51)
1 pM TF Citrate	138 45-259 (96-185)	36 0-151 (18-75)	128 57-286 (94-147)	39 0-106 (20-74)
3 pM TF CTI	46 9-186 26-84)	81 0-189 (30-118)	42 12-161 (23-87)	97 4-132 (71-117)
3 pM TF Citrate	142 46-243 (114-174)	97 1-178 (50-130)	118 60-257 (90-174)	101 3-186 (67-118)
5 pM TF CTI	95 21-240 (50-136)	121 0-244 (92-152)	102 50-213 (71-141)	136 41-168 (93-156)
5 pM TF Citrate	163 50-261 (128-214)	130 0-238 (89-156)	153 101-248 (131-186)	136 22-191 (99-157)
10 pM TF CTI	217 59-327 (170-244)	161 33-286 (143-182)	190 126-299 (159-221)	150 107-200 (142-171)
10 pM TF Citrate	224 74-370 (197-251)	174 82-291 (143-193)	210 143-318 (185-233)	152 106-213 (144-176)
PPP Low CTI	36 1-115 (25-51)	20 0-196 (3-55)	30 13-104 (20-82)	23 0-110 (4-41)
PPP Low Citrate	161 34-294 (117-202)	52 0-206 (15-72)	142 10-295 (111-189)	51 3-133 (23-60)
PPP CTI	212 76-369 (170-266)	189 41-303 (161-212)	192 120-330 (145-276)	168 94-249 (151-207)
PPP Citrate	246 80-424 (214-294)	195 30-307 (158-214)	253 142-344 (195-293)	169 97-255 (138-216)
PPP+TM CTI	140 47-333 (107-193)	146 12-243 (116-178)	151 51-300 (73-253)	143 67-213 (119-153)
PPP+TM Citrate	149 47-348 (114-198)	127 11-220 (82-163)	155 56-299 (100-230)	118 69-195 (84-143)

Data shown are the median and range (inter-quartile range). Units for peak thrombin concentration are nM.

Table 3.17 Peak thrombin concentration using different trigger solutions before and after cardiopulmonary bypass comparing those who bled at a rate of more or less than 200 mL/hr.

End point	<200mL/hr Pre-CPB	<200mL/hr Post-CPB	>200mL/hr Pre	>200mL/hr Post
0.5 pM TF CTI	5 0-28 (1-10)	0.5 0-49 (0-9)	1 0-33 (0-10)	0 0-12 (0-8)
0.5 pM TF Citrate	156 62-273 (121-198)	32 0-159 (10-70)	156 109-196 (150-188)	29 20-79 (23-50)
1 pM TF CTI	13 0-90 (6-22)	12 0-100 (0-37)	4 0-39 (4-16)	1 0-62 (0-30)
1 pM TF Citrate	136 45-286 (93-180)	36 0-151 (17-79)	132 84-177 (108-153)	39 20-92 (21-73)
3 pM TF CTI	43 9-186 (26-79)	83 0-189 (30-118)	72 12-140 (23-91)	97 4-131 (71-117)
3 pM TF Citrate	137 46-257 (99-173)	96 1-178 (50-129)	142 64-209 (110-174)	106 16-186 (71-118)
5 pM TF CTI	94 21-240 (55-136)	123 0-244 (92-155)	121 50-197 (71-154)	135 76-168 (93-146)
5 pM TF Citrate	162 50-261 (125-208)	130 0-238 (89-157)	173 104-223 (144-194)	136 80-191 (99-147)
10 pM TF CTI	216 59-327 (166-240)	160 33-256 (144-180)	190 126-269 (153-211)	144 111-200 (125-172)
10 pM TF Citrate	221 74-370 (194-249)	172 82-291 (143-191)	210 143-263 (186-228)	151 117-213 (130-176)
PPP Low CTI	38 1-115 (25-55)	21 0-196 (4-60)	26 14-104 (19-59)	16 0-57 (1-39)
PPP Low Citrate	160 34-295 (114-201)	52 0-206 (16-72)	164 10-217 (134-189)	47 7-76 (23-56)
PPP CTI	212 76-369 (169-269)	188 41-303 (159-212)	188 120-310 (145-246)	166 137-225 (151-174)
PPP Citrate	249 80-424 (212-294)	194 30-307 (158-215)	232 162-329 (211-272)	164 131-235 (138-206)
PPP+TM CTI	144 47-333 (107-196)	146 12-243 (116-176)	137 51-287 (70-199)	132 67-157 (119-150)
PPP+TM Citrate	149 47-348 (111-211)	127 11-220 (82-162)	155 74-299 (106-218)	115 69-195 (84-136)

Data shown are the median and range (inter-quartile range). Units for peak thrombin concentration are nM.

Table 3.18 Peak thrombin concentration using different trigger solutions before and after cardiopulmonary bypass in those who bled more or less than 2 mL/kg/hr for 2 consecutive hours.

End point	<2mL/kg/hr for 2 hours Pre-CPB	<2mL/kg/hr for 2 hours Post-CPB	>2mL/kg/hr for 2 hours Pre-CPB	>2mL/kg/hr for 2 hours Post-CPB
0.5 pM TF CTI	5 0-19 (1-9)	0.5 0-49 (0-7)	7 0-33 (0.3-11)	1 0-24 (0-9)
0.5 pM TF Citrate	154 62-273 (121-198)	39 0-159 (13-70)	159 96-260 (147-185)	28 0-79 (20-46)
1 pM TF CTI	12 0-57 (6-21)	10 0-100 (0-37)	13 0-90 (4-23)	3 0-62 (0.3-35)
1 pM TF Citrate	135 45-259 (95-180)	38 0-151 (19-80)	134 66-286 (106-159)	39 1-92 (20-53)
3 pM TF CTI	45 9-186 (25-74)	84 0-189 (33-118)	57 12-160 (29-89)	96 4-149 (52-112)
3 pM TF Citrate	133 46-243 (99-173)	98 1-178 (113-169)	142 67-257 (56-129)	98 3-186 (40-112)
5 pM TF CTI	95 21-240 (53-136)	123 0-244 (102-155)	116 50-213 (40-112)	132 41-168 (78-146)
5 pM TF Citrate	163 50-261 (125-208)	131 0-238 (97-157)	161 101-248 (141-184)	129 22-191 (83-145)
10 pM TF CTI	215 59-327 (164-238)	162 33-286 (145-180)	190 126-299 (167-238)	145 107-200 (134-168)
10 pM TF Citrate	218 74-370 (194-246)	174 82-291 (144-191)	219 143-318 (194-255)	151 206-213 (137-168)
PPP Low CTI	36 1-115 (25-52)	21 0-196 (5-55)	33 14-104 (20-71)	11 0-88 (2-40)
PPP Low Citrate	160 34-294 (114-201)	53 0-206 (18-72)	144 10-295 (133-188)	45 1-153 13-58)
PPP CTI	206 76-369 (169-266)	189 41-303 (164-212)	195 120-330 (164-282)	159 94-225 (139-173)
PPP Citrate	246 80-424 (212-293)	196 30-307 (161-215)	258 162-344 (214-292)	159 80-235 (134-191)
PPP+TM CTI	139 47-333 (107-193)	147 12-243 (118-176)	158 51-300 (99-224)	128 45-202 (86-151)
PPP+TM Citrate	148 47-348 (111-198)	128 11-220 (91-162)	157 74-299 (126-243)	114 21-195 (77-141)

Data shown are the median and range (inter-quartile range). Units for peak thrombin concentration are nM.

Table 3.19 Peak thrombin concentration using different trigger solutions before and after cardiopulmonary bypass in those who did and did not require haemostatic treatment.

End point	No Haemostatic Treatment Pre-CPB	No Haemostatic Treatment Post-CPB	Haemostatic Treatment Pre-CPB	Haemostatic Treatment Post-CPB
0.5 pM TF CTI	5 0-18 (1-10)	0.4 0-49 (0-3)	4 0-33 (0.4-10)	0.6 0-35 (0-10)
0.5 pM TF Citrate	156 65-273 (116-199)	30 0-159 (14-70)	156 62-260 (131-193)	32 0-151 (15-50)
1 pM TF CTI	14 0-40 (9-21)	12 0-100 (0-33)	11 0-90 (4-24)	9 0-77 (0-40)
1 pM TF Citrate	138 51-259 (90-195)	39 0-151 (24-79)	131 45-286 (98-153)	35 0-114 (12-74)
3 pM TF CTI	46 9-186 (25-67)	75 0-189 (19-116)	45 12-160 (26-92)	97 1-153 (53-118)
3 pM TF Citrate	132 46-243 (114-171)	94 1-178 (47-122)	144 64-257 (105-175)	107 3-186 (64-135)
5 pM TF CTI	98 21-240 (52-135)	126 0-244 (87-151)	96 25-225 (60-147)	123 41-218 (94-157)
5 pM TF Citrate	166 50-261 (123-216)	133 0-238 (105-157)	160 65-256 (132-185)	127 22-228 (85-152)
10 pM TF CTI	216 93-327 (171-238)	161 33-286 (138-180)	200 59-300 (160-240)	159 107-233 (142-180)
10 pM TF Citrate	228 117-370 (195-247)	169 82-291 (141-191)	217 74-318 (189-249)	164 106-244 (147-190)
PPP Low CTI	36 9-107 (26-53)	20 0-196 (5-56)	34 0.6-115 (20-57)	23 0-95 (3-48)
PPP Low Citrate	159 34-294 (124-200)	53 0-206 (13-70)	164 10-295 (113-201)	50 0.6-176 (17-66)
PPP CTI	212 101-369 (171-267)	193 41-303 (159-212)	198 76-330 (164-268)	180 94-276 (157-212)
PPP Citrate	245 110-424 (211-294)	194 41-307 (148-213)	254 80-347 (213-290)	189 30-281 (159-216)
PPP+TM CTI	140 47-333 (109-200)	146 12-243 (117-181)	147 51-300 (99-193)	144 45-239 (117-162)
PPP+TM Citrate	149 47-348 (116-225)	127 11-220 (85-163)	155 56-299 (107-197)	122 21-195 (81-146)

Data shown are the median and range (inter-quartile range). Units for peak thrombin concentration are nM.

Table 3.20 Peak thrombin concentration using different trigger solutions before and after cardiopulmonary bypass in those who did and did not undergo re-operation due to bleeding.

End point	No Re-op Pre-CPB	No Re-op Post-CPB	Re-op Pre-CPB	Re-op Post-CPB
0.5 pM TF CTI	5 0-19 (1-10)	0.5 0-49 (0-8)	1 0-33 (0-16)	0.6 0-24 (0-10)
0.5 pM TF Citrate	154 62-273 (121-193)	31 0-159 (13-69)	193 109-260 (171-197)	39 1-79 (21-46)
1 pM TF CTI	13 0-57 (6-22)	10 0-100 (0-37)	5 0-90 (4-10)	1 0-62 (0-39)
1 pM TF Citrate	133 45-259 (93-175)	38 0-151 (19-79)	137 105-286 (133-161)	39 1-74 (22-67)
3 pM TF CTI	46 9-186 (26-81)	87 0-189 (36-119)	36 12-160 (18-101)	94 4-117 (40-110)
3 pM TF Citrate	133 46-243 (106-169)	98 1-178 (56-129)	164 64-257 (113-192)	98 3-186 (43-112)
5 pM TF CTI	96 21-240 (58-136)	128 0-244 (98-155)	98 44-213 (60-152)	114 41-168 (78-130)
5 pM TF Citrate	162 50-261 (127-198)	131 0-238 (97-157)	174 104-248 (128-203)	110 22-191 (83-138)
10 pM TF CTI	215 59-327 (170-238)	160 33-286 (144-179)	200 126-299 (132-231)	142 121-200 (127-172)
10 pM TF Citrate	218 74-370 (194-248)	174 82-291 (144-191)	220 143-318 (198-243)	151 21-213 (132-157)
PPP Low CTI	36 0.6-115 (25-55)	21 0-196 (4-53)	20 14-98 (17-49)	11 0-91 (4-39)
PPP Low Citrate	159 34-294 (118-198)	51 0-206 (17-69)	189 10-295 (127-237)	50 3-95 (25-56)
PPP CTI	206 76-369 (169-269)	187 41-303 (161-212)	195 120-330 (160-239)	156 94-225 (147-165)
PPP Citrate	246 80-424 (212-293)	194 30-307 (158-215)	242 162-344 (215-285)	159 97-235 (138-171)
PPP+TM CTI	139 47-333 (107-196)	146 12-243 (118-175)	158 56-300 (107-195)	145 67-157 (86-152)
PPP+TM Citrate	148 47-348 (111-198)	126 11-220 (88-162)	203 74-299 (131-223)	115 69-148 (77-141)

Data shown are the median and range (inter-quartile range). Units for peak thrombin concentration are nM.

Table 3.21 Velocity index using different trigger solutions before and after CPB comparing those who bled less or more than 1L.

End point	<1L Pre-CPB	<1L Post-CPB	>1L Pre-CPB	>1L Post-CPB
0.5 pM TF CTI	1 0-5 (0.3-2)	0.3 0-12 (0-2)	0.5 0-8 (0.2-1.5)	0 0-12 (0-1.3)
0.5 pM TF Citrate	71 22-139 (50-88)	9 0-75 (3-23)	76 32-142 (56-81)	11 0-43 (6-16)
1 pM TF CTI	2.3 0-15 (1-5)	2.4 0-43 (0-10)	2.7 0-33 (0.5-4)	0.7 0-38 (0-19)
1 pM TF Citrate	56 15-113 (34-76)	13 0-65 (5-31)	54 16-187 (36-63)	14 0-48 (5-28)
3 pM TF CTI	12 2-67 (7-25)	31 0-93 (9-65)	15 3-68 (7-27)	46 1-78 (21-58)
3 pM TF Citrate	56 16-120 (38-72)	46 2-106 (17-67)	50 16-127 (30-72)	46 1-93 (28-56)
5 pM TF CTI	32 5-119 (16-50)	64 0-161 (43-80)	42 15-106 (22-61)	80 11-96 (56-91)
5 pM TF Citrate	64 15-122 (47-90)	62 0-154 (34-85)	66 31-124 (49-79)	71 6-104 (56-89)
10 pM TF CTI	116 23-180 (78-134)	94 9-182 (82-113)	95 44-179 (73-133)	95 54-130 (85-104)
10 pM TF Citrate	121 36-201 (99-143)	102 27-191 (84-121)	108 64-223 (90-134)	103 52-132 (88-124)
PPP Low CTI	7 2-56 (6-14)	5 0-43 (1-17)	10 3-84 (5-19)	7 0-32 (1-13)
PPP Low Citrate	52 8-140 (37-81)	16 0-91 (4-22)	53 7-146 (37-68)	16 0-46 (8-18)
PPP CTI	91 32-188 (64-125)	106 14-166 (89-127)	91 42-200 (71-127)	99 35-140 (80-115)
PPP Citrate	113 33-213 (90-140)	100 17-175 (84-126)	120 45-242 (88-144)	100 41-145 (78-116)
PPP+TM CTI	69 22-169 (53-100)	81 3-138 (56-100)	76 24-209 (58-123)	71 29-121 (54-92)
PPP+TM Citrate	92 33-189 (67-125)	63 4-117 (39-91)	118 26-242 (57-125)	64 33-117 (40-73)

Data shown are the median and range (inter-quartile range). Units for velocity index are nM/min.

Table 3.22 Velocity index using different trigger solutions before and after CPB comparing those who bled at a rate of more or less than 200 mL/hr.

End point	<200mL/hr Pre	<200mL/hr Post	>200mL/hr Pre	>200mL/hr Post
0.5 pM TF CTI	1 0-8 (0.3-2)	0.3 0-12 (0-2)	0.2 0-2.3 (0-1)	0 0-3 (0-1.3)
0.5 pM TF Citrate	70 22-142 (50-88)	10 0-75 (3-23)	77 52-87 (62-81)	10 5-29 (9-14)
1 pM TF CTI	3 0-33 (1-5)	2 0-43 (0-10)	1 0-11 (0.4-3)	0.1 0-20 (0-10)
1 pM TF Citrate	55 15-187 (33-72)	12 0-65 (4-32)	55 29-80 (48-70)	15 5-48 (6-25)
3 pM TF CTI	12 2-68 (7-23)	37 0-93 (9-65)	22 3-53 (7-34)	44 1-78 (21-58)
3 pM TF Citrate	56 16-127 (35-72)	44 1-106 (17-66)	62 20-89 (41-72)	48 4-93 (28-56)
5 pM TF CTI	32 5-119 (16-50)	66 0-161 (43-87)	45 17-99 (22-67)	73 25-94 (56-82)
5 pM TF Citrate	61 15-124 (46-88)	64 0-154 (34-87)	72 44-101 (60-94)	69 30-103 (56-86)
10 pM TF CTI	117 23-180 (78-134)	95 9-182 (82-113)	93 44-162 (73-120)	86 54-130 (73-99)
10 pM TF Citrate	120 36-223 (94-143)	104 27-191 (85-121)	106 72-195 (90-132)	90 52-132 (84-126)
PPP Low CTI	9 2-56 (6-14)	5 0-43 (1-17)	8 4-84 (4-22)	6 0-18 (0-13)
PPP Low Citrate	52 8-146 (35-78)	16 0-91 (4-22)	57 7-104 (41-69)	15 1-26 (12-18)
PPP CTI	92 32-200 (65-127)	107 14-166 (87-127)	86 42-157 (71-127)	92 65-118 (80-108)
PPP Citrate	115 33-242 (90-140)	100 17-175 (84-126)	109 45-159 (88-155)	99 57-138 (78-105)
PPP+TM CTI	74 22-209 (54-105)	78 3-138 (56-100)	66 34-139 (56-101)	74 29-103 (54-87)
PPP+TM Citrate	92 33-242 (67-125)	64 4-117 (40-91)	88 26-159 (53-155)	62 35-97 (39-73)

Data shown are the median and range (inter-quartile range). Units for velocity index are nM/min.

Table 3.23 Velocity index using different trigger solutions before and after CPB in those who bled more or less than 2 mL/kg/hr for 2 consecutive hours.

End point	<2mL/kg/hr for 2 hours Pre-CPB	<2mL/kg/hr for 2 hours Post-CPB	>2mL/kg/hr for 2 hours Pre-CPB	>2mL/kg/hr for 2 hours Post-CPB
0.5 pM TF CTI	0.9 0-4.8 (0.3-2)	0.3 0-12 (0-1.5)	0.8 0-8 (0-2)	0 0-6 (0-1.3)
0.5 pM TF Citrate	69 22-139 (50-88)	10 0-76 (3-23)	77 34-142 (62-84)	9 0-29 (5-14)
1 pM TF CTI	2.3 0-15 (1-5)	2.1 0-43 (0-10)	3.2 0-33 (0.5-6)	0.5 0-20 (0-10)
1 pM TF Citrate	55 15-113 (34-72)	13 0-65 (5-33)	55 19-187 (40-70)	11 0-48 (5-19)
3 pM TF CTI	12 2-67 (6-21)	38 0-93 (11-65)	21 3-68 (7-34)	39 1-84 (14-58)
3 pM TF Citrate	56 16-120 (35-72)	47 2-106 (17-66)	53 20-127 (41-72)	41 1-93 (6-56)
5 pM TF CTI	32 5-119 (16-50)	67 0-161 (46-87)	45 16-106 (22-67)	68 11-101 (31-82)
5 pM TF Citrate	64 15-122 (46-88)	64 0-154 (40-87)	68 31-124 (53-94)	63 6-106 (30-86)
10 pM TF CTI	117 23-180 (78-133)	95 9-182 (83-113)	101 44-179 (73-143)	86 54-137 (73-99)
10 pM TF Citrate	120 36-201 (94-142)	105 24-191 (86-121)	111 64-223 (90-150)	90 52-134 (79-126)
PPP Low CTI	9 2-56 (6-14)	5 0-43 (1-17)	11 4-84 (5-22)	3 0-29 (0-13)
PPP Low Citrate	53 8-140 (34-78)	16 0-91 (4-22)	51 7-146 (41-69)	13 0-63 (4-18)
PPP CTI	86 32-188 (64-121)	109 14-166 (91-127)	95 42-200 (71-136)	84 35-135 (71-108)
PPP Citrate	115 33-213 (88-140)	100 21-175 (86-126)	122 45-242 (90-157)	90 17-138 (69-105)
PPP+TM CTI	69 22-169 (53-100)	83 3-138 (58-100)	74 24-209 (58-113)	69 12-121 (45-87)
PPP+TM Citrate	91 33-189 (63-123)	64 4-117 (41-91)	115 26-242 (72-155)	55 5-101 (36-76)

Data shown are the median and range (inter-quartile range). Units for velocity index are nM/min.

Table 3.24 Velocity index using different trigger solutions before and after CPB in those who did and did not require haemostatic treatment.

End point	No Haemostatic Treatment Pre-CPB	No Haemostatic Treatment Post-CPB	Haemostatic Treatment Pre-CPB	Haemostatic Treatment Post-CPB
0.5 pM TF CTI	0.9 0-4.5 (0.2-2)	0.3 0-12 (0-1)	0.8 0-8 (0.2-2)	0.2 0-12 (0-2)
0.5 pM TF Citrate	70 22-139 (48-83)	9 0-68 (3-23)	74 27-142 (57-87)	11 0-75 (2-18)
1 pM TF CTI	2.6 0-9 (1-5)	2.6 0-43 (0-10)	1.9 0-33 (1-6)	1.2 0-30 (0-10)
1 pM TF Citrate	60 16-113 (34-82)	13 0-65 (7-31)	54 15-187 (36-68)	12 0-49 (4-28)
3 pM TF CTI	12 2.5-67 (7-21)	29 0-93 (6-61)	14 3-68 (7-33)	42 0-89 (16-64)
3 pM TF Citrate	55 16-120 (36-70)	44 2-106 (17-63)	56 19-127 (40-75)	48 1-93 (17-69)
5 pM TF CTI	37 6-119 (17-48)	64 0-124 (46-84)	32 5-106 (21-61)	70 11-161 (41-85)
5 pM TF Citrate	69 15-117 (43-91)	61 0-117 (43-86)	61 27-124 (50-85)	67 6-154 (33-87)
10 pM TF CTI	116 33-169 (80-133)	93 9-182 (81-114)	112 23-180 (74-133)	97 54-146 (85-107)
10 pM TF Citrate	122 43-201 (84-144)	100 27-191 (83-122)	114 36-223 (96-140)	105 52-169 (88-122)
PPP Low CTI	9 2-35 (6-14)	5 0-43 (1-18)	8 2-84 (5-17)	6 0-41 (1-13)
PPP Low Citrate	55 9-140 (37-79)	16 0-85 (4-22)	52 7-146 (37-70)	16 0-91 (5-22)
PPP CTI	92 32-188 (64-127)	105 14-160 (87-126)	92 39-200 (68-127)	105 35-166 (83-122)
PPP Citrate	115 33-213 (91-140)	99 21-175 (79-125)	113 45-242 (88-143)	101 17-164 (86-128)
PPP+TM CTI	73 22-169 (57-103)	78 3-135 (56-100)	69 22-209 (54-105)	73 12-138 (54-95)
PPP+TM Citrate	92 33-189 (62-120)	62 4-117 (39-88)	95 26-242 (70-128)	66 5-107 (40-89)

Data shown are the median and range (inter-quartile range). Units for velocity index are nM/min.

Table 3.25 Velocity index using different trigger solutions before and after CPB in those who did and did not undergo re-operation due to bleeding.

End point	No Re-op Pre-CPB	No Re-op Post-CPB	Re-op Pre-CPB	Re-op Post-CPB
0.5 pM TF CTI	1 0-5 (0.3-2)	0.2 0-12 (0-1)	0.2 0-8 (0-0.3)	0 0-7 (0-1.7)
0.5 pM TF Citrate	68 22-13 (50-83)	9 0-75 (3-23)	84 52-142 (77-93)	14 0-29 (6-14)
1 pM TF CTI	3 0-15 (1-6)	2 0-43 (0-10)	1 0-33 (0.4-2)	1 0-19 (0-13)
1 pM TF Citrate	55 15-113 34-70)	13 0-65 (5-33)	56 48-187 (50-66)	14 0-28 (6-25)
3 pM TF CTI	13 5-67 (7-25)	39 0-93 (12-65)	11 3-68 (3-31)	35 1-67 (11-56)
3 pM TF Citrate	56 16-120 (36-71)	47 2-106 (17-66)	67 20-127 (48-82)	35 1-93 (14-64)
5 pM TF CTI	32 5-119 (17-51)	67 0-161 (46-90)	37 11-106 (19-64)	69 11-82 (28-77)
5 pM TF Citrate	62 15-122 (46-89)	65 0-154 (39-87)	73 44-124 (64-90)	59 6-102 (33-83)
10 pM TF CTI	114 23-180 (81-133)	95 9-182 (82-112)	107 44-179 (57-131)	85 54-130 (75-97)
10 pM TF Citrate	118 36-201 (92-143)	104 27-191 (87-124)	122 72-223 (90-141)	84 52-128 (80-99)
PPP Low CTI	9 2-84 (6-15)	5 0-43 (1-15)	5 4-31 (4-12)	5 0-39 (2-15)
PPP Low Citrate	52 7-140 (35-74)	16 0-91 (4-22)	69 37-146 (52-108)	16 0-44 (13-18)
PPP CTI	92 32-188 (65-127)	107 14-166 (87-127)	71 42-200 (59-113)	83 35-118 (68-96)
PPP Citrate	115 33-213 (90-140)	101 17-175 (86-126)	138 61-242 (89-157)	84 41-116 (63-103)
PPP+TM CTI	71 22-169 (55-105)	77 3-138 (56-102)	69 24-209 (45-90)	71 29-95 (44-90)
PPP+TM Citrate	92 26-189 (64-125)	64 4-117 (40-93)	97 40-242 (66-157)	55 33-89 (37-75)

Data shown are the median and range (inter-quartile range). Units for velocity index are nM/min.

The reduction in protein C (55% versus 62%, p=0.023) and free protein S (42% versus 47%, p=0.046) may explain why the reduced clotting factors measured post-cardiopulmonary bypass in those who bled in excess of 2mL/kg/hr for 2 consecutive hours did not translate into reduced thrombin generation. Addition of thrombomodulin to these samples resulted in very similar thrombin generation results in both groups pre and post-CPB as shown in Figure 3.12.

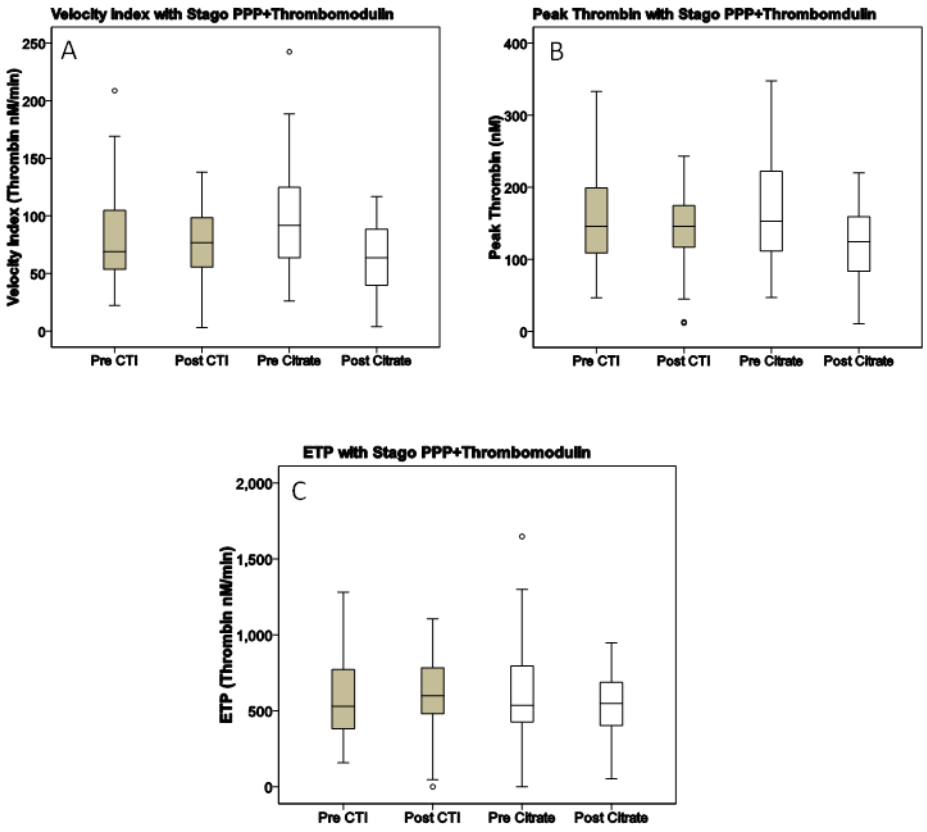


Figure 3.12 Velocity Index (A), Peak thrombin (B) and ETP (C) measured pre and post cardiopulmonary bypass in samples taken into anticoagulant consisting of citrate and CTI or citrate alone. The trigger used was Stago PPP containing thrombomodulin. Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

3.3 Discussion

The aim of this section was to investigate whether any haemostatic tests were useful for predicting progression to post-operative bleeding. Blood loss in excess of 1L at 24 hours or at a rate exceeding 2mL/kg/hr for two consecutive hours in the first six post-operative hours was associated with a significantly lower FXIII and fibrinogen concentration and a lower platelet count. FXIII was also lower in those who bled at a rate exceeding 200mL/hr for 2 consecutive hours in the first 24 hours. A longer time on cardiopulmonary bypass was associated with blood loss in excess of 1L at 24 hours and the need for haemostatic treatment. A longer time on bypass is likely to indicate greater complexity of the operation being performed, something which in itself is also a risk factor for bleeding (181). The observations regarding FXIII falling post-bypass are consistent with other studies (103, 182), although reports of its association with bleeding have been conflicting (108, 110). A lower fibrinogen has also been associated with bleeding (105, 183) although the critical concentration at which replacement is appropriate has not yet been determined (109). Thrombocytopenia has also been previously described as a risk factor for bleeding (11, 184) .

Thrombin generation assays were generally unable to identify patients at risk of excess bleeding regardless of tissue factor concentration or anticoagulant used. The significant reduction of factors II, V and X seen in those who bled in excess of 2mL/kg/hr for two consecutive hours in the first six post-operative hours which would have been expected to reduce thrombin generation and hence increase bleeding, was balanced by a reduction in protein C and S. Activation of Protein C by addition of thrombomodulin to the trigger resulted in near identical thrombin generation, supporting this conclusion.

Previous studies have reported that reduced thrombin generation was associated with an increased risk of bleeding (11, 169). The reasons why the results reported here differ from previous studies may in part be the result of having recruited a patient group undergoing more complex operations where bleeding is more frequent. Previous studies included patients undergoing CABG as the sole procedure, an operation that is associated with a lower incidence of post-CPB bleeding (11, 169). In addition in this study the pattern of abnormalities in clotting factors in those bleeding in excess of 2mL/kg/hr and 1L at twenty four hours indicates that initial clot formation will have been reduced through reduction in fibrinogen and platelet count. The quality of the clot would have been further compromised by the reduction in FXIII resulting in reduced fibrin cross-linkage. Also the longevity of this clot would have been shortened by the reduction in activity of TAFI which was reduced compared to pre-CPB samples and would have comparatively had a greater impact in those where clot stability had already been impaired. This would then result in excess bleeding. As FXIII and TAFI have no influence on thrombin generation, and this study used PPP rather than PRP, thus removing any influence of platelets, it is unsurprising that thrombin generation parameters were unable to detect those at risk of bleeding. Furthermore, the data suggests that correction of these factors in isolation may not be the most effective strategy, which may partly explain the negative result from a recent clinical trial using recombinant FXIII (185).

Tests of platelet function were not performed in this study. It is already well described that following exposure to cardiopulmonary bypass, platelets exhibit reduced aggregation in response to ADP (115, 118). Furthermore, thrombin generation assays performed in platelet rich plasma (PRP) may have been sensitive to any alteration in platelet membrane phospholipid composition, which may be another factor explaining some of the differences in conclusion between this study and that of Bosch et al (169).

A paradoxical increase was observed in median peak thrombin and velocity index observed post-CPB compared to pre-CPB samples taken into CTI when trigger solutions containing lower concentrations of TF were used. This may be due to the reduction in full length TFPI having a greater effect on the TF-FVIIa interaction. Full-length TFPI has previously been shown to significantly influence calibrated automated thrombography (186). Addition of CTI increases the sensitivity of the assay to the initiation phase of coagulation. In the post-CPB samples the lower full-length TFPI concentration may have resulted in a greater amount of FVIIa being generated compared to pre-operative samples. At higher TF concentrations, the FVII concentration becomes rate limiting, therefore post-CPB the reduction in FVII would reduce thrombin generation, thus resulting in the expected pattern of reduced thrombin generation post-cardiopulmonary bypass compared to pre.

In summary, the post-CPB fibrinogen concentration had modest clinical utility in identifying patients who went on to lose more than 1L of blood at 24 hours or at a rate greater than 2 mL/kg/hr for two consecutive hours in the first 6 hours post-surgery. Other coagulation factors, whilst having some clinical utility, in practice would not be available in a timely manner to guide clinical management. Calibrated automated thrombography performed in PPP was unable to predict bleeding regardless of the definition used.

Chapter 4 The effect of CPB on platelet phospholipids

4.1 Introduction

Under resting conditions, the external platelet membrane is composed predominantly of the inert phospholipid phosphatidylcholine (PC). On activation of the platelet, the negatively charged phospholipids phosphatidylethanolamine (PE) (77, 79) and phosphatidylserine (PS) (80) move to the external membrane to support coagulation. Phospholipid exposure on cell membranes is considered to be mediated by three types of transporter: flippase, floppase and scramblase, as discussed in Chapter 1.

Following CPB, platelet aggregation is reduced in response to ADP (115-118). P-selectin, a marker of platelet activation, has generally been reported to be either unchanged (119) or increased (120, 121). The ability of platelets to express P-selectin in response to thrombin receptor stimulation appears unchanged post-CPB where thrombin has been used as the agonist (119), but where thrombin receptor activating peptide-6 (TRAP-6) has been used the response post-CPB has been reported to be reduced (187). Whilst this difference is likely to be related to thrombin being able to activate more types of receptor, such as PAR3 and PAR4, it nevertheless suggests that the ability of platelets to respond to thrombin receptor activation may be different post-CPB.

These observations suggest there may also be changes in the phospholipids expressed on the external membrane of the platelets. Evidence to support this comes from studies which have reported changes in annexin V binding post-CPB. Annexin V binds to negatively

charged phospholipids and therefore has been used as indicator of presence of these lipids. However, the studies have been contradictory, with some reporting an increase in annexin V binding (120) and others reporting a decrease (122).

Recently a novel family of oxidised phospholipids has been described that are rapidly generated by 12-lipoxygenase in platelets activated by thrombin or collagen (99). The phospholipids have a hydroxyl group added to the 12^{th} carbon of arachidonic acid in the Sn2 position of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). These oxidised fatty acids are termed hydroxyeicosatetraenoic acids (HETE) and hence activated platelets generate 12-HETE-PE and 12-HETE-PC. These HETE-phospholipids have been shown to increase thrombin generation (99). The effect of CPB on these novel lipids is currently unknown. The structures of the platelet phospholipids measured in this Chapter are shown in Figure 4.1 for PE and PS species, Figure 4.2 for 12-HETE-PE species and Figure 4.3 for PC and 12-HETE-PC species.

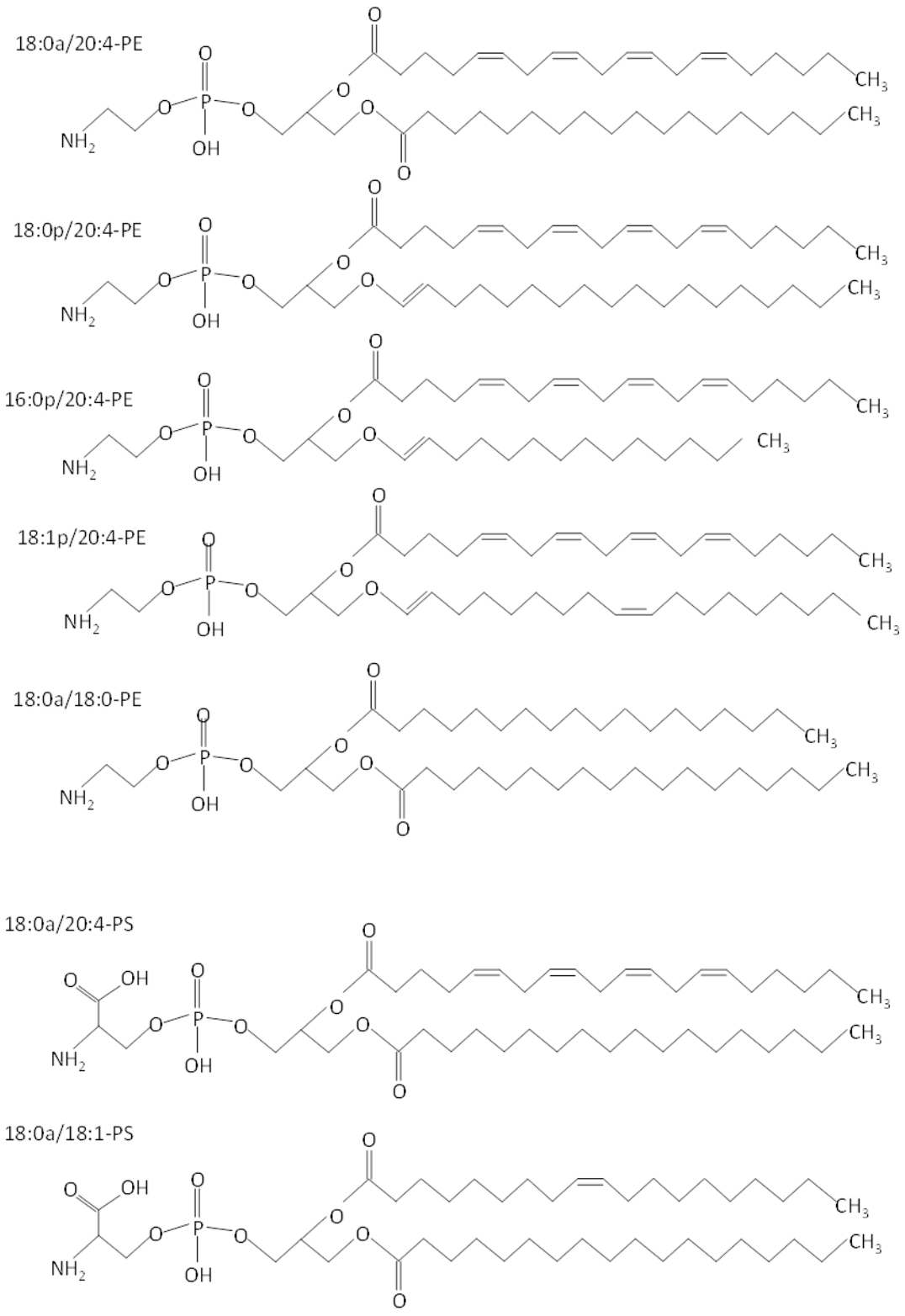


Figure 4.1 Structures of phosphatidylethanolamine (PE) species and phosphatidylserine (PS) species measured in this Chapter.

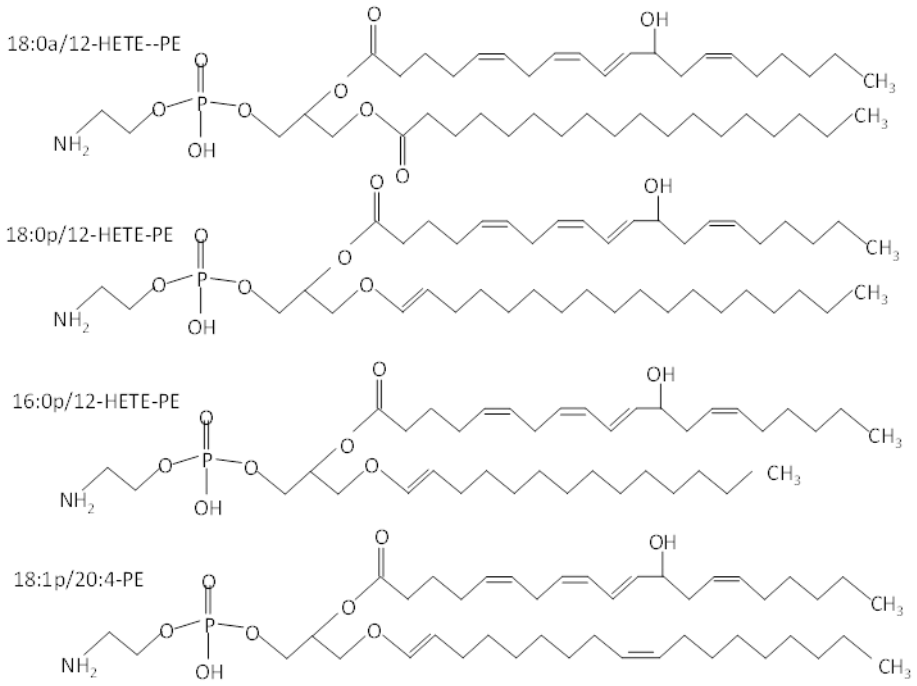


Figure 4.2 Structures of the 12-HETE-PE phospholipids measured in this Chapter. They differ from the parent PE phospholipids shown Figure 4.1 by the addition of a hydroxyl group to the 12th carbon atom of arachidonic acid.

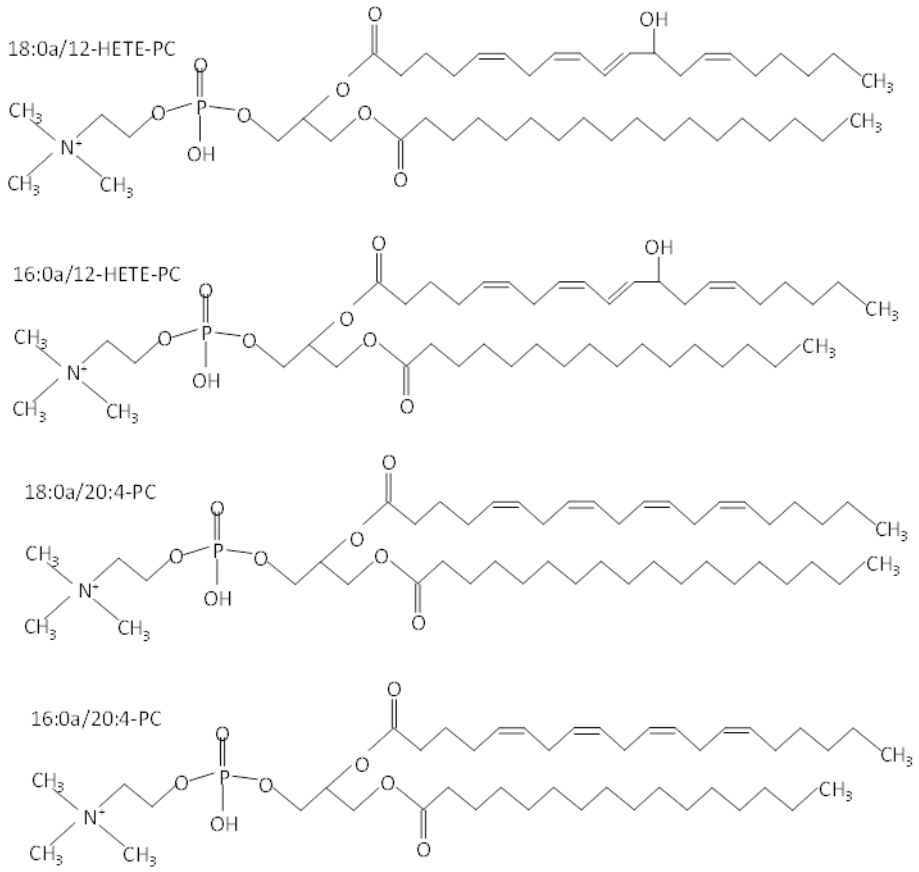


Figure 4.3 Structures of the PC and 12-HETE-PC phospholipids measured in this Chapter. The 12-HETE-PC species differ from the parent PC phospholipids by the addition of a hydroxyl group to the 12th carbon atom of arachidonic acid.

The purpose of the experiments described in this Chapter was firstly to investigate whether the externalisation of negatively charged phospholipids and the generation of oxidised phospholipids was altered by CPB. The second objective was to investigate their effect on thrombin generation and whether their inclusion in trigger solutions would alter the ability of calibrated automated thrombography to identify those at risk of bleeding.

4.2 Results

4.2.1 Flowcytometry

Platelets were identified by initially gating on forward and side scatter, with CD61 being used as a platelet marker to identify platelets co-expressing Annexin V. Annexin V expression was increased in washed platelets compared to whole blood ($p=0.012$) as shown in Figure 4.4. There was no significant difference in the percentage of platelets positive for Annexin V post-CPB compared to pre-CPB ($p=0.484$ for washed platelets; $p=0.169$ for platelets in whole blood). The percentage of Annexin V positive platelets did not correlate with the amount of individual species of externalised PE and PS, nor did it correlated with the total amount of externalised PE and PS (Figure 4.5).

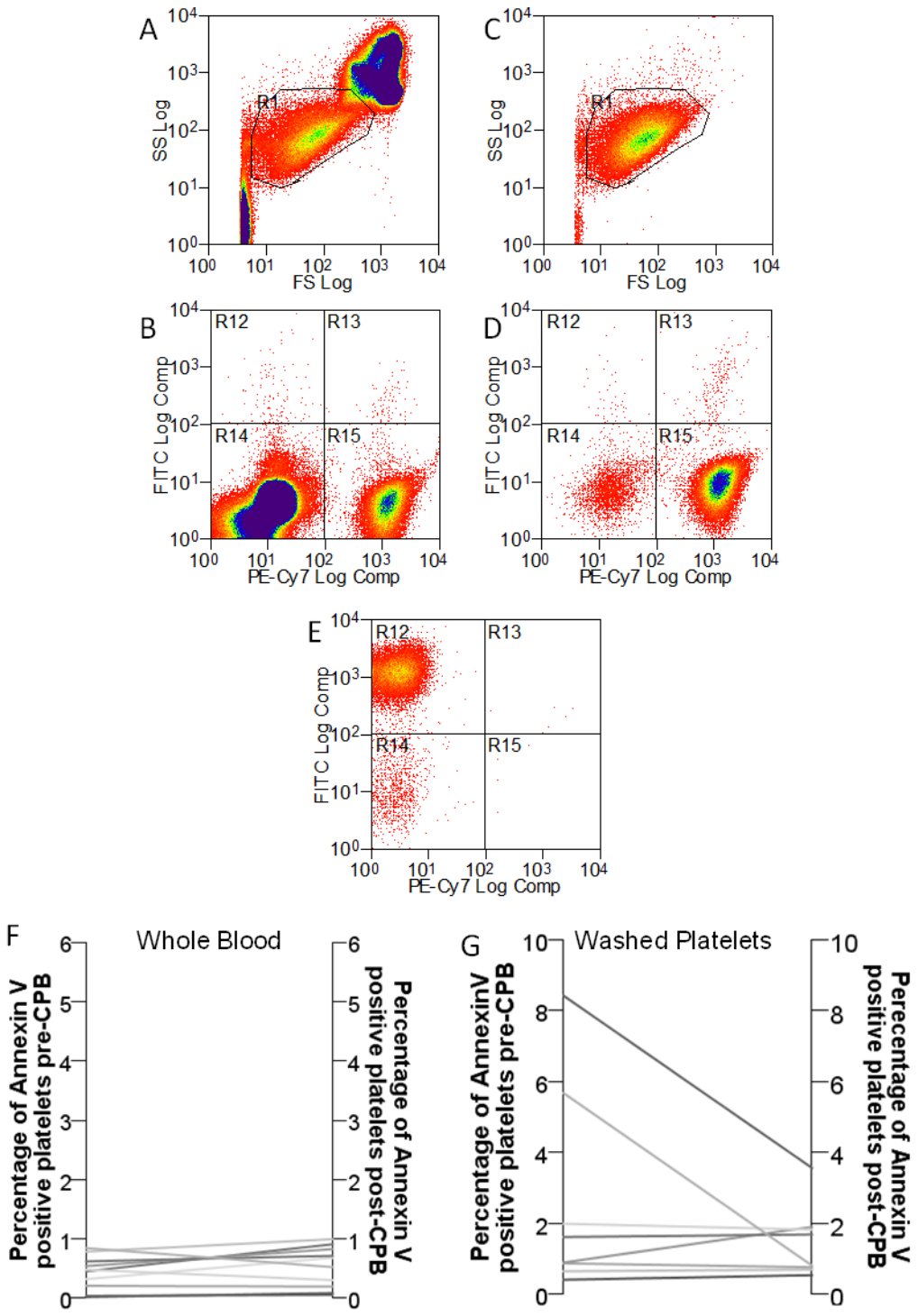


Figure 4.4 The gating strategy is summarised using plots from a representative sample. The region where platelets were expected to be present was initially identified using forward and side scatter (A, whole blood and C, washed platelets). This population was then gated upon comparing CD61 positive events (PE-Cy7 fluorochrome) with Annexin V positive events (FITC fluorochrome) in both whole blood (B) and washed platelets (D). A positive control using fixed washed platelets stained only for Annexin V is included for comparison (E). The percentage of Annexin V positive platelets was lower in whole blood (F) than in washed platelets (G).

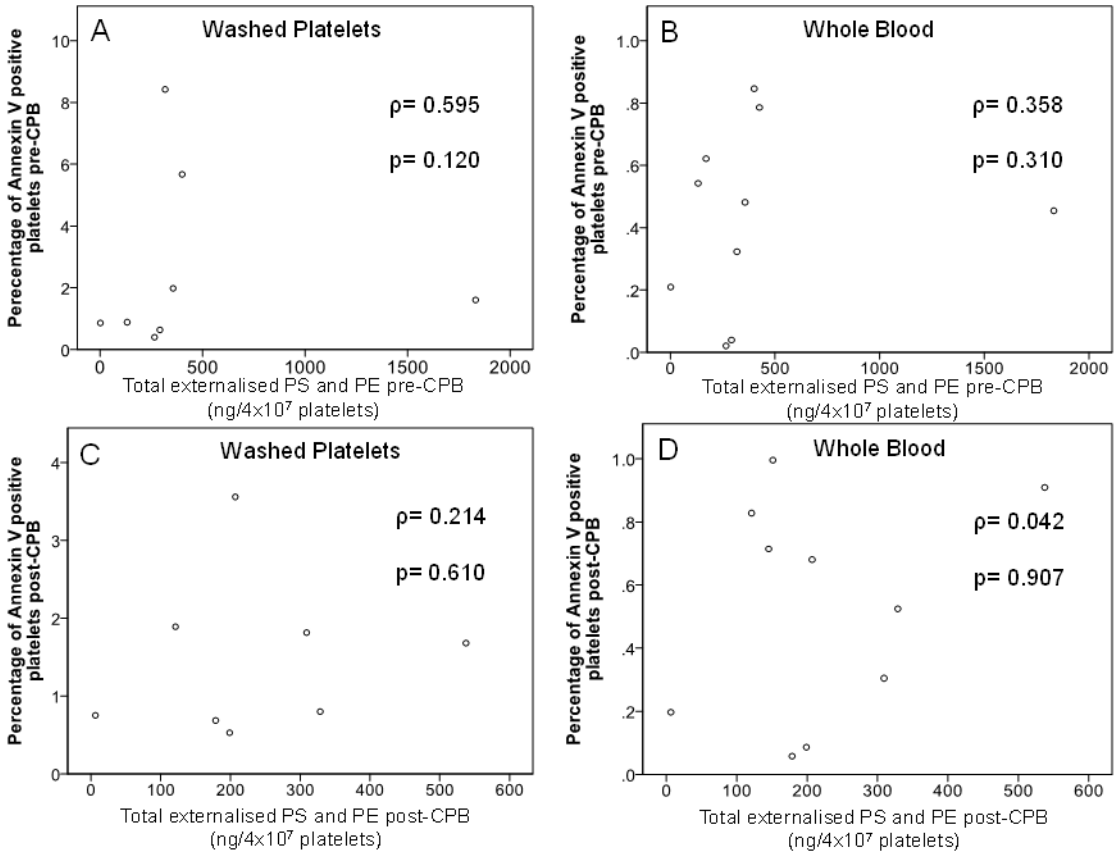


Figure 4.5 Correlation plots comparing total amounts of externalised PS and PE with the percentage of Annexin V positive platelets from washed platelets (A, C) and whole blood (B,D) pre and post-CPB. Spearmen's correlation coefficient (ρ) and p-value are shown. There was no correlation in any of the four conditions.

4.2.2 Effect of CPB on externalisation of PE and PS

As expected, thrombin and collagen both induced the externalisation of PE (Figure 4.6) and PS (Figure 4.7). Post-CPB the amount of PE and PS externalised in response to stimulation was reduced compared to pre-CPB. This reduction in externalisation suggests that scramblase function within the platelet membrane is partially inhibited. The total amount of PE and PS did not change comparing pre and post CPB.

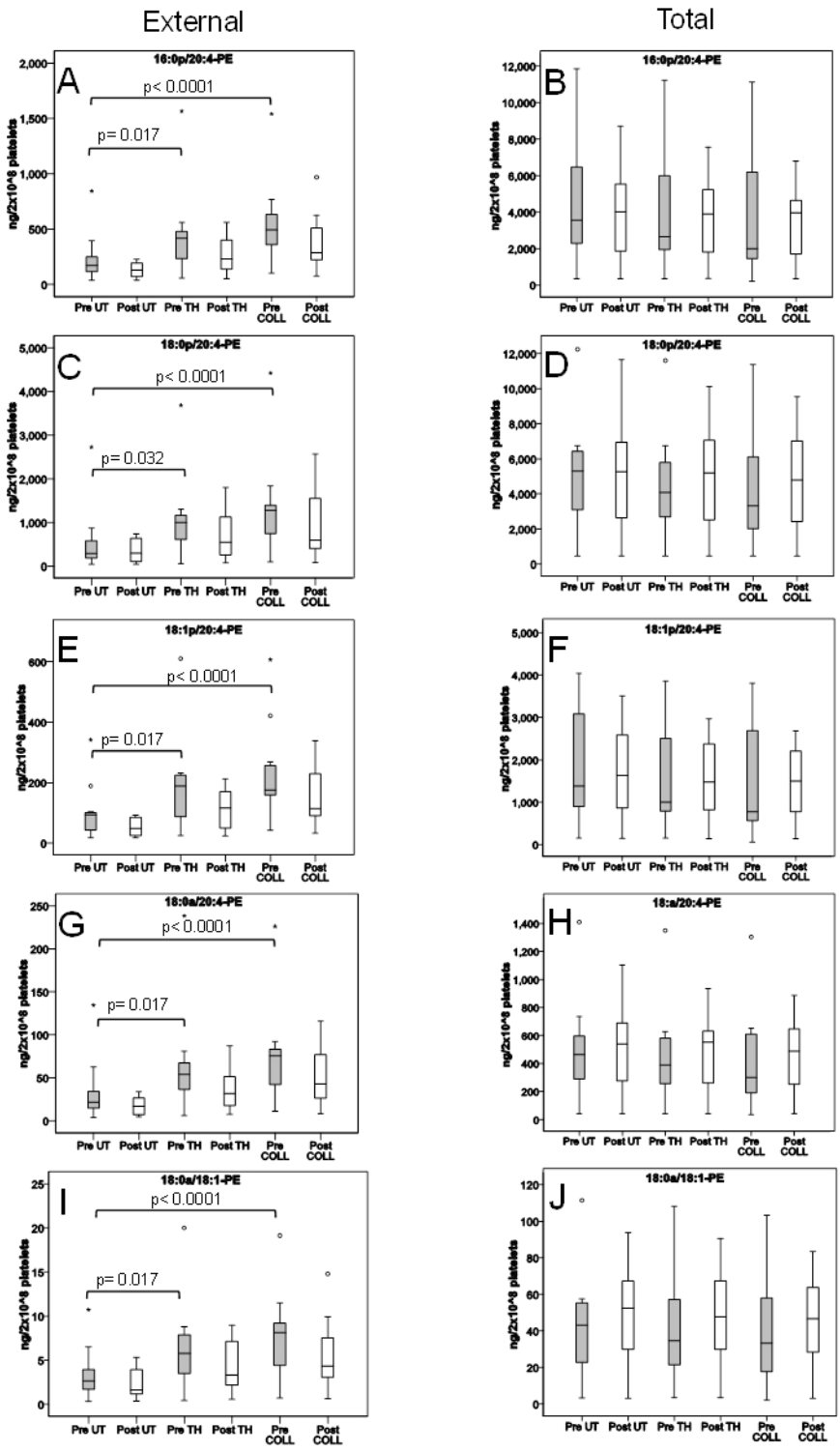


Figure 4.6 Median concentrations of PE species on the external platelet membrane increased in response to collagen and thrombin. Post-CPB the levels were lower (A,C,E, G, I). Total concentrations remained similar pre and post-CPB (B, D, F, H, J) Data shown are median values (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles) (n=11).

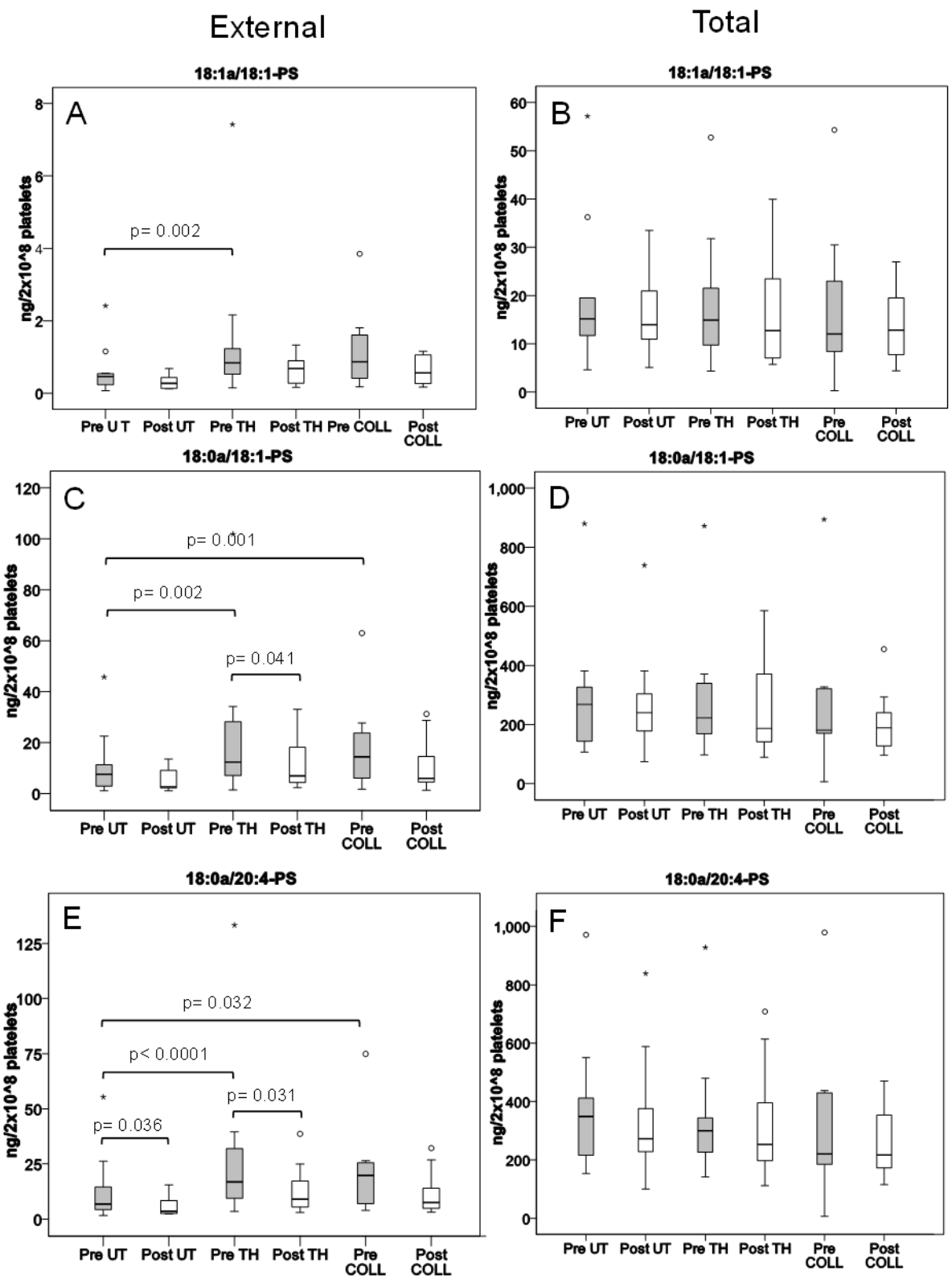


Figure 4.7 PS was externalised in response to thrombin and collagen. Post-CPB, externalisation was reduced (A, C, E). Total concentrations remained similar (B, D, F). Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles) (n=11).

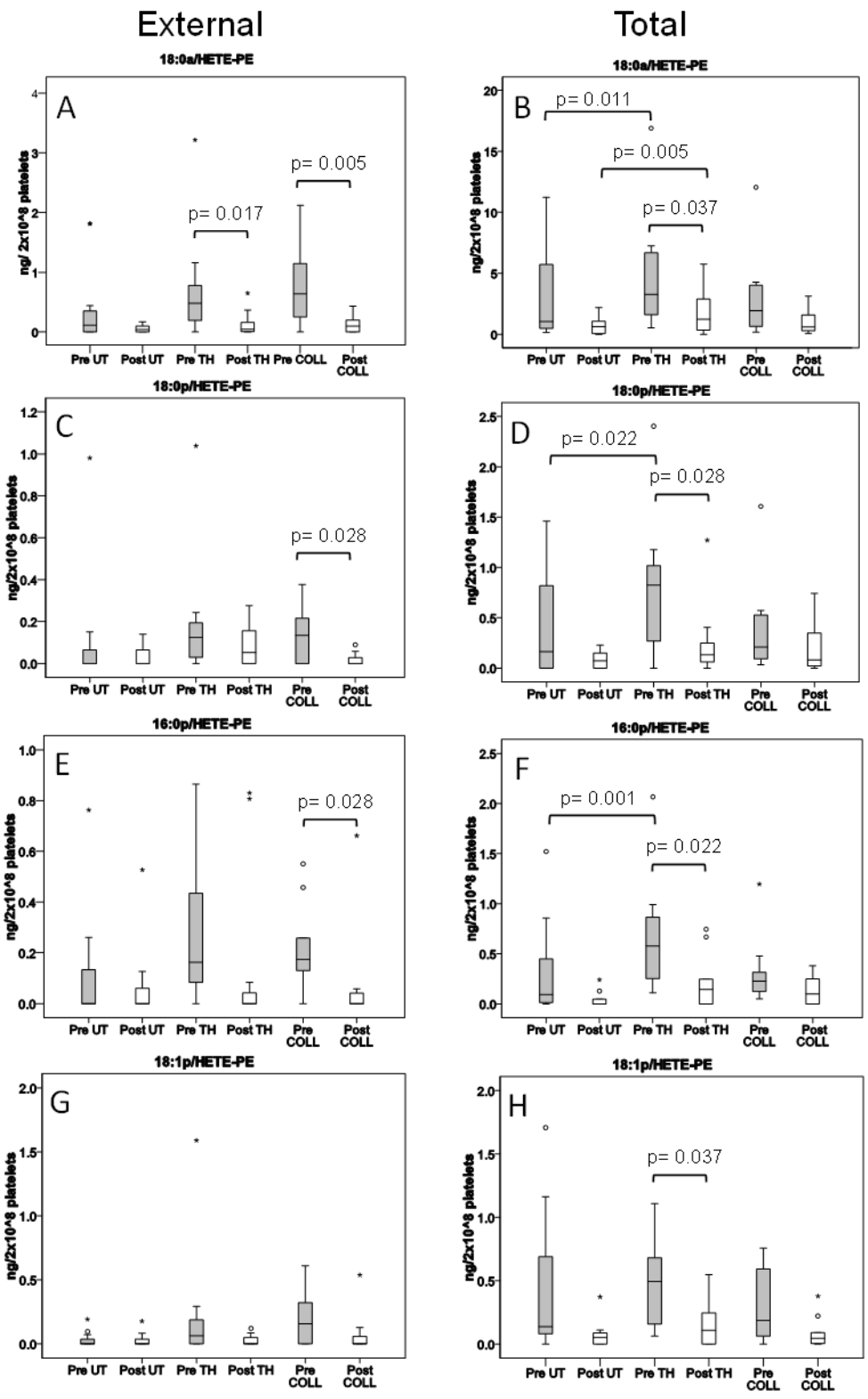


Figure 4.8 Concentrations of 12-HETE-PE species increased in response to thrombin and collagen; post-CPB they were reduced (A, C, E, G). Total levels post-CPB were lower than pre-CPB, although there was still evidence to of a response to stimulation with thrombin (B, D, F, H). Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles) (n=11).

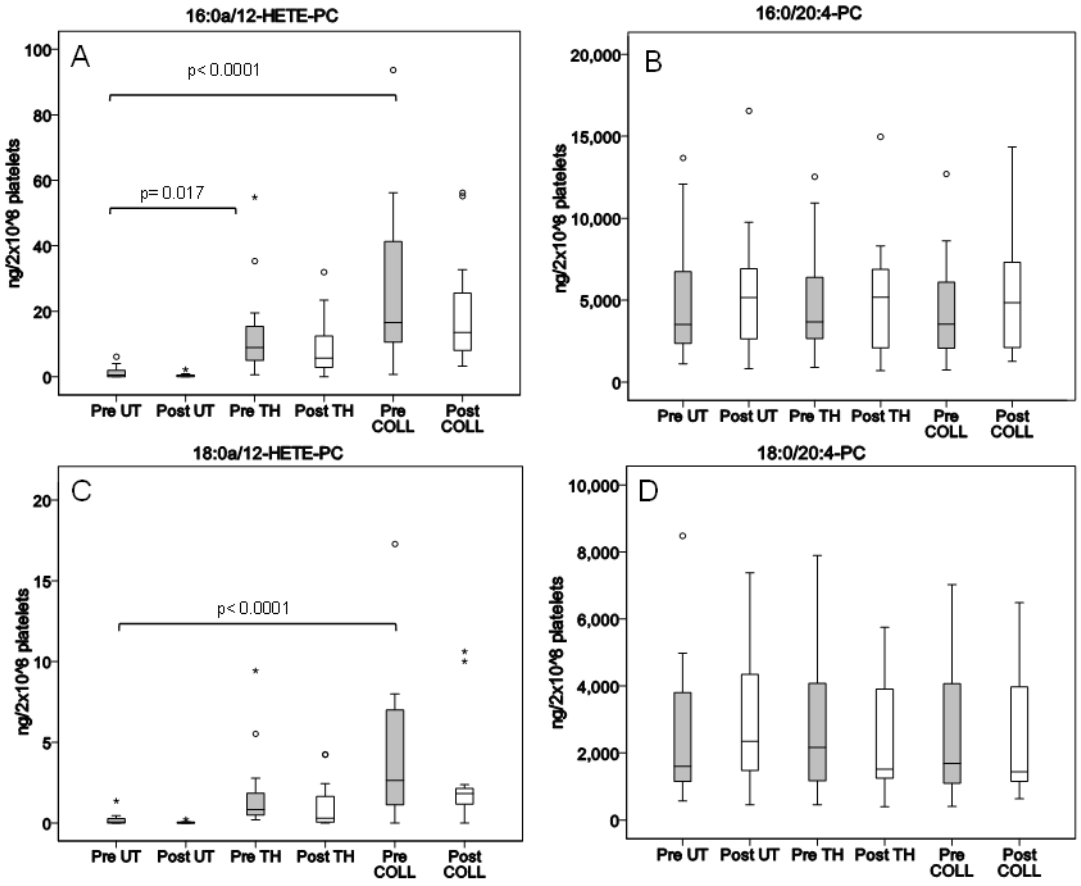


Figure 4.9 12-HETE-PC increased in response to stimulation with thrombin and collagen, with a reduction post-CPB (A,C). Amounts of parent species of PC were unchanged pre and post-CPB (B,D). Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles) (n=11).

4.2.3 Effect of CPB on 12-HETE-PE and 12-HETE-PC

Following CPB less 12-HETE-PE was externalised and less was synthesised in response to stimulation with thrombin or collagen (Figure 4.8). In addition, post-CPB less 12-HETE-PC was synthesised in response to thrombin and collagen, although the difference was not statistically significant (Figure 4.9). These results suggest that 12-LOX, the lipoxygenase present in platelets responsible for oxygenation of arachidonic acid to HETE, has reduced function post-CPB.

Figure 4.10 summaries the effect of CPB on the synthesis of the overall amounts of 12-HETE-PC and 12-HETE-PE (Figure 4.10A and Figure 4.10B) and the externalisation of the overall amounts of 12-HETE-PE (Figure 4.10C), PE (Figure 4.10D) and PS (Figure 4.10E).

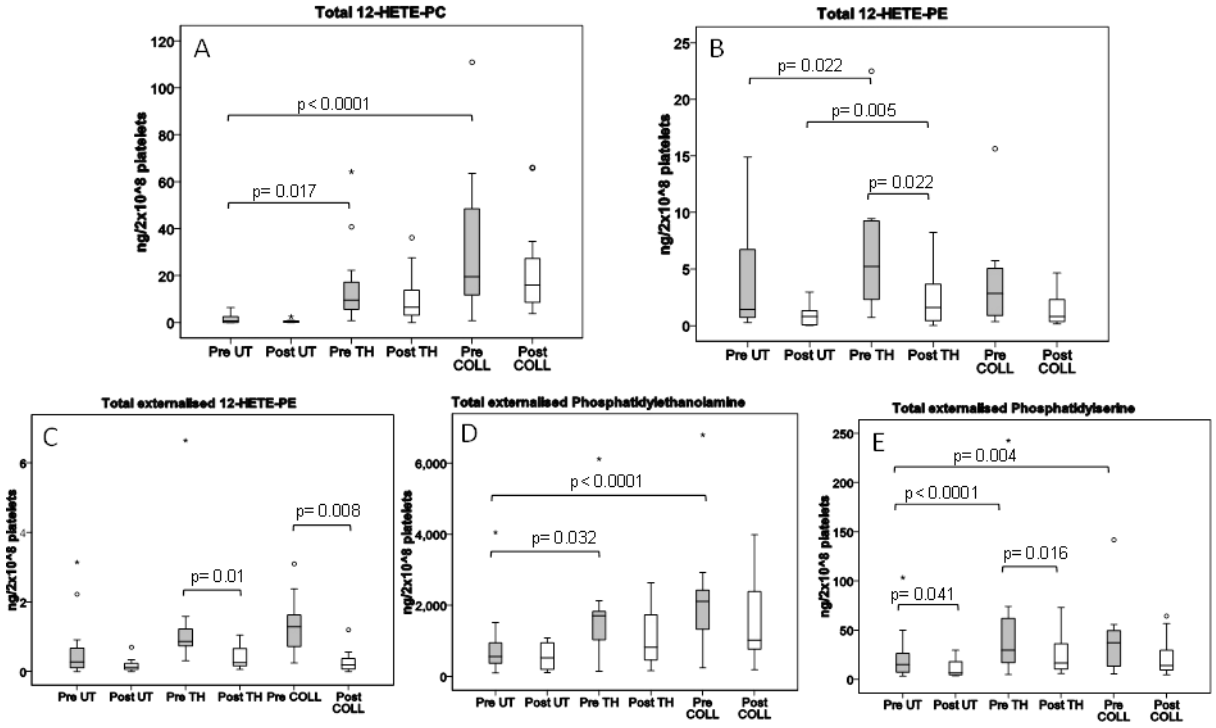


Figure 4.10 Total amounts of 12-HETE-PC (A) and 12-HETE-PC (B) were lower post-CPB. Total externalised 12-HETE-PE (C), PE (D) and PS (E) were lower post CPB. Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles) (n=11).

4.2.4 Effect of 12-HETE-PE and PC on thrombin generation

In order to investigate the effect of the 12-HETE-PE and 12-HETE-PC on thrombin generation it was necessary to generate liposomes that would only reflect the lipids added. 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), as used in standard thrombin generation tests, are lipids that have not been

reported in human platelets. For these experiments, they were substituted for phospholipid species previously identified in platelets**:** 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC), 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phospho-L-serine (SAPS) and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamines (SAPE), at proportions better reflecting those previously reported in the literature (188).

Innovin as a source of TF has the disadvantage of already containing phospholipids which may interfere with experiments investigating the effects of changing the phospholipid composition of liposomes. Using human full length recombinant TF avoids this potential problem. However, incorporating this into the liposomes required a different method as when using the method described in Chapter 2.9.2, very little thrombin generation was observed. In order for a complete thrombin generation curve to be obtained the trigger solution required 20 pM of TF (final in-well concentration). A freeze-thawing step was introduced prior to passing the lipid and TF mixture through the extruder device, which resulted in liposomes which supported more thrombin generation (Figure 4.11 A). In five patients a comparison was made of the effect of freeze-thawing on the preparation of liposomes using Innovin as the source of tissue factor. These also showed enhanced thrombin generation when a freeze-thawing step was used (Figure 4.11 B-E).

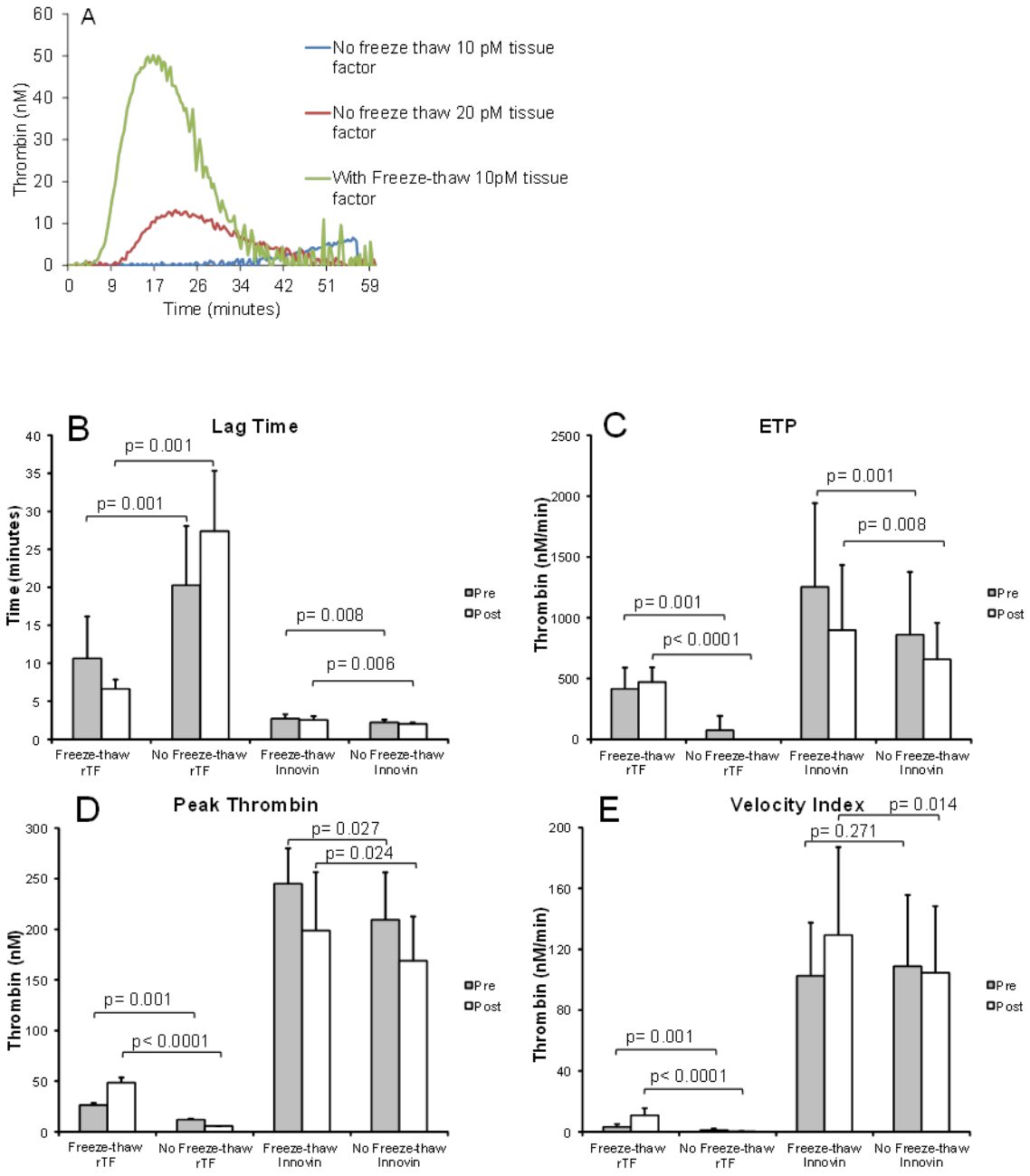


Figure 4.11 A freeze-thaw step was necessary to demonstrate any tissue factor induced thrombin generation (A). Concentrations of tissue factor refer to the final in-well concentration when running the thrombin generation assay. Making liposomes using rTF (10 pM final in-well concentration) without freeze-thawing resulted in an increase in lag time (B) and a reduction in measured ETP (C), Peak thrombin (D) and Velocity Index (E). Using Innovin (10 pM final in-well concentration) as the source of tissue factor and making the liposomes without a freeze-thaw step resulted in a similar pattern. Data shown in B-E are mean (columns) and standard deviation (error bars), n=5.

In addition it was necessary to use a version of buffer A with either a lower pH of 7.05 or lower sodium chloride concentration of 100 mmol/L, when making the initial liposome preparation (Figure 4.12) prior to freeze-thawing. After this step, all liposomes were diluted in standard buffer A (see Chapter 2) to obtain the final working concentration. This suggests that the lower pH or lower sodium chloride concentration enhanced the incorporation of the recombinant tissue factor into the liposome rather than having any effect on coagulation factor interaction with tissue factor or the phospholipid membrane.

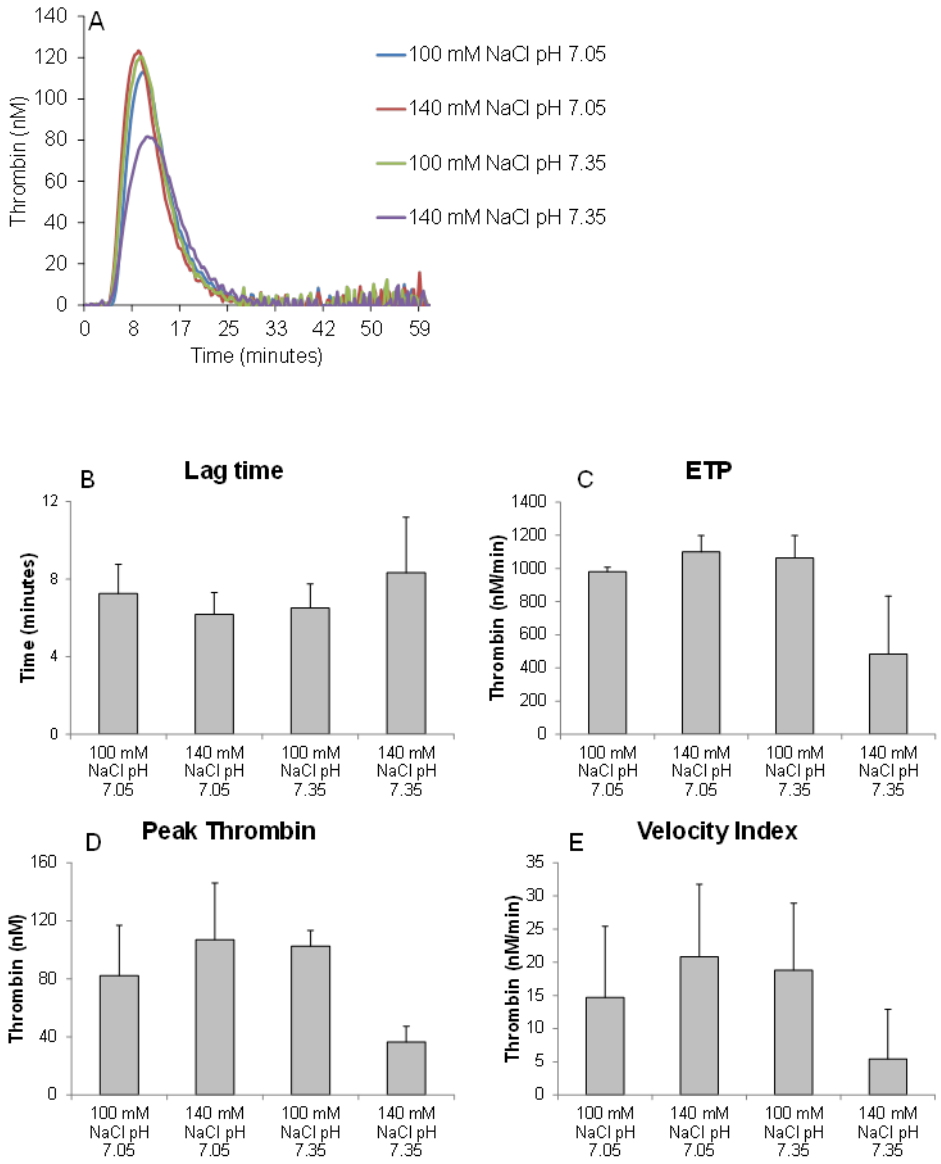


Figure 4.12 Effect of sodium chloride concentration and pH on thrombin generation results. Reducing the pH or the sodium chloride concentration of the buffer used to make the liposomes improved the thrombin generation (A). The mean values (columns) and standard deviation (error bars) of thrombin generation parameters measured in three separate experiments are shown in panels B-E (n=3).

Whilst interesting, these observations would only be of clinical interest if the effect of 12-HETE-PE or 12-HETE-PC could be shown to influence thrombin generation. Therefore liposomes were constructed where 10% of the total lipids were replaced by 12-HETE-PE or 12-HETE-PC, with the proportion of SAPE and SAPC being reduced accordingly. Addition of

12-HETE-PE or 12-HETE-PC to liposomes resulted in an increase in thrombin generation compared with un- oxidised phospholipids in both pre and post-CPB samples as shown in Figure 4.13.

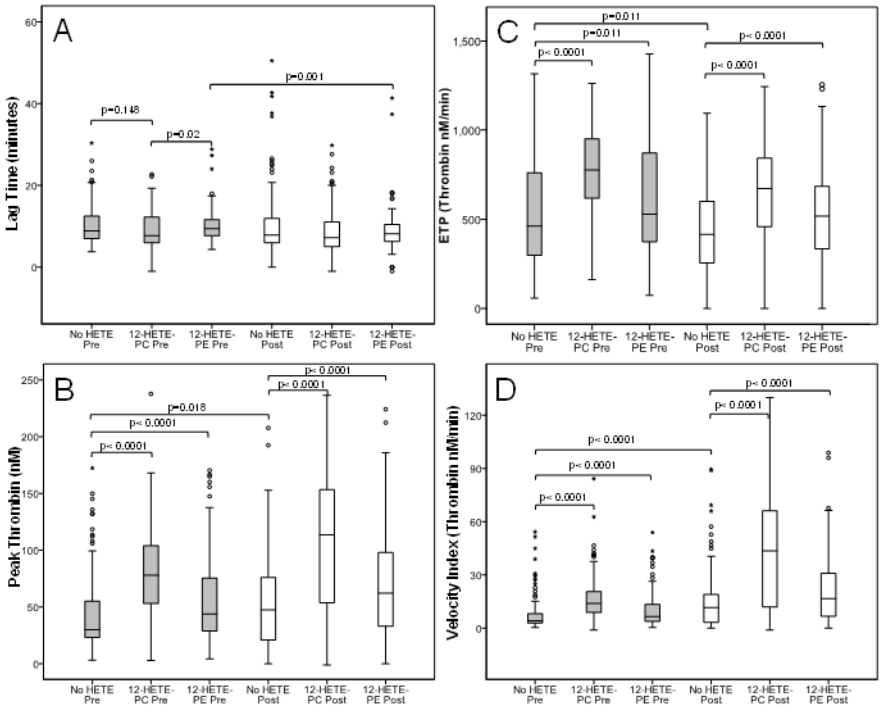


Figure 4.13 The lag time (A) was shorter and ETP (B), peak thrombin (C) and velocity index (D) were increased when measured using liposomes containing either 12-HETE-PE or 12-HETE-PC, and also in all samples post-CPB compared to pre-CPB (n=87). In five patients who had previously shown a decrease in thrombin generation parameters post-CPB, the effect of the change in method of producing the liposomes was investigated. Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles).

The fold-increase in thrombin generation in response to the presence of 12-HETE-PE or 12-HETE-PC was less in those patients who went on to require haemostatic treatment post-CPB (Figure 4.14). This suggests that the interaction of the coagulation factors and the lipids in these individuals was different to those who did not bleed.

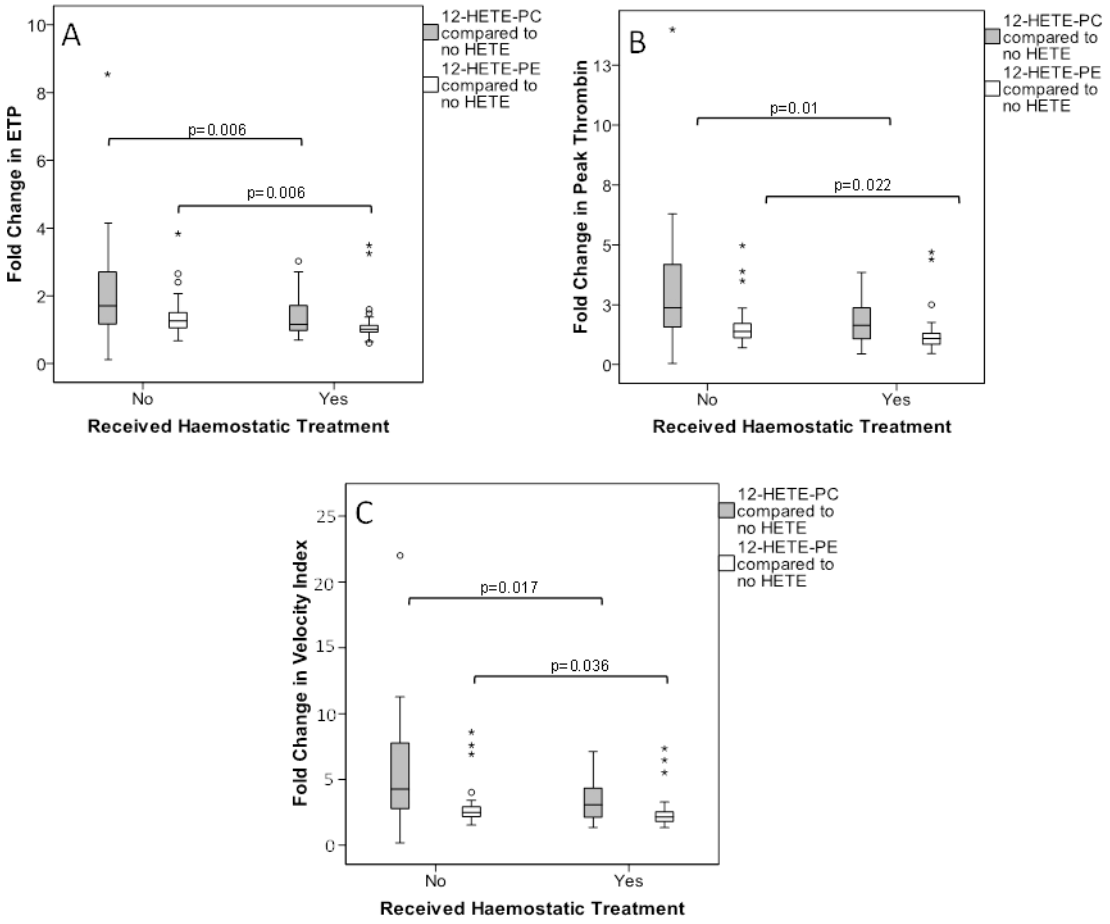


Figure 4.14 Fold change in ETP (A), Peak Thrombin (B) and Velocity Index (C), measured in pre-operative samples using triggers with and without 12-HETE-PC (pale grey boxes) and 12-HETE-PE (unshaded boxes) was significantly less in those who went on the receive haemostatic treatment following the end of cardiopulmonary bypass (n=87).

A ROC analysis was preformed to assess the clinical use of these tests in predicting bleeding. The results are shown in Table 4.1. Only the fold change in thrombin generation in response to 12-HETE-PC measured pre-CPB had any predictive value, but this was only modest and in clinical practice would be of limited clinical utility.

Table 4.1 ROC curve values for the fold change in discriminating those who needed haemostatic treatment post-CPB.

Fold change in thrombin generation parameter	ROC area pre-CPB (95% confidence interval)	ROC area post-CPB (95% confidence interval)
ETP with 12-HETE-PC	0.63 (0.51-0.76)	0.53 (0.40-0.66)
ETP with 12-HETE-PE	0.62 (0.5-0.75)	0.54 (0.41-0.66)
Peak Thrombin with 12-HETE-PC	0.64 (0.52-0.76)	0.55 (0.42-0.68)
Peak Thrombin with 12-HETE-PE	0.60 (0.42-0.72)	0.54 (0.41-0.66)
Velocity Index with 12-HETE-PC	0.65 (0.53-0.77)	0.55 (0.42-0.68)
Velocity Index with 12-HETE-PE	0.59 (0.47-0.72)	0.54 (0.42-0.67)

ROC curve area values for fold-change in thrombin generation are shown pre and post-CPB with 95% confidence intervals.

4.3 Discussion

As might have been anticipated from previous reports of reduced platelet responses to thrombin and collagen post-CPB, the ability of platelets to externalise PE, PS and 12-HETE-PE in response to thrombin was reduced. This implies either a reduction in flippase activity or a reduction in scramblase activity, or both. The ability to synthesise 12-HETE-PE was also diminished and there was a trend towards a reduction in synthesis of 12-HETE-PC. This finding suggests the activity of 12-LOX is reduced post-CPB. There were insufficient numbers of patients to make any conclusions about the risk of bleeding associated with these changes, although from the experience with patients with Scott's Syndrome, it can be implied that these changes would lead to a reduction in thrombin generation and consequently an increased risk of bleeding. The observed changes in phospholipids may explain why Bosch et al found significantly lower thrombin generation measured in platelet rich plasma (PRP) pre and post-CPB in patients who bled excessively, but only in post-CPB samples when measuring thrombin generation in platelet poor plasma (PPP) (169).

The ability of 12-HETE-PE and 12-HETE-PC to enhance thrombin generation supports previous findings (99). The lower fold-change in thrombin generation in those who went on to need haemostatic therapy for bleeding post-CPB suggests there may be differences in either the direct interaction of the coagulation factors of these individuals with the 12-HETE phospholipids, or that the 12-HETE phospholipids are influencing the way the coagulation factors interact with the other phospholipids. As the gla-domain containing clotting factors II,VII, IX and X require the presence of negatively charged phospholipids to interact with cell membranes (74) and thereby allow stable catalytic reactions to take place, changes in these lipids would affect this process and could in turn lead to changes in thrombin generation. FVIII and FV also have phospholipid binding sites located in their C-domains, and mutations induced affecting these sites have been shown to impair their pro-coagulant activity (189-191). Families with FIX mutations have also been described where the FIX activity has been widely different between different APTT reagents, where the only differences identified have been the phospholipid content of those reagents (192). However, the ROC analysis showed that only the fold-change in thrombin generation in response to 12-HETE-PC had any predictive value. However, this was only modest which is likely to be because almost half of the patients received some form of haemostatic treatment. Therefore the overall clinical value of this observation is limited and would need further investigation in an independent cohort of patients.

Other types of lipid, not measured in these experiments, have been reported to influence coagulation such as sphingolipids (193). The degree of saturation of the fatty acid chains of phospholipids has also been shown to influence thrombin generation, with Kung et al reporting that thrombin generation was around twenty times lower when measured using

liposomes composed of PS and PC species that were fully saturated compared to those that were unsaturated (194). Others have reported similar findings (195). Therefore there may be changes in other lipids present in the platelet membrane that influence coagulation and hence bleeding that have not been accounted for in these experiments.

The technical aspects of the thrombin generation assays reported in this chapter suggest that firstly freeze-thawing improves incorporation of tissue factor into liposomes. Incorporation of tissue factor into liposomes using freeze-thawing has previously been reported to result in its random distribution between the external and internal membrane (196), although no studies have reported on the effects on thrombin generation. Freeze-thawing has also been shown to result in a more uniform diameter of liposomes, which may be another contributing factor by providing a larger surface area with a more even distribution of lipids upon which thrombin generation can take place (197). Secondly, lower pH or sodium chloride concentration also enhanced thrombin generation. Whether this relates to incorporation of tissue factor into the membrane or not is uncertain. It has previously been reported that lower sodium chloride concentrations in thromboplastin reagents reduce their sensitivity to lower levels of prothrombin and FV, but had no effect on their sensitivity to FVII or FX (86). It also been reported that increasing sodium chloride concentrations also increase liposome diameter (197) which may result in the size of liposome becoming less favourable to the assembly of the prothrombinase complex due its components binding further apart from each other. Another consideration is that thrombin has a sodium ion binding site. However, this is less likely to explain the experimental findings because binding of sodium ions results in enhanced substrate binding and catalytic activity, leading to enhanced procoagulant activity (198). This would be expected to

increase thrombin generation rather than cause the apparent impairment that was observed.

Overall these experiments indicate the need for further investigation to provide a clearer indication of the normal range for the phospholipids described herein and also to identify the effect of CPB on other platelet membrane lipids. Further investigation is also warranted into polymorphisms in phospholipid binding domains of coagulation and anticoagulant factors.

Chapter 5 The effect on thrombin generation of adding FFP, fVIIa, PCC and inhibiting TFPI *in vitro*

5.1 Introduction

For patients who bleed due to impairment of thrombin generation, the currently available haemostatic options consist of fresh frozen plasma (FFP) and the off-label use of recombinant FVIIa and Prothrombin Complex Concentrate (PCC). FFP is the most commonly used and readily available way to replace coagulation factors. However, the volume of FFP needed to produce a clinically relevant increase in clotting factors has been reported to be as much as 30 mL/kg (124). In the context of surgery involving CPB, this constitutes a considerable volume load in a group of patients who may already have compromised cardiac function. In addition the transfusion of plasma can be complicated by transfusion related acute lung injury, and transfusion of larger the volumes FFP have been associated with an increased risk of stroke in some studies (199).

rFVIIa has been used off label and in clinical trials in the setting of massive haemorrhage but evidence to demonstrate efficacy and safety has been limited (200-202). Two recent meta-analyses were unable to demonstrate a reduction in mortality but did find an increased incidence of thrombotic events (128, 129) . Furthermore the optimal dose is unknown, and lower doses than those used to treat haemophilia-relating bleeding may be preferable to minimise any risk of thrombosis. Successful off label use of PCCs to manage excess bleeding following cardiac has been reported in a number of studies (131-133). However, the optimal dose is unknown and

disseminated intravascular coagulation has been reported when using high concentrations in a porcine trauma model (203), although this may relate to the type of PCC used.

Tissue Factor Pathway Inhibitor (TFPI) is an important regulator of the initiation phase of coagulation. Heparin, the principal anticoagulant used during CPB, induces the release of TFPI from the endothelial surface (64). *In vitro* studies have shown that in the presence of TFPI the rate of thrombin generation is reduced in a concentration dependent manner (165). Recently a number of studies have reported using TFPI-inhibition to prevent bleeding in patients with severe haemophilia A and FVIII inhibitors (204, 205). This raises the possibility that inhibition of TFPI may be a therapeutic target in treating bleeding in other circumstances, including cardiac surgery. The experiments described in this chapter sought to identify the most effective haemostatic agents to correct thrombin generation.

5.2 Results

Samples from the same patients recruited in Chapter 3 were used for these experiments. Ten patients were excluded from analysis because they had heparin anti-Xa levels greater than 0.3 anti-Xa units/mL in the post-CPB samples. This left 92 patients in the analysis.

CAT results are summarised for peak thrombin in Figure 5.1, ETP in Figure 5.2, lag time in Figure 5.3 and velocity index in Figure 5.4. There was a fall in peak thrombin, ETP

and velocity index in the post-CPB samples compared to pre-CPB, whilst lag time increased.

The addition of FFP resulted in a progressive, concentration dependent increase in peak thrombin, ETP and velocity index and shortening of the lag time. The largest effect was seen with a concentration equivalent to 30mL/kg. FFP at 15 mL/kg resulted in an increased peak thrombin to a level significantly greater than both pre and post-operative levels. Exceeding this volume resulted in a further dose-dependent increase. For rFVIIa, 45 µg/kg was sufficient to increase peak thrombin to preoperative levels, but not above preoperative levels, and exceeding this dose produced no further improvement. PCC at 25 units/kg resulted in peak thrombin greater than that seen pre-operatively. There was no increase in peak thrombin between 25 units/kg and 35 units/kg, but there was a small but statistically significant difference comparing doses of 25 units/kg and 50 units/kg. Inhibition of TFPI resulted in a peak thrombin concentration that was much higher than the pre-operative level. Results for ETP were similar to peak thrombin.

FFP at a dose of 15 mL/kg was sufficient to correct the velocity index to pre-operative levels. Exceeding this dose resulted in increases statistically significantly greater than pre-operative levels. For rFVIIa and PCC, 45µg/kg and 25 units/kg respectively were sufficient to return the velocity index to preoperative levels. Exceeding these doses produced no further increase. Inhibition of TFPI produced a dramatic increase in velocity index well exceeding the pre-operative level. Similar results were obtained for

the lag time with the exception that it required 30 mL/kg of FFP before the lag time corrected to pre-operative levels.

Full length TFPI measured post-CPB was inversely correlated with ETP, peak thrombin and velocity index in the absence and presence of all concentrations of FFP, rFVIIa and PCC, the weakest correlation being seen with FFP. Thrombin generation in the presence of the anti-TFPI antibody did not correlate the full length TFPI concentration. Figure 5.5 summarises these findings for ETP. Results for peak thrombin and velocity index were similar.

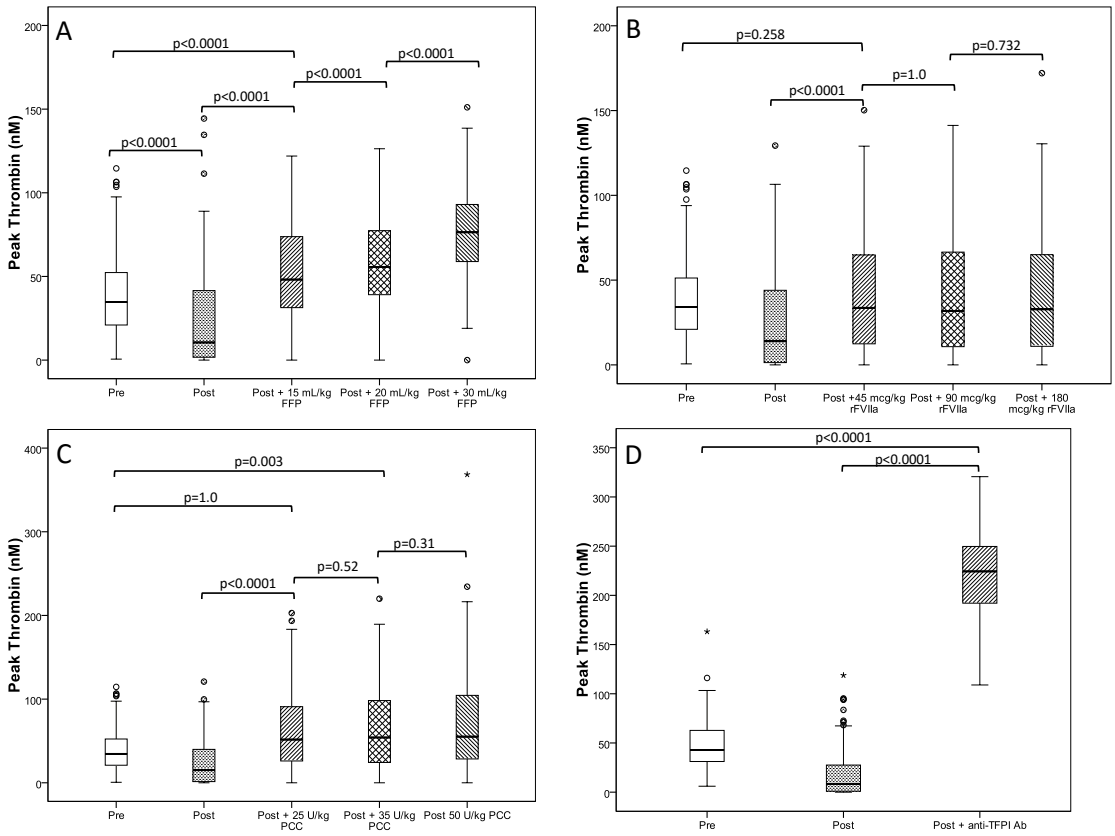


Figure 5.1 Effect on peak thrombin of adding increasing concentrations of FFP (A), rFVIIa (B), PCC (C) and in the presence or absence of an anti-TFPI antibody. Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles). P values shown are for the Wilcoxon ranks test for comparison between pre and post samples, and Friedman's test for comparison between samples at the same time point (i.e. post samples).

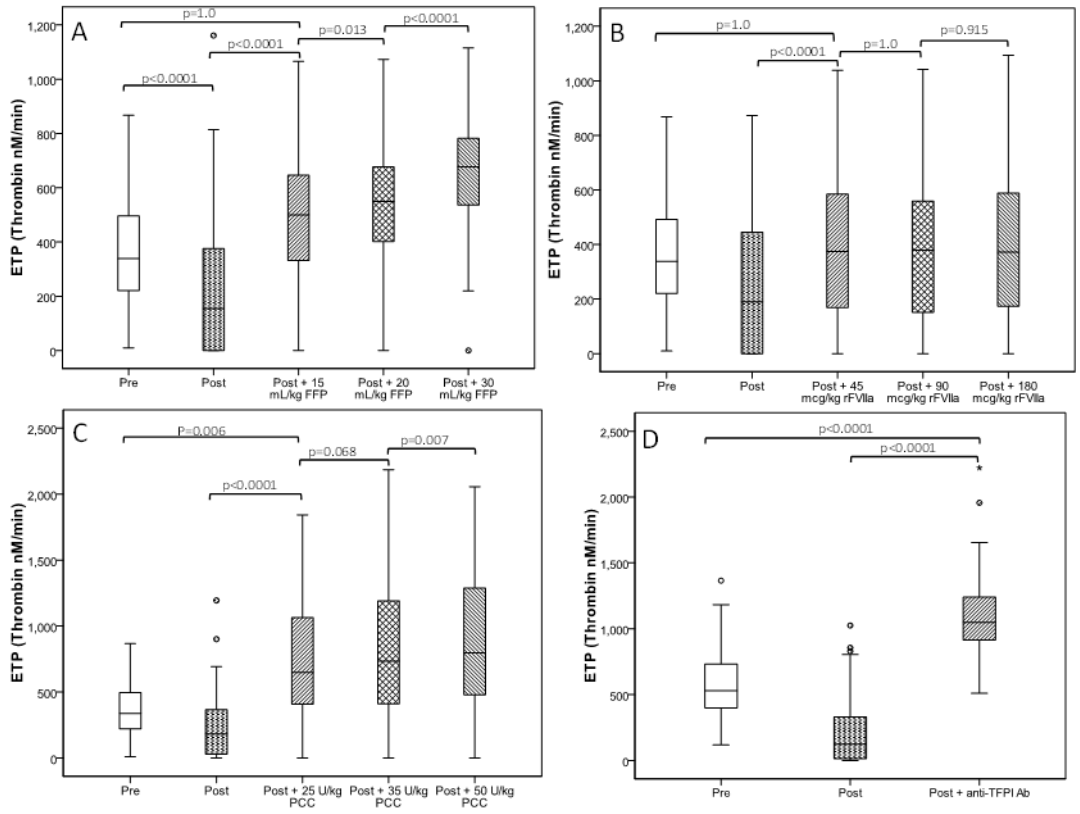


Figure 5.2 Effect on ETP of adding increasing concentrations of FFP (A), rFVIIa (B), PCC (C) and in the presence or absence of an anti-TFPI antibody. Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles). P values shown are for the Wilcoxon ranks test for comparison between pre and post samples, and Friedman's test for comparison between samples at the same time point (i.e. post samples).

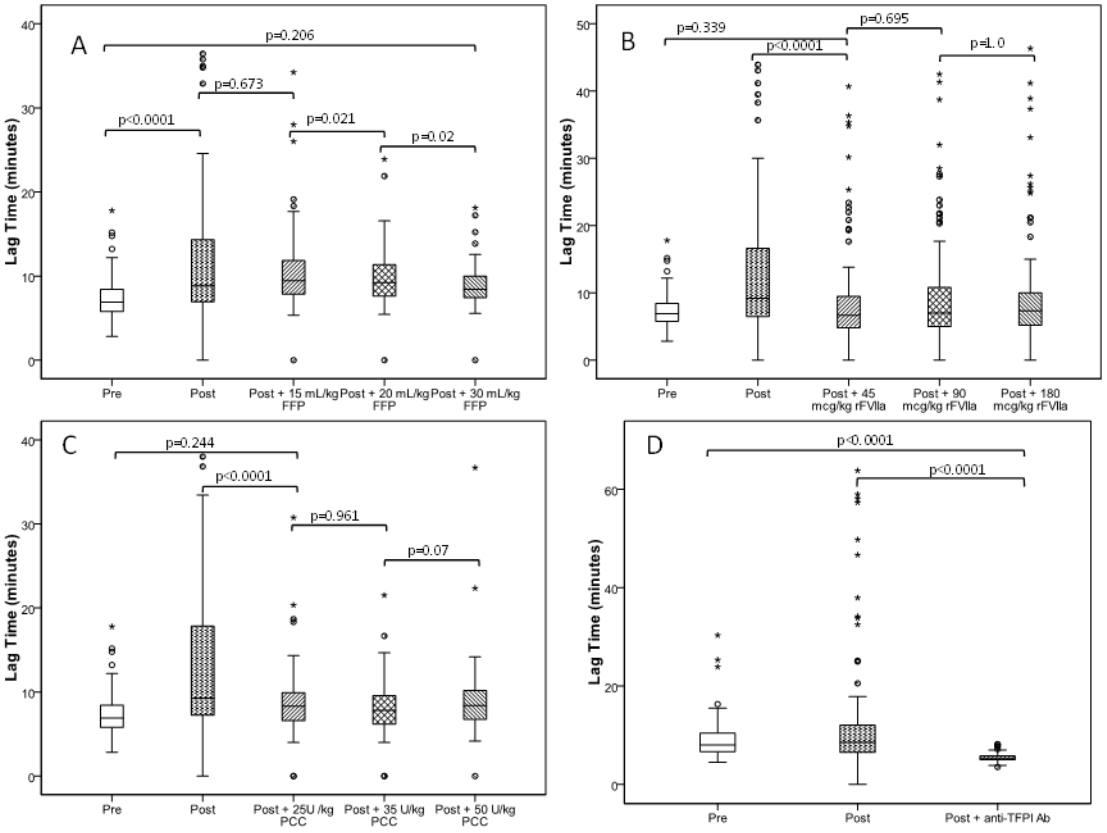


Figure 5.3 Effect on lag time of adding increasing concentrations of FFP (A), rFVIIa (B), PCC (C) and in the presence or absence of an anti-TFPI antibody. Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles). P values shown are for the Wilcoxon ranks test for comparison between pre and post samples, and Friedman's test for comparison between samples at the same time point (i.e. post samples).

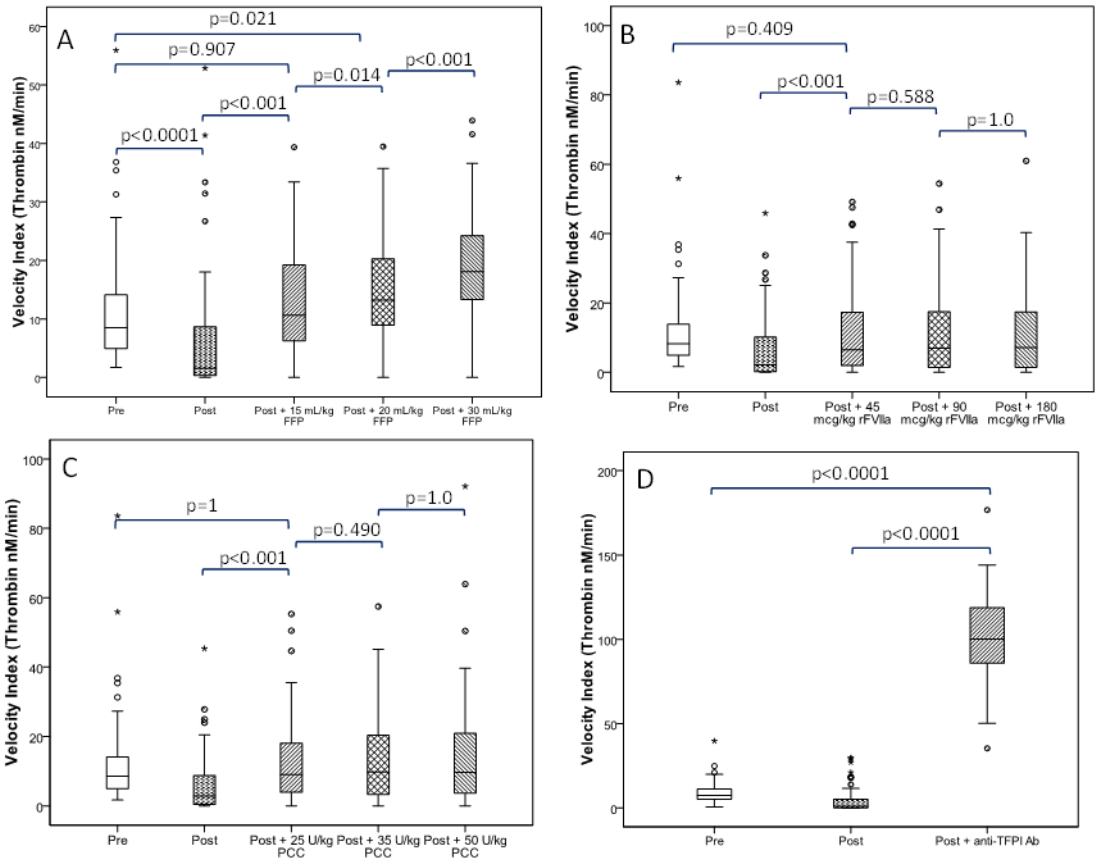


Figure 5.4 Velocity Index in response to increasing concentrations of FFP (A), rFVIIa (B), PCC (C), and inhibition of TFPI (D). Data shown are median (horizontal black bar), interquartile range (box), values with 1.5 times interquartile range (whiskers) and values between 1.5-3 times interquartile range (asterisks and circles). P values shown are for the Wilcoxon ranks test for comparison between pre and post samples, and Friedman's test for comparison between samples at the same time point (i.e. post samples).

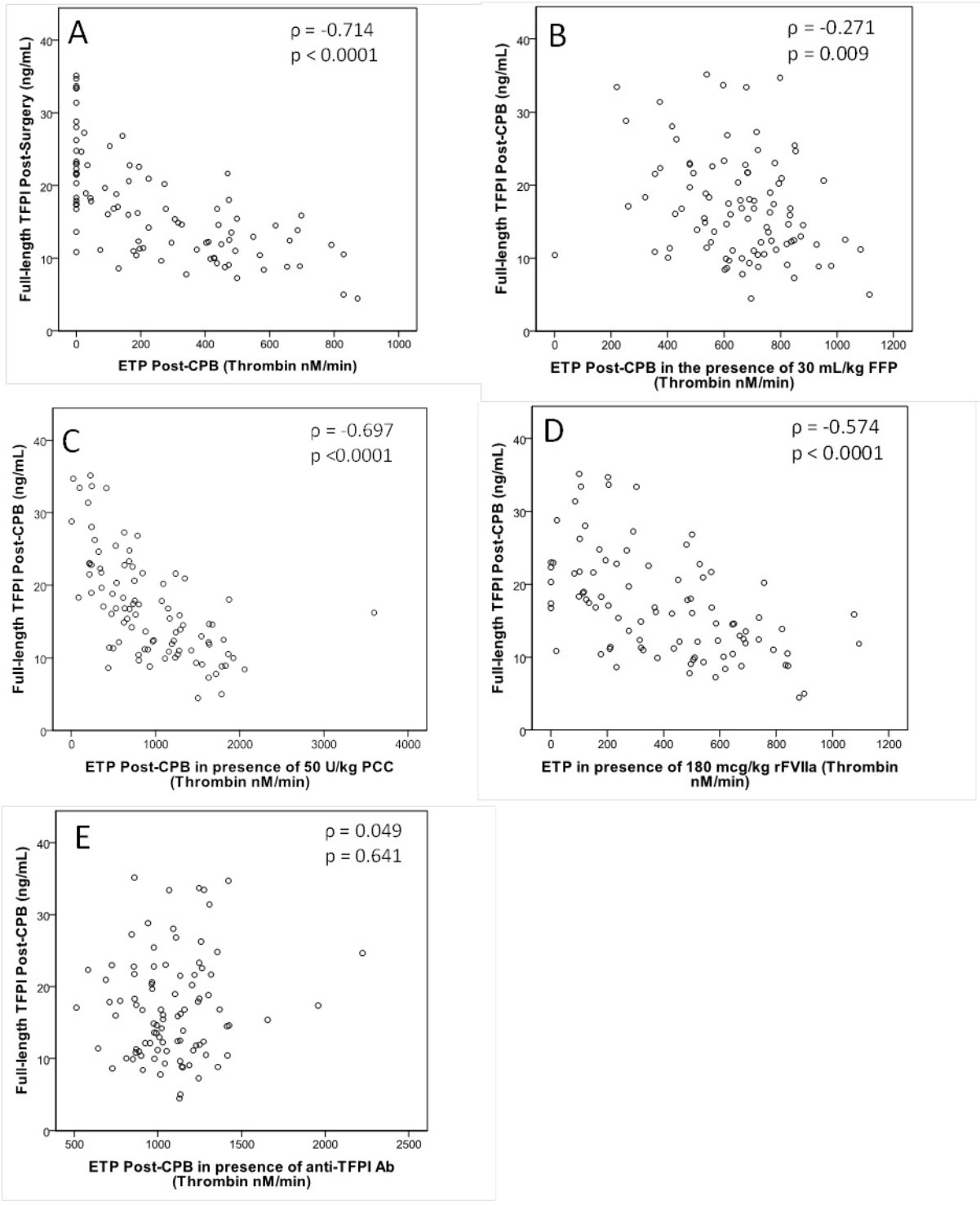


Figure 5.5 Scatter plots comparing full-length TFPI concentration post-CPB with ETP post-CPB in patient plasma alone (A), or with the addition of FFP(B), PCC (C), rFVIIa (D) and an anti-TFPI antibody (E). Spearman's correlation coefficients (ρ) are shown with p- values.

5.3 Discussion

The results show that 15 mL/kg of FFP was sufficient to correct ETP, peak thrombin and velocity index to pre-CPB levels, but 30 mL/kg was required to correct the lag time. Larger volumes of FFP improved thrombin generation above baseline. rFVIIa at a dose of 45 μg/kg was sufficient to correct all measured thrombin generation parameters to pre-CPB levels but there was no further improvement by exceeding this dose. Similarly, 25 units/kg of PCC was required for the same effect and 50 units/kg enhanced thrombin generation further. Inhibition of TFPI markedly increased the ETP, peak thrombin, and velocity index beyond pre-operative levels and shortened the lag time.

FFP remains the main treatment available to most clinicians for correcting any coagulopathy. The optimum dose of FFP to return the majority of thrombin generation parameters to pre-CPB levels was 15 mL/kg. Some studies have reported a correlation with lag time and bleeding (206) and 30 mL/kg was required to achieve this. The full-length TFPI concentration correlated with all thrombin generation parameters post-CPB and in the presence of FFP, rFVIIa and PCC. This supports the view that TFPI is an important determinant of thrombin generation. FFP had weaker correlation with thrombin generation parameters than rFVIIa and PCC. This may be because FFP contains both pro-coagulant factors and inhibitors and would tend to normalise both where rFVIIa and PCC only affected the pro-coagulant pathways. There was no correlation between full length TFPI and thrombin generation in the presence of an anti-TFPI inhibitor further supporting the importance of this inhibitor in thrombin generation assays activated with low concentrations of TF.

The enhancing effect of FFP observed on thrombin generation may be overestimated by the *in vitro* nature of the assay. Patient plasma was contacted inactivated with CTI and so after addition of rFVIIa, PCC and TFPI antibody thrombin generation was activated through TF. In contrast, the added FFP was not CTI inhibited and would therefore have been prone to contact activation in addition to TF activation. Contact activation has previously been shown to result in higher ETP and peak thrombin and shorter lag time when using lower concentrations of TF as used in these experiments (156, 207).

rFVIIa made no significant difference to thrombin generation parameters beyond doses of 45µg/kg, indicating that lower doses are as effective as higher doses in enhancing thrombin generation *in vitro*. This would be predicted given the global reduction in clotting factors observed in this patient cohort. A similar result was observed by Altman and colleagues (208) using a model of dilutional coagulopathy. This suggests that rFVIIa may be inappropriate to use in isolation to treat bleeding. Whilst there are a number of studies which have reported safety using rFVIIa in paediatric patients undergoing CPB (209), some studies have reported an increase in thrombotic events in adult patients (168). Studies looking at adult patients from a broader population have also shown an increase in arterial events (210), something that would be particularly deleterious in the typical adult patient population undergoing cardiac surgery. Therefore, based on these *in vitro* assays, if rFVIIa is used, lower doses may offer the best balance of achieving a beneficial effect whilst possibly reducing the chance of an adverse event.

PCC corrected thrombin generation parameters to pre-operative levels at a concentration of 25 units/kg. There was some further statistically significant improvement at concentrations of 50 units/kg although this was so small it is unlikely to be clinically significant. This finding is consistent with other *in vitro* studies where PCC has been shown to enhance thrombin generation (211). *In vivo* PCC has been shown to be effective in reducing blood loss in human studies (132, 212). As well as different numbers of clotting factors, different PCCs contain different amounts of heparin, with some containing more heparin than the PCC used in this study, which in turn has been shown to affect thrombin generation (130). While a four factor PCC was used in this study, others have shown similar effects on thrombin generation using a 3 factor PCC (211). PCC has the advantage of additional safety over FFP for transmission of infection and smaller volumes in patients with cardiac compromise. However, large doses have been associated with disseminated intravascular coagulation in some studies (203).

Inhibition of TFPI had a marked effect on enhancing thrombin generation. TFPI *in vivo* is increased by administration of heparin (64), the anticoagulant routinely used in CPB and therefore may be a good target in this group of patients. TFPI inhibition has been reported to enhance thrombin generation in plasma from people with haemophilia (204). However, a number of studies have reported higher ETP and peak thrombin concentration in patients with pro-thrombotic tendencies (213-215). This suggests caution may be required if TFPI inhibition was to be used as a therapeutic target especially in cardiac patients where arterial thrombosis is often already a significant risk.

A weakness of studies investigating thrombin generation in plasma is that it takes no account of other important factors that require correction in controlling bleeding following cardiac surgery: fibrinogen concentration, platelet count and function and fibrinolysis. A low fibrinogen has been associated with excessive post-operative bleeding in a number of studies (105, 183). Thrombocytopenia has also been described as a risk factor for bleeding following CPB (11, 184). Clot formation and durability requires sufficient thrombin to cleave fibrinogen to fibrin, FXIII to cross-link fibrin monomers, platelets and reduced fibrinolysis. Therefore correction of thrombin generation forms only one component in the management of bleeding.

In summary these results suggest that comparatively low doses of FFP, rFVIIa and PCC may be sufficient to correct thrombin generation in patients who have undergone surgery requiring CPB. Inhibition of TFPI may offer a future therapeutic strategy for managing bleeding in this group of patients. However, these *in vitro* results will need to be validated *in vivo*.

Chapter 6 Modelling the effect of changes in lipids and coagulation factors on in vitro thrombin generation

6.1 Introduction

The results presented thus far demonstrate that whilst coagulation and anticoagulant factor concentrations change between pre and post-CPB, there are also changes in the phospholipids that platelets generate and externalise. This raises the question whether if these measured changes were accounted for in the thrombin generation assay, this would lead to significant differences in the thrombin generation parameters between groups of patients who bled excessively.

The inability of platelets to externalise phospholipids reduces thrombin generation as demonstrated in patients with Scott's Syndrome, where thrombin generation is reduced compared to normal controls (216). An expectation that there may be differences in thrombin generation between pre and post-CPB liposomes is inferred by the findings of Bosch et al (169). They reported that when thrombin generation was measured in PRP there was a statistically significant difference between those who bled and those who did not, in samples taken both pre and post-CPB. This finding, coupled with the results presented in Chapter 4, suggests that differences in thrombin generation might be observed due to changes in platelet phospholipids.

Another factor that may influence thrombin generation is the source of tissue factor. Current concepts of coagulation are based upon the initiation phase of coagulation taking place on a tissue factor expressing surface, before the reaction then moves to the surface of an activated

platelet. Under normal circumstances *in vivo*, the cells expressing tissue factor would be in the sub-endothelial matrix, such as smooth muscle cells, fibroblasts and macrophages. Monocytes are also able to express tissue factor and this can be measured through sampling of the peripheral blood. Following CPB, TF expression on leucocytes has been reported to increase (217, 218). The monocyte count has also been reported to increase (219). It has also been shown that in a model of cardiopulmonary bypass, monocyte activation markers and tissue factor expression increases (220). Monocytes can also support thrombin generation on their cell membrane through exposure of negatively charged phospholipids on activation (221, 222). However, it is generally considered that monocytes and other tissue factor expressing cells provide the surface for the initiation of coagulation after which the propagation phase moves to the platelet membrane (223, 224). Therefore inclusion of monocytes in the thrombin generation assay might increase the information provided by better reflecting these different stages.

Monocytes can be induced to express tissue factor using lipopolysaccharide (LPS) (225, 226). Monroe et al have previously reported on a model for thrombin generation using LPS stimulated monocytes as a source of tissue factor (227). In this model, washed platelets, purified factors II, V, VIIa, VIII, IX and X, along with TFPI and antithrombin were added to wells of a cell culture plate containing monocytes. Thrombin generation was then measured by repeatedly sampling the supernatant and using a chromogenic assay. Such a model is laborious and due to the constant loss of substrate may result in a misrepresentation of true thrombin generation. If it were adaptable to continuous measurement of thrombin generation using a method such as that of Hemker et al (154), such a model might offer opportunities to further explore the interaction of coagulation factors and phospholipids under conditions more relevant to the *in vivo* situation.

The objectives of the experiments described in this chapter were firstly to identify whether there were any differences in thrombin generation using liposomes reflecting pre and post-CPB phospholipid proportions. The second objective was to investigate the feasibility of developing a model of thrombin generation using purified coagulation factors and monocytes as a source of tissue factor, similar to that described by Monroe et al (227). Platelets were replaced by liposomes composed of phospholipids reflecting changes observed pre and post-CPB. Protein S was included because of its activity as a cofactor for TFPI (228, 229).

6.2 Results

The total amounts of the different species of PE, PS, PC, 12-HETE-PE and 12-HETE-PC measured in thrombin stimulated platelets pre and post-CPB in Chapter 4 were converted into moles and then summed to give a figure for the total number of moles of phospholipid measured. The proportions of this number contributed to by the total moles of PE, PS, PC and the respective 12-HETE phospholipids were then expressed as a percentage and liposomes reflecting this were constructed with 60 pM rTF to achieve a final in-well concentration of 4 μM phospholipids and 10 pM rTF. The proportions of the phospholipids are shown in Table 6.1. There was a reduction in the proportion of PC post-CPB, which may reflect a loss of membrane during CPB.

Table 6.1 Proportions of phospholipids measured pre and post-CPB and used to construct liposomes.

Phospholipid	Pre-CPB (%)	Post-CPB (%)
PE	50.85	52.93
PS	2.99	2.99
PC	46	44
12-HETE-PE	0.06	0.02
12-HETE-PC	0.1	0.06

(PE= phosphatidylethanolamine; PS= phosphatidylserine; PC= phosphatidylcholine; 12-HETE-PE= 12-hydroxyeicosatetraenoic-PE; 12-HETE-PC= 12- hydroxyeicosatetraenoic-PC).

Thrombin generation assays were then performed in pooled normal (4 donors) PPP collected into citrate and CTI. Thrombin generation curves showed subtle differences between pre and post-CPB liposomes (Figure 6.1 A). Peak Thrombin and velocity index were lower using the post-CPB liposome, although these differences were not statistically significant (Figure 6.1 B-E). Lag time was slightly shorter using the post-CPB liposome (mean 7.94 minutes, range 6.11 to 10.11 minutes pre; mean 7.69 minutes, range 6.56 to 9.22 minutes post) whilst the ETP was slightly higher, but again these differences were not statistically significant.

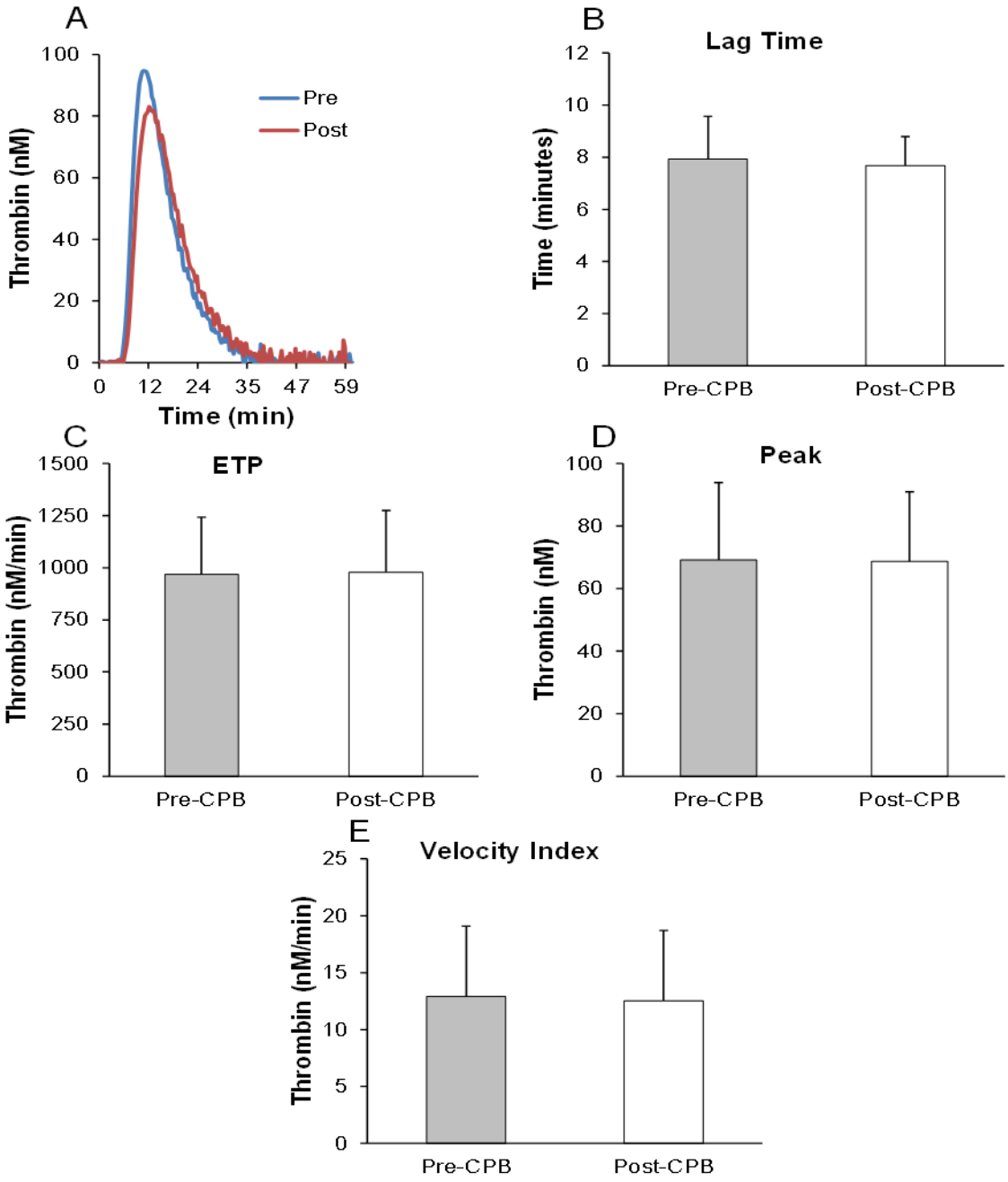


Figure 6.1The effect on thrombin generation of using liposomes reflecting measured phospholipid concentrations pre and post-CPB. Panel A shows a representative thrombin generation curve from a single patient. Panels B-E show the mean values for the thrombin generation parameters. Error bars represent the standard deviation (n=5).

However, these differences when coupled with observed changes in coagulation factor and anticoagulant concentrations might result in clearer differences in thrombin generation parameters between those who did and did not bleed excessively.

6.2.1 Monocyte based thrombin generation model

A tissue factor expressing surface, provided *in vivo* by activated monocytes and other cells in the sub-endothelium, is considered to be the initiator of coagulation. Therefore to try to replicate this, a model was designed where monocytes were used to coat the wells of a 96-well plate as described in Chapter 2 (Materials and Methods). After incubation for 18 hours in RPMI media with or without LPS, the media was removed, and liposomes were added followed by pooled normal PPP. Thrombin generation was then measured using calibrated automated thrombography (with all conditions measured in quadruplicate). The first series of experiments aimed to establish the optimal cell concentration to use for coating the wells and the concentration of LPS to use for stimulation of the monocytes to induce tissue factor expression. Increasing concentrations of cells in the original cell suspension resulted in an improvement in the coefficient of variation (CV) for all thrombin generation parameters (Figure 6.2 A-D). The $1x10^7$/mL cell concentration produced the lowest CV, therefore this concentration was selected for the next series of experiments. Thrombin generation was then measured after stimulating the cells with varying concentration of LPS. In absence of LPS there was considerable variation between wells (in excess of 20 %) in all thrombin generation parameters apart from lag time, where the CV was less than 10%. In the presence of all concentrations of LPS, the CV was below 10%, with overall 0.5 µg/mL showing the least variation (Figure 6.2 E-H).

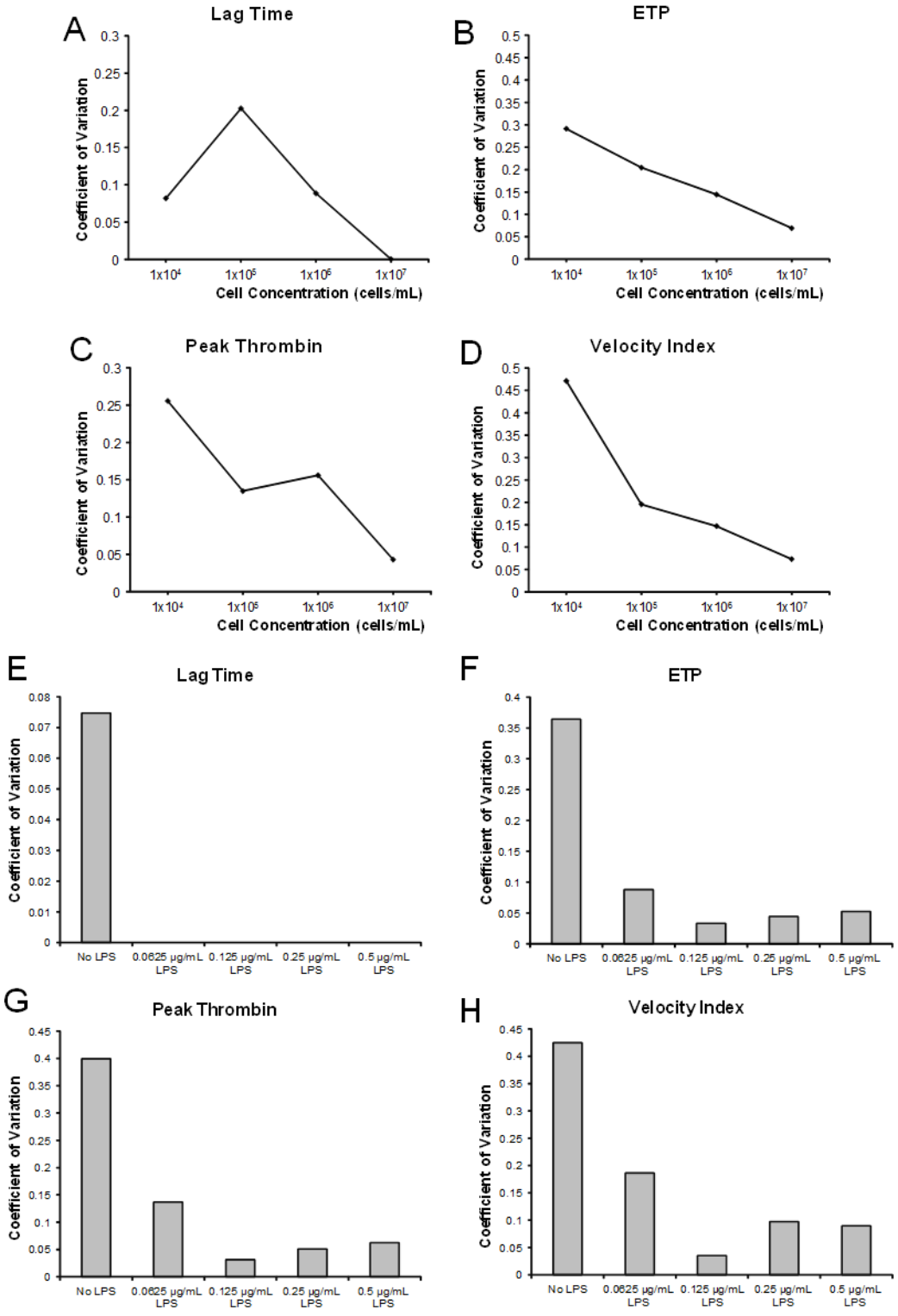


Figure 6.2 Intra-assay coefficients of variation in thrombin generation parameters using different concentrations of cells (A-D), and different concentrations of LPS (E-H). Panels A-D and E-H represent separate experiments (n=1 in each case, each condition performed in quadruplicate).

Adhesion to the culture plate was used to select monocytes in this model. Whilst this is a widely used method to select monocytes, the concentration of cells in the original cell suspension did not necessarily indicate the number that had adhered to the plate and were present for the thrombin generation measurement. In an attempt to obtain a measure of how evenly cells were distributed between wells, the cells were fixed, nuclear material was stained using crystal violet and the cells where then lysed. Light absorbance at 590 nm was measured. Absorbance was higher (indicating a greater number of cells present) in wells where the cells had been incubated with media without LPS, but the standard deviation was lower than in the wells not exposed to LPS (Figure 6.3).

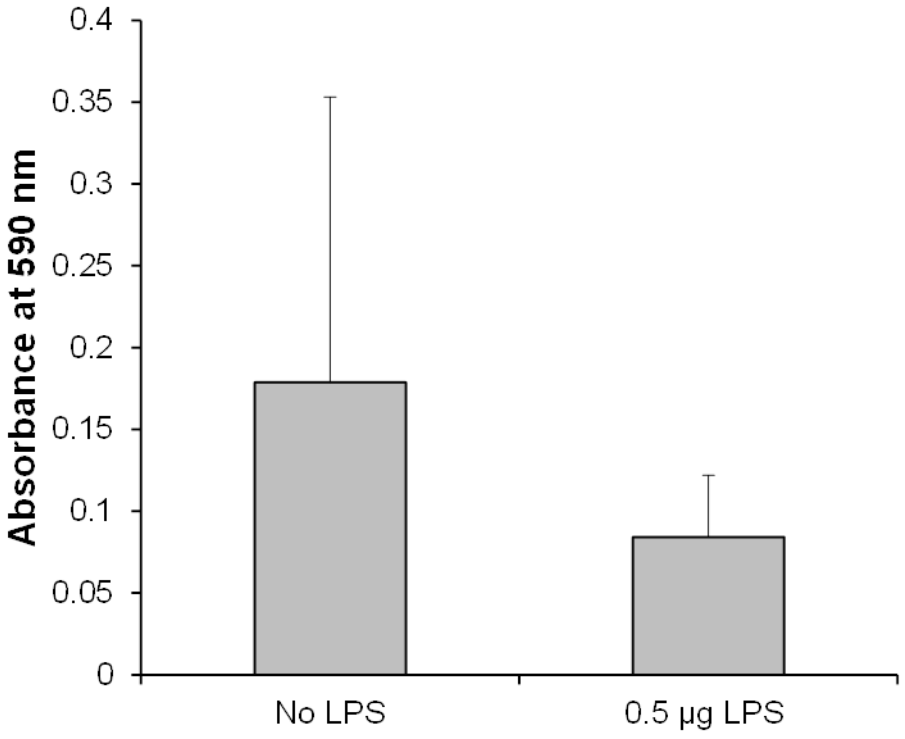


Figure 6.3 Light absorbance at a wave length of 590 nm measured from cell lysates after staining with crystal violet. Absorbance was lower in wells exposed to LPS, suggesting fewer cells were present, although the standard deviation was lower indicating less variation between wells. Columns represent the mean readings, error bars the standard deviation (n=3).

The next series of experiments used plates coated with cells from a cell suspension with a concentration of $1x10^7$ cells per mL and exposed to 0.5 μg/mL LPS. Thrombin generation increased in response to LPS compared to wells containing monocytes that were not exposed to LPS (Figure 6.4 A). However, even in the absence of liposomes there was still considerable thrombin generation with LPS stimulated monocytes (Figure 6.4 B). Whilst there was a trend toward the ETP and peak thrombin concentration being lower using the post-CPB liposome, there was no statistically significant difference between pre and post-CPB liposomes (Figure 6.4 B-E).

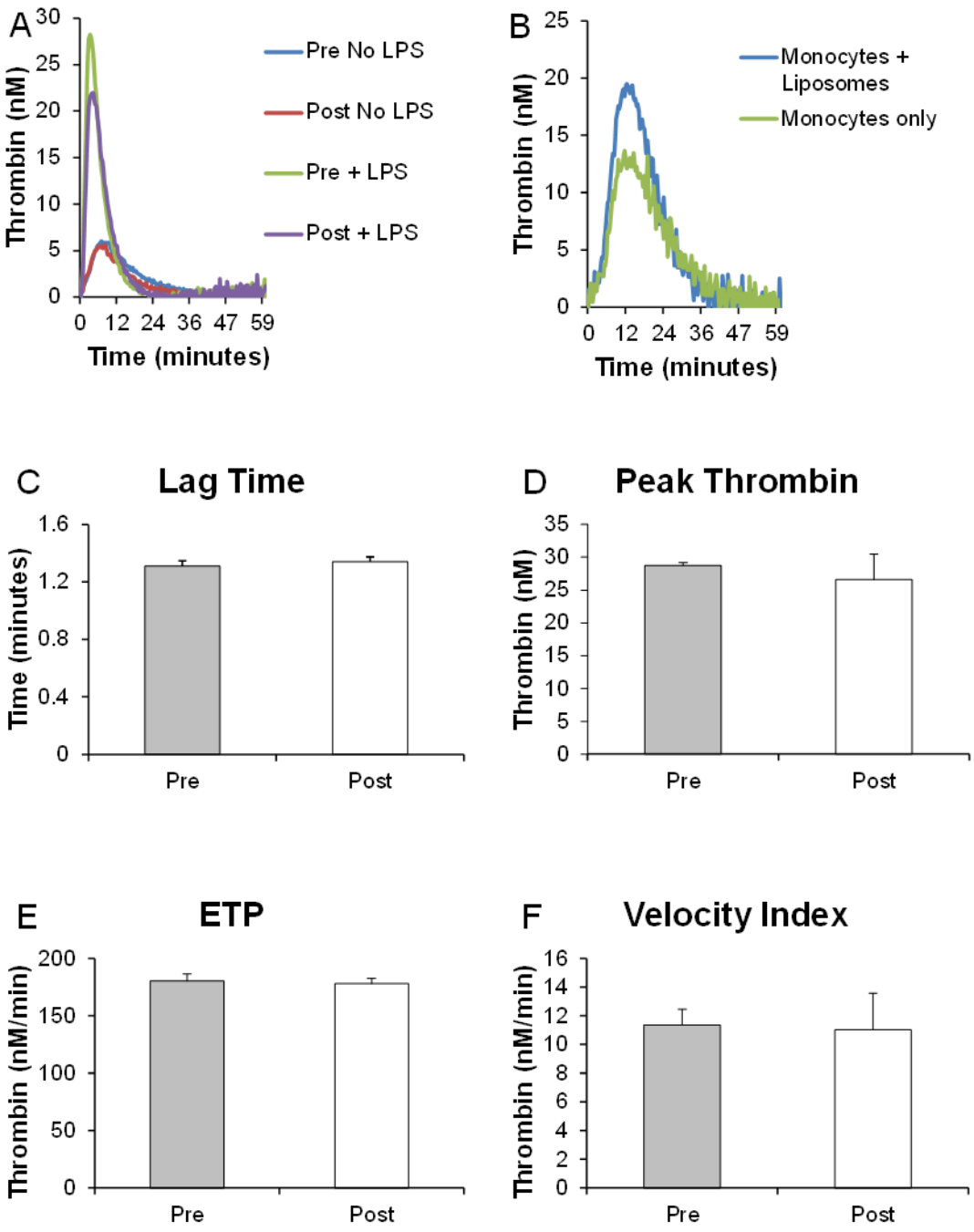


Figure 6.4 Thrombin generation parameters measured using monocytes stimulated with 0.5 µg/mL LPS, liposomes reflecting phospholipids measured pre and post-CPB, and pooled normal PPP collected into CTI/citrate. Panel A shows a representative thrombin generation curve; even in the absence of LPS stimulation, there was detectable thrombin generation. Panel B shows a representative thrombin generation curve comparing monocytes stimulated with LPS in the presence and absence of liposomes. Panels C-F summarise the thrombin generation parameters measured. There was no significant difference between pre and post-CPB liposomes. Columns represent mean and error bars the standard deviation (n=3).

6.2.2 Thrombin generation model using purified coagulation factors and anticoagulants

The monocyte model appeared unlikely to be sensitive enough to detect subtle differences in thrombin generation. Therefore this model was abandoned in favour of one where median concentrations of FII, FV, FVII, FVIII, FIX, FX, protein S, antithrombin and full length TFPI were added to wells of a 96 well plate to replicate the levels measured pre and post CPB in the following: those who did or did not bleed >1 L; those who did or did not bleed >2 mL/kg/hr; those who did or did not need haemostatic treatment. These endpoints were chosen based on those endpoints where there had either been a significant difference in coagulation or anticoagulant factors or thrombin generation parameters.

The ETP, peak thrombin concentration and velocity index were lower in the post-CPB setting for all end points, whilst the lag time increased (Figure 6.5). However, there was no significant difference between conditions reflecting those who did and did not bleed.

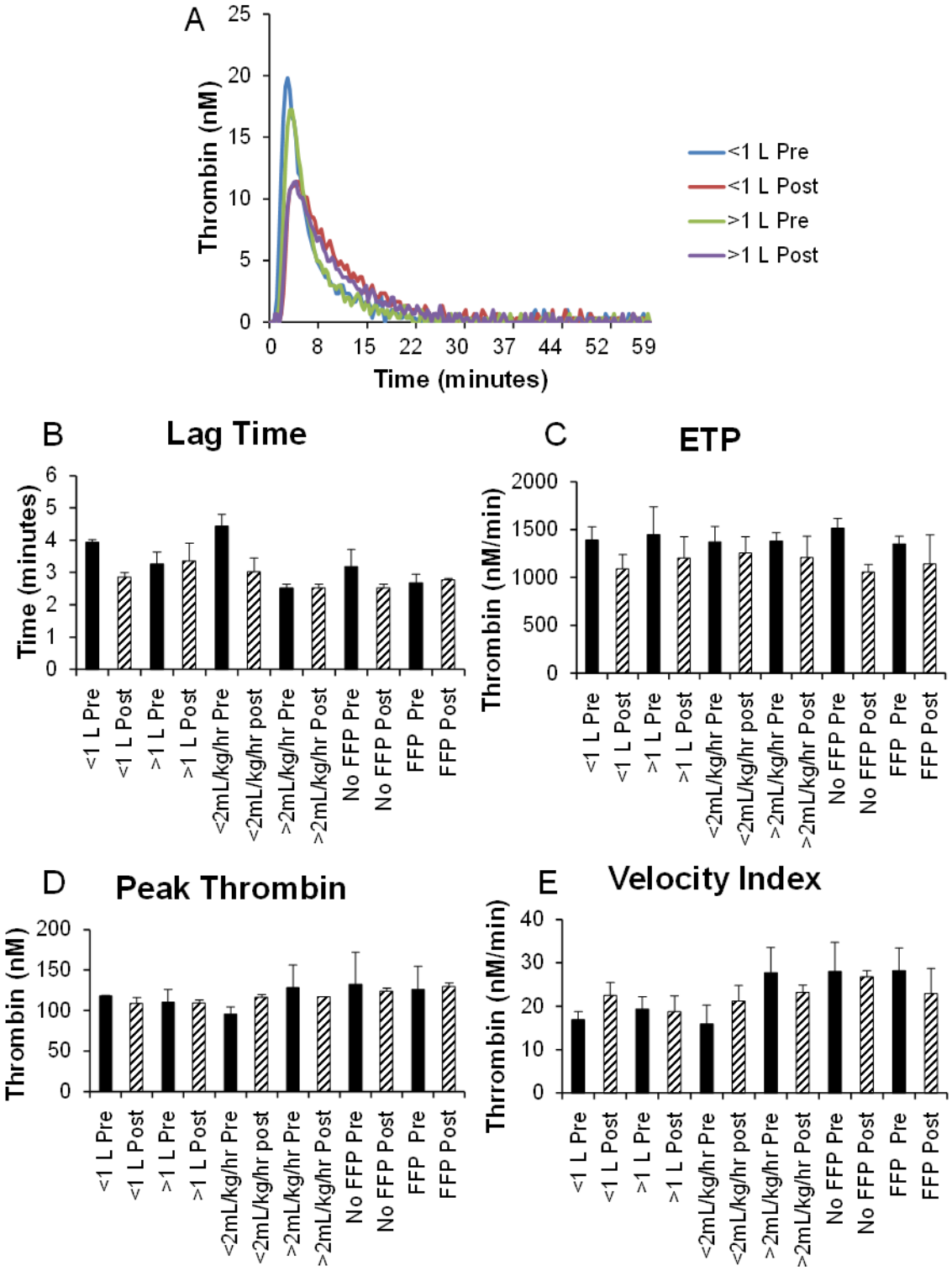


Figure 6.5 Thrombin generation parameters measured in a system containing liposomes reflecting measured phospholipids pre and post CPB, containing 10 pM rTF (final in-well concentration), and median concentrations of II, V, VII, VIII, IX, X, AT, PS and full length TFPI. There was no significant difference between any parameter for any of the bleeding end points listed (n=3).

6.3 Discussion

The absence of any demonstrable difference in these experiments in thrombin generation parameters between bleeding end points may have a number of explanations. The monocyte model had a number of weaknesses, including uncertainty of how pure the original cell suspension was, and also the inability to directly quantify the number of cells adherent to the wells of the 96 well plate at the point of measuring thrombin generation. Whilst some of these problems could potentially be overcome by using flow cytometry to assess the purity of the cell suspension, it would still not be possible to be certain of the number of cells that remained adherent to the 96-well plate at the point of measuring thrombin generation. It may also be that with as much as 50% of the measured thrombin generation being contributed by the tissue factor expressing monocytes alone (Figure 6.4B), the limits of sensitivity of the thrombin generation assay had been reached and the chance of any small differences conferred by the addition of liposomes being measurable was low.

The absence of a significant difference in thrombin generation using the purified model reflects the absence of a difference observed using the standard liposomes described in Chapter 3. In addition this model excluded the contribution of variations in individual coagulation factors and also excluded other plasma proteases, such as protein Z, which may influence thrombin generation (230).

Finally, the liposomes used in both models were unable to fully replicate the function of platelets because of the lack of any ability to change membrane conformation or composition in response to activation. As shown in Chapter 4, it appears there are differences between individuals in how their coagulation factors and anticoagulant factors interact with oxidised phospholipids. There is published evidence demonstrating that variations in the C2 domains of

FVIII and FV alter their binding to phospholipids (231, 232), and there have also been reports of mutations in FIX influencing the results of the APTT depending on the phospholipid used (192). Ultimately measuring thrombin generation in PRP would be a superior method of taking into account changes in platelet phospholipids and variations in coagulation factors. However, this would make the clinical application of calibrated automated thrombography more difficult given the technical skills and staff time required to prepare and process PRP.

In conclusion, the experiments described in this chapter demonstrate the feasibility of modifying the calibrated automated thrombin generation assay to investigate thrombin generation in the setting of cell based tissue factor presentation. This model provides a basis to further investigate the influence of different TF expressing cells on thrombin generation.

Chapter 7 General Discussion

The primary objective of this thesis was to test the hypothesis that thrombin generation assays were useful in identifying patients at risk of increased bleeding post-CPB. A variety of definitions of bleeding were used: these were based on total blood loss at 24 hours, rate of blood loss, need for re-exploration or the need for haemostatic treatment. The basis of this hypothesis was two previous studies which found that calibrated automated thrombography in PPP measured post-CPB predicted bleeding (11, 169). In one of those studies, pre-CPB thrombin generation was also predictive of bleeding and it was this finding that provided the catalyst for this thesis (11). In contrast, the results presented in Chapter 3 show that thrombin generation measured in PPP using calibrated automated thrombography was unable to identify those at risk of bleeding, irrespective of the definition of bleeding, the concentration of tissue factor in the trigger solution and whether or not CTI was used to prevent contact activation.

The reason of the absence of a difference in this study may relate to the patient population. The patients in the previous studies either exclusively or predominantly underwent coronary artery bypass grafting as the sole surgical procedure, whereas the patients recruited in this thesis all underwent surgery to repair or replace heart valves or part of the ascending aorta. This type of surgery is more complex and is associated with a higher incidence of post-CPB bleeding compared to operations where CABG is the sole procedure (233, 234). Therefore it may be that in the lower risk setting of CABG, excessive bleeding post-CPB may be more influenced by impaired thrombin generation than in higher risk surgery where surgical bleeding is more frequent and bypass times are longer, which in itself is a risk factor for bleeding (181). Furthermore thrombin generation in PPP takes no account of other important

factors that have been associated with bleeding, depending on the definition used, both in this thesis and in other studies: the fibrinogen concentration (105, 183) and thrombocytopenia (11, 184). A further but minor aim was to investigate whether thrombin generation assays could be performed as a near patient test. As shown in Chapter 3, the assay is too complex and even with training there was a poor correlation between the results obtained from the central laboratory.

The use of thrombin generation to assess the effect of available haemostatic agents in correcting thrombin generation post-CPB was investigated in Chapter 4. The results show that 15 mL/kg of FFP was sufficient to correct the majority of thrombin generation parameters to pre-CPB levels, but 30 mL/kg was required to correct all. rFVIIa at a dose of 45 µg/kg was sufficient to correct all measured thrombin generation parameters to pre-CPB levels. Experiments using PCC showed that 25 units/kg was required for the same effect. Inhibition of TFPI not only corrected thrombin generation but enhanced it beyond the levels measured pre-CPB. TFPI inhibition is a theoretically attractive therapeutic strategy because it would be potentially quicker to administer and represent a smaller fluid load compared to FFP. However, increased thrombin generation has been reported in patients with pro-thrombotic phenotypes (213-215), so the risk of inducing thrombosis would need to be balanced against any potential benefit. This is especially so in cardiac patients where arterial thrombosis is often already a significant risk. Whilst these *in vitro* results need to be validated *in vivo* before they could be applied directly to patient management, they do provide a basis on which to select doses for use in such studies.

Traditional tests of platelet function such as light transmission aggregometry were not performed in this thesis. Reduced platelet aggregation in response to ADP following CPB has

previously been described in a number of studies (115, 118). Instead, a novel approach was taken to investigate the effect of CPB on platelet phospholipids. The data presented in Chapter 5 demonstrates that post-CPB platelets are less able to externalise PS and PE. As discussed in Chapter 1, proteins with flippase, floppase and scramblase activity are believed to control this process, therefore targeting these proteins may lead to new therapeutic strategies in managing bleeding and thrombosis.

The experiments in Chapter 5 also found that post-CPB the ability of platelets to externalise 12-HETE-PE and to synthesise 12-HETE-PC and 12-HETE-PE was decreased. This is likely to further impair haemostasis because they were shown to enhance thrombin generation, a finding consistent with previous studies (99). The finding that the effect of 12-HETE-PE and 12-HETE-PC in increasing thrombin generation was less in those who went on to need haemostatic therapy for bleeding post-CPB, suggests that either the direct interaction of the coagulation factors of these individuals with the 12-HETE phospholipids may be different, or that the in the presence of 12-HETE phospholipids the interaction of their coagulation factors with PE and PS was impaired. This explanation is supported by the finding that mutations in the phospholipid binding sites located in the C-domain of FVIII and FV impair their pro-coagulant activity (189-191) and that mutations in FIX which result in discrepant activity when measured using APTT reagents containing different phospholipids (192).

However, the differences observed were small and given that nearly half of patients needed haemostatic treatment, the value of this observation can be questioned. Equally it may be that the decision to give haemostatic treatment was based on the clinical team caring for the patient correctly responding to haemostatic failure. Giving FFP, which was received by all these patients, would correct any haemostatic defect due to the patient's own coagulation factors

interacting less effectively with phospholipids on the platelet surface and thus improve haemostasis. Whether this is a plausible argument rests not only on whether the observation can be repeated in an independent cohort of patients, but what the true prevalence of such mutations in coagulation factors are and what the normal range of phospholipid expression in platelets is in the wider population.

The effects of 12-HETE-PE and 12-HETE-PC in enhancing thrombin generation also indicate the importance of carefully considering the composition of phospholipids used in any haemostatic assay. When this is coupled with the other findings reported in Chapter 5 of the effects of source of tissue factor, the use of freeze-thawing in liposome production and the effects of pH and sodium chloride concentration on thrombin generation, the importance of carefully considering how the choice of experimental conditions determine the results obtained can be clearly seen.

The experiments reported in Chapter 6 attempted to address whether using liposomes reflecting the proportions of the phospholipids measured in Chapter 5 would influence the ability of calibrated automated thrombography to identify patients at risk of bleeding post-CPB. As there were insufficient volumes of patient plasma remaining this necessitated using purified human coagulation factors and anticoagulants to model a "typical patient." Based on the likelihood that *in vivo* variations in the interaction of these proteins with phospholipids influence thrombin generation it is unsurprising that the experiments were unable to demonstrate any differences. However, the experiments where monocytes were used to provide tissue factor represent a novel adaptation of calibrated automated thrombography that allows the real-time measurement of thrombin generation initiated by tissue factor expressing cells. This model provides the possibility to investigate whether differences exist in

the amount of thrombin generated between different cells types. Theoretically this could allow an individualised thrombin generation assay utilising plasma, platelets and tissue factor producing cells from the same patient, although this would be highly labour intensive.

This thesis has raised a number of questions which require further work. Firstly a larger study investigating the effect of CPB on phospholipid externalisation and oxidised phospholipid synthesis is required to validate these findings in an independent patient cohort and provide a population wide overview. This also needs to be combined with genomic studies looking for polymorphisms in coagulation factor and anticoagulant genes in regions that encode for their phospholipid binding domains. The question also arises that as the phospholipids investigated in this thesis are important in enhancing thrombin generation, could a reduction in their externalisation or synthesis explain some cases of hitherto unclassified bleeding disorders? Conversely, could an increase in externalisation or synthesis contribute to cases of venous or arterial thrombosis where no other cause can be identified, or could they influence the risk of ischaemic stroke or the phenotype in anti-phospholipid syndrome? Furthermore to what extent do differences in other membrane lipids, such as cholesterol or sphingolipids, influence thrombin generation and hence bleeding or thrombosis? Cholesterol has been reported to enhance the effect of phospholipids in activating protein C (235), which would in turn affect thrombin generation *in vivo*. Sphingolipids have been reported to reduce thrombin generation (193). Increasing saturation of the fatty acid chains of phospholipids has also been shown to reduce thrombin generation (194, 195). These observations suggest that changes in other lipids present in the platelet membrane, beyond those investigated in this thesis, warrant further investigation.

Finally, the cell-based model of thrombin generation lends itself to exploring the contribution of different tissue factor bearing cell types to thrombin generation. Tissue factor can be expressed on a variety of cell types including monocytes, eosinophils and endothelial cells (236). At present little is known about the composition of the cell membrane upon which they express tissue factor. The techniques used in Chapter 4 to investigate phospholipids in platelets could also be applied to these cells types to answer this question.

In summary, the original hypothesis that thrombin generation assays are useful for identifying patients at increased risk of excess bleeding following CPB and can be used in clinical practice to inform blood product usage, can be rejected on the basis of this thesis, at least for patients who are undergoing valve replacement or surgery on the aorta. The experiments in this thesis also demonstrate that CPB alters the externalisation of PE, PS and 12-HETE-PE suggesting that either scramblase or flippase activity, or both, are impaired. CPB also reduces the synthesis of 12-HETE-PE and 12-HETE-PC indicating a reduction in 12-LOX activity. 12-HETE-PE and 12-HETE-PC enhance thrombin generation and there is the suggestion that the magnitude of this effect differs between patients. For the management of bleeding, 30 mL/kg FFP, 45mcg/kg rFVIIa and 25 units/kg PCC may be sufficient to correct thrombin generation, but this would need to be confirmed in *in vivo* studies. Finally, this thesis provides insight into how the choice of phospholipids can influence thrombin generation and raises a number of questions for further study.

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Appendix 1 Data collection proforma

Patient Trial Number:

Age:

Weight:

Nature of operation: Date of operation

Anti platelet agents: Yes Specify: Date stopped:

No

Anticoagulants: Yes Specify: Date Stopped:

No

Intra-operative data:

Tranexamic acid -Dose : Pre CPB----gms On CPB----gms Post CPB ---- gms

Heparin Dose:--------Units Time Given :

Bypass: Time on: Time off:

Aortic cross clamp: Time on : Time off:

Skin closure time:

Time of heparin reversal: Protamine dose: ----mg

Haemoglobin (g/dL): pre op: post (on admission to CITU)

Platelet count (10x10/L): pre post (on admission to CITU)

ACT pre: ACT post reversal of heparin:

Fluids infused (including salvaged red cells and blood products)

Type of Fluid	Volume	Time

Patient Trial Number:

Post-operative data:

Volume of Blood loss from drains for first 24 hours or until removal:

Time post operation	Volume of blood lost (or record if drain removed)
1 hr	
2 hrs	
3 hrs	
4 hrs	
5 hrs	
6 hrs	
7 hrs	
8 hrs	
9 hrs	
10 hrs	
11 hrs	
12 hrs	
13 hrs	
14 hrs	
15 hrs	
16 hrs	

17 hrs	
18 hrs	
19 hrs	
20 hrs	
21 hrs	
22 hrs	
23 hrs	
24 hrs	

Total volume of Blood loss: **HCT of the lost blood:**

Fluids infused (including blood products) in CITU

Type of Fluid	Volume	Time

Patient Trial Number:

Length of time ventilated (days):

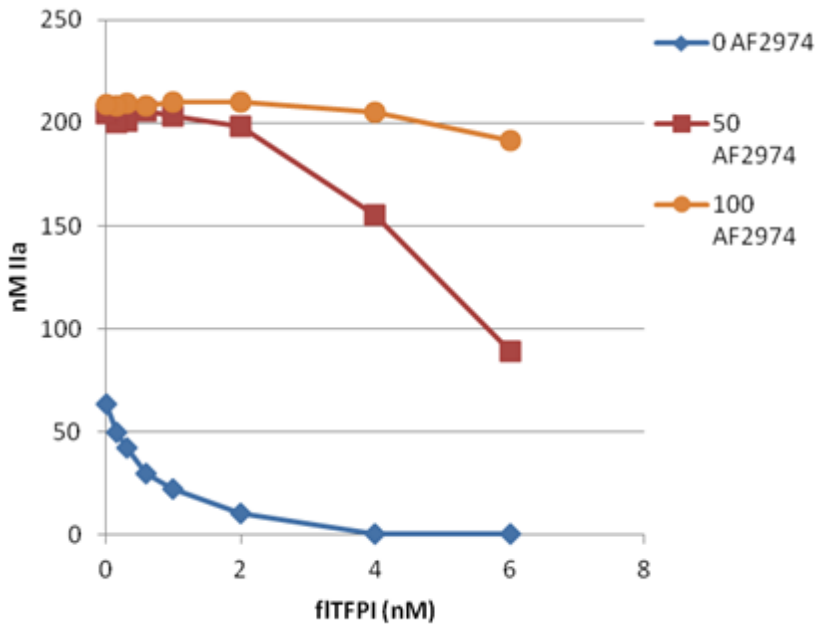
Duration of Level 2/3 Care (days):

Duration from operation to discharge (days):

Major complications of cardiac surgery:

- myocardial infarction (defined as an elevated cardiac troponin) ☐
- significant haemodynamic instability (defined as use of two or more inotropic drugs or intra-aortic balloon pump) ☐
- occurrence of atrial fibrillation lasting for more than 24 hours ☐
- reoperation for bleeding or any cause ☐
- prolonged ventilation (requirement for positive pressure ventilation(including NIV) for >48 hours) ☐
- sepsis ☐
- renal impairment requiring renal replacement therapy ☐

Appendix 2 Titration of anti-TFPI antibody (AF2974) to inhibit TFPI



This graph plots the effect on ETP (y-axis) of spiking plasma with different concentrations of full length TFPI (x-axis). The blue line indicates no anti-TFPI antibody, red line 50 nmol/L anti-TFPI antibody and orange line 100 nmol/L anti-TFPI antibody. Courtesy of Mr Erwin Panholzer, Baxter Biosciences, Vienna, Austria.

Appendix 3 Tissue factor concentrations in trigger solutions

TF activity in thrombin generation trigger solutions was measured using an IMUBIND ® ELISA kit purchased from Sekisui Diagnostics, Stamford, CT, USA. The lyophilised TF standards (50, 100,200,500 and pg/mL) were reconstituted by adding 1 mL of distilled water to each vial and agitating gently for 3 minutes. A vial of lyophilised biotinylated anti-human tissue factor antibody was reconstituted by adding 5.5 mL of distilled water and agitating gently for 3 minutes. The lyophilised contents of the vial of enzyme conjugate diluent provided in the kit was reconstituted by adding 20 mL distilled water and agitation gently. The packet of wash buffer (PBS, 0.1% Triton X-100, pH 7.4) was dissolved in 1 L of distilled water. A sample dilution buffer was prepared by making a solution of 1% (w/v) BSA dissolved in an appropriate volume of the wash buffer. Aliquots of the trigger solutions were diluted 1 in 4 and 1 in 8 using the sample dilution buffer described. All samples were run in triplicate. The results are shown in the table below (measured concentration is mean value).

Supposed Trigger TF concentration	**Measured Concentration (pM)**	**Standard Deviation**
3 pM	4.3	1.2
6 pM	11.9	7.9
18 pM	20.6	1.3
30 pM	36	3.6
60 pM	78.2	8.4
60 pM rTF	72.2	11.1