An Investigation into Coagulation Activation during Extracorporeal Circulation

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A thesis submitted to the University of London for the degree of PhD

University College London, 1998
Abstract

The objective of this thesis was to investigate coagulation activation during extracorporeal circulation. Two groups were selected for study: patients undergoing elective cardiac surgery requiring cardiopulmonary bypass (CPB) and critically ill patients in acute renal failure requiring continuous venovenous haemofiltration (CVVH).

It was suspected that contact activation by the CVVH circuitry might cause the observed generation of thrombin during haemofiltration. However, there was no evidence of contact activation during CVVH. I therefore investigated activation of the tissue factor pathway. Initially during CVVH FVIIa generation was suppressed due to the release of TFPI by heparin. However, the TFPI response to heparin is tachyphylactic, as TFPI levels fell FVIIa generation occurred, coincidental to thrombin generation.

This prompted the investigation of the tissue factor pathway during CPB where mechanisms of thrombin generation are not understood. During CPB, although there was an initial increase in FVIIa, the heparin released TFPI, which in contrast to CVVH was maintained throughout the procedure, suppressed FVIIa generation. Like CVVH, there was no evidence for contact activation. This suggested that thrombin generation during CPB did occur, but was not related to activation of either the contact system or tissue factor pathway. I therefore examined other potential mechanisms of thrombin generation at surfaces not detectable using conventional assays.

The activation of FXII on the surface of lipoproteins and blood cells was investigated in vitro as a possible mechanism for coagulation activation which might lead to thrombin generation during CPB. FXIIa generation was demonstrated on triglyceride-rich lipoprotein particles and leucocyte surfaces, where it may be protected from inhibition by serpines.
Enhanced activation and reduced inhibition of the tissue factor pathway was also demonstrated in blood from the pericardial cavity during CPB. Reinfusion of this blood to the patient was deemed likely to contribute to systemic coagulation activation during CPB.
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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>$\alpha_2$MG</td>
<td>$\alpha_2$macroglobulin</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATIII</td>
<td>Antithrombin III</td>
</tr>
<tr>
<td>$Ca^{2+}$</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>C1-INH</td>
<td>C1-esterase inhibitor</td>
</tr>
<tr>
<td>CPB</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td>CVP</td>
<td>Central venous pressure</td>
</tr>
<tr>
<td>CVVH</td>
<td>Continuous veno-venous haemofiltration</td>
</tr>
<tr>
<td>ECC</td>
<td>Extracorporeal circulation</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>FVII</td>
<td>Factor VII</td>
</tr>
<tr>
<td>FVIIa</td>
<td>Activated factor VII</td>
</tr>
<tr>
<td>FX</td>
<td>Factor X</td>
</tr>
<tr>
<td>FXa</td>
<td>Activated factor X</td>
</tr>
<tr>
<td>FXI</td>
<td>Factor XI</td>
</tr>
<tr>
<td>FXII</td>
<td>Factor XII</td>
</tr>
<tr>
<td>FXIIa</td>
<td>Activated factor XII</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCF</td>
<td>Mean channel fluorescence</td>
</tr>
<tr>
<td>MODS</td>
<td>Multi-organ dysfunction syndrome</td>
</tr>
<tr>
<td>MOF</td>
<td>Multi-organ failure</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Protein C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>pNA</td>
<td>p-nitroanaline</td>
</tr>
<tr>
<td>PS</td>
<td>Protein S</td>
</tr>
<tr>
<td>SBTI</td>
<td>Soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
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Publications arising from this thesis


Acknowledgements

I would like to express my gratitude to the following people who have helped me to accomplish this study.

My supervisor, Dr. Ian Mackie (Haematology, UCL), for his valuable advice, criticism and encouragement. Professor Sam Machin (Haematology, UCL) for his support and advice. Dr. Mervyn Singer (Bloomsbury Institute of Intensive Care Medicine, UCL) for his helpful advice, criticism and infectious enthusiasm. Helen McGloin (Intensive Care, UCL Hospitals) for collecting samples from haemofiltration patients, especially those arriving out of hours. Mr. Jonathan Unsworth-White (Cardiothoracic Surgery, UCL Hospitals) for allowing me access to his patients. Professor EDG Tuddenham (RPMS Hammersmith) for providing soluble recombinant tissue factor. Dr. Rob Ford (Shield Diagnostics Ltd.) for providing numerous FXIIa kits. Mr P Lumb (St. Thomas’ Hospital) for performing lipoprotein fractionations. Ms N Chavda (UCL Hospitals) for helpful advice on flow cytometry.

All the patients who kindly agreed to take part in the various studies in this thesis.

My colleagues at the Haemostasis Research Unit, UCL for their constant encouragement.
General Introduction

1.1 Normal Haemostasis

Haemostasis is the mechanism by which blood loss is prevented. When a blood vessel is ruptured or severed, haemostasis is achieved by several different but inter-related mechanisms: (1) Initially the blood vessel will go into vascular spasm, thus reducing blood flow from the ruptured vessel, (2) there is formation of a platelet plug, (3) there is activation of blood coagulation with clot formation, and (4) there is eventual clot retraction and growth of fibrous tissue to repair the injury site.

It is traditionally believed that the coagulation system is activated by two separate routes: the tissue factor (extrinsic) and contact factor (intrinsic) pathways (Figure 1.1), although such a division is unlikely to exist in vivo. Activation of these pathways leads to the generation of thrombin and subsequent fibrin formation. Most coagulation factors are identified by roman numerals, the active form being denoted by the lower case 'a' (Table 1.1). They generally circulate in an inactive zymogen form and become active after proteolytic cleavage; most are serine proteases related to trypsin. However, factor XIII (FXIII) is an exception, having transglutaminase activity which cross links proteins, while other factors such as tissue factor, factor V (FV), factor VIII (FVIII) and high molecular weight kininogen (HK) act as co-factors.

Naturally, if this system was kept unchecked it would lead to generalised coagulation activation and thrombosis. However, there are a number of mechanisms which localise coagulation to the site of injury. Most steps in the coagulation cascade require the formation of an activation complex comprising an enzyme, a substrate, a surface which organises the complex and makes the reaction more likely to occur (lowers the $K_m$), and a co-factor which acts a catalyst (increases the $V_{max}$). The surface is usually phospholipid and this may be provided by the membrane of activated or damaged cells. Availability and quality of both surface and co-factor influence the
reaction. There are also positive and negative feedback loops where products of one reaction influence an earlier process. Finally there are a series of inhibitors and inactivators that act at various steps in the coagulation cascade and shut down the system. Thus to maintain blood in its fluid state requires a balance between procoagulant and anticoagulant mechanisms, and any shift in this balance can have pathological consequences.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Synonym</th>
<th>MW</th>
<th>Plasma Concentration</th>
<th>Plasma Half-Life (hours)</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Fibrinogen</td>
<td>340,000</td>
<td>1.5-4.0 g/l</td>
<td>96-120</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
<td>72,000</td>
<td>100-150 mg/l</td>
<td>72</td>
</tr>
<tr>
<td>III</td>
<td>Tissue factor</td>
<td>43,000</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>V</td>
<td>Proaccelerin</td>
<td>330,000</td>
<td>10mg/l</td>
<td>12-15</td>
</tr>
<tr>
<td>VII</td>
<td>Proconvertin</td>
<td>50,000</td>
<td>0.5mg/l</td>
<td>4-6</td>
</tr>
<tr>
<td>VIII</td>
<td>Anti-haemophilic factor</td>
<td>330,000</td>
<td>0.1mg/l</td>
<td>10-18</td>
</tr>
<tr>
<td>IX</td>
<td>Christmas factor</td>
<td>56,000</td>
<td>5-10mg/l</td>
<td>36</td>
</tr>
<tr>
<td>X</td>
<td>Stuart-Prower factor</td>
<td>58,000</td>
<td>10mg/l</td>
<td>10-20</td>
</tr>
<tr>
<td>XI</td>
<td>Thromboplastin antecedent</td>
<td>160,000</td>
<td>5mg/l</td>
<td>50-70</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman factor</td>
<td>80,000</td>
<td>40mg/l</td>
<td>50-70</td>
</tr>
<tr>
<td>XIII</td>
<td>Profibrinoligase</td>
<td>320,000</td>
<td>10mg/l</td>
<td>100-120</td>
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<tr>
<td>Prekallkrein</td>
<td>Fletcher factor</td>
<td>86,000</td>
<td>50mg/l</td>
<td>N/A</td>
</tr>
<tr>
<td>HMW</td>
<td>HMW kininogen</td>
<td>110,000</td>
<td>70mg/l</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Table 1.1 Coagulation Factors*

NA = not applicable
Figure 1.1 The Classical Coagulation Cascade Leading to Thrombin Generation
(abbreviations as in text)
1.1.1 Platelet Activation

The formation of the primary platelet plug following vascular injury is a crucial step in haemostasis. Central to this function are the platelet reactions adhesion, secretion and aggregation as well as their procoagulant activity.

Following blood vessel injury, platelets adhere to the exposed subendothelial tissues. Platelet integrin receptors are capable of binding von Willebrand factor (vWF), fibrinogen, fibronectin, vitronectin, thrombospondin, collagen and laminin. vWF bound to the underlying connective tissue matrix reacts with platelet membrane GPIb promoting platelet adhesion and the exposure of the platelet GPIIb/IIIa receptor complex which forms a secondary binding site with vWF, promoting further platelet adhesion.

Upon activation, platelets lose their discoid shape, become relatively irregular in form and extend long pseudopodia as well as bulky surface protrusions. The cell organelles are contracted towards the centre, and are enclosed by a tight fitting ring of newly reassembled microtubules and microfilaments. The plasma membrane invaginates into the platelet interior to form an open membrane (canalicular) system which provides a large surface on which coagulation reactions may take place. The contents of the secretory organelles are extruded by the surface canalicular system. Collagen exposure or thrombin action results in the secretion of platelet granule contents which include ADP, serotonin, fibrinogen, β-thromboglobulin (βTG), and platelet factor 4.

Platelet aggregation is thought to proceed via three distinct mechanisms. The first is mediated by ADP released from platelet granules. The second involves the generation of prostaglandin endoperoxides and thromboxane A₂ (TXA₂) which requires the liberation of arachidonate from membrane phospholipids. TXA₂ lowers platelet cyclic AMP levels and thus lowers the threshold for platelet release and aggregation; in addition, TXA₂ is a potent vasoconstrictor. The third mechanism is independent of the others, possibly involving platelet activating factor (PAF-acether).

Released ADP and TXA₂ cause additional platelets to aggregate at the injury site, resulting in further release reactions and the formation of a platelet plug large enough to
fill the area of injury. During the activation of platelets exposure of phospholipids on the platelet membrane provides a suitable surface on which the components of several coagulation reactions can assemble and thus enhance the action of the platelet plug by fibrin clot formation.

1.1.2 The Tissue Factor Pathway of Coagulation

The tissue factor pathway is considered to be the principal initiating pathway of coagulation *in vivo*. The critical component is tissue factor (TF), which functions as a co-factor. TF is a cell surface glycoprotein consisting of a 219 residue extracellular domain comprising two immunoglobulin-like domains. In addition, TF consists of a 23 residue transmembrane and 21 residue cytoplasmic domain (Ruf & Edgington, 1994). TF is expressed by cells in the subendothelium of blood vessels, organ capsules, and cells of epithelial surfaces and the nervous system (Drake *et al*, 1982). Thus TF is expressed at sites which are physically separated from the circulating blood. Peripheral blood cells do not normally express TF, but expression by monocytes and endothelial cells can be induced *in vitro* by a variety of stimuli including endotoxin, TNF-α, IL-1, and immune complexes (Osterud, 1995; Wada *et al*, 1995). There is also some evidence that neutrophils and macrophages may express TF under certain conditions (Higure *et al*, 1997; Petit *et al*, 1997), although this remains controversial.

Initiation of the pathway is achieved when TF is exposed following trauma or cell damage and binds plasma factor VII (FVII) or activated FVII (FVIIa). It is currently not understood which forms the initial complex. Plasma FVII is unusual in that it possesses weak proteolytic activity in the zymogen form and FVIIa, in contrast to other active coagulation enzymes, appears to persist in the circulation. Indeed, with recent advances in the measurement of FVIIa (Morrissey *et al*, 1993) it has been possible to show that plasma FVIIa represents approximately 1% of total FVII levels.

The FVII:TF catalytic complex has been shown to exert sufficient protease activity to activate factor X (FX) and the FXa formed may then rapidly convert the FVII:TF complex to FVIIa:TF, thus potentiating the system. Alternatively, any
circulating FVIIa binding to TF may directly activate FX. Human FVII and FVIIa have been shown to bind to TF with identical dissociation constants (Broze, 1982) and may even co-operate in binding to TF (Fair & MacDonald, 1987). The fact that TF requires phospholipid for full activity and that it has a large transmembrane domain help to retain the TF:FVIIa:FX activating complex at the cell surface, ensuring that coagulation is localised to the site of injury.

The FXa formed remains phospholipid bound and forms a complex with factor V and prothrombin (the prothrombinase complex). FXa then cleaves prothrombin to form thrombin which is free from the phospholipid surface. Thrombin has a positive feedback on the coagulation cascade by the activation of factors V, VIII and possibly XI. During the conversion of prothrombin to thrombin a small peptide is released, prothrombin fragment F1+2, and this has gained recent attention as a marker of thrombin generation in vivo. In addition to activating FX, the FVIIa:TF complex can also cleave FIX to form FIXa which can then itself activate FX. This illustrates the lack of division of the intrinsic and tissue factor pathways in vivo.

1.1.3 The Intrinsic (contact factor) Pathway of Coagulation

The contact factor pathway is initiated when plasma factor XII (FXII) binds to a negatively charged surface resulting in autoactivation of FXII to form αFXIIa. Such a surface may be provided by foreign material, collagen, sulphatides or endotoxin. Alternatively, FXII may be activated proteolytically by cell proteases, plasmin, or complement proteases. It is unclear whether FXII has to be cleaved or merely undergoes a conformational change on surface binding which exposes the active site of this serine protease. Alternatively, the contact system can be activated proteolytically by cell proteases, plasmin and complement proteases.

Prekallikrein (PKK) and factor XI (FXI), both of which circulate as a bimolecular complex with high molecular weight kininogen (HK) facilitating their surface binding, are cleaved by αFXIIa to kallikrein (KK) and FXIa respectively. Kallikrein cleaves FXII, amplifying αFXIIa generation, whilst HK is cleaved causing
loss of surface binding properties and liberating the vasoactive peptide bradykinin. Further action of KK on αFXIIa produces βFXIIa, a low molecular weight fragment which retains the serine centre, but loses its surface binding domain. These two forms of FXIIa differ in their properties: βFXIIa does not have surface binding capacity and cannot activate FXI, but retains activity on PKK and, in some instances, can activate FVII. FXIa acts on FIX to produce FIXa, which forms a complex with FVIII, FX and phospholipid and activates FX, leading to the subsequent generation of thrombin.

Proteins of the contact system may also interact with fibrinolytic and complement pathways as well as cellular surfaces. The classical pathway of complement can be activated by βFXIIa by interacting with macromolecular C1. This activation is apparently a direct enzymatic reaction that does not require PKK or plasminogen (Ghebrehiwet et al., 1981).

The contact system may participate in fibrinolysis: plasma KK (Colman, 1969) can activate plasminogen directly as can FXIa (Mandle et al., 1977) and both α and βFXIIa (Goldsmith et al., 1978), although to a lesser extent than kallikrein. The physiological significance of these mechanisms are not fully understood, as the contact factors are far less able to activate plasminogen than urokinase (although this may depend upon in vitro conditions). However, kallikrein itself can activate single-chain urokinase (Ichinose et al., 1986), bradykinin formed during contact activation can release t-PA from endothelial cells (Smith et al., 1985) and contact activation enhances fibrin clot lysis in the presence and absence of urokinase. Furthermore, a severe deficiency of FXII may be associated with an increased risk of thromboembolism (Mannhalter et al., 1987; Lammle et al., 1991; Rodeghiero et al., 1992; Halbmayer et al., 1992) although this remains controversial.

Platelets may participate in activation of the intrinsic pathway: FXI antigen and FXI-like activity are found in or on platelets and HK is contained within platelet α granules (Schmaier et al., 1986). In addition, platelets may regulate contact factor activation by secreting plasma protease inhibitors such as C1-INH (Schmaier et al., 1985), α1-antitrypsin and α2-macroglobulin (Nachman et al., 1976).
Contact factor proteins interact with neutrophils, endothelial cells and monocytes. Kallikrein (Wachtfogel et al, 1983) and αFXIIa (Wachtfogel et al, 1986) can stimulate neutrophil aggregation and degranulation, and kallikrein is chemotactic for neutrophils (Kaplan et al, 1972). Indeed, PKK, FXII, HK and FXI have been shown to exist on the surface of human neutrophils (Henderson et al, 1994) which raises the possibility that all components of the contact system may be present and functional on cell-surfaces.

Endothelial cells can bind (Iwaarden et al, 1988) and express HK (Schmaier et al, 1988) in vitro. LPS-induced IL-1 production by monocytes is enhanced by α and βFXIIa (Toossi et al, 1992) and this can be inhibited by corn trypsin inhibitor, which has relative specificity for FXIIa (Ratnoff et al, 1995). In addition, αFXIIa reduces the expression of FcγRI receptors, the binding site for monomeric IgG, on monocytes (Chien et al, 1988). Thus FXIIa may serve to regulate expression of this receptor during inflammation.

Inherited deficiency of factor II, V, VII, X, IX or VIII results in a bleeding diathesis, while homozygous FXI deficient patients are only likely to bleed after trauma or surgery. In contrast, FXII and PKK deficiency are only rarely associated with a bleeding tendency and thus deficiencies in the contact factors may be compensated by other mechanisms in order for thrombin generation to occur. One such mechanism may be the activation of FXI by thrombin, although thrombin is a very poor activator of FXIa in the absence of glycosaminoglycan cofactors e.g. dextran sulphate (Broze & Gailani, 1993).

The tissue factor pathway is considered to play the pivotal role in the mitigation of thrombin generation following vessel trauma in vivo. The role of the contact system remains unclear, but it may be important for enhancing thrombin generation after the initial burst initiated by tissue factor, or if the processes opposing coagulation are particularly active (e.g. fibrinolysis or inhibition of coagulation factors). In addition, the contact system may have a role to play in the generation of thrombin in situations where there is no major tissue disruption, such as infection or influx of foreign bodies (or
tumour cells) which must be surrounded by fibrin as part of the host defense mechanism. Some believe that the major role of the contact system is not in the generation of thrombin, but in maintaining the anticoagulant environment of the intravascular compartment by its anti-thrombotic and profibrinolytic actions (Schmaier, 1997).

The contact system may have an important role to play in several pathological situations. When blood comes into contact with a suitable foreign surface such as during cardiopulmonary bypass, haemodialysis, or when an artificial implant is used, activation of FXII may occur resulting in coagulation activation (Salzman et al, 1994). In patients with ARDS - the acute respiratory distress syndrome (Velasco et al, 1986) and hyperlipidaemia (Carvalho et al, 1976), levels of FXII and prekallikrein are reduced suggesting an underlying degree of contact activation in these patients. However, a recent report suggests that FXII production by hepatocytes may be downregulated by interleukin-6 (Citarella et al, 1997). This would imply that reduced FXII levels seen in some conditions may be part of the acute-phase response and not due to consumption during contact activation, although this remains controversial.

There is however, a lack of division between the contact system and tissue factor pathway as the TF:VIIa complex can cleave FIX to FIXa and FIXa can itself activate FVII. In addition, FXIIa can activate FVII in vitro (Radcliffe et al, 1977) although it is not clear if this mechanism operates in vivo.

1.1.4 Fibrin Formation and Fibrinolysis

Fibrinogen is a hetero-dimer consisting of two each of three polypeptide chains, α, β, and γ, linked by multiple disulphide bonds. Thrombin binds to the fibrinogen central domain liberating fibrinopeptides A & B from the α and β chains of fibrinogen respectively, to form fibrin monomer which undergoes end-to-end polymerisation to form long fibrin strands which become insoluble. The resulting fibrin clot is stabilised by the FXIIIa-dependent formation of covalent bonds between, α and γ chains of adjacent fibrin strands. Blood cells become trapped in the developing fibrin clot, and the
strands bind to and intersperse platelet aggregates as they form to give a primary haemostatic plug.

![Figure 1.2 Fibrin formation and fibrinolysis](image)

The fibrin clot is broken down into progressively smaller soluble fragments by the action of plasmin. Plasmin cleaves both fibrin and cross-linked fibrin at a number of sites liberating fibrin degradation products (FDP's), but if cross-linked fibrin is cleaved, then larger products are cross-linked (D-dimers, X-oligomers). Plasmin is generated from plasminogen by several mechanisms. Plasminogen is activated by tissue plasminogen activator (tPA) in the presence of fibrin, by urokinase-type plasminogen activator (uPA, urokinase), by a contact factor dependent mechanism or by certain neutrophil secreted proteases such as elastase.
1.1.5 Inhibition of Coagulation

In addition to the tissue factor pathway being partly limited by the requirement of a phospholipid surface on which several of the reactions take place, control is also provided by plasma protease inhibitors. Tissue factor pathway inhibitor (TFPI), previously called extrinsic pathway inhibitor or lipoprotein associated coagulation inhibitor, appears to be the main effector in controlling TF mediated coagulation. TFPI binds reversibly to the active site of FXa, inhibiting the protease, and this TFPI:FXa complex then interacts with TF:FVIIa forming an inactive quaternary complex.

TFPI is present in three circulating pools: approximately 80% (50-150ng/ml) is associated with plasma lipoproteins (notably LDL, and to a lesser extent, HDL), 10% (10ng/ml) is sequestered in platelets and the remaining 10% exists as a free form in plasma (Novotony, 1994). However, the majority of TFPI is believed to be bound to the endothelium via heparan sulphate and/or other glycosaminoglycans and injection of intravenous heparin results in a 2-10 fold increase in plasma TFPI levels (Sandset et al, 1988). The precise mechanism of TFPI release by heparin is unknown. TFPI is constitutively expressed by monocytes (McGee et al, 1994) and endothelial cells in culture (Ameri et al, 1992), but its expression is not increased by the same inflammatory mediators that induce TF expression in these cells (van der Logt et al, 1994; Ameri et al, 1992).

TFPI is a member of the kunitz-type family of protease inhibitors, which includes aprotinin. TFPI contains three kunitz domains linked in series. The first kunitz domain is responsible for binding FVIIa, the second for binding FXa, the third is not essential for inhibition of TF mediated coagulation but is thought to contain a low-affinity heparin binding site and may be involved in interactions with lipoproteins (Girard et al, 1989). The carboxyl terminus of TFPI contains a high-affinity heparin binding site (Harenberg et al, 1995), interacts with lipoproteins (Valentin et al, 1993) also interacts with FXa and is required for the full anticoagulant potential of TFPI (Nordfang et al, 1991). The third kunitz domain of TFPI bears remarkable amino acid sequence homology with aprotinin.
The role of TFPI in controlling thrombin generation *in vivo* has not been fully elucidated. Individuals with congenital TFPI deficiency have not been identified and thrombosis is not associated with reduced TFPI levels (Novotony *et al.*, 1991). However, mice that are homozygous for a deletion in the TFPI gene which results in the TFPI molecule lacking kunitz domain 1, die in utero (Huang & Broze, 1997) which would imply that severe TFPI deficiency is not compatible with life.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-III</td>
<td>Thrombin, FXa, FIIa, FIXa, FXIa, Kallikrein, TF:FVIIa</td>
</tr>
<tr>
<td>HClII</td>
<td>Thrombin, Meizothrombin, Cathepsin G</td>
</tr>
<tr>
<td>C1-INH</td>
<td>FXIIa, Kallikrein, C1s</td>
</tr>
<tr>
<td>PCI</td>
<td>APC, Kallikrein, t-PA</td>
</tr>
<tr>
<td>α2-antiplasmin</td>
<td>Plasmin</td>
</tr>
<tr>
<td>α2-antitrypsin</td>
<td>FXIa, neutrophil elastase</td>
</tr>
<tr>
<td>α2-macroglobulin</td>
<td>Kallikrein, FXIIa, FXa, Thrombin, Plasmin</td>
</tr>
<tr>
<td>PAI-1&amp;2</td>
<td>t-PA, uPA</td>
</tr>
<tr>
<td>TFPI</td>
<td>FXa, TF:FVIIa</td>
</tr>
</tbody>
</table>

*Table 1.2. Naturally occurring inhibitors of coagulation and their target enzymes*

Control of coagulation proteins is also provided by the serine protease inhibitor (SERPIN) super family of inhibitors which includes antithrombin III (AT-III), heparin co-factor II (HClII), α2-antiplasmin, plasminogen activator inhibitors 1&2, protein C inhibitor (PCI), α2-antitrypsin and C1-esterase inhibitor (C1-INH). The serpins form a 1:1 irreversible complex with their target protease, which is subsequently removed from the circulation and catabolised. The predominant target proteases of the serpins and other coagulation inhibitors is shown in Table 1.2.

AT-III is considered to be the major inhibitor of thrombin *in vivo*, and its action is potentiated approximately 1000-fold by heparin - the natural counterpart of this is
probably heparan sulphate. AT-III is also capable of inhibiting the FVIIa:TF complex (Lawson et al, 1993), kallikrein, Fxa, FIXa and FXIa. In vitro models suggest that HCII is also an important inhibitor of thrombin, but may be most relevant at cell surfaces and in extravascular tissues, where its glycosaminoglycan cofactor, dermatan sulphate may be more abundant (Tollefsen, 1995). In addition, HCII has a much greater ability than AT-III to inhibit meizothrombin (the first intermediate formed during conversion of prothrombin to α-thrombin) even when bound to phospholipid or in the prothrombinase complex (Han & Tollefsen, 1997).

Additional control of thrombin is provided by the naturally occurring anticoagulants protein C and S which lead to the degradation of the co-factors Va and VIIIa, greatly reducing thrombin generation. Thrombin binds with high affinity to thrombomodulin, a cellular receptor expressed at the endothelial cell surface which redirects the action of thrombin, blocking its fibrinogen and platelet actions, but potentiating its anticoagulant functions. The thrombin-thrombomodulin complex binds protein C, which is cleaved by thrombin in a reaction catalysed by thrombomodulin to produce activated protein C (APC). Protein S then acts as a co-factor for the APC mediated inactivation of factors Va and VIIIa.

Regulators of the contact factor pathway include C1-INH, α2-macroglobulin, and α1-antitrypsin. C1-INH contributes more than 90% of the inhibitory capacity in normal plasma towards both α and βFXIIa (Pixley et al, 1985). C1-INH also contributes approximately 42% of plasma inhibitory capacity against kallikrein, an additional 50% being provided by α2-macroglobulin (Van de Graff et al, 1983).

The fibrinolytic system is controlled by various forms of plasminogen activator inhibitors (PAI 1-3). PAI-1 is the predominant inhibitor of tPA and uPA in normal plasma. Approximately 90% of the circulating pool is contained within platelets, however, the main source of PAI-1 is probably endothelial cells. PAI-2 is primarily a placental protein, not normally detectable in plasma, but levels increase during pregnancy and may be important in regulating haemostasis at the placental interface during pregnancy. PAI-2 may be synthesised and secreted by monocytes in normal individuals.
(Ritchie et al, 1997). PAI-3 (PCI) is mainly involved in the inhibition of activated protein C and is unlikely to play an important role in the physiological inhibition of tPA and uPA.

1.1.6 The Complement Cascade

Although usually considered as a separate plasma protease system to the coagulation cascade, the complement system interacts with coagulation proteins at several levels and is fundamentally involved in acute inflammatory reactions. The complement system comprises a series of plasma (Table 1.3) and cell membrane (Table 1.4) proteins which are involved in the inflammatory and immune responses.

<table>
<thead>
<tr>
<th>Activation</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classical pathway</strong></td>
<td></td>
</tr>
<tr>
<td>First component (C1) - C1q, C1r, C1s</td>
<td>C1-inhibitor</td>
</tr>
<tr>
<td>Second component (C2)</td>
<td>C4 binding protein</td>
</tr>
<tr>
<td>Fourth component (C4)</td>
<td>Factor I</td>
</tr>
<tr>
<td><strong>Alternative pathway</strong></td>
<td></td>
</tr>
<tr>
<td>Factor B</td>
<td>Factor H</td>
</tr>
<tr>
<td>Factor D</td>
<td>Clusterin (SP40-40)</td>
</tr>
<tr>
<td>Factor P</td>
<td>Carboxypeptidase N</td>
</tr>
<tr>
<td><strong>Terminal sequence</strong></td>
<td></td>
</tr>
<tr>
<td>Third component (C3)</td>
<td></td>
</tr>
<tr>
<td>Fifth component (C5)</td>
<td></td>
</tr>
<tr>
<td>Sixth component (C6)</td>
<td></td>
</tr>
<tr>
<td>Seventh component (C7)</td>
<td></td>
</tr>
<tr>
<td>Eighth component (C8)</td>
<td></td>
</tr>
<tr>
<td>Ninth component (C9)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3 Serum proteins of the complement system

In a similar manner to the coagulation cascade, complement proteins may function as enzymes, inhibitors or cofactors and some circulate in an inactive form. Traditionally the complement system is thought to be activated by two routes: the classical pathway
(antibody dependent) and the alternative pathway (antibody-independent). However, antibody-independent activation of the classical pathway is also believed to occur.

During complement activation anaphylotoxins are released from the activated complement components, complement components bind to the surface of microorganisms to prepare them for phagocytosis or intracellular killing, and a multimeric cytolytic membrane attack complex is assembled and inserted into the membranes of micro-organisms to destroy them directly. Protection of host cells against complement mediated attack is conferred by a series of membrane-bound proteins which regulate both the early stages of complement activation and the insertion of the membrane attack complex.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement receptor 1 (CR1, C3b/C4bR)</td>
<td>CD59 (MIRL)</td>
</tr>
<tr>
<td>Complement receptor 2 (CR2, C3dR)</td>
<td>decay-accelerating factor (DAF)</td>
</tr>
<tr>
<td>Complement receptor 3 (CR3, iC3bR)</td>
<td>membrane cofactor protein (MCP)</td>
</tr>
<tr>
<td>C3a/C4a receptor</td>
<td>homologous recognition factor (HRF)</td>
</tr>
<tr>
<td>C5a receptor</td>
<td></td>
</tr>
<tr>
<td>C1q receptor</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.4 Membrane-bound proteins of the complement system*

The pivotal complement protein is C3. C3-convertase produced by the classical pathway is generated after activation of C1 by IgG or IgM antibodies complexed to their respective antigens (Figure 1.3), although certain viruses and gram-negative bacteria can also activate this pathway. Recognition takes place through a circulating complex of plasma proteins called C1 comprised of Cq, Cr, Cs (see Table 1.3). Following binding to antibody C1q undergoes a conformational change, which results in the activation of C1r (Colomb *et al*, 1984) to C1r. C1r cleaves its natural substrate C1s to its active form (C1s) which in turn has two substrates: C4 and C2. C1s cleaves a small fragment (C4a) from C4 exposing the major product C4b. C2 binds to C4 to form pro-convertase
before being cleaved by C1s into C2b. The remaining protein (C2a) forms a complex with C4b which is the classical pathway C3 convertase, cleaving C3 to produce a small peptide (C3a). Some C3b binds to C4b in the C4b2a complex and acts as a receptor for C5. This C4b2a3b complex is the classical pathway C5 convertase which releases a small peptide from C5 (C5a) and makes the major cleavage product (C5b) available for the assembly of the membrane attack complex.

Figure 1.3 The Classical Pathway of Complement Activation

Activation of the alternative pathway can occur in the absence of immunoglobulin, C4 or C2 but requires C3 and involves three processes: initiation, amplification and recognition. Initiation is thought to occur as the result of spontaneous hydrolysis of C3.
to form a molecule C3(H2O) which has C3b-like properties. Activators of the alternative pathway tend to be molecules with repeating chemical structures such as lipopolysaccharides and teichoic acid present on the surfaces of bacteria and other pathogens. Amplification occurs by the positive feedback loop which is regulated by factors H and I. C3b formed by alternative pathway activation binds to any surface and recognition only occurs after C3b binding.

The complement cascade is controlled by a number of processes: the intrinsic decay of various enzyme complexes, labile reactive sites, dissociation of incompletely assembled membrane attack complex from cell membranes, and regulatory proteins that either circulate in serum or are membrane bound and prevent uncontrolled activation and consumption of complement proteins. Of note is Cl inhibitor (C1-INH) which binds to the active sites of C1s and C1r thus preventing activation of C4 and C2 proteins (Harpel & Cooper, 1975). C1-INH also interacts locally with the Cl zymogen molecule preventing spontaneous Cl activation (Cooper, 1985).

This illustrates one level at which the complement and coagulation cascades are related, as C1-INH is also involved in regulating FXII dependent coagulation activation (see section 1.1.5). In addition, βFXIIa can activate the classical pathway by interacting with C1 (Ghebrehiwet et al, 1981), FXII can interact with C3, kallikrein can cleave C5 directly to produce C5a (Wiggins et al, 1981) and can also cleave C1, plasmin can activate C1s (Ratnoff & Naff, 1961) and cleave C3 (Ward, 1967) and protein S anchors C4 binding protein to cell surfaces.

1.2 Cardiopulmonary Bypass

Extracorporeal circulation is the use of an artificial circuit outside the body, through which the blood flows. It is used during cardiopulmonary bypass (CPB), haemodialysis and related procedures such as haemofiltration. Despite advances in the safety of CPB, its use is still associated with significant morbidity and mortality. CPB initiates a ‘whole body inflammatory response’ by the patient, the pathogenesis of which is likely to be multifactorial in origin involving the kallikrein-kinin, haemostatic, complement and
humoral systems as well as cellular mediators. The understanding of the pathophysiology of these systems during CPB and their interactions may enable the development of therapeutic strategies to help reduce the deleterious effects associated with its use.

1.2.1 Historical Perspective

Early cardiac surgery was extremely limited. Surgeons cooled the entire body to reduce tissue oxygen requirements, enabling them to operate for only a few minutes on the heart itself. The procedures which could be carried out in such a short time were naturally very restricted. The introduction of the ‘heart-lung’ or CPB machine for clinical use revolutionised cardiac surgery; for the first time the body could be maintained without the lungs or heart functioning, enabling surgeons to operate on the heart for far longer periods of time.

CPB was first used clinically in 1951 (Dennis et al, 1951), but, unfortunately, the patient died on the table. The first successful use of CPB was in 1953 (Gibbon, 1954), and although one patient survived, many others died and Gibbon, who was instrumental in developing the CPB machine, gave up the idea that it could be used routinely. However, others pursued the work and successfully applied the techniques which he had developed (Kirklin et al, 1955). Since its first use, there have been many refinements in the components of the CPB circuit and there is a continual drive to improve the biocompatibility of the CPB circuit.

1.2.2 The Cardiopulmonary Bypass Circuit

The CPB machine consists of one or more venous cannulae, a venous reservoir, an oxygenator/heat exchanger, a pump, a filter and an arterial cannula (Figure 1.3). The machine is built from non-toxic metals and synthetic materials such as polycarbonate, polyvinylchloride, Teflon, polyethylene, silicone rubber and polyurethanes.

The venous cannula is placed in the right atrium, vena cavae or a large peripheral vein to bring blood into the circuit. The oxygenator/heat exchanger removes carbon
dioxide, adds oxygen and controls body temperature. There are two basic types of oxygenator: the bubble and membrane oxygenator. In bubble oxygenators, small oxygen bubbles are directly exposed to venous blood. At the bubble surface, oxygen diffuses outward and carbon dioxide diffuse into the bubble which then escapes.

Figure 1.4 Cardiopulmonary Bypass Circuit
In membrane oxygenators, which are now more generally used due to enhanced biocompatibility, oxygen and carbon dioxide move across a solid or microporous membrane that separates blood and gas elements.

Two different kinds of pump can be used to drive blood around the CPB circuit. The roller pump, which was introduced first, has two roller heads 180 degrees apart that compress blood filled tubing against a rigid raceway. Centrifugal pumps either have nested cones or blades that rotate to generate flow.

Both types of pump cause some haemolysis due to turbulence and shear forces. A filter in the arterial line removes gaseous and particulate emboli before the oxygenated blood re-enters the body through a cannula inserted in the aorta or other large artery. A cross clamp placed across the aorta prevents back flow to the coronary vessels and left ventricle. Blood flow to the heart and through the pulmonary vasculature during this period is effectively halted.

During surgery a cardiotomy sucker is used to return heparinised blood aspirated from open cardiac chambers and the surgical field to the CPB circuit. This blood is filtered and added directly to the venous side of the perfusion system. In some systems, a ‘cell saver’ recovers diluted blood in the surgical field. A centrifuge within the saver concentrates the red cells which are then returned to the perfusate. These systems significantly reduce the requirements for homologous blood. Over half of first time open-heart operations do not require homologous blood.

During CPB blood is routinely diluted to a haematocrit of approximately 20-30%. This is achieved by the ‘priming’ of the CPB circuit with crystalloid solutions that include 5% dextrose, saline and Ringer’s lactate. Added colloids include albumin, dextran and starch solutions; homologous blood is used sparingly.

1.2.3 Anticoagulation
Exposure to the foreign surface of the CPB circuit provides a massive stimulus for blood to clot. To prevent this, high doses of unfractionated heparin, universally the
anticoagulant of choice, are used to anticoagulate blood during CPB. However, for a number of reasons, heparin is not an ideal anticoagulant for this purpose.

The anticoagulant action of heparin depends upon a specific pentasaccharide sequence which binds with high affinity to AT-III and potentiates its activity by approximately 1000-fold (Lindahl et al., 1979) thus accelerating thrombin inhibition. Heparin also accelerates inhibition of FXa (anti-Xa activity) by AT-III. Due to the destruction of some of the AT-III binding sequences by the depolymerisation process, low molecular weight (LMW) heparins have a much greater anti-Xa activity than antithrombin activity.

Monitoring heparin therapy during CPB is extremely important. Adequate anticoagulation must be provided, but there is a risk of post-operative bleeding if the patient is over-anticoagulated. There are two basic modes of monitoring heparin: global coagulation tests and anti-FXa assays. Global tests include the whole blood clotting time (WBCT), activated clotting time (ACT) or activated partial thromboplastin time (APTT) which are all prolonged by heparin.

The ACT is normally carried out on fresh whole blood in the operating theatre so that anticoagulation can be adjusted during the operation. The APTT may be performed by the coagulation laboratory, normally in conjunction with measurement of the prothrombin time (relatively unaffected by heparin). However, aprotinin can prolong the WBCT, ACT and APTT which may result in an overestimation of heparin levels, although this effect can be overcome by using clotting tests not dependent on the contact system e.g. ‘Heptest’ (Webb et al., 1995). LMW heparins cannot be monitored using global tests due to their high anti-Xa: anti-thrombin activity ratio.

The most specific assays for heparin are the anti-Xa assays. These are based on the ability of plasma to inhibit excess FXa added to it, the residual FXa being detected by clotting or amidolytic assay. These methods are suitable for measuring both unfractionated and LMW heparins and are not affected by aprotinin or other coagulation variables.
Before CPB, heparin is added to the bypass circuit prime solution (usually 2000IU/l) and the patient is anticoagulated with an initial dose in the region of 300IU/kg unfractionated heparin. This dose is aimed to achieve an ACT of 400-500s during CPB and additional heparin is given to maintain the ACT at this level, which is deemed safe for CPB (Colvin & Barrowcliffe, 1993). To put this level of anticoagulation into perspective, patients receiving heparin for prophylaxis against venous thrombosis should have their plasma heparin level maintained at 0.02-0.05 U/ml, and for treatment of an acute thrombotic event at 0.2-0.5 U/ml (Salzman et al, 1994). During cardiopulmonary bypass plasma heparin levels frequently exceed 1 U/ml, often reaching 3-5 U/ml. When CPB is discontinued heparin is neutralised by the administration of protamine sulphate, assuming that 1mg of protamine neutralises 100U heparin.

Despite these high doses of heparin, thrombin is still generated during CPB (Boisclair et al, 1993; Brister et al, 1993). Thrombin is not a desirable enzyme to produce during CPB, it has a number of effects not all of which are beneficial, as discussed in more detail in section 1.2.13. In addition, heparin has a number of side effects. Firstly, it is associated with bleeding in approximately 5% of CPB patients (Woodman & Harker, 1990). Secondly, heparin-induced thrombocytopenia is a postoperative complication in some (2-5%) patients (King & Kelton, 1984); heparin should therefore not be use in patients suspected of previously developing this reaction. In addition, the use of heparin necessitates the concurrent use of protamine sulphate to neutralise it at the end of CPB. Approximately 5% of patients develop an adverse reaction to protamine sulphate, characterised by haemodynamic instability (Harrow, 1985). Furthermore, in vitro, heparin can enhance platelet aggregation (Thompson et al, 1973; Zucker, 1975; Michalski et al, 1977; Chen et al, 1991), leucocyte aggregation, and can promote contact activation by reducing the inhibition of βFXIIa by C1-INH (Gallimore et al, 1991).

The undesirable side effects of the use of heparin during CPB has led to the search for alternative anticoagulants and substances to neutralise heparin. Ancrod, an anticoagulant derived from the venom of the Malayan pit viper, acts by depleting plasma
of fibrinogen (Reid et al, 1963). It has been used successfully as an alternative to heparin during CPB (Zulys et al, 1989) although its use is associated with significantly greater post-operative blood loss than heparin.

Orgaran is an anticoagulant which does not contain heparin, but is a mixture of glycosaminoglycans (heparan, dermatan and chondroitin sulphate). In patients with heparin-induced thrombocytopenia who require CPB, Orgaran has been used successfully as an alternative anticoagulant to heparin (Doherty et al, 1990; Chong & Magnani, 1992). However, it is unclear what effect Orgaran has on the incidence of post-operative bleeding and its use during CPB in routine cases remains speculative.

LMW heparins have been successfully used as an anticoagulant in models of CPB (Koza et al, 1993; Bagge et al, 1994) and in clinical CPB in conjunction with a heparin-bonded circuit (Ganjoo et al, 1996). However, there are two main problems associated with the use of LMW heparins: the need for frequent anti-Xa assays for accurate monitoring and the inability to achieve complete reversal of anticoagulation at the end of CPB.

Other possible alternative anticoagulants include the direct thrombin inhibitor hirudin which has been used in a canine model of CPB (Walenga et al, 1991) but its use is associated with a significant increase in fibrin deposition and a tendency towards greater blood loss compared to heparin and it cannot be neutralised. Dermatan sulphate, which catalyses thrombin inhibition by heparin cofactor II, has also been evaluated as a possible anticoagulant in a porcine model of CPB (Brister et al, 1994) with promising results. Finally, Iloprost (a stable and potent PGI₂ analogue) has been used to prevent platelet activation and subsequent coagulation (Kappa et al, 1987; Kraenzler et al, 1988) during CPB. Unfortunately its use is associated with significant hypotension due to its vasodilatory action.

When the use of heparin during CPB is contraindicated due to heparin-induced thrombocytopenia or adverse reactions to protamine, an alternative choice of anticoagulant must be used. However, most of the possibilities listed above are either associated with an increased risk of post-operative bleeding or other side effects.
compared to heparin. Moreover, given that coagulation activation occurs during CPB despite heparin administration, further investigations into other anticoagulant options are warranted. It would also be prudent to assess these not only in terms of global clotting tests, post-operative blood loss or anti-IIa and anti-Xa activity but also in wider studies on haemostatic function.

1.2.4 Protein Adsorption

The adsorption of protein onto an artificial surface is considered to be the first major event induced by blood biomaterial contact and exerts a strong influence on subsequent blood interactions. When an artificial surface is exposed to blood in an ex vivo chamber, there is a lag period of approximately one minute before platelets adhere to it (Petschek et al., 1968). During this period, some kind of 'conditioning' of the artificial surface occurs, involving deposition of a layer of plasma proteins approximately 100nm thick.

The manner and extent of protein attachment depends upon the nature of the artificial surface. For glass, electrostatic adsorption is important (Chan & Brash 1981) whereas for polymers, the interaction may be hydrophobic resulting from the interaction of non-polar protein and non-polar surface groups in the polar aqueous medium. In general, protein adsorption and retention are greater with hydrophobic rather than hydrophilic surfaces (Hoffman, 1974; Brash et al., 1974; Chuang et al., 1978).

Fibrinogen is prominent among the proteins adsorbed onto artificial surfaces exposed to blood. On many surfaces it is the first and the predominant protein adsorbed (Vroman et al., 1971) and on some polymers it is present in excess of its proportional concentration in plasma (Brash & Davidson, 1976). Fibrinogen is preferentially adsorbed as compared to albumin, immunoglobulin, lipoproteins and coagulation factors (Vroman et al., 1972). There are, however, time dependent changes in the adsorbed fibrinogen layer, such that by 30-60 seconds it is no longer recognised by antifibrinogen antibody (Vroman & Adams, 1969). Presumably this is due to either a conformational change in fibrinogen molecules, that fibrinogen is no longer present on the surface or that it is masked by another protein. There is some evidence to suggest that fibrinogen is
displaced by high molecular weight kininogen and, to some extent, FXII after a short contact with the artificial surface (Vroman et al, 1980).

The adsorption of fibrinogen to the artificial surface is important due to its ability to interact with both platelets and leucocytes. Surface-bound fibrinogen strongly attracts platelets, although the time dependent changes in the fibrinogen layer make it less reactive to platelets (Vroman et al, 1980). The role of fibrinogen in platelet adhesion to the artificial surface is illustrated by the fact that defibrinated or afibrinogenaemic plasma does not support platelet accumulation unless fibrinogen is added (Zucker & Vroman, 1969). During clinical CPB platelets interact with the adsorbed fibrinogen layer (see section 1.2.7).

In addition, fibrinogen is known to bind to receptors on monocyte and neutrophil surfaces (Altieri et al, 1988; Wright et al, 1988; Simon et al, 1993). Moreover, binding of LPS stimulated monocytes to fibrinogen may enhance tissue factor expression by these cells (Fan & Edginton, 1991). Prior adsorption of albumin inhibits platelet and leucocyte adhesion onto foreign surfaces (Salzman et al, 1969) and limits thrombus formation (Packham et al, 1969). This phenomenon is important in improving the biocompatibility of extracorporeal circuits.

The coating of an artificial surface with γ-globulin has been reported to enhance platelet adhesion and stimulate the platelet release reaction (Evans & Mustard, 1968) and γ-globulin adsorption may be followed by leucocyte adhesion (Adams et al, 1978). In addition, exposing a plasma-coated artificial surface to an antibody against IgG abolishes the enhancement of platelet activity normally seen (Lindon et al, 1988). This would indicate that IgG is, at least in part, responsible for this effect.

The adsorption of proteins belonging to the intrinsic coagulation pathway is of particular significance in relation to cardiopulmonary bypass. FXII can be activated on binding to a negatively charged surface, such as the synthetic surface of a CPB circuit, resulting in activation of the contact system of coagulation (see section 1.1.3). Preliminary work has shown that high molecular weight kininogen and FXII may replace adsorbed fibrinogen on artificial surfaces (Vroman et al, 1980). Further studies
indicated that the active form of high molecular weight kininogen (HKa) produced by cleavage by FXIIa or kallikrein, and not the inactive form produced by further proteolytic cleavage of HKa by FXIa, is principally involved in fibrinogen displacement. However, FXII deposition or a FXII mediated reaction, is also necessary for this displacement to occur (Brash et al, 1988). More recently, FXII has been shown to be adsorbed from circulating blood onto biomedical polymer surfaces by radioimmunoassay and electron microscopy (Ziats et al, 1990). The latter authors also showed adsorption of fibronectin and FVIII/vWF which may also play a role in platelet adhesion to ‘conditioned’ artificial surfaces. It remains unclear how contact factors adsorbed onto artificial surfaces could interact with other proteins, and whether they become activated during this process.

One limitation of many of the early studies on protein adsorption to artificial surfaces was that they were conducted in vitro using single protein solutions or, at best solutions containing several proteins. Whether these proteins react with such surfaces in a similar manner when whole blood or plasma come into contact with them is questionable. In addition, protein adsorption occurring during the circulation of blood through an extracorporeal circuit in vitro may be sufficiently different to that taking place in a clinical setting. The adsorption of plasma components onto artificial surfaces and their interactions with other proteins and cells warrants further investigation.

1.2.5 Complement Activation
There is now a large body of evidence to suggest that complement activation is involved in the pathogenesis of post-CPB organ dysfunction. The pivotal studies of Chenoweth et al, in the 1980’s led investigators to believe that complement activation was the key to understanding some of the deleterious effects of CPB.

The first indication was the demonstration that the anaphylotoxins C3a and C5a are generated during CPB (Chenoweth et al, 1981). A further study confirmed that this increase in C3a is not seen in cardiac operations that do not use CPB (Kirklin et al, 1983). The latter authors also performed a multivariate analysis to identify risk factors
associated with pulmonary, cardiac and renal dysfunction following the use of CPB. They discovered that higher levels of C3a, longer duration of CPB and a younger age at operation were all associated with postoperative cardiac and pulmonary dysfunction. The same factors except bypass time are risk factors for renal dysfunction. This was the first study to relate complement activation with post-CPB organ dysfunction.

C5a, a more potent anaphylotoxin than C3a, is also generated during CPB (Chenoweth et al, 1981; Masteroroberto et al, 1994). However, the fact that C5a has an extremely short half-life \textit{in vivo} due to binding to neutrophil receptors, has made detecting C5a generation \textit{in vivo} difficult. A new assay based on a monoclonal antibody specific for a neoepitope exposed upon C5a activation has also demonstrated significant generation of C5a during CPB (Mollnes et al, 1991). It is generally accepted that complement activation observed during extracorporeal circulation proceeds by alternative pathway mechanisms (Chenoweth, 1988). However, there is some indication that the classical pathway may also be activated during CPB (Wachtfogel et al, 1989).

As well as antigen-antibody complexes, polyanion and polycation interactions (such as protamine and heparin) can activate complement by the classical pathway (C4a) in vitro (Rent et al, 1975). This finding prompted studies to investigate whether the reversal of heparin during CPB using protamine sulphate would result in complement activation. The administration of protamine sulphate results in the generation of C4a, evidence that these complexes do activate complement by the classical pathway (Cavarocchi et al, 1985). A further study concluded that serum containing protamine alone or heparin alone does not activate complement, but together heparin-protamine complexes activate the classical complement pathway with generation of C3a, C4a and C5a (Kirklin et al, 1986). The haemodynamic effect of protamine in some patients may thus be explained, at least in part, by the additional activation of complement by the classical pathway.

The pro-inflammatory activities of complement are mainly the result of the generation of the anaphylotoxins C4a, C3a and C5a. All three cause mast cells to degranulate and therefore increase vascular permeability (Johnson et al, 1975). In
addition, C5a is a potent chemotactic agent for monocytes and neutrophils (Fernandez et al, 1978) and is also a powerful activator of neutrophils increasing adhesiveness, stimulating the respiratory burst and degranulation (Tonnensen et al, 1984). It is believed that the effect of activated complement components on leucocyte activation may play an important role in post-CPB organ dysfunction and this will be discussed further in section 1.2.12.

Attempts to ameliorate complement activation during CPB generally focus on three areas: bypass circuitry design, ultrafiltration during CPB and the use of specific pharmaceutical agents. Two groups report higher C3a levels in patients undergoing CPB using bubble oxygenators compared to membrane oxygenators (Cavarrochi et al, 1986; Tamiya et al, 1988) one of which suggests that complement is activated by the alternative pathway using membrane oxygenators and via the classical pathway using bubble oxygenators (Tamiya et al, 1988). However, other workers have failed to find a difference in complement activation between the two oxygenator types (Moore et al, 1988; Nilsson et al, 1990), probably reflecting differences in patient populations and assay methodologies.

In a porcine model of CPB, complement activation was attenuated by the use of a heparin-bonded circuit (Nilsson et al, 1990b) yet, in an in vitro extracorporeal circuit, heparin bonding of the circuit had little effect on complement activation (Svennevig et al, 1993). During clinical CPB, there was no difference in terms of complement C3 and C4 consumption (Hamulu et al, 1996) but, 30 minutes post-operatively C3a levels were reported to be lower with the use of heparin-bonded circuits (Pekna et al, 1994). Given that it is the increase in C3a levels post-CPB that is a risk factor for post-operative organ failure, this finding may be of clinical significance.

Ultrafiltration is occasionally used after CPB in infants to remove extravascular fluid accumulating due to increased capillary permeability. It has been demonstrated that the use of haemofiltration in paediatric patients during the rewarming stage of CPB can reduce levels of C3a and C5a (Andreasson et al, 1993; Journois et al, 1995); indeed the presence of complement fragments in the ultrafiltrate suggests that the haemofilter may
actually remove these from the circulation and thus have beneficial effects in terms of the inflammatory actions of CPB.

During CPB complement is activated via the alternative pathway during the period of extracorporeal circulation and this may be enhanced by activation of the classical pathway following the administration of protamine. The effect of activated complement proteins augmenting leucocyte activation may be partly responsible for some of the deleterious effects of CPB, but these are more likely to result from activation of all arms of the inflammatory cascade as well as other protease systems.

1.2.6 Cytokine and Endotoxin Induction

In addition to complement activation, several other mediators are likely to play a role in the development of 'the whole body inflammatory response' associated with CPB. These include endotoxin and cytokines such as tumour necrosis factor (TNF) and members of the interleukin family. The release of cytokines during CPB could have numerous effects: the expression of endothelial or leucocyte adhesion molecules may be altered, leucocyte chemotaxis and chemokinesis may be enhanced and cells may be stimulated to release other cytokines or cytotoxic substances. Cytokines may therefore be important in the inflammatory response to CPB.

Interleukin-8 (IL-8) is a cytokine that belongs to a superfamily of neutrophil-attracting and activating peptides. It is known that both the length of time on bypass and the sequestration of neutrophils in the pulmonary microvasculature are critical factors in the development of post CPB pulmonary insufficiency (Ratliff et al, 1973). It is possible that the release of IL-8 during CPB may enhance neutrophil sequestration within the pulmonary vasculature.

An elevation of plasma IL-8 has been demonstrated during CPB (Jorens et al, 1993; Kawamura et al, 1993; Fin et al, 1993) which may be attenuated by pretreatment with methylprednisolone (Kawamura et al, 1995) and enhanced by CPB conditions (Ohata et al, 1995). Others have failed to observe such an increase in plasma IL-8, yet found a significant increase in the IL-8 and IL-1β content of leucocytes (Kalfin et al,
Furthermore, IL-8 mRNA is reported to be increased in myocardial and skeletal muscle during paediatric CPB (Burns et al, 1995), which may result in high local IL-8 concentrations contributing to tissue injury after CPB.

TNF-α levels are reported not to change during CPB (Kawamura et al, 1993; Steinberg et al, 1995; Ohata et al, 1995), yet other studies demonstrate an increase in levels following the removal of the cross-clamp during CPB (Jansen et al, 1992) and after the termination of CPB (Hill et al, 1995b). Additional evidence for TNF-α release during CPB, comes from a study investigating TNF-α mRNA content of leucocytes which is increased by the end of the procedure, especially in those patients with long bypass times (Hattler et al, 1995). In addition to regulating other cytokine production, TNF may also play a role in the activation of coagulation (Bauer et al, 1989; Van der Poll et al, 1990).

IL-1 is the prototypic ‘multifunctional cytokine’; it affects nearly every cell type, often in concert with other cytokines or small mediator molecules (Dinarello, 1996). IL-1 plasma levels are reported to be increased at 24 hours following CPB but not during the procedure itself (Haefner et al, 1989). However, other studies demonstrated no significant changes in IL-1 levels during or after CPB (Kalfin et al, 1993; Steinberg et al, 1993; Ohata et al, 1995; Tarbardel et al, 1996). This may be due to differing patient populations and sensitivities of IL-1 assays used as IL-1 is found in plasma at much lower concentrations than IL-6 or TNF-α.

Serum IL-6 levels have been shown to be increased during CPB (Steinberg et al, 1993; Kawamura et al, 1993) and may be partly attenuated by preadministration of methylprednisolone (Hill et al, 1995b). Interestingly, the use of haemofiltration during paediatric CPB was associated with a reduction in IL-6 and TNF-α levels (Millar et al, 1993; Joumois et al, 1995b), possibly due to removal of these cytokines by the haemofiltration system, a function of haemofiltration that is discussed in section 1.3.3.

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine which inhibits the release of IL-8 and TNF- by activated macrophages and polymorphonuclear leucocytes. During CPB IL-10 rises moderately and transiently and this may be enhanced by
preaministration of corticosteroids (Tabardel et al, 1996). Interestingly, this increase in IL-10 was not seen when blood was circulated through an in vitro extracorporeal circuit (McBride et al, 1995), illustrating differences in the immune response to CPB in vivo.

Endotoxin is a recognised potent activator of various cellular and humoral pathways involved in the inflammatory response (Williams & Maier, 1992). Endotoxin has been shown to directly activate the contact system (Morrison et al, 1974) and cause depletion of C1-INH (Triantaphyllopoulos & Cho, 1986). Endotoxin is difficult to assay reliably, partly due to the possibility of environmental contamination, and partly as the commonly used Limulus amoebocyte lysate assay has not been standardised and is fairly non-specific. In addition, systemic endotoxaemia may be pulsatile in nature. Another approach to assessing endotoxin exposure is to assay cross reactive antibodies directed against the core of Gram-negative bacterial endotoxin molecules (EndoCab), which are comprehensively found in adults (Barclay, 1990). If endotoxin is exposed, these antibodies will bind to it and therefore there will be a reduction in the plasma levels of EndoCab.

Endotoxin has been detected in the plasma of patients undergoing CPB (Andersen et al, 1987; Rocke et al, 1987; Nilsson et al, 1990; Riddington et al, 1996). The source of this endotoxin remains speculative. Solutions used to prime the CPB machine and drugs infused into the patient during CPB have been shown to contain endotoxin. Levels of EndoCab antibodies are reduced during CPB indicating exposure to endotoxin (Mythen et al, 1993). The latter authors also observed a relationship between gut hypoperfusion, decreased levels of EndoCab antibodies and increased contact activation and hypothesised that as a result of loss of gut barrier function endotoxin may be translocated from the gut to the systemic circulation during CPB. Others have failed to relate the degree of gut permeability with endotoxaemia and subsequent outcome (Riddington et al, 1996).
1.2.7 Platelet Activation

The use of CPB is associated with a bleeding diathesis in some patients (Woodman & Harker, 1990). In addition to changes in coagulation proteins, platelet dysfunction is believed to contribute significantly to post-operative bleeding in these patients. During CPB platelets adhere to binding sites located on the α chain and C-terminal domain of the γ-chain of surface-adsorbed fibrinogen (Wenger et al, 1989). The adherence of platelets to the extracorporeal circuit is believed to be primarily mediated via platelet GPIIb/IIIa receptor complexes binding to adsorbed fibrinogen (Gluszko et al, 1987).

The number of platelets attaching to the surface of the extracorporeal circuit depends upon the concentration of adsorbed fibrinogen and also the exact chemical and physical nature of the circuitry (Lindon et al, 1986).

Electron microscopy studies of platelets from CPB patients show that some platelets undergo shape change (Zilla et al, 1989) and loss of granule contents (Harker et al, 1980) consistent with platelet activation. This evidence of platelet activation is supported by reports that concentrations of serum β-thromboglobulin, platelet factor 4 and thromboxane B₂ are increased during CPB (Mezzano et al, 1986; Faymonville et al, 1986; Zilla et al, 1989; Metzelaar et al, 1993). Furthermore, platelet serotonin levels are reduced during CPB suggesting that serotonin is released from platelet dense granules during this period (Aznavoorian et al, 1983).

Many studies have shown that platelet sensitivity to aggregation agonists in vitro is reduced in blood from CPB patients (Edmunds et al, 1982; Zilla et al, 1989, Harker et al, 1980; Boldt et al, 1994a), although others have failed to find any such changes (Holloway et al, 1988; Wenger et al, 1989). The reduced aggregatory response of platelets to ADP was reported to have a time course distinct from platelet activation (Rinder et al, 1991a).

The aggregatory response of platelets from CPB patients to agonists may be affected by a variety of factors: the nature of the pump priming solution and plasma volume expanders (Boldt et al, 1992; Boldt et al, 1993; Tabuchi et al, 1995), the temperature at which CPB is carried out (Boldt et al, 1993b), the anticoagulant regimen
hypothermic CPB conditions, high dose heparin and the use of a cell separator all decrease the aggregatory response of platelets from CPB patients to agonists in vitro.

The use of inotropic support prior to CPB does not effect the platelet count or response to aggregation agonists (Boldt et al, 1992b; Kikura et al, 1995). However the preoperative use of aspirin even if withdrawn 7 days prior to surgery, is associated with further reductions in platelet responses to the agonists ADP, collagen and adrenaline (Boldt et al, 1992c). The fall in platelet numbers during CPB was partly attenuated by adenosine infusion during the procedure (Sollevi et al, 1985).

There have been numerous studies investigating platelet receptors during clinical cardiopulmonary bypass with conflicting results. The percentage of platelets positively expressing an $\alpha$-granule membrane protein P-selectin (GMP-140, CD62P), which is expressed on the platelet surface membrane after activation is increased during CPB (Rinder et al, 1991a; Metzelaar et al, 1993; Wu et al, 1993). One study reports no change in P-selectin expression (Holada et al, 1996) but the latter authors express their results in terms of relative fluorescence, whereas the previous authors noted an increase in positive platelets and not relative fluorescence. In addition, the percentage of platelets positive for the lysosomal integral membrane protein CD63 are increased during CPB (Metzelaar et al, 1993), suggesting that there is release of platelet lysosomal contents including potent acid hydrolases and proteases which could contribute to the systemic inflammatory response seen during CPB.

Some (George et al, 1986; Wenger et al, 1989; Rinder et al, 1991b), but not all (Abrahms et al, 1990; Tanaka et al, 1993; Kestin et al, 1993; Holada et al, 1996) studies have reported defects in the platelet surface GPIIb/IIIa complex. One study using a platelet GPIIb/IIIa inhibitor in a canine model of CPB, showed improved platelet count, response to ADP induced aggregation and blood loss following surgery (Uthoff et al, 1994) which would indicate that changes in this receptor complex may be important in platelet dysfunction during bypass. Furthermore, some (George et al, 1986; Rinder et al, 1991b; Tanaka et al, 1993) but not other studies (Vanderveld et al, 1991; Kestin et al,
1993; Metzelaar et al, 1993; Holada et al, 1996) have reported defects in platelet surface glycoprotein GPIb complex.

The differing results between these studies may be due to differences in patient populations, the length and nature of the CPB operation, or methodologies for studying platelet surface antigens. Using whole blood flow cytometric techniques which should minimise changes in platelet surface receptors by cell handling ex vivo, platelet P-selectin, GPIIb/IIIa and GPIb expression are not altered during CPB (Kestin et al, 1993). Moreover, in this study changes in P-selectin, GPIIb/IIIa and GPIb-IX complex could be induced in platelets in vitro from the blood of patients during CPB, indicating that the platelet defect during CPB may be extrinsic to the platelet rather than intrinsic.

During clinical bypass platelets may form aggregates which can circulate and form microemboli (Edmunds et al, 1982). Platelets also form aggregates with monocytes and, to a lesser extent, neutrophils, but not with lymphocytes during CPB (Rinder et al, 1992). CPB also abolishes platelet force development (the force generated by platelets during plasma clot retraction), which does not return to normal by the end of the procedure at which point the percentage recovery of platelet force development inversely correlates with post-operative blood loss (Greilich et al, 1995).

There have been some investigations into the use of prostacyclin infusion during CPB to improve platelet function. The increase in plasma β-thromboglobulin and platelet factor-4 during clinical CPB is reduced by the infusion of prostacyclin (Aren et al, 1984), which preserves the platelet response to ADP and collagen (Jestice et al, 1990). In both of these studies, the effect seen was not dependent upon whether the infusion was started before or on commencement of CPB, which would imply that this effect is independent of the initial ‘conditioning’ of the CPB circuit.

Platelet aggregation, adhesion and destruction as well as haemodilution are the major causes of thrombocytopenia during CPB; platelet numbers decrease during CPB by 30-50% (Woodman & Harker, 1990). At the end of CPB the platelet population is extremely heterogeneous and this is probably dependent upon the individual patient as well as the nature of the bypass circuitry used. Some platelets show pseudopodia
formation while the majority appear morphologically normal (Zilla et al, 1989), some patients show only a modest increase in CD62 positive platelets, whereas in others the increase is marked (Rinder et al, 1991). In addition, at the end of CPB, some larger platelets are present (Laufer et al, 1975).

As a result of thrombocytopenia and platelet dysfunction, bleeding times during and after CPB are increased, returning to normal 4-12 hours after surgery (Woodman & Harker, 1990). Whether the generation of thrombin during CPB is the major stimulus for platelet activation remains unanswered. Thrombin, plasmin, ADP and heparin may all play a role in stimulating platelets during CPB. Whatever the mechanisms of platelet activation during CPB, clinical cardiopulmonary bypass induces a constellation of platelet defects which have not been fully characterised and are fundamental in contributing to the bleeding diathesis associated with its use.

1.2.8 Contact Activation

The artificial surface of the CPB circuit and the gross tissue trauma provide a massive stimulus for coagulation activation during cardiac surgery. Obviously coagulation must take place to prevent blood loss at the wound site, but it is not desirable for this to occur in a generalised manner. High doses of heparin are used to prevent the extracorporeal circuit from clotting during CPB, but this fails to prevent thrombin generation. Thrombin generation in vivo cannot be measured directly due to its rapid complexing to plasma inhibitors, but thrombin-antithrombin (TAT) complexes as well as prothrombin fragment 1+2 (the small peptide released when prothrombin is cleaved to thrombin) levels are increased during CPB (Boisclair et al, 1993; Brister et al, 1993) which would imply that thrombin is being generated. Activation of the contact system of coagulation has traditionally been considered to play a pivotal role in thrombin generation during CPB. When this thesis was undertaken, there were two basic lines of evidence for activation of the contact system: a fall in the levels of FXII and PKK zymogen proteins and an increase in contact factor:inhibitor complexes during CPB. As the contact system
was studied in chapter 5 of this thesis, literature pertaining to this subject area will be discussed there.

### 1.2.9 The Tissue Factor Pathway

During open heart surgery gross surgical trauma occurs. It is conceivable that this would result in the exposure of tissue factor with the potential for activation of the tissue factor pathway of coagulation. The use of the CPB circuit provides further trauma to the blood. As monocytes and endothelial cells can be induced to express tissue factor by various inflammatory mediators, these cells may also provide a source of tissue factor during CPB.

Monocyte expressed tissue factor can be assessed by a variety of methods: clotting based assays, chromogenic based assays or flow cytometric techniques. Techniques which use isolated mononuclear cells may be complicated by the fact that cell separation procedures can activate the cells which are to be studied. An advantage of flow cytometric methods is that a whole blood technique can often be employed, thus reducing these artefactual changes.

In a model of CPB, using flow cytometry on fixed isolated monocytes, there is an increase in monocyte expressed tissue factor after 2-6 hours of recirculation of blood (Kappelmayer *et al*, 1993). Whether monocyte tissue factor expression is increased during clinical CPB is a source of contention. Two groups report that, using whole blood flow cytometry, monocyte tissue factor is not increased at the end of CPB (Parratt & Hunt, 1996; Ernofsson *et al*, 1997) but, one did observe a significant increase 20 hours post-operatively. However, others using similar techniques have shown an increase in monocyte tissue factor by the end of CPB (Barstad *et al*, 1996). One factor that may be different between these studies is the time spent on bypass. Monocyte procoagulant activity has been shown to increase during (Chung *et al*, 1996) and following clinical CPB (Nilsen *et al*, 1993).

Interestingly, the expression of tissue factor on cells recovered from CPB circuits is greater than those in the systemic circulation (Kappelmayer *et al*, 1993;
Barstad et al, 1996; Chung et al, 1996). This is reported to be partly attenuated by the use of heparin-bonded circuits (Barstad et al, 1996). The latter authors also observed a reduction of monocyte tissue factor in circulating monocytes at the end of CPB with such circuits although others have failed to find any difference between coated and non-coated circuits (Ernofsson et al, 1997).

Clearly if there is upregulation of tissue factor expression on monocytes during CPB, it is slow to occur. The reason for this may be many-fold. Firstly, in vitro, monocyte tissue factor expression induced by LPS is maximal after 3-6 hours incubation and requires de novo synthesis of the protein (van der Logt et al, 1994). Secondly, it is reported that hypothermic conditions suppress tissue factor expression by stimulated endothelial cells, but rewarming results in an increase in tissue factor expression (Johnson et al, 1995). Finally, heparin has been demonstrated to inhibit tissue factor production by monocytes (Gori et al, 1996). Perhaps a similar phenomenon is seen during CPB, where heparin and hypothermic conditions help to suppress tissue factor expression on monocytes until the end of surgery. Activated monocytes may provide a source of tissue factor during CPB, but this does not appear to correlate with thrombin generation (Ernofsson et al, 1997). However, monocytes may become adhered to CPB circuitry where they are unavailable for assay and able to promote coagulation reactions.

Relatively little attention has been paid to the activation of the tissue factor pathway of coagulation during CPB. This is mainly due to a lack of laboratory methods to carry out such studies. Previously it has not been possible to measure tissue factor in plasma samples, indeed the membrane-bound nature of tissue factor suggests that this would not be present free in plasma. However, it appears that the extracellular region may be present in plasma and can be detected by a specific ELISA (Koyama et al, 1994). The interpretation of plasma tissue factor assays is complicated by the fact that it is not clear whether tissue factor detected by this method is the functional form. As tissue factor requires phospholipid for its activity, soluble tissue factor in plasma may
not reflect the potential for clotting activation (Francis et al, 1995). There is currently no published literature employing this assay during CPB.

When this thesis was undertaken, methods to assay FVIIa directly were not available. Previously FVIIa has been measured using clotting assays for FVII (FVII:C) which are a function of both FVII zymogen and FVIIa (Miller et al, 1994). Two FVII clotting assays are performed with different thromboplastins, one having a greater sensitivity to FVIIa than the other, the ratio of the two giving an index of FVIIa activity. Alternatively a ratio of FVII zymogen measured by immunoassay to FVII:C can be used. However, an assay for FVIIa has recently been developed which uses a recombinant mutant form of tissue factor that has selectively lost its ability to promote factor VII activation while retaining cofactor function for the FVIIa catalysed activation of factor X (Morrissey et al, 1993). One of the aims of this thesis was to establish this method in our research laboratory to enable the activation of the tissue factor pathway during CPB to be examined more closely. Literature published regarding FVIIa and CPB will therefore be discussed in chapter 5 of this thesis.

1.2.10 Fibrinolysis

Changes in the fibrinolytic system during and after cardiac surgery could clearly contribute to bleeding complications observed postoperatively. This could be due to an increase in fibrinolytic activity, to a reduction in inhibitory capacity, or a combination of both of these factors.

There is an increase in fibrinolytic activity, as evidenced by an increase in plasma levels of D-dimer or other fibrin degradation products (FDPs), during CPB (Rifon et al, 1990; Giuliani et al, 1991; Teufelsbauer et al, 1992; Ray et al, 1994; Lu et al, 1994). Following CPB there is a decrease in D-Dimer levels until two days postoperatively, after which levels increase again until about seven days postoperatively (Lu et al, 1994). This presumably reflects clot dissolution and wound healing at surgical sites. Consistent with the formation of fibrin during CPB, there is a decrease in plasma fibrinogen levels.
Evidence that plasmin is being generated during CPB comes from studies that report a
decrease in plasminogen levels (Paramo et al, 1991) and an increase in plasmin
complexed to \( \alpha_2 \)-antiplasmin (Teufelsbauer et al, 1992; Tanaka et al, 1993;
Mastroroberto et al, 1995) during clinical bypass. Consistent with these findings, during
CPB there is a fall in \( \alpha_2 \)-antiplasmin, the main plasma inhibitor of plasmin (Ray et al,
1994).

The enhanced fibrinolytic response during CPB is thought to be primarily
mediated through the release of tPA from the endothelium (Stibbe et al, 1984). There is
a rapid increase in both tPA functional activity (Paramo et al, 1991) and antigenic levels
(Giuliani et al, 1991; Valen et al, 1994; Lu et al, 1994) during clinical CPB whereas total
urokinase levels are not altered (Valen et al, 1994; Spannagl et al, 1995). It is not clear
what happens to PAI-1 activity during CPB procedures, although most studies report
that levels do not change (Giuliani et al, 1991; Paramo et al, 1991; Valen et al, 1994) but
are increased postoperatively (Paramo et al, 1991; Ray et al, 1994; Valen et al, 1994).
However, individual variations in the fibrinolytic response in terms of PAI-1 and tPA
levels can be observed in different subjects during CPB (Chandler et al, 1995).

The slow onset of PAI-1 response is consistent with the production of PAI-1 by
the liver or endothelium in response to inflammatory mediators. It is uncertain what the
stimulus for tPA release by the endothelium during CPB is - there are numerous
possibilities: adrenaline and other \( \beta \)-adrenergic agonists (Downing & Edmunds, 1992),
bradykinin (Brown et al, 1997), platelet activating factor, thrombin, FXa and other
coagulation proteins (Emeis, 1992).

Whether activation of the contact system during CPB contributes to increased
fibrinolytic activity remains unclear. Plasma KK (Colman, 1969), FXIa (Mandle et al,
1977) and both \( \alpha \) and \( \beta \)FXIIa (Goldsmith et al, 1978), can activate plasminogen directly
and kallikrein can cleave single chain urokinase to the active two chain form. In addition,
cleavage of high molecular weight kininogen by kallikrein results in the liberation of
bradykinin which could release tPA from the endothelium. Reduced levels of plasmin-
\( \alpha_2 \)-antiplasmin complexes and FDPs have been demonstrated in two patients with FXII
deficiency undergoing CPB (Chung et al, 1994). However this may be due to these patients exhibiting a fibrinolytic response which is not 'typical' of CPB patients and not because of a lack of FXII. Further investigation into the clinical significance of varying patterns of fibrinolytic response, the role of the contact system in fibrinolytic activity, and the correlation between fibrinolytic parameters during CPB and postoperative blood loss are clearly needed.

1.2.11 Coagulation inhibition

Some inhibitors of coagulation proteins have been dealt with in preceding sections but many have been poorly studied during CPB. Since AT-III, HCII and thrombomodulin were studied in chapter 5 of this thesis, these will be discussed in more detail there.

1.2.12 Neutrophil activation

Neutrophil activation can result in tissue damage by the release of enzymes, oxygen metabolites and arachidonic acid metabolites (Sibille & Reynolds, 1990). Adherence to the endothelium is a crucial step in both neutrophil transmigration and neutrophil-mediated endothelial injury and is dependent upon interactions between endothelial and leucocyte adhesion molecules.

The $\beta_2$ integrins are a family of adhesion molecules involved in the adhesion of leucocytes to the endothelium (Figure 1.5). They share a common $\beta$ chain (CD18) but differing $\alpha$ chains (CD11a, b and c). CD11b is stored in secondary and tertiary granules of neutrophils and peroxidase positive granules of monocytes. Upon activation these granules can be rapidly translocated to the cell surface (Miller et al, 1987). To this end, the expression of CD11b on the surface of leucocytes has been used as a marker of activation of these cells.

Neutrophil expressed CD11b has been shown to be mobilised during simulated ECC (Kapplemeyer et al, 1993; Moat et al, 1993) which is consistent with observations in vivo (Rinder et al, 1992; Gillinov et al, 1993b). There is some evidence that products of complement activation may enhance neutrophil adhesion molecule expression during
CPB (Tennenberg et al, 1986; Gillinov et al, 1994) but others have failed to draw such conclusions (Finn et al, 1996). The increase in neutrophil CD11b during CPB may be partly attenuated by the preadministration of glucocorticoids (Hill et al, 1994) and other anti-inflammatory agents (Gillinov et al, 1994).

Figure 1.5 Neutrophil-endothelial interactions

In addition to being a ligand for leucocyte adhesion, CD11b has been shown to function as a receptor for the inactivated complement receptor C3bi (Wright et al, 1983), fibrinogen (Altieri et al, 1988a; Wright et al, 1988; Simon et al, 1993), factor X (Altieri et al, 1988b), HK (Wachtfogel et al, 1991) and in stimulated monocytes engagement of CD11b by one of its ligands results in enhanced tissue factor expression (Fan & Edgington, 1991). There is also some evidence that adherence via CD11b primes neutrophils to degranulate (Richter et al, 1990) and produce the respiratory burst.
(Shappel et al, 1990). Thus during CPB there is upregulation of a cellular receptor that not only mediates leucocyte adhesion and transmigration but can result in the release of cytotoxic substances and the modulation of procoagulant, fibrinolytic and complement pathways.

Activated neutrophils release a variety of enzymes and cytotoxic substances. Neutrophil elastase is released during clinical CPB (Wachtfogel et al, 1987; Faymonville et al, 1991). Elastase release from neutrophil azurophilic granules can be stimulated by several coagulation proteins including kallikrein (Wachtfogel et al, 1983), FXIIa (Wachtfogel et al, 1986), and thrombin (Cohen et al, 1991), all of which may be generated during CPB. The potential relevance of neutrophil elastase to haemostasis is that elastase is a potent serine protease which has many effects on coagulation and fibrinolysis. Elastase can degrade fibrinogen and most coagulation factors except prothrombin (Schmidt et al, 1975) and can cleave the inhibitors AT-III (Jochum et al, 1981), HCII (Sie et al, 1987), protein C (Eccle et al, 1991), protein S (Oates et al, 1991) and TFPI (Higuchi et al, 1992). Interestingly, elastase can cleave prekallikrein to kallikrein (Shibuya et al, 1991), a possible positive feedback mechanism for further elastase production. Neutrophil elastase also enhances fibrinolysis (Plow, 1982).

The upregulation of neutrophil adhesion molecules and release of neutrophil enzymes is thought to play a fundamental role in post-CPB organ dysfunction and will be discussed in chapter 5.

1.2.13 Monocyte activation

Monocytes may also become activated during CPB. There is some ambiguity as to whether CD11b is upregulated on the surface of monocytes during CPB. Some investigators have demonstrated mobilisation of monocyte CD11b during clinical bypass procedures (Rinder et al, 1992; Parrat & Hunt, 1996). The latter authors also suggest that the increased CD11b receptor expression leads to the direct activation of factor X by monocytes during CPB. When activated, monocytes can release a number of cytokines (see section 1.2.6) and express tissue factor (see section 1.2.9).
1.2.14 Consequences of Blood Activation during CPB

In the preceding sections we have seen that, during CPB the coagulation, complement and fibrinolytic systems become activated as do cellular components of blood. This may have various effects as outlined in Figure 1.6.

Figure 1.6 The deleterious effects of CPB

The overall mortality for CPB procedures is 1-4% (Allen, 1988) but depends upon the type of operation being performed. One of the problems associated with the use of CPB is the risk of excessive post-operative bleeding which occurs in approximately 3-5% of
patients (Woodman & Harker, 1990). Nonsurgical bleeding problems associated with CPB are usually related to the use of heparin, a decrease in platelet number and function, and an increase in fibrinolytic activity. Dilution of coagulation factors and their inhibitors may also contribute to post-operative bleeding.

CPB produces a variety of emboli that are not completely removed by filters in the CPB circuit. The sources of these include fibrin, denatured lipoproteins and other fat emboli, platelet and leucocyte aggregates, tissue debris and even suture material. These emboli may contribute to post-operative neurological dysfunction and stroke (Edmunds, 1995). The incidence of stroke post-CPB ranges between 1-5%.

During CPB, thrombin is generated. In addition to its direct effects on coagulation (activating platelets, fibrin formation, and activating various procofactors and proenzymes), thrombin activity extends through anticoagulation, stimulation of fibrinolytic reactions, activation of white blood cells, regulation of vascular tone and wound healing (Figure 1.7).

![Figure 1.7 The multiple roles of thrombin](Image)
It is difficult to assess the degree of post-operative organ dysfunction due to CPB from that due to the underlying disease. Nonetheless, CPB temporarily impairs lung function and some patients (approximately 2%) develop a reaction indistinguishable from ARDS (Fowler et al, 1983). The sequestration of neutrophils in the microvasculature of the lung where they can release cytotoxic substances is a fundamental process in CPB-associated pulmonary dysfunction (See chapter 5). In addition, haemodilution, hypothermia, low perfusion, microemboli and haemolysis during CPB as well as periods of low cardiac output after CPB can contribute to renal insufficiency. Given the gross trauma that occurs to the patient during surgical procedures that require the use of CPB, it is remarkable that so few patients develop clinically significant post-operative organ dysfunction.

1.2.15 Aprotinin

Although none of the patients studied in this thesis received aprotinin therapeutically, no discussion of modern cardiac surgery would be complete without mentioning the use of aprotinin to reduce post-operative blood loss.

Aprotinin is a naturally occurring serine protease inhibitor isolated from bovine pancreas with a molecular weight of 6512 (Davis & Whittington, 1995). Its action results from the formation of reversible stoichiometric complexes with serine proteases. Some of the effects of aprotinin on haemostatic reactions have been realised for decades, but the first definitive use of a high-dose regimen during clinical CPB was published in 1987 (Royston et al, 1987). This study was designed to attempt to reduce complement activation and pulmonary injury related to CPB, but the most striking finding was a dramatic reduction in postoperative bleeding in patients treated with aprotinin. The findings of this study were confirmed by numerous centres around the world.

The mechanism by which aprotinin reduces blood loss following CPB is not fully understood. Aprotinin has a broad spectrum of activity as outlined in Table 1.5. It is likely that a combination of several of these actions play an important role in blood conservation by aprotinin.
Table 1.5 Reported effects of aprotinin on haemostasis

<table>
<thead>
<tr>
<th>Category</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinolysis</td>
<td>Inhibits plasmin directly</td>
</tr>
<tr>
<td></td>
<td>Inhibits tPA release</td>
</tr>
<tr>
<td>Coagulation</td>
<td>Inhibits plasma kallkrein</td>
</tr>
<tr>
<td></td>
<td>Prevents loss of C1-INH activity by heparin</td>
</tr>
<tr>
<td></td>
<td>Inhibits the TF:FVIIa complex</td>
</tr>
<tr>
<td></td>
<td>Inhibits FXIIa generation</td>
</tr>
<tr>
<td></td>
<td>Binds and inhibits thrombin</td>
</tr>
<tr>
<td></td>
<td>Inhibits activated protein C</td>
</tr>
<tr>
<td>Platelets</td>
<td>Prevents loss of GPIb</td>
</tr>
<tr>
<td></td>
<td>Prevents thrombin-induced aggregation</td>
</tr>
<tr>
<td></td>
<td>Shortens bleeding time</td>
</tr>
<tr>
<td></td>
<td>Prevents impaired platelet function due to FDP</td>
</tr>
<tr>
<td></td>
<td>Reduces heparin binding</td>
</tr>
<tr>
<td>Endothelium</td>
<td>Reduces platelet adherence</td>
</tr>
<tr>
<td></td>
<td>Enhances vWF synthesis</td>
</tr>
<tr>
<td>Neutrophil function</td>
<td>Inhibits upregulation of adhesion molecules</td>
</tr>
<tr>
<td></td>
<td>Reduced cytokine release</td>
</tr>
<tr>
<td></td>
<td>Ameliorates elastase release</td>
</tr>
</tbody>
</table>

1.2.16 Heparin-bonded circuits

The desire to improve the biocompatibility of CPB circuits led to the introduction of CPB circuits which are coated with heparin. These are often used in conjunction with reduced systemic heparin administration. There have been numerous studies investigating the blood interactions with heparin-coated compared to standard circuits. However, comparison and interpretation of these studies is complicated by the fact that many studies differ in the circuitry used (some use complete heparin-bonded circuits and others only parts of a circuit e.g. an oxygenator) and systemic heparin concentrations. Nonetheless, heparin-coated circuits may afford improved biocompatibility in terms of complement, cellular, cytokine and coagulation activation.

In a porcine model of CPB, complement activation was attenuated by the use of a heparin-bonded circuit (Nilsson et al, 1990b), and similar effects have been observed using in vitro circuits (Videm et al, 1991b). During clinical CPB, heparin-coated circuits reduced complement activation (Videm et al, 1992) and C3a levels 30 minutes post-
operatively were reported to be lower with the use of heparin-bonded circuits (Pekna et al., 1994). Given that it is the increase in C3a levels post-CPB that is a risk factor for post-operative organ failure, this finding may be of therapeutic significance. In addition, the use of a covalently bonded heparin CPB circuit, including membrane oxygenator and tubing, is associated with reduced levels of IL-6, IL-8 (Steinberg et al., 1995) and TNF-α (Yamada et al., 1996) compared to standard circuits.

Granulocyte activation may be partly attenuated by the use of heparin-coated circuits. Using an in vitro heparin-coated circuit the upregulation of CD11b may be partly attenuated (Videm et al., 1991), and the release of the neutrophil granule proteins elastase (Takano et al., 1992; Wendel et al., 1994), myeloperoxidase and lactoferrin (Moen et al., 1996) is reduced. During clinical CPB, heparin-bonded circuits resulted in less free radical, myeloperoxidase and lactoferrin release from neutrophils (Bozayi et al., 1996).

Heparin-bonded circuits appear to have little effect on platelet loss or function. There is reported to be less platelet loss (Videm et al., 1991b) and βTG release (Takano et al., 1992; Moen et al., 1996) using in vitro circuits coated with heparin and less platelet loss and improved function in an animal model (Tong et al., 1990). However, in clinical CPB, βTG production and platelet count are not altered by the use of heparin-coated circuits (Van der Kamp & Van Oeveren, 1993; Wagner et al., 1994; Boonstra et al., 1994; Ovrum et al., 1995), although platelet adhesion to the CPB circuit may be reduced (Gorman et al., 1996).

Using an ex vivo model, heparin-bonded circuits partly attenuate the rise in KK and fall in FXII during CPB and may thus reduce contact activation (Wendel et al., 1994). In clinical CPB, no significant difference was observed with heparin-coated circuits in terms of FXIIa amidolytic generation or FXIIa:Cl-INH complexes (Van der Kamp & Van Oeveren, 1993). However, the later authors did note decreased binding of FXII to the heparin-coated circuits. During clinical CPB the use of heparin-bonded circuits was associated with a reduction in the expression of tissue factor on circulating...
monocytes and monocytes stuck to the CPB circuitry (Barstad et al, 1996). However, others have failed to observe any such differences (Ernofsson et al, 1996).

Heparin-coated circuits appear to offer little benefit over standard circuits in terms of thrombin generation or fibrinolytic activity. During clinical CPB there were no significant difference in levels of TAT complexes and prothrombin fragment 1+2 (Wagner et al, 1994; Gorman et al, 1996). Indeed, one group reported higher levels of both markers of thrombin generation in heparin-coated versus non-coated groups (Ovrum et al, 1995). During clinical CPB there was no significant difference in levels of D-dimer, fibrinopeptide A, tPA, PAI-1 or plasmin-antiplasmin complexes (Wagner et al, 1994; Boonstra et al, 1994; Ovrum et al, 1995; Gorman et al, 1996) which would suggest that the heparin-coating of CPB circuits had no effect on fibrinolysis during CPB. This is in keeping with the finding in most of the studies above that post-operative blood loss is also not significantly different.

1.3 Haemofiltration

There are several renal replacement therapies available for clinical use in renal failure: these include peritoneal dialysis, haemodialysis and haemofiltration. The choice of therapy is based upon clinical need but is essentially required to perform the function of a healthy kidney, i.e. removal of uraemic toxins such as creatinine and urea, electrolyte convection, and the maintenance of fluid balance.

In critically ill patients, the underlying disease processes pose particular problems for instituting extracorporeal renal replacement. The majority of patients in the intensive care unit have multiple rather than single organ failure, often in combination with sepsis. Many patients are catabolic and/or have intra-abdominal sepsis making peritoneal dialysis unsuitable or ineffective. Conventional haemodialysis is frequently complicated by haemodynamic instability and is thus unsuitable for many critically ill patients.

The use of haemofiltration was first described in 1977 (Kramer et al, 1977) when Kramer accidentally inserted a catheter into the femoral artery instead of vein. He
left the catheter in place, and observed that the arterial pressure was sufficient to achieve satisfactory filtration without the use of a blood pump. Continuous arteriovenous haemofiltration (CAVH) has two main advantages over conventional dialysis for renal replacement in the intensive care patient: it permits accurate control of fluid balance and also affords improved haemodynamic stability (Schetz et al, 1989). However, CAVH does depend upon an adequate arterial driving pressure to maintain blood flow through the circuit so a haemofiltration system has been developed that uses venous access and a pumped extracorporeal system i.e. continuous veno-venous haemofiltration (CVVH), thus eliminating this requirement (Wendon et al, 1989). CVVH is now a well established supportive treatment in acute renal failure and is used in all haemofiltration subjects in this thesis.

The published literature available detailing haemostatic changes during haemofiltration is very limited and for this reason will be discussed in the relevant chapters on haemofiltration. This probably does not reflect a lack of interest in or the significance of this subject area, but more likely that unlike CPB and haemodialysis, haemofiltration is not an elective procedure, making patient recruitment and sample collection difficult. In addition, in comparison to CPB and dialysis which are performed on large numbers of patients, very few patients are haemofiltered (less than 50 annually in the largest of intensive care units).

There is substantial literature published regarding haemodialysis and haemostasis, however it must be stressed that haemodialysis and haemofiltration differ in several fundamental aspects: the physical principle used for solute transfer, treatment-timing, blood flow, and anticoagulation regimens. Haemofiltration patients are critically ill, often with multi-organ dysfunction syndrome and are continuously on extracorporeal circulation. Haemodialysis patients, on the other hand, tend to have only single organ failure and are usually dialysed intermittently for a short period of time. Although activation of coagulation, complement and inflammatory pathways may be common to all types of extracorporeal circuit, because of these differences it is inappropriate to extend the results of studies in CPB and dialysis to haemostasis during haemofiltration.
1.3.1 Haemofiltration circuits

One of the principle differences between haemofiltration and haemodialysis is the mechanism by which solutes are transferred (Figure 1.8).

![Diagram of Haemodialysis and Haemofiltration](image)

*Figure 1.8 Principles of solute transfer in haemodialysis and haemofiltration*

Haemodialysis uses diffusion through a semi-permeable membrane down a concentration gradient. Removal of fluid is achieved by ultrafiltration, where the driving force is a hydrostatic pressure gradient. Haemofiltration uses convection whereby solutes are carried along with the bulk flow of fluid through a highly permeable membrane down a pressure gradient (similar to glomerular filtration). Tubular function is partially replaced by infusion of a substitution fluid. Fluid balance is achieved by balancing ultrafiltration and substitution.
Figure 1.9 Typical circuits used for haemodialysis and haemofiltration

Abbreviations: CAVH: continuous arteriovenous haemofiltration; CAVHD: continuous arteriovenous haemodialysis; CVVH: continuous venovenous haemofiltration; CVVHD: continuous venovenous haemodialysis.
Due to these differing mechanisms of solute removal, haemodialysis provides better clearance of small solutes, whereas haemofiltration is independent of molecular weight up to the cut-off of the membrane (approximately 25-30 Kd) resulting in better clearance of middle molecules (MW 0.5-10 Kd). Haemodialysis requires the presence of a dialysate whereas haemofiltration requires the intravenous administration of large volumes of sterile, non-pyrogenic (and hence expensive) substitution fluid.

The main types of haemodialysis and haemofiltration circuits can be seen in Figure 1.9. In continuous arteriovenous haemofiltration (CVAH) and haemodialysis (CVAHD) a catheter is inserted into an artery (or fistula) and arterial pressure drives the blood around the circuit which is then returned to the patient by cannulation of a large vein. In continuous venovenous haemodialysis (CVVHD) or haemofiltration (CVVH) vascular access is via cannulation of a large vein (usually subclavian, jugular or femoral) and blood is pumped around the extracorporeal circuit and then returned to the patient by cannulation of a large vein. Haemofilters in clinical use are generally made from polysulphone, polyamide or polyacrylonitrile.

1.3.2 Anticoagulation
To prevent haemodialysis and haemofiltration circuits from clotting, anticoagulants are infused directly into the circuit and generally consist of unfractionated heparin. In patients treated by haemodialysis the risk of bleeding is low and the aim of anticoagulation is therefore to prevent fibrin formation and platelet activation which would otherwise occur on a regular basis in these patients. A plasma heparin concentration (measured by anti-Xa activity) of >0.5U/ml has been shown by numerous studies to provide adequate anticoagulation during haemodialysis in chronic renal failure as determined by suppression of FPA levels (Ireland et al, 1989). This translates in most patients to a loading dose of 1-5000U and a maintainance dose of 1-2000U/hour of unfractionated heparin (Colvin & Barrowcliffe, 1993).

In acutely ill patients, who may be have a high risk of bleeding, a heparin regimen should be employed that does not pose too great a risk of haemorrhage. This
necessitates tight control and monitoring of heparin, and a degree of clotting may have to be accepted. In haemofiltration, anticoagulants are infused directly into the arterial side of the haemofilter. Generally this consists of unfractionated heparin infused at a rate of 300-1000IU/hour. However, the heparin dosage is not standardised but based upon pre-filtration global clotting tests, platelet count and the clinical condition of the patient. In critically ill patients in particular, the requirement for adequate anticoagulation must be balanced with the risk of bleeding. This results in most haemofiltration patients receiving smaller doses of heparin compared with haemodialysis patients. Indeed, some patients are not anticoagulated at all due to underlying coagulopathies.

Alternatives to unfractionated heparin for anticoagulation during haemofiltration include LMW heparin, prostacyclin, citrate, Orgaran and dermatan sulphate. Unfortunately most of these have problems associated with their use and there have been few studies detailing their use in continuous haemofiltration. LMW heparins cannot be monitored using global clotting tests and require anti-Xa assays for adequate control. Prostacyclin may result in peripheral vasodilation and consequent hypotension, but this may be attenuated by careful control of fluid balance during haemofiltration. Indeed, one group reported improved haemodynamic profiles and filter lifespan when patients were anticoagulated with a combination of PGI\textsubscript{2} and unfractionated heparin compared to either anticoagulant used alone (Sibylle et al, 1994). Prostacyclin has a distinct advantage over heparin in the critically ill patient as its use is associated with significantly less haemorrhagic problems (Davenport et al, 1994).

Sodium citrate can be used as an anticoagulant during haemofiltration (Ahmad et al, 1990), but the large sodium load requires a reduction in the sodium content of haemofiltration substitution fluid which usually has a fixed sodium concentration. In addition, the metabolism of citrate to bicarbonate can result in metabolic alkalosis. Careful monitoring of calcium is therefore required with additional supplementation of calcium if necessary.
1.3.3 Possible beneficial effects of haemofiltration

Patients with acute renal failure have a poor prognosis with a mortality of at least 50% (Barton et al, 1993). The use of continuous renal replacement therapies may result in a better outcome of these patients, although this is not established. Haemofiltration may have a beneficial effect on patient outcome in the intensive care unit due to the removal of inflammatory mediators by the haemofilter. Evidence for this hypothesis comes from short-term studies measuring levels of cytokines and inflammatory mediators in both the plasma and ultrafiltrate of patients during continuous haemofiltration.

TNF-α is detectable in the plasma of septic patients before haemofiltration commences and some (Bellomo et al, 1993; Tonnensen et al, 1993; Sander et al, 1995), but not all (Hoffman et al, 1995) studies report detectable levels in the ultrafiltrate after haemofiltration has commenced. Given that the fully active form of TNF-α has a molecular weight of 56kD and the molecular weight cut off for most haemofiltration systems is around 30kD, perhaps one would not expect TNF-α to be removed by haemofiltration. The fact that some studies observe TNF-α in the ultrafiltrate may be a function of the assay system used to measure TNF-α, as some ELISAs clearly recognise split-off products as well as the full TNF-α molecule. In all of these studies however, there were no significant changes in plasma levels of TNF-α during haemofiltration.

IL-1α (Tonnensen et al, 1993), IL-1β (Bellomo et al, 1993; Hoffman et al, 1995), IL-6 (Sander et al, 1995; Bellomo et al, 1995) and IL-8 (Bellomo et al, 1995) have also been detected in the ultrafiltrate of haemofiltration patients, although others have failed to detect IL-6 in the ultrafiltrate (Hoffman et al, 1995). Although the molecular weight of IL-6 is 25kD and should thus be removed by haemofiltration, it can form a complex with α2-macroglobulin and would thus be retained in plasma. With all of these studies there are no significant changes in plasma levels of the cytokines. This does not however preclude their removal from blood by haemofiltration as their rate of release, short half-life and their distribution between receptor-bound and free forms must be taken into account.
Using an *in vitro* circuit, haemofiltration has also been shown to clear platelet activating factor from blood (Ronco *et al*, 1995). In addition, there is some evidence that haemofiltration can remove complement components C3a (Hoffman *et al*, 1995) and factor D (Gasche *et al*, 1996). The latter was not found in the ultrafiltrate but was removed due to adsorption to the PAN membrane of the haemofilter.

It is difficult to assess exactly what mediators haemofiltration removes and how this may improve the status of the patient. However, the fact that the ultrafiltrate from septic haemofiltration patients can induce mononuclear cells to produce TNF-α would suggest that there are some form of immunoregulatory molecules contained within the ultrafiltrate. The nature of these molecules needs to be identified and further studies undertaken if haemofiltration is to be instituted as a treatment in septic patients who do not have renal failure. A recent report suggests that haemofiltration can increase IL-6 clearance, yet not alter plasma levels of this cytokine, in patients with systemic inflammatory response syndrome irrespective of their renal function (Sander *et al*, 1997).

These findings also pose an interesting use of haemofiltration during CPB. If during CPB generation of activated complement components and cytokines occurs and it is possible that haemofiltration can remove these substances from the circulation, could a haemofilter be incorporated into the CPB circuit to help ameliorate these changes? Indeed, in paediatric patients, haemofiltration is occasionally used during the rewarming stage of CPB to maintain fluid balance and this is reported to be associated with lower plasma levels of C3a, C5a, IL-6, TNF-α and also with less blood loss (Journois *et al*, 1994b).
1.4 Aims of thesis

The objective of this thesis was to investigate the mechanisms involved in activation of the coagulation system during the use of extracorporeal circulation. Two groups of patients were selected for study: patients undergoing elective cardiac surgery requiring cardiopulmonary bypass and critically ill patients in acute renal failure requiring continuous venovenous haemofiltration.

The possibility that activation of the contact system of coagulation was responsible for the generation of thrombin during haemofiltration was examined (Chapter 3). Having found no evidence for contact activation during haemofiltration this led to an investigation into activation of the tissue factor pathway of coagulation during haemofiltration and its relation to thrombin generation (Chapter 4).

The finding that activation of the tissue factor pathway occurred during haemofiltration, and that thrombin generation was known to occur during CPB but the mechanisms for this were not understood, promoted an investigation into possible activation of this pathway during CPB (Chapter 5). Observations from this chapter suggested that thrombin generation during CPB did occur, but was not related to activation of either the contact or tissue factor pathway of coagulation.

This led to the notion that activation of coagulation proteins could be occurring on surfaces not detectable in conventional assays and hence other possible surfaces for supporting coagulation activation were investigated in vitro. The activation of FXII on the surface of lipoproteins and peripheral blood cells was investigated as a possible mechanism for coagulation activation (Chapter 6).

Finally, the activation of the tissue factor pathway in blood shed in the surgical field that is reinfused into the patient was examined as a mechanism of enhancing systemic coagulation activation during CPB (Chapter 7).
General Methods

A list of addresses of the companies used to supply reagents, equipment and consumables used in this thesis is given in appendix 1. Therefore in this chapter they will be cited by name only.

2.1 Collection and Separation of Blood Samples

2.1.1 Blood collection tubes
Tubes containing dipotassium ethylenediamine tetra-acetic acid (EDTA) were obtained from Becton Dickinson Ltd. These gave a final concentration of 1.0mg/ml EDTA in blood. Plastic tubes containing 0.106M tri-sodium citrate (Merck Ltd) were used for collection of citrated blood (1 part anticoagulant to 9 parts blood). Plain glass tubes were used for the collection of serum, and blood was allowed to clot at 37°C before sample separation for serum.

2.1.2 Sample collection
Blood was collected from apparently healthy normal subjects, patients undergoing cardiopulmonary bypass procedures and critically ill patients requiring haemofiltration. Collection of samples from patients is described in the relevant chapter. Blood from normal subjects was collected from the ante-cubital fossa with minimal stasis, using a 21-gauge needle and plastic disposable syringe.

2.1.3 Separation
Plasma was separated within 2 hours of collection. Platelet poor plasma was prepared by centrifuging anticoagulated blood samples at 2000g for 15 minutes at room temperature.
Plasma was removed with a disposable plastic pasteur pipette, aliquoted into polypropylene tubes and frozen at -70°C.

2.2 Full Blood Counts

Full blood counts were performed on blood anticoagulated with EDTA using a Coulter STAKS cell counter (Coulter Electronics Ltd).

2.3 Amidolytic Substrate Assays

2.3.1 General Principle

Amidolytic substrate assays are based on the use of synthetic peptide substrates which are covalently linked to a chromophore, frequently p-nitroaniline (pNA). Proteases cleave the substrate with liberation of pNA. The rate of pNA release is measured photometrically at 405nm and the change in absorbance/minute is proportional to the enzyme activity (and thus reflects the concentration of the protease under study or its inhibition). Alternatively, the substrate is incubated for a set time and then the reaction stopped by the addition of acid, and then the absorbance measured (end-point determination). The amino acid sequence of the synthetic substrate confers specificity to the substrate and this may be enhanced by the inclusion of inhibitors or by choosing buffers of a certain pH or ionic strength in the assay.

2.3.2 Standardisation

All amidolytic assays were standardised using Reference Plasma 100% (Immuno Ltd) unless otherwise indicated. Results were corrected to allow for the potency of the standard and each new batch of standard was cross calibrated with the previous batch to ensure continuity of studies. Where a potency was not provided by the manufacturer, the material was assumed have 1.00U/ml of the relevant parameter, since it has been obtained from a large pool of donors and was checked for quality against local fresh pooled plasma from 20 healthy normal subjects.
2.3.3 Quality Control

A quality control plasma was included in all amidolytic substrate assays performed. The reference range for this plasma was defined as the mean ± 2 standard deviations from assays performed on at least 20 separate occasions. Results from assays where the QC plasma fell outside of this reference range were rejected. Assays were performed in duplicate unless otherwise stated. If the CV between duplicates was greater than 10% then the results were rejected.

2.3.4 Factor XII Assay

**Principle** Based on the method of Gallimore *et al*, 1987. Factor XII in dilute plasma is converted to factor XIIa with an activator preparation (containing kallikrein, high molecular weight kininogen and a soluble activator). Kallikrein activity is inhibited by the addition of soybean trypsin inhibitor (SBTI). FXIIa generated then cleaves a chromogenic substrate, liberating p-nitroaniline (pNA), which can be measured photometrically. Plasmas are acetone treated to prevent interference in the assay by C1-INH. Methylamine is included in the buffer to prevent formation of FXIIa: α₂-macroglobulin complexes *in vitro*, which cleave the chromogenic substrate.

**Reagents** Reagents were from Merck Ltd unless otherwise stated.

- 1mM Factor XII amidolytic substrate 2AcOH.H-D-CHT-Gly-Arg-pNA (Unicom Diagnostics Ltd) reconstituted according to the manufacturer’s instructions
- Factor XII Activator comprising kallikrein, HMWK and a soluble activator (Unicorn Diagnostics Ltd) reconstituted according to the manufacturer’s instructions
- Kallikrein Inhibitor (Unicorn Diagnostics Ltd) reconstituted according to the manufacturer’s instructions
• Methylamine buffer, pH 7.9 - 0.05M Tris, 0.12M methylamine, 0.01M EDTA
• Reference Plasma 100% (Immuno Ltd) reconstituted according to the manufacturer’s instructions
• Acetone
• 1M citric acid

**Acetone Treatment of Plasmas**

Test and reference plasmas were acetone treated by adding 1:3 volumes of acetone to plasma in a polypropylene microfuge tube, mixing and incubating for 15 minutes at 4°C. Any precipitate formed was removed by centrifuging for 4 minutes at 10,000g. Acetone treated reference and test plasmas were diluted in methylamine buffer. Reference plasma was assayed at 5 dilutions: 1:2.66, 1:4, 1:5.33, 1:8, 1:16. Test plasmas were assayed at 1:4 dilution.

**Method**

The factor XII substrate and kallikrein inhibitor were warmed to 37°C. Dilute test and reference plasma (25μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Factor XII activator (25μl) was then added to each well, mixed and incubated at 37°C for 10 minutes followed by the addition of kallikrein inhibitor (75μl) to each well, which was then mixed and incubated at 37°C for 1 minute. Factor XII substrate 50μl was then added to each well, mixed and incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 50μl of 1M citric acid. The optical density was then read using an automated plate reader (Anthos 2001, Labtech International Ltd) at 405nm with a reference wavelength of 492nm. Factor XII concentrations of samples were derived from a linear curve of A405 against percentage factor XII. Results were corrected for the potency of the reference standard. A plasma sample of known factor XII concentration was assayed on each occasion to ensure reliability of assay.
2.3.5 Factor XIIa-like activity

**Principle** A modification of the FXII assay, but omitting the FXII activator step, it detects FXIIa present in plasma. The absence of methylamine in the assay buffer means that the FXIIa detected in this assay is predominantly FXIIa bound to α₂macroglobulin.

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

- Kallikrein inhibitor 20mg/ml (Unicom Diagnostics Ltd) reconstituted according to the manufacturer’s instructions
- 1mM Factor XII amidolytic substrate 2AcOH.H-D-CHT-Gly-Arg-pNA (Unicom Diagnostics Ltd) reconstituted according to the manufacturer’s instructions
- Assay buffer - 0.05M tris, 0.1M sodium chloride, pH 7.9. 1ml of kallikrein inhibitor was added to 49ml assay buffer.
- Acetone

**Method** Plasmas were diluted 1:11 in assay buffer containing kallikrein inhibitor. The factor XII substrate was warmed to 37°C. To the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) 100μl of diluted plasma was added and incubated at 37°C for 3 minutes. Then 100μl of factor XII substrate was added, mixed and incubated at 37°C for 20 minutes. The reaction was stopped by the addition of 100μl acetic acid and the optical density read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm.

2.3.6 Prekallikrein Assay

**Principle** Based on the method of Gallimore *et al*, 1982. Prekallikrein is converted to kallikrein by an activator mixture containing FXII, high-molecular-weight kininogen and ellagic acid. Kallikrein liberated then cleaves a chromogenic
substrate liberating p-nitroaniline (pNA), which can be measured photometrically.

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

- Prekallikrein Activator comprising FXII, HMWK and a soluble activator (Unicom Diagnostics Ltd) reconstituted according to manufacturer’s instructions
- Kallikrein substrate - MBz-Pro-Phe-Arg-pNA (Unicom Diagnostics Ltd) reconstituted according to manufacturer’s instructions
- Buffer - 0.05M Tris-HCl, pH 7.9
- Reference Plasma 100% (Immuno Ltd) reconstituted according to the manufacturer’s instructions
- Acetone
- 1M citric acid

**Method** Test and reference plasmas were acetone treated as in section 2.3.4 and diluted in assay buffer. Reference plasma was assayed at 5 dilutions: 1:40, 1:60, 1:80, 1:120, 1:240. Test plasmas were assayed at a 1:60 dilution. The kallikrein substrate and prekallikrein activator were warmed to 37°C. Dilute test and reference plasma (50μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Prekallikrein activator (50μl) was added, mixed and incubated at 37°C for 5 minutes. Kallikrein substrate (50μl) was added, mixed and incubated at 37°C for 20 minutes. The reaction was stopped by the addition of 50μl of 1M citric acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. Prekallikrein concentrations of samples were derived from a linear curve of A405 against percentage prekallikrein. Results were corrected for the potency of the reference standard. A plasma sample of
known prekallikrein concentration was assayed on each occasion to ensure reliability of assay.

2.3.7 Factor VII Assay

**Principle** This method is based on a two stage principle first described by Seligsohn *et al.*, 1978. In the first stage factor X is activated in the presence of thromboplastin, factor VII and calcium ions to factor Xa. In the second stage factor Xa generated hydrolyses the amidolytic substrate S-2765 liberating p-nitroaniline (pNA), which can be measured photometrically. Factor VII is completely converted to factor VIIa and hence there is no interference of preactivated factor VII. The assay is not effected by heparin in plasma up to a concentration of 10IU/ml.

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

- TBS, pH 7.5 - 0.05M Tris, 0.15M sodium chloride
- 0.025M calcium chloride
- Bovine factor X (Enzyme Research Ltd) diluted to 1.4U/ml in TBS
- Rabbit brain thromboplastin (Diagnostic Reagents Ltd)
- 2mM chromogenic substrate S-2765 (Chromogenix Ltd)
- Reference Plasma 100% (Immuno Ltd)

**Method** An equal volume of calcium chloride was diluted with substrate and warmed to 37°C. Doubling dilutions of reference plasma from 1:100 to 1:3200 were prepared and test plasmas were diluted 1:200. Dilute test and reference plasma (50μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Dilute thromboplastin (50μl) was added, mixed and then immediately 50μl of bovine factor X was added, mixed and incubated at 37°C for 3 minutes. S-2765/calcium chloride (50μl) was then added and incubated at 37°C for 7 minutes. The reaction was stopped by the addition of 50μl of 1M citric
acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. Factor VII concentrations of samples were derived from a linear curve of A405 against percentage factor VII. Results were corrected for the potency of the reference standard. A plasma with known factor VII concentration was assayed on each occasion to ensure reliability of test.

2.3.8 FVII - Sysmex CA-6000 version

During the undertaking of this thesis the FVII amidolytic substrate method above was modified slightly in order to automate the method on a Sysmex CA-6000 random access analyser. The principle is the same but bovine FX was replaced by human FX and the source of thromboplastin was changed to Innovin as this is more easily stored than the thromboplastin used above.

Reagents All reagents were from Dade: Sysmex UK Ltd unless otherwise stated.

- Owren's Veronal Buffer
- 0.025M calcium chloride in water (Merk Ltd)
- Thrombostatin: Innovin reconstituted according to manufacturer’s instructions then diluted 1/5 in 0.025M calcium chloride
- Human FX (Sigma-Aldrich Chemical Co.) - reconstituted with 1ml water then diluted to 1.0U/ml in Owren’s veronal buffer
- 2mM S2765 chromogenic substrate (Chromogenix Ltd)
- Reference Plasma 100% (Immuno Ltd).

Method The CA-6000 aspirates neat plasma and makes a 1/40 dilution of the sample in two stages using Owren’s veronal buffer. To 40µl of dilute sample the analyser then adds an equal volume of dilute FX. This mixture is incubated at 37°C for three minutes prior to the addition of 40µl of dilute Innovin. After a further one minute incubation period, 40µl of S2765 is added and the reaction monitored kinetically at 405nm for the liberation of pNA.
Factor VII concentrations of samples were derived from a log/log curve of
delta A405 against percentage factor VII. Results were corrected for the
potency of the reference standard. A plasma with known factor VII
concentration was assayed on each occasion to ensure reliability of test.

2.3.9 Tissue Factor Pathway Inhibitor (TFPI) Assay

*Principle* This assay is based upon that described by Sandset *et al*, 1987. Factor VII (FVII) in the presence of tissue factor (TF) and calcium forms FVIIa: TF complex which can then initiate coagulation via factors IX and X. TFPI is a physiological factor Xa dependent inhibitor of the FVIIa:TF complex. This assay is based on the ability of a test sample to inhibit FVIIa:TF activation of factor X in the presence of factor Xa. The inclusion of hexadimethrine bromide in the assay buffer neutralises any heparin up to 10 IU/ml that may be present in plasmas. Samples are heat treated to remove fibrinogen to prevent clotting occurring during the assay procedure.

*Reagents* All reagents were from Merck Ltd unless otherwise stated.

- Human Factor X (Sigma-Aldrich Chemical Co.) - diluted to 0.60 U/ml and 0.03 U/ml in assay buffer
- Factor VII concentrate (BPL Ltd) - diluted to 0.013 U/ml in assay buffer
- Thromborel S human thromboplastin (Behring Ltd) reconstituted according to the manufacturer’s instructions and diluted 1/25 in assay buffer immediately prior to use
- 0.075M calcium chloride
- 2mM chromogenic substrate S-2222 (Chromogenix Ltd)
- Assay buffer - 0.1M sodium chloride, 0.05M Tris, 0.01M tri-sodium citrate, 0.1% (w/v) bovine serum albumin, 0.02% (w/v) hexadimethrine bromide (polybrene), pH 8.0
• Reference Plasma 100% (Immuno Ltd)
• 50% (v/v) Acetic acid

**Preparation of working reagents.**

• Combined reagent - 1 part dilute thromboplastin, 1 part 0.075m calcium chloride, 1 part 0.013 U/ml factor VII, 1 part 0.03 U/ml factor X. Prepared immediately before use, placed in plastic universal and incubated at 37°C for 15 minutes.

• Factor X/S-2222 substrate - 1 part 0.60 U/ml factor X, 1 part S-2222

**Method**

Test and reference plasmas were heat treated by placing 0.4ml plasma in a polypropylene microfuge tube into a water bath at 56°C for 15 minutes. The samples were then rapidly cooled in ice water for 2 minutes before centrifuging at 2500g for 10 minutes at 4°C. Test and reference plasmas were diluted in assay buffer on ice. Reference plasma was assayed at 5 doubling dilutions from 1:5 to 1:80. Test plasmas were assayed at 1:20 dilution. Dilute test and reference plasma (25μl) and combined reagent (100μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd), mixed and incubated at 37°C for 10 minutes. Factor X/S-2222 (50μl) was then added, mixed and incubated at 37°C for 25 minutes. The reaction was stopped by the addition of 50μl of 50% acetic acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. TFPI concentrations of samples were derived from a linear curve of A405 against percentage TFPI. Results were corrected for the potency of the reference standard. A plasma with known TFPI concentration was assayed on each occasion to ensure reliability of test.
2.3.10 Antithrombin III (AT-III) Assay

**Principle**  Based on the method of Odegaard *et al.*, 1975. Excess thrombin is added to plasma diluted in buffer containing heparin, which potentiates the action of AT-III. Remaining thrombin cleaves the chromogenic substrate, liberating p-nitroaniline (pNA), which can be measured photometrically. Thrombin inhibition is proportional to AT-III present in the sample. The assay is not influenced by heparin cofactor II since this protein shows no inhibitory capacity for bovine thrombin and in addition the heparin inclusion in the assay buffer will potentiate the action of AT-III but not heparin cofactor II.

**Reagents**  All reagents were from Merck Ltd unless otherwise stated.

- 1mM Thrombin substrate -2AcOH.H-D-CHG-Gly-Arg-pNA (Unicom Diagnostics Ltd) - reconstituted according to the manufacturer’s instructions
- Bovine thrombin (Unicom Diagnostics Ltd) - reconstituted according to the manufacturer’s instructions
- Assay buffer - 0.175M sodium chloride, 0.05M Tris, 0.0075M disodium EDTA, pH 8.4 containing 3IU/ml (fc) unfractionated heparin (Monoparin, CP Pharmaceuticals Ltd).
- Reference plasma 100% (Immuno Ltd)
- 1M Citric acid

**Method**  Test and reference plasmas were diluted in assay buffer with heparin. Reference plasma was assayed at 5 dilutions: 1:204, 1:240, 1:320, 1:480, 1:960. Test plasmas were assayed at a 1:240 dilution. The thrombin substrate was warmed to 37°C. Dilute test and reference plasmas (100μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Bovine thrombin (25μl) was then added, mixed and incubated at 37°C for 1 minute. Thrombin substrate (50μl) was then added, mixed and incubated at 37°C for
2 minutes. The reaction was stopped by the addition of 75μl of 50% acetic acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. AT-III concentrations of samples were derived from a linear curve of A405 against percentage AT-III. Results were corrected for the potency of the reference standard. A plasma with known AT-III concentration was assayed on each occasion to ensure reliability of test.

2.3.11 Heparin Cofactor II (HCII) Assay

**Principle** Based on the method of Abildgaard et al, 1984. Dermatan sulphate binds to HCII and acts as a cofactor, accelerating the inhibition of thrombin. Excess thrombin is added to dilute plasma in the presence of dermatan sulphate. Thrombin is inhibited by the HCII:dermatan sulphate complex in proportion to the level of HCII present in the plasma. Remaining thrombin cleaves the chromogenic substrate, liberating p-nitroaniline (pNA), which can be measured photometrically. The assay is not influenced by AT-III as dermatan sulphate is used which potentiates HCII but not AT-III activity. The inclusion of hexadimethrine bromide in the assay buffer neutralises any heparin that may be present in plasmas.

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

- 1mM Thrombin substrate (Unicorn Diagnostics Ltd) - reconstituted according to the manufacturer’s instructions
- Human thrombin (Unicorn Diagnostics Ltd) - reconstituted according to the manufacturer’s instructions
- Dermatan sulphate (Unicorn Diagnostics Ltd) - reconstituted according to the manufacturer’s instructions
- Assay buffer - 0.15M sodium chloride, 0.05M Tris, 0.0075M disodium EDTA, 0.02% (w/v) hexadimethrine bromide (polybrene), pH 8.2
Method

Test and reference plasma were diluted in assay buffer. Reference plasma was assayed at 5 dilutions: 1:60, 1:80, 1:107, 1:160, 1:320. Test plasmas were assayed at a 1:80 dilution. The thrombin substrate was warmed to 37°C. Dilute test and reference plasmas (50μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and 50μl of dermatan sulphate added, mixed and incubated at 37°C for 2 minutes. Human thrombin (50μl) was then added, mixed and incubated at 37°C for 5 minutes. Thrombin substrate (50μl) was then added, mixed and incubated at 37°C for 3 minutes. The reaction was stopped by the addition of 50μl of 1M citric acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. HCII concentrations of samples were derived from a linear curve of A405 against percentage HCII. Results were corrected for the potency of the reference standard. A plasma with known HCII concentration was assayed on each occasion to ensure reliability of test.

2.3.12 C1-Esterase Inhibitor Assay

Principle

C1-esterase is added in excess to dilutions of test and reference plasma. C1-esterase inhibitor present in plasma forms a complex with C1-esterase and any remaining C1-esterase that is unbound cleaves the chromogenic substrate liberating pNA which is measured photometrically.

Reagents

All regents were part of a kit from Immuno Ltd, unless otherwise stated.

- Human C1-esterase, reconstituted according to the manufacturer’s instructions.
- 1.2mM C1-esterase amidolytic substrate
- Reference Plasma 100%
- sample buffer, pH 7.4 - 0.05M Tris, 0.25M sodium chloride
Method

Test and reference plasma were diluted in sample buffer. Reference plasma was assayed at 6 dilutions: 1:6.66, 1:8, 1:10, 1:7.5, 1:5, 1:2.5. Test plasmas were assayed at a 1:10 dilution. The Cl-esterase substrate was warmed to 37°C. Dilute test and reference plasmas (25μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Cl-esterase (25μl) was then added, mixed and incubated at 37°C for 5 minutes. Cl-esterase substrate (125μl) was then added, mixed and incubated at 37°C for 3 minutes. The reaction was stopped by the addition of 50μl of 50% acetic acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. Cl-esterase inhibitor concentrations of samples were derived from a linear curve of A405 against percentage Cl-esterase inhibitor. Results were corrected for the potency of the reference standard. A plasma with known Cl-esterase inhibitor concentration was assayed on each occasion to ensure reliability of test.

2.3.13 α2-macroglobulin

Principle

Excess trypsin is added to dilute plasma and α2-macroglobulin present in plasma binds to trypsin forming a complex. Remaining trypsin is inhibited by the addition of soybean trypsin inhibitor (SBTI). Trypsin complexed to α2-macroglobulin is then able to cleave small chromogenic substrates liberating pNA.

Reagents

All reagents were from Merck Ltd unless otherwise stated.

- Trypsin substrate [Bz-Val-Lys-Arg-pNA] (Unicorn Diagnostics Ltd) - reconstituted according to the manufacturer’s instructions
- Porcine trypsin (Unicorn Diagnostics Ltd) reconstituted in 1mM hydrochloric acid according to the manufacturer’s instructions
• 2mg/ml SBTI (Sigma-Aldrich Chemical Co.)
• Assay buffer - 0.05M Tris, 0.05M sodium chloride, pH 8.1
• Reference Plasma 100% (Immuno Ltd)
• 50% Acetic acid

**Method**

Test and reference plasma were diluted in sample buffer. Reference plasma was assayed at 5 dilutions: 1:53.3, 1:80, 1:106.7, 1:160, 1:320. Test plasmas were assayed at a 1:80 dilution. The trypsin substrate was warmed to 37°C. Dilute test and reference plasmas (50μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Trypsin (50μl) was then added, mixed and incubated at 37°C for 2 minutes. SBTI (50μl) was then added, mixed and incubated at 37°C for 2 minutes before the addition of trypsin substrate (50μl) and a further incubation at 37°C for 5 minutes. The reaction was stopped by the addition of 50μl of 50% acetic acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. α₂macroglobulin concentrations of samples were derived from a linear curve of A405 against percentage α₂macroglobulin. Results were corrected for the potency of the reference standard. A plasma with known α₂macroglobulin concentration was assayed on each occasion to ensure reliability of test.

2.3.14 Protein C

**Principle**

Protein C activator, which is purified from the venom of the Copperhead snake, is added to dilute plasma and converts inactive protein C to the active enzyme. The latter may be measured by the cleavage of a tripeptide chromogenic substrate and measurement of the released pNA.

**Reagents**

All reagents were from Merck Ltd unless otherwise stated.
• Protein C Activator (Unicom Diagnostics Ltd) reconstituted according to the manufacturer's instructions
• Protein C substrate 2AcOH.H-D-Lys(Cbo)-Pro-Arg-pNA (Unicom Diagnostics Ltd) reconstituted according to the manufacturer's instructions
• Tris imidazole buffer - 0.029M Tris, 0.029M NaCl, pH 8.4
• 1M Citric Acid
• Reference Plasma 100% (Immuno Ltd)

Method

Test and reference plasma were diluted in tris imidazole buffer. Reference plasma was assayed at 5 dilutions: 1:2, 1:3, 1:4, 1:6, 1:12. Test plasmas were assayed at a 1:3 dilution. The Protein C substrate was warmed to 37°C. Dilute test and reference plasmas (12.5μl) were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Protein C activator (25μl) was then added, mixed and incubated at 37°C for 5 minutes. Buffer (100μl) and Protein C substrate (50μl) was then added, mixed and incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 25μl of citric acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference wavelength of 492nm. Blanks for each plasma were performed by substituting buffer for protein C activator to allow for endogenous protease activity. Protein C concentrations of samples were derived from a linear curve of A405 (after subtracting blanks) against percentage protein C activity. Results were corrected for the potency of the reference standard. A plasma with known protein C concentration was assayed on each occasion to ensure reliability of test.
2.3.15 Heparin Assay

**Principle** Excess factor Xa is added to plasma followed by an amidolytic substrate for factor Xa. Heparin binds to antithrombin III which competes with the substrate for factor Xa. The substrate is cleaved by factor Xa, liberating pNA which can be measured photometrically. Plasma samples containing low levels of AT-III were supplemented with a clinical concentrate of AT-III to approximately 1.00 IU/ml.

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

- Bovine factor Xa (Unicom Diagnostics Ltd) reconstituted according to the manufacturer’s instructions.
- Factor Xa substrate (Unicom Diagnostics Ltd) reconstituted according to the manufacturer’s instructions.
- Unfractionated heparin (Monoparin, CP Pharmaceuticals Ltd) diluted to 100U/ml in saline (0.150M sodium chloride)
- Pooled normal plasma
- 1M citric acid
- AT-III clinical concentrate, (BPL Ltd)

**Method** Heparin was diluted 1:10 in normal plasma and this predilution was then further diluted in normal plasma to achieve final concentrations of heparin ranging from 0.05-0.8U/ml. The factor Xa substrate was warmed to 37°C. Test plasmas and standard heparin dilution (12.5μl) of were added to the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd) and incubated at 37°C for 2 minutes. Factor Xa substrate (100μl) was then added, mixed and immediately 100μl of factor Xa was then added, mixed and incubated at 37°C for 8 minutes. The reaction was stopped by the addition of 100μl of 1M citric acid. The optical density was then read using an automated plate reader (Anthos 2001) at 405nm with a reference
wavelength of 492nm. Heparin concentrations of samples were derived from a log/linear curve of A405 against heparin concentration.

2.4 Enzyme Linked Immunosorbant Assays (ELISA)

2.4.1 General Principle
All the ELISAs described in this section employ a direct double antibody method. Plastic microtitre plates are coated with an antibody against the protein of interest by passive adsorption (the capture antibody). Unbound antibody is then removed by washing the plate and then unknown antigen solution (diluted plasma sample) is added and incubated. Unbound antigen is removed by washing before a second antibody directed against the protein of interest is added. This detecting antibody is conjugated to an enzyme, usually peroxidase or alkaline phosphatase. The reaction is visualised by the addition of a substrate which is colourless, but is cleaved by the enzyme conjugate to produce a coloured product which can then be measured spectrophotometrically. The amount of colour produced is therefore proportional to the amount of antigen bound to the plate.

2.4.2 Quality Control
A quality control plasma was included in all ELISA assays performed. In ELISA kits the QC plasma was usually provided, if it was not then an ‘in house’ QC plasma was prepared using plasma from a pool of at least 20 heathly subjects. The reference range for this plasma was defined as the mean ± 2 standard deviations from assays performed on at least 20 separate occasions. Results from assays where the QC plasma fell outside of this reference range were rejected. Assays were performed in duplicate unless otherwise stated. If the CV between duplicates was greater than 10% then results were rejected.
2.4.3 Neutrophil Elastase: $\alpha_1$-Proteinase Inhibitor Complexes

**Principle**  A Solid phase ELISA using a polyclonal sheep anti-human neutrophil elastase antibody as the capture antibody and a polyclonal sheep anti-human $\alpha_1$-antitrypsin antibody conjugated to peroxidase as the detecting antibody. The assay is standardised using neutrophil elastase: $\alpha_1$-antitrypsin complexes prepared in plasma.

**Reagents**  All reagents were from Merck Ltd unless otherwise stated.

- PBS, pH 7.2 - 0.01M phosphate, 0.145M sodium chloride
- PBS with tween - PBS containing 0.1% (v/v) Tween 20
- Blocking agent - PBS containing 1% (w/v) bovine serum albumin (Sigma-Aldrich Chemical Co.)
- Sheep ant-human neutrophil elastase (Serotec Ltd)
- Sheep anti-human $\alpha_1$-antitrypsin peroxidase conjugate (Serotec Ltd)
- Standard plasma containing neutrophil elastase: $\alpha_1$-antitrypsin complex (388 µg/l)
- 3,3’,5,5’-tetramethylbenzidine (TMB) 0.1 mg/ml in phosphate-citrate buffer with urea hydrogen peroxide (Sigma-Aldrich Chemical Co.).
- 2M Sulphuric acid

**Preparation of Neutrophil elastase: $\alpha_1$-antitrypsin complex standard**

Human granulocyte neutrophil elastase was isolated from fresh buffy coat preparations obtained from 15 units of blood from normal healthy donors. Purified enzyme was obtained by Sepharose R - Trasylol affinity chromatography (Baugh & Travis, 1976). Neutrophil elastase: $\alpha_1$-antitrypsin complexes were generated by adding neutrophil elastase diluted in PBS to normal plasma and incubating for 10 minutes at 23°C (Brower &
The complexes generated were calibrated with a commercially available standard (Merck Ltd).

**Method**
Sheep anti-human neutrophil elastase diluted 1 in 1000 in PBS (180μl) was added to each well of a microtitre plate (Nunc Maxisorb, Life Technologies Ltd). The plate was covered and left at 4°C overnight. Unbound antibody was then removed by washing each well 3 times with 200μl of PBS-tween. Blocking agent (180μl) was then added to each well and incubated for 1 hour at room temperature. The wells were washed once with PBS-tween and then 180μl of test and standard plasma diluted in PBS-tween were added to the wells and incubated for 2 hours at room temperature. Standard plasma was assayed at 7 doubling dilutions from 1:25 to 1:1600 and test plasmas were assayed at 3 doubling dilutions from 1:50 to 1:200. Unbound antigen was removed by washing 3 times with 200μl PBS-tween per well. Sheep anti-human α₁-antitrypsin peroxidase conjugate was diluted 1:1000 in PBS-tween and 180μl was added to each well and incubated for 2 hours at room temperature. After washing 3 times with 200μl PBS-tween, 200μl of TMB substrate was added to each well. Colour development was stopped by the addition of 50μl of 2M sulphuric acid to each well. The absorbance at 450nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a log/log standard curve. A plasma with known neutrophil elastase: α₁-antitrypsin complex concentration was assayed on each occasion to ensure reliability of assay. Any test plasmas with concentrations outside the standard curve were reassayed at a higher dilution.

2.4.4 Thrombin-Antithrombin (TAT) Complexes

**Principle**
A solid phase ELISA using a rabbit antibody against human thrombin as the capture antibody. TAT complexes are then detected using a polyclonal
rabbit anti-human AT-III antibody conjugated to peroxidase. The assay is standardised using plasma containing a range of TAT complexes.

**Reagents** All reagents were part of a kit from Behring Ltd unless otherwise stated.

- Microtitre plate coated with rabbit anti-human thrombin antibody
- Rabbit-anti-human antithrombin III peroxidase conjugated antibody - diluted according to manufacturer’s instructions
- Standard plasma containing 2-60µg/l TAT - reconstituted according to manufacturer’s instructions
- TAT control plasma - reconstituted according to manufacturer’s instructions
- Washing solution - PBS containing tween
- Sample buffer
- Substrate - o-phenylenediamine dihydrochloride in citrate-phosphate buffer containing hydrogen peroxide
- 0.5M Sulphuric acid

**Method** Sample buffer (50µl) and standard, control or test plasma (50µl) was added to the wells of the microtitre plate and incubated at 37°C for 15 minutes. Unbound antigen was removed by washing three times with 200µl of washing solution per well. 100µl of anti-antithrombin III conjugate was then added to the wells of the microtitre plate and incubated at 37°C for 15 minutes. Unbound antibody was removed by washing three times with 200µl of washing solution per well. Substrate (100µl) was then added to the wells of the microtitre plate and incubated at room temperature for 30 minutes in the dark. The reaction was stopped by the addition of 100µl of sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a log/log standard curve.
2.4.5 Prothrombin Fragment F1+2

**Principle**
Prothrombin fragment F1+2 (ProF1+2) are the small peptides released when prothrombin is cleaved by factor Xa. This assay is a solid phase ELISA, the plates are coated with a rabbit antibody to human Pro F1+2. The detection antibody is a rabbit anti-human prothrombin conjugated to peroxidase. The assay is standardised with human Pro F1+2.

**Reagents**
All reagents were part of a kit from Behring Ltd unless otherwise stated.

- Microtitre plate precoated with rabbit antibodies to human Pro F1+2
- Rabbit-anti-human prothrombin peroxidase conjugated antibody - diluted according to manufacturer’s instructions
- Standards containing 0.04-10.00nM/l Pro F1+2 - reconstituted according to manufacturer’s instructions
- Pro F1+2 control plasma - reconstituted according to manufacturer’s instructions
- Washing solution - PBS containing tween (1.8% w/v)
- Sample buffer - tris buffer containing tween
- Substrate - o-phenylenediamine dihydrochloride in citrate-phosphate buffer containing hydrogen peroxide
- 0.5M Sulphuric acid

**Method**
Sample buffer (50μl) and standard, control or test plasma (50μl) was added to the wells of the microtitre plate, mixed gently and incubated at 37°C for 30 minutes. Unbound antigen was removed by washing three times with 200μl of washing solution per well. Anti-prothrombin conjugate (100μl) was then added to the wells of the microtitre plate and incubated at 37°C for 15 minutes. Unbound antibody was removed by washing three times with 200μl of washing solution per well. Substrate (100μl) was then added to the wells of the microtitre plate and incubated at room temperature for 15 minutes in the dark. The reaction was stopped by the addition of 100μl of
sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a log/log standard curve of A492 against Pro F1+2 concentration. Results were accepted if the level of Pro F1+2 in the control plasma was within the desired range.

2.4.6 Soluble Thrombomodulin

**Principle**  A solid phase ELISA employing two monoclonal antibodies against human thrombomodulin, one conjugated to peroxidase. The assay is standardised using purified human thrombomodulin.

**Reagents**  All reagents were part of a kit from Diagnostica Stago Ltd unless otherwise stated.

- Microtitre plate coated with monoclonal mouse anti-human thrombomodulin
- Monoclonal mouse anti-thrombomodulin peroxidase conjugate - diluted according to manufacturer’s instructions
- Washing solution
- Dilution buffer - phopshate buffer containing bovine albumin and tween-20
- 100ng/ml Thrombomodulin standard reconstituted according to manufacturer’s instructions
- Substrate - o-phenylenediamine dihydochloride in citrate-phosphate buffer containing hydrogen peroxide
- 3M sulphuric acid (Merck Ltd)

**Method**  Test samples and standard thrombomodulin were diluted with dilution buffer. The reference standard was assayed at 6 doubling dilutions from 100 to 3.13ng/ml, test plasmas were assayed at a dilution of 1:5. Dilute test plasma or reference thrombomodulin (200μl) was added to the wells of the
microtitre plate and incubated at room temperature for 2 hours. Unbound antigen was removed by washing five times with 300μl of washing solution per well. 200μl of anti-thrombomodulin conjugate was then added to the wells of the microtitre plate and incubated at room temperature for 2 hours. Unbound antibody was removed by washing five times with 300μl of washing solution per well. Substrate (200μl) was then added to the wells of the microtitre plate and incubated at room temperature for 8 minutes. The reaction was stopped by the addition of 50μl of sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a log/log standard curve.

2.4.7 Endothelin-1 (ET-1)

**Principle**
A solid phase ELISA employing two antibodies against human endothelin-1, one conjugated to peroxidase. The assay is standardised using human recombinant endothelin-1. An extraction procedure is employed to improve the specificity of the assay by reducing cross-reacting substances.

**Reagents**
All reagents were part of a kit from R&D Systems unless otherwise stated.

- Microtitre plate coated with rat antibody to human ET-1
- anti-human ET-1 antibody conjugated to peroxidase
- Substrate solution - tetramethylbenzidine in buffer solution
- Stop solution - 1M hydrochloric acid
- synthetic human ET-1 standards
- Wash buffer
- sample diluent
- parameter control - QC sample
- extraction solvent: acetone:1M HCl:water (40:1:5)
The following extraction procedure was used: 1.0ml of EDTA plasma was added to 1.5ml of extraction solvent in a polypropylene tube, mixed by inversion and then centrifuged for 20 minutes at 2000g at 4°C. The supernatant was removed to a polypropylene tube and dried down in a centrifugal evaporator for a minimum of 4 hrs at 37°C. The resulting pellet was then reconstituted in 0.25ml of sample diluent. Anti-ET-1 conjugate (100µl) was added to the precoated microtitre wells, followed by 100µl of standard, control or reconstituted sample extract and incubated at room temperature for 1 hour. Unbound material was removed by washing and aspiration of each well with 200µl of wash buffer 6 consecutive times. Substrate solution (100µl) was then immediately added to each well and incubated at room temperature for 30 minutes after which 100µl of stop solution was added to each well. The absorbance at 450nm with a correction wavelength of 620nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear standard curve and corrected to allow concentration occurring during the extraction procedure.

2.4.8 Soluble E-Selectin

Principle A solid phase ELISA employing two antibodies against human E-Selectin, one conjugated to peroxidase. The assay is standardised using recombinant human E-Selectin.

Reagents All reagents were part of a kit from R&D Systems unless otherwise stated.

- Microtitre plate coated with murine antibody to human E-Selectin
- anti-human E-Selectin antibody conjugated to peroxidase
- Substrate solution - tetramethylbenzidine in buffer solution
- Stop solution - 1M hydrochloric acid
- recombinant human E-Selectin standards
Method  
Serum samples and control serum were diluted 1/20 in sample diluent. Anti-E-selectin conjugate (100µl) was added to the precoated microtitre wells, followed by 100µl of standard, control or reconstituted sample extract and incubated at room temperature for 1.5 hours. Unbound material was removed by washing and aspiration of each well with 300µl of wash buffer 6 consecutive times. Substrate solution (100µl) was then immediately added to each well and incubated at room temperature for 30 minutes after which 100µl of stop solution was added to each well. The absorbance at 450nm with a correction wavelength of 620nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear standard curve and corrected to allow for dilution of samples.

2.4.9 Soluble Tissue Factor

Principle  
A solid phase ELISA employing a murine anti-human tissue factor antibody for antigen capture. The bound TF is detected using a biotinylated antibody fragment against tissue factor. The assay is standardised using recombinant tissue factor.

Reagents  
All reagents were part of a kit from American Diagnostica Inc.
- microtitre plate precoated with murine anti-human tissue factor antibody
- lyophilised tissue factor standards 0-1000pg/ml - reconstituted according to manufacturer’s instructions.
- Biotinylated detection antibody - reconstituted according to manufacturer’s instructions.
- Enzyme conjugate - streptavidin-horse radish peroxidase - reconstituted according to manufacturer’s instructions.
• Substrate - TMB
• Wash buffer - PBS containing 0.1% (v/v) Triton X-100
• 0.5M sulphuric acid

**Method**

Citrated plasma samples were diluted 1:10 in wash buffer containing 1% (w/v) BSA. Tissue factor standard or diluted test plasmas (100μl) were added to the wells of the precoated microtitre plate, covered and incubated at room temperature for 3 hours. Unbound antigen was removed by washing the wells of the micotitre plate four times with 200μl of wash buffer. Detection antibody (100μl) was then added to each well, the plate covered and incubated for one hour at room temperature. Unbound antibody was removed by washing the wells of the micotitre plate four times with 200μl of wash buffer. Enzyme conjugate (100μl) was then added to each well, the plate covered and incubated for one hour at room temperature. Unbound conjugate was removed by washing the wells of the micotitre plate four times with 200μl of wash buffer. Substrate solution (100μl) was then added to each well, the plate covered and incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 50μl of sulphuric acid to each well. The absorbance at 450nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear standard curve of A450 against tissue factor concentration. Results were corrected to allow for the dilution of plasma in the assay.

**2.4.10 Activated Factor XII (FXIIa)**

**Principle**

A solid phase ELISA employing a mouse monoclonal antibody against human FXIIa (which does not recognise FXII zymogen) as the capture antibody. A sheep polyclonal antibody conjugated to alkaline phosphatase against human FXIIa is used as detecting antibody. The assay is standardised using purified βFXIIa in buffer.
**Reagents**

All reagents were part of a kit from Shield Diagnostics Ltd unless otherwise stated.

- Microtitre plate coated with mouse monoclonal antibody to human factor XIIa
- Sheep polyclonal anti-human Factor XIIa antibody conjugated to alkaline phosphatase
- Substrate solution - phenolphthalein monophosphate in buffer solution
- Stop solution - carbonate buffer containing sodium hydroxide and EDTA
- 0-20ng/ml Factor XIIa standards
- Wash buffer

**Method**

Test plasma or factor XIIa standard (100µl) was added to the wells of the microtitre plate and incubated at room temperature for 1 hour. Unbound antigen was removed by washing five times with 200µl of washing solution per well. 100µl of anti-factor XIIa conjugate was then added to the wells of the microtitre plate and incubated at room temperature for 1 hour. Unbound antibody was removed by washing five times with 200µl of washing solution per well. Substrate (100µl) was then added to the wells of the microtitre plate and incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 100µl of stop solution to each well. The absorbance at 540nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear standard curve of A540 against FXIIa concentration.

**2.4.11 Protein C**

**Principle**

A solid phase ELISA employing two polyclonal antibodies against human protein C, one conjugated to peroxidase. The assay is standardised using reference plasma.
Reagents

All reagents were from Merck Ltd unless otherwise indicated.

- Buffer A - 0.01M phosphate, 0.15M sodium chloride, pH 7.4
- Buffer B - 0.01M phosphate, 0.5M sodium chloride, 1mM disodium EDTA, 0.1% v/v Tween-20, pH 7.4
- Buffer C - Buffer B containing 3% w/v polyethylene glycol mw 8000
- Polyclonal rabbit anti-human protein C antibody (Dako Ltd)
- Polyclonal rabbit anti-human protein C antibody conjugated to peroxidase (Dako Ltd)
- 1,2-phenylenediamine dihydrochloride (OPD) 0.63mg/ml in phosphate-citrate buffer with urea hydrogen peroxide (Sigma-Aldrich Chemical Co.)
- Reference Plasma 100% (Immuno Ltd)

Method

Doubling dilutions of standard plasma were prepared from 1:10 to 1:640 and test plasmas from 1:20 to 1:80 in buffer C. Rabbit anti-human protein C antibody (100μl) diluted 1 in 500 in buffer A was added to each well of a microtitre plate (Nunc Maxisorb, Life Technologies Ltd). The plate was covered and left at 4°C overnight. Unbound antibody was then removed by washing each well 3 times with 150μl of buffer B. Diluted test and standard plasmas (100μl) were added to the wells and incubated for 2 hours at room temperature. Unbound antigen was removed by washing 3 times with 150μl buffer B per well. Rabbit anti-human protein C peroxidase conjugate was diluted 1:1000 in buffer C and 100μl was added to each well and incubated for 2 hours at room temperature. After washing 3 times with 150μl buffer B, 100μl of OPD substrate was added to each well. Colour development was stopped by the addition of 100μl of 2M sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear/log
standard curve. A plasma with known protein C concentration was assayed on each occasion to ensure reliability of assay.

2.4.12 Total Protein S

**Principle**  Protein S exists in two forms in plasma: free and bound to C4b-binding protein. This assay measures total protein S (i.e. both forms) by solid phase ELISA employing two polyclonal antibodies against human protein S, one conjugated to peroxidase. The assay is standardised using reference plasma.

**Reagents**  All reagents were from Merck Ltd unless otherwise indicated.

- Buffer A - 0.01M phosphate, 0.15M sodium chloride, pH 7.4
- Buffer B - 0.01M phosphate, 0.65M sodium chloride, 0.1% v/v Tween-20, pH7.4
- Buffer C - Buffer B containing 3% w/v polyethylene glycol MW 6000
- Polyclonal rabbit anti-human protein S antibody (Dako Ltd)
- Polyclonal rabbit anti-human protein S antibody conjugated to peroxidase (Dako Ltd)
- 1,2-phenylenediamine dihydrochloride (OPD) 0.63mg/ml in phosphate-citrate buffer with urea hydrogen peroxide (Sigma-Aldrich Chemical Co.).
- NIBSC 7th standard for Blood Coagulation Factors (NIBSC)
- 2M Sulphuric Acid

**Method**  Doubling dilutions of standard plasma were prepared from 1:200 to 1:12800 and test plasmas from 1:400 to 1:1600 in buffer C. Rabbit anti-human protein S antibody (100μl) diluted 1 in 6000 in buffer A was added to each well of a microtitre plate (Nunc Maxisorb, Life Technologies Ltd). The plate was covered and left at 4°C overnight. Unbound antibody was then removed by washing each well 3 times with 150μl of buffer B. Diluted test and standard plasmas (100μl) were added to the wells and incubated for
2 hours at room temperature. Unbound antigen was removed by washing 3 times with 150µl buffer B per well. Rabbit anti-human protein S peroxidase conjugate was diluted 1:3000 in buffer C and 100µl was added to each well and incubated for 2 hours at room temperature. After washing 3 times with 150µl buffer B, 100µl of OPD substrate was added to each well. After 10 minutes incubation colour development was stopped by the addition of 100µl of 2M sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear/log standard curve. A plasma with known protein S concentration was assayed on each occasion to ensure reliability of assay.

2.4.13 Free Protein S

**Principle** Free protein S can be measured in plasma using a solid phase ELISA employing a cocktail of two monoclonal antibodies directed against epitopes only found on free human protein S as the capture antibody. A polyclonal antibody against protein S conjugated to peroxidase is used as the detecting antibody. The assay is standardised using reference plasma.

**Reagents** All reagents were from Merck Ltd unless otherwise indicated.

- Buffer A - 0.01M phosphate, 0.15M sodium chloride, pH 7.4
- Buffer B - 0.01M phosphate, 0.65M sodium chloride, 0.1% v/v Tween-20, pH7.4
- Buffer C - Buffer B containing 3% w/v polyethylene glycol MW 6000
- Monoclonal mouse anti-human protein S antibodies clones 15C4 and 34G2 (Kordia Ltd)
- Polyclonal rabbit anti-human protein S antibody conjugated to peroxidase (Dako Ltd)
1,2-phenylenediamine dihydrochloride (OPD) 0.63mg/ml in phosphate-citrate buffer with urea hydrogen peroxide (Sigma-Aldrich Chemical Co.).

- NIBSC 7th standard for Blood Coagulation Factors (NIBSC)
- 2M Sulphuric Acid

**Method**

Doubling dilutions of standard plasma were prepared from 1:200 to 1:12800 and test plasmas from 1:800 to 1:3200 in buffer C. Mouse anti-human protein S monoclonal antibodies were diluted 1 in 2500 (of each clone) in buffer A and 100μl was added to each well of a microtitre plate (Nunc Maxisorb, Life Technologies Ltd). The plate was covered and left at 4°C overnight. Unbound antibody was then removed by washing each well 3 times with 150μl of buffer B. Diluted test and standard plasmas (100μl) were added to the wells and incubated for 2 hours at room temperature. Unbound antigen was removed by washing 3 times with 150μl buffer B per well. Rabbit anti-human protein S peroxidase conjugate was diluted 1:2000 in buffer C and 100μl was added to each well and incubated for 2 hours at room temperature. After washing 3 times with 150μl buffer B, 100μl of OPD substrate was added to each well. After 10 minutes incubation colour development was stopped by the addition of 100μl of 2M sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear/log standard curve. A plasma with known protein S concentration was assayed on each occasion to ensure reliability of assay.

2.4.14 Total TFPI

**Principle**

TFPI exists in plasma both in association with lipoproteins, in a free form and complexed to coagulation proteins. This assay detects both forms as well as the complexes TFPI:FXa, TFPI:TF:FVIIa and TFPI:FXa:TF:FVIIa.
(see section 1.1.5) and the heparin releasable form of TFPI. This is therefore a measure of total TFPI protein.

**Reagents**
All reagents were part of a kit from American Diagnostica Ltd unless otherwise indicated.

- Microtitre plate precoated with a rabbit anti-human polyclonal antibody
- TFPI depleted plasma
- TFPI standard 10ng/ml (reconstituted in sample buffer containing 5% (v/v) TFPI depleted plasma)
- TFPI QC plasma
- Detection antibody - biotinylated anti-human monoclonal antibody (against kunitz-1 domain) reconstituted according to the manufacturer’s instructions
- Enzyme conjugate - streptavidin-peroxidase reconstituted according to the manufacturer’s instructions
- TMB substrate
- Wash buffer (PBS containing 0.1% (v/v) Triton X-100
- Sample buffer (wash buffer containing 1% (w/v) bovine serum albumin (A-7030 Grade V, Sigma-Aldrich Chemical Company)
- 0.5M Sulphuric Acid

**Method**
A standard curve was prepared by double-diluting the 10ng/ml TFPI standard in sample buffer containing 5% (v/v) TFPI depleted plasma. Samples were diluted 1:20 in sample buffer. Samples from patients receiving heparin therapy were diluted at an appropriately higher dilution. Diluted test and standard plasmas (100μl) were added to the wells of the precoated microtitre plate and incubated overnight at 4°C. Unbound antigen was removed by washing 4 times with 200μl of wash buffer per well. Detection antibody (100μl) was added to each well and incubated for 1 hour
at room temperature. After washing 4 times with 200μl wash buffer, 100μl of enzyme conjugate was added to each well and incubated for 1 hour at room temperature. After washing 4 times with 200μl wash buffer, TMB substrate (100μl) was added to each well and incubated for 20 minutes at room temperature. Colour development was stopped by the addition of 50μl of 0.5M sulphuric acid to each well. The absorbance at 450nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear standard curve. Results were corrected to allow for the plasma dilution used.

2.4.15 β₂Glycoprotein-I

**Principle** This assay measures β₂Glycoprotein-I by solid phase ELISA employing two polyclonal antibodies against human β₂Glycoprotein-I, one conjugated to peroxidase. The assay is standardised using a pooled normal plasma which has been calibrated against purified β₂Glycoprotein-I.

**Reagents** All reagents were from Merck Ltd unless otherwise indicated.

- Buffer A - 0.01M phosphate, 0.15M sodium chloride, pH 7.4
- Buffer B - 0.01M phosphate, 0.15M sodium chloride, 0.1% v/v Tween-20, pH 7.4
- Polyclonal rabbit anti-human β₂Glycoprotein-I antibody (Dako Ltd)
- Polyclonal rabbit anti-human β₂Glycoprotein-I antibody conjugated to peroxidase (Dako Ltd)
- 1,2-phenylenediamine dihydrochloride (OPD) 0.63mg/ml in phosphate-citrate buffer (35mM citric acid, 67mM di-sodium hydrogen orthophosphate dodecahydrate, pH 5.0) containing 0.0025% (v/v) hydrogen peroxide
- PNP standard
• 2M Sulphuric Acid

**Method**

Doubling dilutions of standard plasma were prepared from 1:3200 to 1:102400 and test plasmas from 1:6400 to 1:25600 in buffer B. Rabbit anti-human $\beta_2$Glycoprotein-I antibody (180$\mu$l) diluted 1:1000 in buffer A was added to each well of a microtitre plate (Nunc Maxisorb, Life Technologies Ltd). The plate was covered and left at 4°C overnight. Unbound antibody was then removed by washing each well 3 times with 200$\mu$l of buffer B. Diluted test and standard plasmas (180$\mu$l) were added to the wells and incubated for 2 hours at room temperature. Unbound antigen was removed by washing 3 times with 200$\mu$l buffer B per well. Rabbit anti-human $\beta_2$Glycoprotein-I peroxidase conjugate was diluted 1:1000 in buffer B and 180$\mu$l was added to each well and incubated for 2 hours at room temperature. After washing 3 times with 200$\mu$l buffer B, 200$\mu$l of OPD substrate was added to each well. After 12 minutes incubation colour development was stopped by the addition of 50$\mu$l of 2M sulphuric acid to each well. The absorbance at 492nm was read using an automated plate reader (Anthos 2001). The concentrations of test samples were derived from a linear/log standard curve. A plasma with known $\beta_2$Glycoprotein-I concentration was assayed on each occasion to ensure reliability of assay.

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**2.5 Clotting Assays**

**2.5.1 Preparation of Factor VII Deficient Plasma**

**Principle**

Adsorption of plasma with aluminium hydroxide leads to the removal of factors VII, II, IX and X.

**Reagents**

All reagents were from Merck Ltd unless otherwise stated

- citrated platelet poor plasma
- 25%(w/v) Aluminium moist gel suspended in distilled water
Method

1/10 the volume of aluminium hydroxide suspension was added to plasma in a plastic beaker and stirred at room temperature for 3 minutes. The plasma was then centrifuged at 2000g for 15 minutes at room temperature for 10 minutes. This adsorption process was repeated twice more. Then 100mg barium sulphate per ml of plasma was added and mixed for 10 minutes at room temperature. The plasma was then centrifuged at 2000g at room temperature for 15 minutes. Factor II, IX, X concentrate was added to the plasma to achieve a final concentration of Factor II and X of 0.5-1.0U/ml. The plasma was aliquoted into plastic tubes and stored at -70°C.

2.5.2 Preparation of Bovine Thromboplastin

Reagents All reagents were from Merck Ltd unless otherwise indicated.

- acetone treated dried bovine brain extract (a kind gift from Ken Denson, Diagnostic Reagents Ltd)
- physiological saline - 0.9% (w/v) sodium chloride

Method A 5% (w/v) suspension of dried bovine brain was made in saline and heated at 45°C for 45 minutes and then allowed to cool to room temperature. The suspension was then centrifuged at 1000g for 15 minutes and the supernatant removed, aliquoted into plastic tubes and stored at -70°C.

2.5.3 Factor VII Clotting Assay

Reagents All reagents were from Merck Ltd unless otherwise indicated.

- FVII deficient substrate plasma (see section 2.5.1)
- Prothrombin/Fibrinogen reagent (Instumentation Laboratory Ltd, Warrington)
• Imidazole buffer - 0.05M imidazole, 0.1M sodium chloride, pH 7.3
• Reference plasma 100% (Immuno Ltd, Vienna).

**Method**

The assay was automated using an ACL-300 coagulometer (Instumentation Laboratory Ltd, Warrington). A 1/10 dilution of reference plasma in imidazole buffer was placed in the pool position of the autosampler tray of the ACL and dilutions of test plasmas (1/10, 1/20, 1/40 in imidazole buffer) in positions 1-12. Imidazole buffer was placed in the diluent and 13 position and FVII deficient plasma in position 15. The prothrombin/fibrinogen reagent with stir bar was placed in reagent reservoir 1 of the ACL-300. The analyser was run in the single factor mode for FVII and the results calculated from the standard curve using the software installed in the ACL.

**2.5.4 Factor VIIa assay using ratio of two FVII clotting assays**

**Principle**

FVII clotting activity is measured using both rabbit and bovine brain thromboplastin which have differing sensitivities to the amount of activated FVII present in the sample. The ratio of FVII measured using bovine thromboplastin to rabbit thromboplastin gives an index as to the level of FVIIa in the sample.

**Reagents**

As for 2.5.3 and in addition:

- Rabbit brain thromboplastin (Diagnostic Reagents Ltd) diluted 1/4 in imidazole buffer and then an equal volume of 25mM calcium chloride added
- Bovine brain thromboplastin (see section 2.5.2) diluted 1/4 in imidazole buffer and then an equal volume of 25mM calcium chloride added.

**Method**

A FVII clotting assay was performed as in section 2.5.3 except that the prothrombin/fibrinogen reagent was replaced by the rabbit thromboplastin-calcium chloride reagent. The assay was then repeated using bovine
thromboplastin reagent. The ratio of FVII (Bovine):FVII (rabbit) was calculated as the index for FVIIa.

2.5.5 Factor VIIa assay using soluble tissue factor - validation

When this thesis was initiated there was no direct method for measuring FVIIa in plasma. FVII was previously assessed by the ratio of FVII clotting activity using two different thromboplastins, one more sensitive to FVIIa than the other, or the ratio FVII antigen (total FVII):FVII:C (a measure of FVII and FVIIa). However, during the course of this thesis a method was developed by Dr. J Morrissey that measures FVIIa in plasma independent of FVII zymogen. As this assay was not available commercially, it was necessary to establish this method to advance the studies in this thesis. Since this assay is clotting based and was to be used to measure FVIIa in patients receiving heparin therapy, it was also necessary to determine the effect of heparin on the assay and establish appropriate ways of neutralising heparin if necessary.

Method

All reagents were from Merck Ltd unless otherwise stated.

Comparison of FVIIa methods: 18 plasma samples covering a range of FVIIa concentrations were assayed for FVIIa using methods 2.5.4 and 2.5.6.

Reference range Citrated plasma collected from 44 healthy subjects was used to determine the reference range.

Effect of unfractionated heparin: Citrated plasma was prepared from three apparently healthy normal subjects as described in section 2.1. The plasmas were spiked with heparin by diluting unfractionated heparin (Multiparin, CP Pharmaceuticals Ltd) to 0, 10, 50, 100, 500, and 1000 U/ml in PBS then diluting these 1/100 in plasma to give a final concentration of 0, 0.1, 0.5, 1, 5 and 10U/ml.

Heparin neutralisation: The effect of neutralising heparin with either protamine sulphate, hexadimethrine bromide (polybrene) or triethylaminoethyl (TEAE) cellulose was investigated. Protamine sulphate (CP Pharmaceuticals) or hexadimethrine bromide (Sigma) were diluted to 0, 0.1, 0.5, 1, 5 and 10mg/ml in saline and then these were
diluted 1/100 in plasma to give 0, 1, 5, 10, 50, and 100 µg/ml final concentrations which were predicted to neutralise 0, 0.1, 0.5, 1, 5, and 10 U/ml of heparin respectively. Plasma samples were treated with TEAE cellulose (Sigma) by adding 10mg of TEAE cellulose to 1ml of plasma (of all heparin concentrations) and mixing by gentle inversion for 10 minutes at room temperature. After centrifugation at 1200g for 5 minutes, the supernatant plasma was removed. Samples were assayed for FVIIa as described in section 2.5.6 and heparin using an anti-factor Xa amidolytic substrate assay standardised using unfractionated heparin (Multiparin) as described in section 2.3.

**Results** There was a good correlation between FVIIa levels measured by the Morrissey assay and by the ratio of two FVII:C assays (Figure 2.1).

![Figure 2.1 Correlation between FVIIa measured using methods 2.5.4 and 2.5.6.](image)

FVIIa Morrissey (ng/ml)

FVII:C Bovine/Rabbit ratio

\[ r = 0.80 \]

\[ p = 0.001 \]
Effect of Heparin  The heparin assayed in the plasma samples spiked with 0, 0.1, 0.5, 1.0, 5.0, 10.0 U/ml was <0.05, 0.09±0.02, 0.63±0.12, 1.09±0.17, 5.63±0.51, 10.31±0.23 (mean±SD) respectively. After treatment with polybrene, protamine sulphate or TEAE cellulose, heparin levels were below 0.05 U/ml in all samples. The effect of heparin and heparin neutralisation on the FVIIa assay is shown in Table 2.1.

<table>
<thead>
<tr>
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<th>Heparin 0 U/ml</th>
<th>0.1 U/ml</th>
<th>0.5 U/ml</th>
<th>1 U/ml</th>
<th>5 U/ml</th>
<th>10 U/ml</th>
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<tbody>
<tr>
<td>Heparin only</td>
<td>111.0 ± 6.6</td>
<td>111.5 ± 6.2</td>
<td>110.5 ± 6.9</td>
<td>112.0 ± 7.5</td>
<td>no clot</td>
<td>no clot</td>
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<tr>
<td>Heparin + Protamine</td>
<td>111.0 ± 6.6</td>
<td>112.3 ± 4.6</td>
<td>119.8 ± 7.6</td>
<td>128.0 ± 6.0</td>
<td>131.1 ± 7.1</td>
<td>135.5 ± 22.6</td>
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<tr>
<td>Heparin + Polybrene</td>
<td>111.0 ± 6.6</td>
<td>112.8 ± 9.3</td>
<td>116.6 ± 9.6</td>
<td>119.7 ± 10.3</td>
<td>130.8 ± 7.6</td>
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<tr>
<td>Heparin + TEAE</td>
<td>109.2 ± 6.0</td>
<td>108.5 ± 6.9</td>
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</tbody>
</table>

Table 2.1 The Effect of Heparin and its Neutralisation with Protamine Sulphate, Polybrene or TEAE Cellulose on the Clotting Time (s) in the Assay for Factor VIIa. Results are expressed as mean ± sd, n=3.

Heparin had little effect on the FVIIa assay up to a concentration of 1 U/ml, but at a concentration of 5 and 10 U/ml there was a gross prolongation of the clotting time. Polybrene and protamine sulphate neutralisation of heparin partially corrected this, but some prolongation of the clotting time occurred despite apparently adequate neutralisation of heparin. In the absence of heparin protamine sulphate lengthened the clotting time at 5μg/ml and above and polybrene shortened the clotting time at 10μg/ml and above (Table 2.2). When heparin spiked plasmas were treated with TEAE cellulose, heparin had little effect on the clotting time up to at least 10 U/ml. In the absence of heparin TEAE treatment of plasma showed only small changes in the clotting time (110.0 ± 6.6 - 109.2 ± 6.0, Table 2.1). TEAE cellulose was therefore used to neutralise heparin in clinical plasma samples containing heparin before assaying for FVIIa.
Table 2.2. The Effect of Protamine Sulphate or Polybrene in the absence of heparin on the Clotting Time (s) in the Assay for Factor VIIa.

Results are expressed as mean ± sd, n=3.

<table>
<thead>
<tr>
<th>(µg/ml)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polybrene</td>
<td>103 ± 99.7 ± 93.7 ± 92.5 ± 87.0 ± 86.0 ± 7.2 7.4 10.9 6.4 1.6 12.9</td>
<td>Polybrene</td>
<td>103.5 ± 102.2 ± 109.2 ± 114.2 ± 126.5 ± 131.5 ± 6.2 10.4 12.5 8.8 15.8 15.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Reference range and assay performance** The distribution of FVIIa in the normal subjects studied was non-Gaussian with positive skew. Data was normalised by natural log transformation. The reference range was 0.60-5.90ng/ml. The intra-assay performance was assessed by assay of a single blood sample 10 times and the coefficient of variation determined and found to be 4.3% The inter-assay coefficient was assessed by assaying the same plasma on 20 separate occasions and found to be 9.1%.

**2.5.6 Factor VIIa assay using soluble tissue factor - final method**

**Principle** This assay was adapted from that first performed by Morrissey *et al*, 1993 and uses a mutant form of tissue factor which has selectively lost its ability to promote FVII activation while retaining cofactor function for the FVIIa-catalysed activation of FX. A set quantity of tissue factor-219 is mixed with phospholipid (a cofactor for the TF:FVIIa activation of FX) and the test sample is then added, recalcifying to initiate coagulation. To standardise for other haemostatic factors which may influence the clotting time, test plasmas are diluted 1/10 in VII deficient plasma. The assay is standardised using human recombinant FVIIa.

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

- Tissue factor mutant-219 (TF-219) stock solution at 47µg/ml stored at -70°C (a kind gift from Prof EDG Tuddenham, RPMS Hammersmith)
• Phospholipid reagent ('Bell and Alton' platelet substitute, Diagnostic Reagents Ltd) reconstituted according to manufacturer's instructions

• TBSA - tris buffered saline pH 7.4 containing 1% bovine serum albumin (fraction V, A-7030 Sigma-Aldrich Chemical Co.)

• Human recombinant FVIIa (Novo Nordisk Ltd) stock solution at 6μg/ml in TBSA stored at -70°C.

• FVII deficient plasma (Diagnostic Reagents Ltd)

• In house QC plasma prepared by cold activating citrated normal plasma for 18 hours at 4°C.

• 0.025M calcium chloride

• Triethylaminoethyl (TEAE) cellulose, (Sigma-Aldrich Chemical Co.)

**Heparin neutralisation of samples**

The following procedure was used to neutralise heparin in plasma samples after preliminary optimisation experiments detailed above. 10 mg of TEAE cellulose was added to 1ml of plasma in a plastic tube and mixed by gentle inversion until all the powder was in suspension, then mixed at room temperature for 10 minutes. The plasma was then spun at 1200g for 5 minutes at room temperature and the plasma then removed.

**Method**

Citrated plasmas were thawed at 37°C and then kept at room temperature to avoid artificially raising FVIIa levels. Plasma from cardiopulmonary bypass patients were first treated with TEAE cellulose to remove heparin. A working tissue factor reagent was prepared by adding 500μl of phospholipid to 900μl TBSA and 100μl of TF-219. The FVIIa stock standard was diluted 1/100 in TBSA to give 60ng/ml FVIIa. 100μl was then added to 500μl TBSA to give 10ng/ml FVIIa and this was serially diluted 1/1.66 to give 6.02, 3.63, 2.18, 1.32 ng/ml FVIIa standards. Plasmas and rFVIIa standards (10μl) test were diluted with 90μl FVII deficient plasma
in the microvolume sample cups of an ACL-300 analyser (Instrumentation Laboratory Ltd). The TF-219 working reagent was placed in reagent reservoir 2 and calcium chloride in reagent reservoir 3 of the ACL-300. The analyser was run in research mode using the following parameters: sample volume (50μl), reagent volume position 2 (75μl), reagent volume position 3 (50μl), activation time (64 seconds), inter ramp interval (1 second), delay time (0 seconds), acquisition time (250 seconds), speed (1200rpm). Data generated was analysed using Windows software provided by the manufacturer. A standard curve was constructed by plotting log FVIIa concentration against clotting time. The FVIIa concentration of the test plasmas were then read from the curve.

2.5.7 **Prothrombin Clotting Assay (Taipan method)**

*Principle* Taipan snake venom (*Oxyuranus scutellatus*) cleaves prothrombin at the same site as FXa in a reaction requiring calcium ions and phospholipid, but independent of FVa. A set quantity of absorbed ox plasma (as a source of fibrinogen), phospholipid and the test sample are mixed together. Taipan venom containing calcium ions is then added to initiate coagulation. The time taken to form a fibrin clot is indirectly proportional to the prothrombin concentration. The assay thus measures prothrombin independently of other clotting factors in the plasma sample. Unfractionated heparin does not affect the assay up to a concentration of 5IU/ml.

*Reagents* All reagents were from Merck Ltd unless otherwise stated.

- Taipan venom containing calcium chloride (Diagnostic Reagents Ltd) reconstituted according to manufacturer’s instructions
- Phospholipid reagent (‘Bell and Alton’ platelet substitute, Diagnostic Reagents Ltd) reconstituted according to manufacturer’s instructions
- Absorbed ox plasma (Diagnostic Reagents Ltd) reconstituted according to manufacturer’s instructions
• Glyoxyline buffer - 0.05M imidazole, 0.100M sodium chloride, pH 7.3
• Reference plasma 100% (Immuno Ltd)

**Method**
Test and standard plasmas were diluted 1:80, 1:160, and 1:320 in glyoxyline buffer and kept on ice. Each dilution was transferred (100μl) in duplicate to a glass tube and kept on ice. To each glass tube 100μl of absorbed ox plasma and 100μl of platelet substitute was added. The first two tubes were warmed in a 37°C waterbath for 2 minutes, 200μl of taipan venom was then added and the time for a visible clot to form recorded. The last step was repeated for each pair of tubes. A standard curve was constructed by plotting log prothrombin concentration against log clotting time. The prothrombin concentration of the test plasmas were then read from the curve. Results were corrected to allow for the potency of the standard.

---

### 2.6 Flow Cytometry Techniques

#### 2.6.1 Neutrophil and monocyte CD11b expression - development

It was necessary to establish a direct whole blood method to quantify CD11b expression on the surface of both monocytes and neutrophils to avoid artefactual changes in CD11b expression induced by *ex vivo* cell handling. In this method after incubation of whole blood with primary conjugated antibody, red blood cells are lysed with dilute formic acid, the sample is then brought back to physiological pH and buffered with a mixture of bases, and finally fixed with paraformaldehyde before analysis.

**Method**
All reagents were from Sigma-Aldrich Chemical Co, unless otherwise stated. Venous blood from apparently healthy subjects was collected into tri-sodium citrate (see section 2.1) for the following optimisation experiments.
**Antibody titration:** The concentration of R-phycoerythrin conjugated antibody against CD11b or IgG irrelevant control was varied from 1-10μl of antibody per 100μl of whole blood to determine the saturating concentration of primary antibody.

**CD11b Mobilisation:** F-Met-Leu-Phe (FMLP) or lipopolysacharide (LPS) were used to stimulate whole blood before the addition of a constant concentration of antibody to observe the effect on the mobilisation of intracellular stores of CD11b. FMLP 0.1M in DMSO was diluted at least 1/100 in PBS and 1 volume added to 100 volumes of whole blood to achieve a final concentration of 1nM-10μM. LPS 1mg/ml in PBS was diluted 1/100 in whole blood and then further diluted in whole blood to give a final concentration of 1ng-10μg/ml. Samples were then incubated at 37°C for 10 minutes before continuing with protocol 2.6.2.

**Effect of sample handling:** A whole blood sample was divided into three aliquots and kept at either room temperature (24.5°C), on ice or at 37°C. At selected intervals a sample was taken from each aliquot and analysed for CD11b expression (section 2.6.2). All samples were run on the flow cytometer in the same batch.

**Storage of processed samples:** A sample which had been processed for analysis of CD11b expression was stored in the dark at 4°C and sub-sampled at various time points to observe the efficacy of the fixing procedure and to ascertain how long fixed samples could be kept before analysis.

**Reference range:** After optimising conditions for CD11b expression analysis, these were used to determine a reference range from 25 normal subjects.

**Results**  
**Antibody titration:** A saturating concentration of antibody appeared to be reached at 2μl of antibody per 100μl whole blood. To allow for individual variation of the subjects cells and slight differences in antibody batch variation, at least double this concentration should be used routinely. Therefore in all further experiments 5μl of antibody was used per 100μl of whole blood.

**CD11b Mobilisation:** The effect of stimulating whole blood with FMLP is shown in figure 2.2 and with LPS in Figure 2.3.
Figure 2.2. The effect of FMLP on CD11b expression. (result of a single representative experiment)

Figure 2.3. The Effect of LPS on CD11b expression (result of a single representative experiment)
As little as 1nM FMLP produced substantial and near maximal mobilisation of CD11b from intracellular stores in both monocytes and neutrophils. A concentration of 1μM was selected for any further experiments as this was convenient. LPS caused a dose dependent increase in the mobilisation of CD11b in both monocytes and neutrophils. A concentration of 10μg/ml was selected for any further experiments.

Effect of sample handling: Figure 2.4 shows the effect of sample handling before processing on a) monocyte and b) neutrophil expressed CD11b. Keeping the sample at 24.5 or 37°C produced an increase in the expression of CD11b on both monocytes and neutrophils. Although keeping samples on ice appeared to have little effect on CD11b expression by 1 hour, after this period CD11b expression appeared to be reduced on both monocytes and neutrophils. Samples obtained for CD11b analysis were therefore processed immediately to avoid any changes occurring due to sample handling.

Storage of processed samples: There was little change in the mean channel fluorescence of samples 24 hours after processing, but the MCF gradually declined after this. All samples for CD11b analysis were therefore analysed no later than 12 hours following sample processing.

Reference range: Table 2.3 shows a summary of the results of CD11b expression analysis in normal subjects. The distribution of both monocyte and neutrophil expressed CD11b was normal and therefore the reference range was taken to be the mean ± 2 standard deviations.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>2SD</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>monocyte CD11b</td>
<td>25</td>
<td>4.56</td>
<td>2.12</td>
<td>2.44-6.68</td>
</tr>
<tr>
<td>neutrophil CD11b</td>
<td>25</td>
<td>3.86</td>
<td>2.06</td>
<td>1.80-5.92</td>
</tr>
</tbody>
</table>

Table 2.3 CD11b expression in normal subjects
Figure 2.4. The effect of sample handling on CD11b expression on a) monocytes and b) neutrophils (result of a single representative experiment).
The intra-assay performance was assessed by assay of a single blood sample 10 times and the coefficient of variation determined and found to be 3.4% for neutrophil CD11b and 2.2% for monocyte CD11b. The inter-assay coefficient of variation could not be assessed as a single sample could not be kept over a period longer than 24 hours.

2.6.2 Neutrophil and monocyte CD11b expression - final method

**Principle**  Whole blood is incubated with either an antibody against human CD11b conjugated to R-phycoerythrin or irrelevant control antibody. Red cells in the sample are then lysed and the sample fixed. The amount of fluorescence is quantified using a flow cytometer and is proportional to the expression of CD11b on cell surfaces in the sample. The following conditions were used based on optimisation experiments detailed above. Citrated blood samples were processed immediately once taken as significant elevations of CD11b during handling and temperature changes have been observed (Repo et al, 1993).

**Reagents**  All reagents were from Merck Ltd unless otherwise stated.

- Lysing reagent A - 0.12% (v/v) Formic acid
- Stabilising reagent B - 0.057M sodium carbonate, 0.248M sodium chloride, 0.220M sodium sulphate
- Fixing reagent C - 1% (w/v) paraformaldehyde in PBS
- Mouse anti-human CD11b R-phycoerythrin conjugate (Dako Ltd)
- Fluorescin and isotype matched control antibody (Dako Ltd)
- Mouse anti-human CD14 FITC conjugate (Dako Ltd)

**Method**  Antibody to either CD11b or control antibody (5μl) were added to a plastic tube and 100μl of citrated whole blood was added to each, mixed and left on ice for 30 minutes. Red blood cells were lysed and the remaining cells fixed by the sequential addition of 600μl reagent A, 265μl reagent B, 100μl reagent C with 5 seconds mixing between each addition. Samples were
stored in the dark at 4°C and analysed within 12 hours on a Coulter Epics XL-MCL flow cytometer (Coulter Electronics Ltd). Neutrophils and monocytes were gated by virtue of their forward vs side scatter characteristics and gating around monocytes was confirmed by dual staining with anti-CD14. Data from 5,000 monocytes and 10,000 neutrophils were collected. A control mouse IgG1 antibody conjugated to R-phycoerythrin was used to estimate any changes in non-specific binding. The mean channel fluorescence of the control sample was subtracted from the test sample.

2.7 Lipid assays and lipoprotein separation

2.7.1 Total cholesterol

Principle  Cholesterol is measured by the use of the following enzymatic reactions:

1. Cholesterol esters + H₂O \[\text{esterase}\] → cholesterol + fatty acids
2. Cholesterol + O₂ \[\text{oxidase}\] → cholesterol + fatty acids
3. 2H₂O₂ + 4-aminoantipyrine \[\text{peroxidase}\] → quinoneimine dye + 4H₂O + p-hydroxybenzene sulphonate

Reagents  All reagents were from Sigma-Aldrich Chemical Co.

- Cholesterol reagent 352-20. Reconstituted according to instructions on vial
- Cholesterol calibrator (200mg/dl, C-7921)
- Cardiolipid control level 2 (essentially normal, C-4696)

Method  A series of tubes were labelled blank, standard, control and tests. 1ml of cholesterol reagent was added to each tube followed by 10μl of distilled water, cholesterol standard, cardiolipid control or test samples. The tubes were mixed by gentle inversion and incubated at room temperature for 10
minutes. The absorbance of each tube was read at 500nm against distilled water. The total cholesterol was determined using the following equation:

\[
\text{Cholesterol (mg/dl)} = \frac{A_{500,\text{test}} - A_{500,\text{blank}}}{A_{500,\text{standard}} - A_{500,\text{blank}}} \times \text{concentration of calibrator (mg/dl)}
\]

To convert to SI units (mmol/l) the cholesterol concentration was multiplied by 0.0259. Results were only accepted if the cholesterol concentration of the control sample was within the acceptable range.

### 2.7.2 Total triglycerides

**Principle** Triglycerides were measured using the following enzymatic reactions:

1. Triglycerides \(\xrightarrow{\text{lipoprotein lipase}}\) glycerol + fatty acids
2. Glycerol + ATP \(\xrightarrow{\text{glycerokinase}}\) Glycerol-1-phosphate + ADP
3. Glycerol-1-phosphate + O\(_2\) \(\xrightarrow{\text{GPO}}\) dihydroxyacetone phosphate + H\(_2\)O
4. H\(_2\)O\(_2\) + 4-aminoantipyrine \(\xrightarrow{\text{peroxidase}}\) quinoneimine dye + H\(_2\)O

**Reagents** All reagents were from Sigma-Aldrich Chemical Co.

- Triglyceride reagent (GPO-Trinder, 339-20) reconstituted according to instructions on vial.
- Glycerol standard (250mg/dl, G-1394)
- Cardiolipid control level 2 (essentially normal, C-4696)

**Method** A series of tubes were labelled blank, standard, control and tests. 1ml of triglyceride reagent was added to each tube followed by 10\(\mu\)l of distilled water, glycerol standard, cardiolipid control or test samples. The tubes were mixed by gentle inversion and incubated at room temperature for 15 minutes. The absorbance of each tube was read at 540nm against distilled water. The total triglyceride was determined using the following equation:
Triglyceride (mg/dl) = \frac{A_{540}\text{test}-A_{540}\text{blank}}{A_{540}\text{standard}-A_{540}\text{blank}} \times \text{concentration of standard (mg/dl)}

To convert to SI units (mmol/l) the triglyceride concentration was multiplied by 0.0113. Results were only accepted if the triglyceride concentration of the control sample was within the acceptable range.

2.7.3 Lipoprotein separation by preparative ultracentrifugation

**Principle**  Lipoproteins have lower hydrated densities than the other plasma proteins, permitting their isolation from plasma by flotation ultracentrifugation. The density of the plasma is increased by the addition of potassium bromide and during ultracentrifugation, lipoproteins float to the surface depending on their density and the prevailing small-solute density of the solution. Individual lipoprotein classes can be isolated by sequentially increasing the plasma density.

**Reagents**  All reagents were from Merck Ltd unless otherwise stated.

- 1.33 g/ml stock salt solution - 0.262M sodium chloride, 2.97M potassium bromide containing 0.34μM EDTA.
- 1.006 g/ml stock salt solution - 0.15M sodium chloride
- 1.071 g/ml salt solution - 20.06 ml 1.33 g/ml stock solution + 79.94 ml 1.006 g/ml stock solution
- 1.125 g/ml salt solution - 36.72 ml 1.33 g/ml stock solution + 63.28 ml 1.006 g/ml stock solution
- 1.239 g/ml salt solution - 71.91 ml 1.33 g/ml stock solution + 28.09 ml 1.006 g/ml stock solution.

**Method**  3 ml 1.006 g/ml solution were gently overlaid on 2 ml EDTA plasma in polyallomer centrifuge tube (Beckman Ltd). The tubes were then centrifuged at 100,000g in a 50.3 Ti fixed angle rotor (Beckman Ltd) for 16 hours. The supernatant (VLDL) was removed using a tube slicer (Spinc
Tube Slicer, Beckman Ltd), transferred to a 2 ml volumetric flask and the
volume was returned to 2 ml by addition of 1.006 g/ml solution. The
pelleted material was discarded and the infranatant was transferred to a 4 ml
volumetric flask and the volume adjusted to 4 ml with 1.019 g/ml solution.
This solution was then transferred to a further centrifuge tube and 1 ml of
1.239 g/ml solution was added to make a final density of 1.063 g/ml for
isolation of LDL. After mixing, the tubes were centrifuged at 100,000g for
20 hours. The supernatant (LDL) was transferred to a 2 ml volumetric flask
and the volume was returned to 2 ml by addition of 1.063 g/ml solution.
The infranatant (HDL) was transferred to a 4 ml volumetric flask and the
volume adjusted to 4 ml with 1.063 g/ml solution.

2.8 D-dimer by latex agglutination

**Principle**  D-dimers are formed by plasmin degradation of FXIIIa cross-linked fibrin.
This method is a latex agglutination assay for the semi-quantitative
determination of D-dimer in plasma. Latex beads coated with a mouse
monoclonal antibody against D-dimer are mixed with plasma and after a set
incubation if D-dimer is present in plasma agglutination occurs. The
technique can be semiquantitated in positive plasmas by repeating the test at
increasing dilutions until agglutination no longer takes place. Specificity is
provided by employing a monoclonal antibody that does not cross-react
with fibrinogen or fragment D of fibrinogen.

**Reagents**  All reagents were part of a kit from Sigma-Aldrich Chemical Co unless
otherwise stated.

- D-dimer latex - a suspension of latex beads coated with mouse
  monoclonal antibody against D-dimer in hepes buffer pH 8.2
  containing 0.02% (w/v) sodium azide
- Buffer - buffered saline, pH 7.3, containing 0.02% (w/v) sodium azide
• mixing sticks
• test cards
• negative control plasma, reconstituted according to manufacturers instructions
• positive control plasma, reconstituted according to manufacturers instructions

Method

Undiluted test, positive and negative control plasmas (20μl) were placed in separate circles on a test card. Mixed D-dimer latex (20μl) was placed in a nearby area of each circle. The samples were quickly mixed using a stick and the test card rocked back and forth for 3 minutes. A positive result was assessed as the presence of aggregated particles in a clear or milky solution. Samples that tested positive were repeated at 1:2, 1:4 and 1:8 dilutions. D-dimer concentrations were established from Table 2.4.

<table>
<thead>
<tr>
<th>D-dimer (ng/ml)</th>
<th>Undiluted</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;250</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>250-500</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>500-1000</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1000-2000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&gt;2000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2.4 D-dimer concentrations determined by latex agglutination

2.9 Preparation of washed neutrophils

Principle

Neutrophils are separated from platelets by centrifugation, red cells by dextran sedimentation, and from mononuclear cells by density gradient centrifugation.

Reagents

All reagents were from Merck Ltd unless otherwise stated.

• Dextran T500 (Pharmacia Ltd) dissolved in 0.9% sodium chloride to give a 10% w/v solution
- Dubelcco's phosphate buffered saline without sodium bicarbonate, magnesium or calcium (Life Technologies Ltd) - for all diluting and washing steps
- Dubelcco's phosphate buffered saline without sodium bicarbonate (Life Technologies Ltd), containing 5.55mM D-glucose for final suspension
- Density gradient media - Histopaque 1077 (Sigma-Aldrich Chemical Co)
- sodium chloride 3.5% (w/v)
- unfractionated heparin (Multiparin, CP Pharmaceuticals)

**Method**

All steps were carried out at room temperature. Whole blood was anticoagulated with 10IU/ml (final concentration) of heparin and then centrifuged at 170g for 10 minutes and the resultant platelet-rich plasma removed and discarded. The remaining blood was diluted 1:2 in PBS and dextran added to give a ratio of blood:dextran of 9:1. The sample was then sedimented for 1 minute/ml for a maximum of 30 minutes. The leucocyte-rich plasma was removed and centrifuged at 170g for 5 minutes. The supernatant was discarded and the cells resuspended in 10ml PBS and underlayered with 5ml of Histopaque. After centrifugation at 400g for 20 minutes, the supernatant and mononuclear cell layer were discarded and the neutrophil pellet resuspended in 2ml of PBS. Remaining red cells were removed using hypertonic lysis by the addition of 6ml ice-cold sterile water for 45 seconds, followed rapidly by the addition of 2ml of saline to restore ionic strength. The cells were washed 3 times in PBS at 170g for 5 minutes and finally resuspended in PBS with calcium, magnesium and glucose. Purity was checked by Romanowsky staining and viability by trypan blue exclusion (0.1% in saline).


2.10 Reference Ranges

A reference range for each assay was performed by assaying samples from at least 20 apparently healthy normal volunteers. If the parameter measured was normally distributed the reference range was defined as the mean ± 2 standard deviations. For non-gaussian data with positive skew the data was normalised by log transformation. The mean ± 2 standard deviations of the log data was antilogged to provide the reference range. Thus for lognormal data the reference range was defined as the geometric mean with 95% confidence interval. The reference range for assays described in this chapter are shown in table 2.5.

2.11 Assay Performance

To assess the performance of each assay the intra (within) and inter (between) assay coefficient of variation (CV) were calculated. The intra-assay CV was calculated from ten measurements of a single plasma on one occasion. The inter-assay CV was calculated from the measurement of a single plasma on at least 20 different occasions over several months. The intra- and inter-assay CVs for assays described in this chapter are shown in table 2.2.

2.12 Computer Hardware and Software

The Anthos 2001 automated plate reader was connected to a PC and data analysed using ANELISA 1.21 (Epsilon Technology Ltd). An Apple Macintosh Performa 6200 Power PC was used to prepare this thesis. Data was initially organised using Excel 5.0 (Microsoft Corp.). Routine manual checks were carried out to ensure that spreadsheet and statistical package software transformation of data was correct.
2.13 Statistical Analysis

The exact nature of the statistical analysis of data was dependant upon the number of observations and the distribution of the data obtained, therefore each chapter will contain its own statement on statistical analysis. Statistical analysis was carried out using Statview II software for Macintosh, Abacus Concepts Inc, Berkley, CA. A probability value of less than 0.05 was taken as being statistically significant.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Units</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.4 Factor XII</td>
<td>U/ml</td>
<td>0.50-1.50</td>
</tr>
<tr>
<td>2.3.6 Prekallikrein</td>
<td>U/ml</td>
<td>0.70-1.30</td>
</tr>
<tr>
<td>2.3.7 Factor VII</td>
<td>U/ml</td>
<td>0.65-1.45</td>
</tr>
<tr>
<td>2.3.9 Tissue factor pathway inhibitor</td>
<td>U/ml</td>
<td>0.40-1.90</td>
</tr>
<tr>
<td>2.3.10 Antithrombin III</td>
<td>IU/ml</td>
<td>0.80-1.20</td>
</tr>
<tr>
<td>2.3.11 Heparin cofactor II</td>
<td>U/ml</td>
<td>0.65-1.45</td>
</tr>
<tr>
<td>2.3.12 C1-esterase inhibitor</td>
<td>U/ml</td>
<td>0.70-1.30</td>
</tr>
<tr>
<td>2.3.13 α2-macroglobulin</td>
<td>U/ml</td>
<td>0.70-1.50</td>
</tr>
<tr>
<td>2.3.14 Protein C</td>
<td>IU/ml</td>
<td>0.70-1.30</td>
</tr>
<tr>
<td>2.4.3 Neutrophil elastase</td>
<td>μg/l</td>
<td>30-180</td>
</tr>
<tr>
<td>2.4.4 TAT complexes</td>
<td>μg/l</td>
<td>1.00-4.10</td>
</tr>
<tr>
<td>2.4.5 Prothrombin Fragment 1+2</td>
<td>nM</td>
<td>0.40-1.10</td>
</tr>
<tr>
<td>2.4.6 Soluble thrombomodulin</td>
<td>ng/ml</td>
<td>5-55</td>
</tr>
<tr>
<td>2.4.7 Endothelin-1</td>
<td>pg/ml</td>
<td>0.30-0.90</td>
</tr>
<tr>
<td>2.4.8 Soluble E-Selectin</td>
<td>ng/ml</td>
<td>29-64</td>
</tr>
<tr>
<td>2.4.9 Soluble tissue factor</td>
<td>pg/ml</td>
<td>64-129</td>
</tr>
<tr>
<td>2.4.10 Activated factor XII</td>
<td>ng/ml</td>
<td>0.34-2.90</td>
</tr>
<tr>
<td>2.4.11 Protein C</td>
<td>IU/ml</td>
<td>0.70-1.30</td>
</tr>
<tr>
<td>2.4.12 Total protein S</td>
<td>male</td>
<td>0.70-1.40</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>0.50-1.25</td>
</tr>
<tr>
<td>2.4.13 Free Protein S</td>
<td>male</td>
<td>0.65-1.75</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>0.55-1.10</td>
</tr>
<tr>
<td>2.4.14 Total TFPI</td>
<td>ng/ml</td>
<td>14-60</td>
</tr>
<tr>
<td>2.4.15 β2-glycoprotein-1</td>
<td>μg/ml</td>
<td>126-273</td>
</tr>
<tr>
<td>2.5.6 Activated factor VII</td>
<td>ng/ml</td>
<td>0.60-5.90</td>
</tr>
<tr>
<td>2.5.7 Prothrombin</td>
<td>U/ml</td>
<td>0.50-1.50</td>
</tr>
<tr>
<td>2.6.2 Neutrophil expressed CD11b</td>
<td>MCF</td>
<td>1.80-5.90</td>
</tr>
<tr>
<td>2.6.2 Monocyte expressed CD11b</td>
<td>MCF</td>
<td>2.40-6.90</td>
</tr>
</tbody>
</table>

Table 2.5 Reference Ranges
<table>
<thead>
<tr>
<th>Assay</th>
<th>Level assayed</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.4 Factor XII</td>
<td>0.7 U/ml</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>2.3.6 Prekallikrein</td>
<td>0.7 U/ml</td>
<td>3.1</td>
<td>5.9</td>
</tr>
<tr>
<td>2.3.7 Factor VII</td>
<td>0.7 U/ml</td>
<td>2.7</td>
<td>4.8</td>
</tr>
<tr>
<td>2.3.9 TFPI</td>
<td>0.9 U/ml</td>
<td>7.4</td>
<td>9.7</td>
</tr>
<tr>
<td>2.3.10 Antithrombin III</td>
<td>0.8 IU/ml</td>
<td>3.8</td>
<td>5.1</td>
</tr>
<tr>
<td>2.3.11 Heparin cofactor II</td>
<td>0.7 U/ml</td>
<td>4.8</td>
<td>5.6</td>
</tr>
<tr>
<td>2.3.12 C1-esterase inhibitor</td>
<td>0.7U/ml</td>
<td>3.1</td>
<td>5.9</td>
</tr>
<tr>
<td>2.3.13 α₂-macroglobulin</td>
<td>0.8 U/ml</td>
<td>3.3</td>
<td>6.9</td>
</tr>
<tr>
<td>2.3.14 Protein C</td>
<td>0.8 U/ml</td>
<td>3.6</td>
<td>6.3</td>
</tr>
<tr>
<td>2.4.3 Neutrophil elastase</td>
<td>80 µg/l</td>
<td>4.3</td>
<td>9.1</td>
</tr>
<tr>
<td>2.4.4 TAT complexes</td>
<td>60 µg/l</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td>2.4.5 Prothrombin F1+2</td>
<td>0.2 ng/ml</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td>2.4.6 Soluble thrombomodulin</td>
<td>30 ng/ml</td>
<td>4.2</td>
<td>6.7</td>
</tr>
<tr>
<td>2.4.7 Endothelin-1</td>
<td>14.4 pg/ml</td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td>2.4.8 Soluble E-Selectin</td>
<td>20 ng/ml</td>
<td>5.0</td>
<td>9.1</td>
</tr>
<tr>
<td>2.4.9 Soluble tissue factor</td>
<td>100 pg/ml</td>
<td>4.2</td>
<td>N/A</td>
</tr>
<tr>
<td>2.4.10 Activated factor XII</td>
<td>2.8 ng/ml</td>
<td>4.7</td>
<td>6.9</td>
</tr>
<tr>
<td>2.4.11 Protein C</td>
<td>1.00U/ml</td>
<td>3.3</td>
<td>7.8</td>
</tr>
<tr>
<td>2.4.12 Total Protein S</td>
<td>0.96U/ml</td>
<td>5.1</td>
<td>6.5</td>
</tr>
<tr>
<td>2.4.13 Free Protein S</td>
<td>0.95U/ml</td>
<td>4.8</td>
<td>6.3</td>
</tr>
<tr>
<td>2.4.14 Total TFPI</td>
<td>2.0 ng/ml</td>
<td>7.1</td>
<td>7.3</td>
</tr>
<tr>
<td>2.4.15 β₂glycoprotein-1</td>
<td>200µg/ml</td>
<td>7.9</td>
<td>9.1</td>
</tr>
<tr>
<td>2.5.6 Activated factor VII</td>
<td>2.2 ng/ml</td>
<td>4.3</td>
<td>9.1</td>
</tr>
<tr>
<td>2.6.2 Neutrophil CD11b</td>
<td>5.1</td>
<td>4.4</td>
<td>N/A</td>
</tr>
<tr>
<td>2.6.2 Monocyte CD11b</td>
<td>5.9</td>
<td>2.2</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Table 2.6 Intra and Inter-Assay Coefficients of variation*
3.1 Introduction

Haemofiltration is well established as a supportive treatment for critically ill patients with acute renal failure. Haemofiltration is usually preferred to haemodialysis in these patients as it affords improved haemodynamic stability and also permits control of fluid balance. The principal differences between haemodialysis and haemofiltration as well as general circuit design are discussed in section 1.3.

Although haemofilters have an expected lifespan of at least 48-72 hours, experience in our Intensive Care Unit suggests that patients in the acute phase of multi-organ dysfunction syndrome tend to clot their haemofilters prematurely, sometimes within a few hours of use. This occurs despite administration of exogenous anticoagulation as recommended by the manufacturers of the haemofilter circuit and/or the coexistence of underlying coagulopathies. Although the replacement cost of a new haemofilter circuit is relatively inexpensive (approximately £100), if a patient requires six filter circuits to be changed in a 24 hour period this is time consuming and may add significantly to the overall cost of intensive care. Prevention of premature clotting is also desirable as loss of the haemofilter circuit is not merely a nuisance but involves a significant delay before renal support can be re-instituted, and may necessitate the use of blood products including platelets and clotting factors.

AT-III is essential for the therapeutic or prophylactic anticoagulant activity of heparin (the anticoagulant most commonly used in renal replacement therapy), but is often depleted in critical illness due to consumption and/or reduced production (Deutsch & Thaler, 1979; Hesselvik et al, 1989). Reduced levels of AT-III in critically ill patients may therefore limit the effectiveness of heparin anticoagulation.
Contact and coagulation system activation with generation of vasoactive kinins is a common feature of sepsis (Velasco et al, 1986; Walshe et al, 1987; Martinez-Brotons et al, 1987). The mechanism for contact activation during sepsis is unclear but may be multifactorial in origin and may result from the action of endotoxin. Endotoxin can directly activate FXII (Morrison & Cochrane, 1974). In a baboon model of sepsis, administration of a FXII antibody prior to endotoxin challenge attenuated activation of the contact system and the development of hypotension (Pixley et al, 1992) and also attenuated complement, leucocyte and fibrinolytic responses which may be enhanced by contact activation (Jansen et al, 1996). During septic shock, levels of contact factor proteins are reduced (De la Cadena et al, 1993; Wuillemin et al, 1995), there is formation of complexes of FXIIa, KK, and FXIa with their plasma inhibitors (Nuijens et al, 1988; Wuillemin et al, 1995), and levels of FXIIa are reported to increase (Mesters et al, 1996). Taken together, these data indicate that activation of the contact system occurs during sepsis.

In other types of extracorporeal circuit, such as CPB, exposure of blood to the foreign surface of the circuit is thought to result in activation of the contact system of coagulation with subsequent thrombin and fibrin generation (Edmunds, 1995). During haemodialysis in vivo there is an increase in kallikrein activity (Wardle & Piercy, 1972), a reduction in plasma levels of contact factors (Vicente et al, 1991), and an increase in FXIIa amidolytic activity, reflecting activation of FXII (Svensson et al, 1996). Haemodialysis membranes have also been shown to activate FXII in vitro (Matata et al, 1996).

The hypothesis was therefore examined that exposing patients to haemofilter circuits might result in further contact and coagulation system activation which, in the absence of adequate levels of circulating endogenous anticoagulants, could result in fibrin deposition and premature clotting of the filter circuit.

Evidence of contact activation and thus initiation of intrinsic pathway activation was sought by a fall in plasma levels of the contact factors PKK and FXII and their main inhibitors AT-III, Cl-INH and alpha-2-macroglobulin (A2MG) as well as an
increase in FXIIa antigen. As free thrombin in plasma is rapidly complexed to circulating protease inhibitors, evidence of thrombin generation was sought by measuring the levels of TAT complexes and the thrombin-specific inhibitor heparin cofactor II (HCII).

3.2 Methods

3.2.1 Patients

12 adult patients on the intensive care unit receiving continuous veno-venous haemofiltration for acute renal failure were enrolled. Patients were excluded if they were receiving aprotinin, required haemodiafiltration rather than haemofiltration, or if discontinuation of haemofiltration was necessary for any reason other than clotting of the circuit, e.g. transfer for CT scan. The study was approved by the Medical Ethics Committee of the University College London Hospitals. Informed consent was obtained from patients wherever possible, otherwise assent was sought from their next of kin.

3.2.2 Haemofiltration.

A BSM 22 pumped haemofiltration system (Hospal, Loughborough, UK) with polyacrylonitrile AN69 hollow-fibre haemofilters (Multiflow 100, Hospal, Loughborough, UK) was used. Venous access was gained by a 12G double lumen cannula (Dualyse-Cath, Vygon, Cirencester, Glos, UK) placed in either internal jugular, subclavian or femoral veins. Blood flow through the circuit was set at 150-200 ml/min and filtrate volumes removed at a rate of 1000 ml/hour. Replacement fluid used was either Hemofiltrasol 22 (Gambro, Lund, Sweden) or Hemosol LG2 (Hospal, Loughborough, Leics, UK), the amount given depending on the patient's clinical status and desired hourly fluid balance. Anticoagulation regimens were not standardized but were based on the clinical situation and coagulation variables. Low molecular weight heparin and/or prostaglandins were used when platelet counts were low and heparin-induced thrombocytopenia was possibly implicated. Anticoagulants were infused directly into the circuit pre-filter and generally consisted of either unfractionated heparin
(Monoparin, CP Pharmaceuticals, Wrexham, UK) or low molecular weight heparin (Fragmin, Kabi-Pharmacia, Milton Keynes, Bucks, UK) to maintain bedside activated coagulation times of 150-200 seconds, and/or prostacyclin (PGI₂) or alprostadil (PGE₁) at rates of 2.5-10 ng/kg/min.

3.2.3 Sample collection
Citrated plasma was prepared (section 2.1) immediately from blood taken from the arterial side of the haemofilter circuit before starting haemofiltration, at 15 minutes and 1, 3-4, 8-12, 24 hours after commencement and 24-hour intervals thereafter or until the filter circuit clotted. At the same time points whole blood anticoagulated with EDTA was collected for performance of full blood counts (section 2.2) and ultrafiltrate was also collected and immediately frozen in aliquots.

3.2.4 Measurement of haemostatic factors
FXII, prekallikrein, AT-III, HCII, alpha-2-macroglobulin and C1-INH were measured by amidolytic substrate assays (section 2.3). All amidolytic assays were standardised using Reference Plasma 100% (Immuno Ltd). TAT complexes and activated factor XII were measured using an ELISA technique (see section 2.4).

3.2.5 Statistical analysis
Repeated measures analysis of variance could not be used to assess whether proteins or inhibitor levels changed over the period of haemofiltration since the filter lifespan was not equal in all patients. Therefore changes in variables over the period of filtration were assessed using the Wilcoxon Rank test between prefiltration levels and those prior to clotting. Comparisons between groups were made using the Wilcoxon rank test. Correlations of variables with filter lifespan or thrombin generation were assessed using Spearman’s rank correlation coefficient. *p*<0.05 was considered statistically significant.
### 3.3 Results

The characteristics of the 12 patients studied are shown in Table 3.1. Seven patients had been filtered previously during their ICU admission for periods ranging from 1-25 days. Ten patients received heparin (nine unfractionated, one low molecular weight), three in conjunction with alprostadil. One patient received prostacyclin alone and one received no anticoagulant due to concurrent problems with haemostasis and thrombocytopenia.

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Sex</th>
<th>Presenting condition</th>
<th>Anticoagulation</th>
<th>Previous filtration (days)</th>
<th>Filter lifespan (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>M</td>
<td>Burns</td>
<td>heparin</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>F</td>
<td>Post-op CCF Chronic renal failure</td>
<td>heparin PGE1</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>M</td>
<td>Staphylococcal septicaemia</td>
<td>heparin</td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>F</td>
<td>ARDS after major post-op bleeding</td>
<td>heparin PGE1</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>59</td>
<td>F</td>
<td>Acute on chronic liver failure</td>
<td>none</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>M</td>
<td>Tumour lysis syndrome</td>
<td>prostacyclin</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>75</td>
<td>F</td>
<td>Post aortic valve replacement</td>
<td>heparin</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>76</td>
<td>M</td>
<td>Post CABG, Chronic renal failure</td>
<td>heparin PGE1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>68</td>
<td>M</td>
<td>Pneumonia, Head injury</td>
<td>heparin</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>M</td>
<td>Fournier's Gangrene</td>
<td>heparin</td>
<td>25</td>
<td>68</td>
</tr>
<tr>
<td>11</td>
<td>45</td>
<td>F</td>
<td>Pancreatitis</td>
<td>heparin</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>77</td>
<td>M</td>
<td>Post-op aortic aneurysm</td>
<td>Fragmin</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Abbreviations: PGE1 Prostaglandin E1; CCF congestive heart failure; CABG coronary artery bypass graft

**Table 3.1 Patient Characteristics**
In five patients the filter clotted within 24 hours, one (at 21 hours) being due to mechanical obstruction of the femoral venous cannula following rolling of the patient into a left lateral position. In only four patients did the filter circuit remain patent for more than 48 hours. The median filter survival was 31 hours (range 6-64 hours).

There was no relationship between filter lifespan and the duration of previous haemofiltration, the anticoagulant used, haemodynamic instability as judged by requirement for vasoactive drugs, nor baseline levels of routine coagulation tests (international normalised ratio, platelet count).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Filter life</th>
<th>Hct</th>
<th>Pits</th>
<th>ATIII</th>
<th>HCII</th>
<th>FXII</th>
<th>FXIIa</th>
<th>PKK</th>
<th>A2M</th>
<th>C1-INH</th>
<th>TAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>0.25</td>
<td>59</td>
<td>2.3</td>
<td>0.62</td>
<td>0.71</td>
<td>0.35</td>
<td>10.56</td>
<td>0.25</td>
<td>0.99</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>0.32</td>
<td>268</td>
<td>2.8</td>
<td>0.70</td>
<td>0.52</td>
<td>0.72</td>
<td>5.52</td>
<td>0.71</td>
<td>0.85</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>0.27</td>
<td>239</td>
<td>1.2</td>
<td>0.98</td>
<td>0.93</td>
<td>0.56</td>
<td>6.98</td>
<td>0.39</td>
<td>0.74</td>
<td>1.10</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>0.33</td>
<td>50</td>
<td>1.9</td>
<td>0.34</td>
<td>0.29</td>
<td>0.50</td>
<td>10.87</td>
<td>0.52</td>
<td>0.63</td>
<td>0.90</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>0.29</td>
<td>85</td>
<td>3.8</td>
<td>0.30</td>
<td>0.06</td>
<td>0.40</td>
<td>24.03</td>
<td>0.15</td>
<td>0.54</td>
<td>1.01</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>0.23</td>
<td>45</td>
<td>1.5</td>
<td>0.87</td>
<td>0.67</td>
<td>0.87</td>
<td>12.78</td>
<td>0.55</td>
<td>0.61</td>
<td>0.91</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>0.34</td>
<td>185</td>
<td>1.4</td>
<td>1.28</td>
<td>0.52</td>
<td>0.48</td>
<td>3.40</td>
<td>0.47</td>
<td>0.70</td>
<td>2.06</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>0.29</td>
<td>269</td>
<td>1.4</td>
<td>0.70</td>
<td>0.54</td>
<td>0.61</td>
<td>5.28</td>
<td>0.54</td>
<td>0.60</td>
<td>1.18</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>0.32</td>
<td>134</td>
<td>1.2</td>
<td>0.78</td>
<td>0.64</td>
<td>0.56</td>
<td>8.94</td>
<td>0.40</td>
<td>0.42</td>
<td>1.04</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>0.28</td>
<td>143</td>
<td>1.4</td>
<td>0.48</td>
<td>0.51</td>
<td>0.64</td>
<td>23.24</td>
<td>0.38</td>
<td>0.30</td>
<td>1.23</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>0.34</td>
<td>178</td>
<td>1.4</td>
<td>0.51</td>
<td>0.59</td>
<td>0.67</td>
<td>7.30</td>
<td>0.73</td>
<td>0.34</td>
<td>1.04</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>0.28</td>
<td>71</td>
<td>1.2</td>
<td>0.34</td>
<td>0.45</td>
<td>0.27</td>
<td>15.26</td>
<td>0.51</td>
<td>0.29</td>
<td>0.87</td>
</tr>
</tbody>
</table>

| normal range | 150-1000 | <1.0-10.0 | 0.65-5.0 | <3-30 | 0.7-7.0 | 0.7-7.0 | 1-4.1 |
| units hours % | 10^9/l | IU/ml | U/ml | ng/ml | U/ml | U/ml | mg/l |

Abbreviations: Hct, haematocrit; Pits, platelets; INR, international normalised ratio.

Table 3.2 Pre-haemofiltration coagulation variables
Baseline levels of AT-III and HCII were below the normal reference range in 9 of the 12 patients (Table 3.2). The lowest AT-III and HCII levels were seen in three of the four patients whose filters clotted within the first 24 hours, the fourth patient also having subnormal levels of both. Low baseline levels of AT-III and HCII were significantly related to premature clotting of filters (Table 3.3).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Filter lifespan &lt;24 hours (n =5)</th>
<th>Filter lifespan &gt;24 hours (n =7)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-III</td>
<td>0.34 ± 0.09</td>
<td>0.74 ± 0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>HCII</td>
<td>0.37 ± 0.11</td>
<td>0.62 ± 0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3.3 Relationship between haemofilter lifespan and pre-filtration levels of ATIII and HCII

![Figure 3.1 Change in TAT complexes over the filter lifespan.](image)

Patients in whom filters clotted within 24 hours are indicated by the stippled line. (n=11, the patient in whom the filter blocked due to mechanical obstruction is not shown).
Thrombin-antithrombin complex levels were raised in 8/12 patients at baseline, but only in the four with low ATIII levels whose filters clotted early were there further rises (Figure 3.1). There was a significant relationship between the percentage change in thrombin-antithrombin complex level and filter lifespan (p<0.008) (Fig 3.2).

![Figure 3.2 Relationship between % change in TAT complex level and filter lifespan (hours)](image)

Levels of all substances assayed remained unchanged throughout the study period (Table 3.4) with the exception of thrombin-antithrombin complex levels which rose markedly in four of the five patients whose filters clotted early.

Prefiltration levels of FXII, PKK and α₂-macroglobulin were low in most patients, whereas C1-INH levels were normal or raised. FXIIa levels were elevated in all twelve patients at baseline (table 3.2) but there were no further changes in FXIIa over the lifespan of the filter (table 3.4). There was no evidence of clearance of any of these substances across the filter into the ultrafiltrate.
### Table 3.4 Change in plasma levels of contact system factors and inhibitors over haemofilter lifespan

Results expressed as [Median ± (SEM)].

<table>
<thead>
<tr>
<th>Time</th>
<th>Baseline</th>
<th>15 min</th>
<th>1 hour</th>
<th>3-4 hr</th>
<th>8-12 hr</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATIII</td>
<td>0.80-1.20</td>
<td>IU/ml</td>
<td>0.66</td>
<td>0.60</td>
<td>0.69</td>
<td>0.63</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.08)</td>
<td>(0.08)</td>
<td>(0.09)</td>
<td>(0.07)</td>
<td>(0.07)</td>
</tr>
<tr>
<td>HClII</td>
<td>0.65-1.45</td>
<td>U/ml</td>
<td>0.53</td>
<td>0.53</td>
<td>0.52</td>
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<td>(3.33)</td>
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<td>(6.6)</td>
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<td>(0.08)</td>
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#### 3.4 Discussion

Continuous haemofiltration is a well-established technique in the management of acute renal failure. However, premature clotting of the haemofilter circuit is a frequent problem which, anecdotally, appears to be more common in the sicker patient. Mechanical causes can usually be excluded and, despite good blood flows through the circuit at low pressures, the filter still clots prematurely even though exogenous anticoagulation is administered. In this study one patient appeared to clot his filter early following kinking of the femoral venous line whilst being turned in bed having previously had good flows through the circuit; the remaining patients appeared to have no mechanical or flow...
related problems during the period of haemofiltration. Major haemodynamic disturbances may also lead to clotting of the filter: none of the patients developed a significant haemodynamic disturbance during the course of the study.

Heparin clearance across the haemofilter has previously been studied (Singer et al, 1994). Levels of unfractionated and low molecular weight heparin were not altered significantly by haemofiltration. Despite satisfactory levels of heparin and anti-Xa activity, premature clotting still occurred. It is possible that contact and intrinsic coagulation activation by the haemofilter circuit might provoke premature clotting, or that depletion of natural inhibitors of coagulation might allow clotting to occur.

Exposure of blood to many foreign surfaces, especially those with a negative surface electric charge causes contact activation with generation of Factor XIIa and conversion of prekallikrein to kallikrein (De La Cadena et al, 1994). Activation may also occur at blood-air interfaces such as the bubble-trap in the haemofilter circuit and this may lead to premature clotting of the circuit and, potentially, to haemodynamic instability.

In this study factor XII and prekallikrein levels were below the normal range in most patients and factor XIIa levels were elevated in all patients before haemofiltration had commenced. This would suggest that in these patients the contact system was already activated to some degree. There was no change in the levels of contact factors over the lifespan of the filter, implying that no progressive activation of the contact system occurred during haemofiltration. However, factor XIIa may either become bound to the haemofilter circuit or to its plasma inhibitors (notably C1-esterase inhibitor) and as factor XIIa: C1-esterase inhibitor complexes are not detected by the assay employed here, factor XIIa generation cannot therefore be excluded. Since this thesis was undertaken other workers have also shown that FXIIa levels measured using this method are increased during the course of sepsis (Mesters et al, 1996).

A number of different materials are used for haemofilter membranes including polysulfone, cuprophane, polymethylmethacrylate and polyacrylonitrile. Studies on haemodynamic responses and biocompatibility of these membranes in patients
undergoing haemofiltration (Davenport & Davison, 1993) have shown varying results. The majority of studies on biocompatibility have been carried out on haemodialysis patients or ex vivo and have concentrated on platelet, neutrophil and complement activation, little attention being paid to coagulation activation. Few have been performed in vivo on patients undergoing continuous haemofiltration although a recent study did demonstrate a significant reduction in platelet aggregability (Boldt et al, 1994). The overall impression is that polysulfone and polyacrylonitrile are the most neutral, polymethyl-methacrylate appears to be intermediate whereas cuprophane is relatively hostile until it has been conditioned (Chenoweth et al, 1983; Horl et al, 1985; Markaert et al, 1988; David et al, 1993).

Whether the results of the dialysis biocompatibility studies can be extrapolated to haemofiltration is questionable, especially as the patient population and length of patient exposure to the filter are markedly different. Many critically ill patients will have a pre-existing degree of inflammatory activation due to their underlying disease and the fluid shifts associated with intermittent haemodialysis and continuous venovenous haemofiltration are very different.

The haemofiltration circuit presents a large surface capable of causing contact activation. Heparin-bonded circuits, which are currently being investigated for use during CPB, could possibly attenuate coagulation activation (Arakawa et al, 1991) though no such circuit is available for clinical use for haemofiltration. It can be postulated that deposition of biological material on this surface over time might improve the bio-compatibility of the circuit as a whole and that any activation that did occur would be most pronounced in the early stages. This is analogous to the effects seen with re-usable oxygenators in early cardiac surgery and with the reuse of dialysis filters (Markaert et al, 1988). Although most of the patients studied here showed evidence of contact activation before starting haemofiltration, the lack of change during the study suggests that the polyacrylonitrile membrane is biocompatible, at least as far as the contact and intrinsic coagulation pathways are concerned.

Despite adequate levels of heparin, premature clotting may still occur because
endogenous inhibitors of the coagulation system are depleted. Indeed, heparin acts by potentiation of antithrombin III which inhibits thrombin and factor Xa, and is also an inhibitor of factors IXa, XIIa and kallikrein (Barrowcliffe & Thomas, 1987). Patients with inherited antithrombin III deficiency have antithrombin III levels between 40-70% of normal and a markedly increased risk of thrombo-embolic disease (Thaler & Lechner, 1981). The low levels of antithrombin III found in this study are consistent with previous studies in critically ill patients (Deutsch & Thaler, 1979; Bluhut et al, 1985; Mammen et al, 1985; Hesselvik et al, 1989) and might limit the efficiency of heparin anticoagulation.

The importance of the regulation of coagulation proteins by AT-III during sepsis is highlighted in recent studies showing that although increases in markers of coagulation activation such as TAT complexes and FPA levels are only associated with non-survival during later stages of sepsis, baseline AT-III levels are inversely correlated with mortality and the development of MODS during sepsis (Mesters et al, 1996; Della Valle et al, 1997; Boveda et al, 1997). This implies that measurement of AT-III levels may also be of predictive value.

Heparin cofactor II is also an efficient inhibitor of thrombin and is not known to inhibit any other clotting factors. It has a much weaker affinity than antithrombin III for heparin and is potentiated by a different glycosaminoglycan, dermatan sulphate, probably at the vessel wall though its precise physiological role is uncertain (Pasche et al, 1991). In the patients studied in this chapter, heparin cofactor II levels were reduced with a similar pattern to antithrombin III. There was no consistent pattern of change of alpha-2 macroglobulin, an endogenous protease inhibitor with a broad spectrum of target proteases including kallikrein, FXIIa, thrombin and factor Xa.

Irreversible thrombin-antithrombin complexes have relatively short plasma half-lives of three minutes and although direct measurement of thrombin is impossible, a rising TAT level gives an indirect measure of thrombin generation. Septic patients have increased plasma levels of TAT complexes (Lorente et al, 1993; Asahura et al, 1994; Leithauser et al, 1996) and it is reported that TAT complex levels increase during the
onset of sepsis (Mesters et al, 1996) but may decrease with time after the development of septic shock (Lorente et al, 1993). In addition, patients with chronic renal failure have elevated TAT levels compared to control subjects, and during haemodialysis there is further generation of TAT complexes in some patients (Kolb et al, 1991; Yamazaki et al, 1995).

In the patients studied here, baseline levels of TAT complexes were elevated in most patients, but a further progressive increase in TAT complex levels during haemofiltration only occurred in those patients whose filters clotted in a relatively short time and suggests that activation of coagulation is indeed occurring during this period. Lack of concurrent change in intrinsic pathway factors or inhibitors suggests alternative routes of coagulation activation; for example, the tissue factor pathway or relative deficiencies of the protein C-protein S inhibitory system (Hesselvik et al, 1991; Esmon et al, 1991). Others have suggested that it is impaired regulation of thrombin rather than additional contact activation that is resonsible for thrombogenesis during haemodialysis (Boisclair et al, 1993c). It is possible that a similar phenomenon could occur during haemofiltration.

Since this study was undertaken, there has been another report into the influence of haemostatic variables on CVVH filter running time (Stefanidis et al, 1996). In agreement with observations from this chapter, the latter authors report no relationship between filter running time and heparin dose, prothrombin time, and thrombin time and the ACT only correlated with filter running time if less than 110 seconds. In contrast to the results observed in this chapter, the authors detected no change in the levels of TAT complexes during CVVH. However, in this study a different haemofiltration system was employed and samples were only analysed every three days, which means that the haemofilter circuit had been replaced during sample points in most patients. Indeed, in a recent abstract others have shown an increase in modified antithrombin (a marker of thrombin generation) and D-dimer levels after 30 and 120 minutes of CVVH (Michaut Paterno et al, 1997) which is in keeping with results from this chapter.

The expense of losing a haemofilter circuit is relatively minor in terms of the
overall cost of intensive care but if frequent changes of the haemofilter circuits are necessary, this can result in significant cost implications. If the underlying problem is deficiency of coagulation inhibitors that act with heparin, strategies to avoid this include replacement of deficient factors (Langley et al, 1991), or the use of other anticoagulants.

Factor replacement would be prohibitively expensive and could only be justified realistically if antithrombin III and other protease inhibitors showed significant additional clinical benefits. In addition, proteases bound to cell surfaces may be relatively protected from inhibition (Marciniak, 1973) by antithrombin III; platelet bound factor Xa is inactivated by antithrombin III at a much lower rate than the free enzyme (Miletich et al, 1978). Whether newer anticoagulants such as Hirudin or Orgaran (Chong & Magnani, 1992) would be effective remains speculative. Furthermore, in the light of our findings, heparin-bonded circuits would only appear to offer significant advantages if low antithrombin III levels were first supplemented.

In conclusion, the observations made in this chapter suggest that thrombin generation is occurring during haemofiltration in patients who clot their haemofilters within a short time and this may be related to low pre-filtration levels of the naturally occurring anticoagulants ATIII and HCII. However, this does not appear to be related to activation of the contact system. This led to a revised hypothesis that the stimulus for thrombin generation seen in these patients may not be related to contact activation as expected, but activation of the tissue factor pathway of coagulation. This could occur due to release of tissue factor from damaged endothelium, the expression of tissue factor by activated monocytes or activation occurring on cell or lipid surfaces. This hypothesis was tested by undertaking an additional study which is described in Chapter 4.
4.1 Introduction

In the previous chapter it was demonstrated that haemofilter circuits may clot prematurely despite adequate exogenous anticoagulation and often concurrent thrombocytopaenia. This is particularly prevalent in acutely ill patients in whom as many as six filters have clotted within 24 hours of use. It has been shown that unfractionated heparin is not found in the ultrafiltrate and local plasma levels fall within the desired therapeutic range (Singer et al, 1994), which would imply that significant amounts of heparin are not being cleared across the membrane of the haemofilter, presumably due to its high negative charge.

In other extracorporeal circuits such as cardiopulmonary bypass, the binding of plasma factor XII to the extracorporeal circuit has been implicated in the activation of the contact system of coagulation (Salzman et al, 1994; Edmunds, 1993). It was therefore postulated that activation of the contact system could occur on exposure of blood to the foreign surface of the haemofilter circuit resulting in further activation of the coagulation system and subsequent thrombin generation.

Contrary to expectations, there was no evidence for activation of the contact system over the lifespan of the haemofilter. Plasma levels of the contact system precursors (factor XII or prekallikrein) as well as their inhibitors (C1-esterase inhibitor and α2-macroglobulin) or the activated form of factor XII (factor XIIa) did not change over the filter lifespan. Furthermore, during haemofiltration, platelet and neutrophil counts were unchanged and there was no evidence of neutrophil activation (as measured by elastase: α1-antitrypsin complexes). However, premature filter clotting was related to low plasma levels of the naturally occurring anticoagulants antithrombin III (ATIII) and...
heparin cofactor II (HCII) at the onset of haemofiltration, and with increasing thrombin generation.

These data suggest that although thrombin is being generated in filters which clot prematurely, the stimulus for this is not initiated through activation of the contact system. In addition, these patients may have a reduced thrombin inhibitory capacity prior to haemofiltration. It is possible that the stimulus for this thrombin generation comes from the tissue factor pathway of coagulation, initiated by the exposure of tissue factor following vascular injury or cell damage or by tissue factor expression on activated monocytes. This could lead to thrombin generation particularly if patients have insufficient levels of endogenous anticoagulants to control coagulation activation.

The aim of this study was to investigate the effect of haemofiltration on i) activation and control of the tissue factor pathway and ii) changes in plasma levels of the naturally occurring anticoagulants ATIII, HCII and the protein C (PC) and protein S (PS) system. Proteins and inhibitors of the tissue factor pathway - namely plasma tissue factor, factor VII (FVII), activated FVII (FVIIa), total TFPI (by both immunological and functional assay), truncated TFPI and TFPI:FXa complexes were studied. Thrombin and fibrin formation was assessed by measuring thrombin-antithrombin (TAT) complexes, prothrombin fragment F1+2 (Pro F1+2) and D-dimers. As a possible mechanism of tissue factor exposure, endothelial damage was assessed using plasma forms of tissue factor, E-selectin, thrombomodulin and endothelin-1. Exogenous anticoagulation was assessed by an anti-Xa amidolytic assay for heparin. Baseline levels and changes during haemofiltration in these variables were related to i) the patients circulatory and septic status and ii) filter lifespan and iii) thrombin generation.

When this study was initiated I also wanted to examine monocyte expressed tissue factor during haemofiltration for which I set up a whole blood flow cytometry method of analysis. Unfortunately this technique requires fresh whole blood for analysis and as haemofiltration is not an elective procedure I found obtaining the samples from Intensive Care was difficult. I therefore concentrated my investigations on plasma proteins.
4.2 Methods

4.2.1 Patients

Patients in acute renal failure requiring continuous haemofiltration on the intensive care unit of University College London Hospitals were studied. Patients were excluded if they were receiving aprotinin, required haemodiafiltration rather than haemofiltration, or if discontinuation of haemofiltration was necessary for any reason other than clotting of the circuit, e.g. transfer for CT scan. Informed patient consent (or relative’s assent) was obtained beforehand. Clinical and routine haematological and biochemical laboratory investigations were recorded, including underlying condition, circulatory status and vasoactive drug requirement. Twelve patients previously not haemofiltered were studied: 6 clotted their filters before 24 hours and 6 clotted their filters after 24 hours of use.

4.2.2 Haemofiltration

Either a BSM 22 or Prisma pumped venovenous haemofiltration system (Hospal Ltd) using polyacrylonitrile AN69 hollow-fibre haemofilters (Multiflow 100, Hospal Ltd) were primed with heparin as recommended by the manufacturer. Blood flow through the circuit was set at 150-200 ml/min and filtrate volumes removed at a rate of 1000 ml/hour. Replacement fluid used was either Hemofiltrasol 22 (Gambro Ltd) or Hemosol LG2 (Hospal Ltd), the amount given depending on the patient’s clinical status and desired hourly fluid balance. Unfractionated heparin (Monoparin, CP Pharmaceuticals Ltd.) was infused directly into the circuit pre-filter to maintain bedside activated coagulation times of 150-200 seconds.

4.2.3 Sample collection

Blood samples were taken pre-filtration, and at these times following commencement of CVVH: 15mins, 1 hour, 3-4 hours, 8-12 hours, 24 hours, 48 hours and every 24 hours thereafter until the filter blocked. The sample was taken from the ‘arterial’ limb of the haemofilter circuit i.e. before blood enters the haemofilter. Whole blood was anticoagulated with either citrate or EDTA (see section 2.1) or allowed to clot for serum.
Citrated plasma and serum were separated (section 2.1), removed and aliquoted into plastic tubes, then frozen at -70°C. The EDTA sample was used for a full blood count estimation (See section 2.2).

4.2.4 Measurement of haemostatic factors

FVIIa was measured by clotting assay using recombinant human soluble tissue factor (see section 2.5). FVII, TFPI, HCII, ATIII and heparin were assayed by amidolytic substrate techniques (see section 2.3). PC could not be assayed by amidolytic methods due to the interference of other proteases in the plasma of the subjects studied. Tissue factor, total TFPI, TAT complexes, prothrombin fragment 1+2 (Pro F1+2), PC, PS (both free and total), soluble thrombomodulin (sTM), soluble E-selectin and endothelin-1 were assayed by ELISA (see section 2.4). Prothrombin (FII) was measured by clotting assay using Taipan venom (see section 2.5), fibrinogen by Clauss technique using a Sysmex CA-1000 analyser and D-dimers by latex agglutination (section 2.7).

4.2.5 In vitro experiments

Since the molecular weight of Pro F1+2 (34 Kd) is very close to the approximate molecular weight cut-off of the haemofilter (30 Kd), it is possible that Pro F1+2 is cleared across the membrane of the haemofilter. Although ultrafiltrate samples were not collected from patients studied in this chapter, they were from those in the previous chapter. Therefore to test this hypothesis, ultrafiltrate samples from two subjects studied in the previous chapter who showed an increase in TAT during haemofiltration were assayed for Pro F1+2 by ELISA (section 2.4).

It was also necessary to establish whether under conditions of excess thrombin generation the cleavage of Pro F1+2 by thrombin may result in a loss of recognition by the antibody used in the Pro F1+2 ELISA. To ascertain this, 450µl of purified Pro F1+2 (standards from the ELISA kit used) was incubated for 10 minutes at 37°C with either 50µl buffer or an excess (50µl of 1000U/ml) of bovine thrombin (Diagnostic Reagents Ltd) and then assayed using the Pro F1+2 ELISA.
4.2.6 Statistical analysis

Repeated measures analysis of variance could not be used to assess whether proteins or inhibitor levels changed over the period of haemofiltration since the filter lifespan was not equal for all patients. Therefore changes in variables over the period of filtration were assessed using the Wilcoxon Rank test between prefiltration levels and those prior to clotting. Comparisons between groups were made using the Wilcoxon rank test. Correlations of variables with filter lifespan or thrombin generation were assessed using Spearman’s rank correlation coefficient. \( p<0.05 \) was considered statistically significant.

4.3 Results

As the distribution of the variables studied was non-Gaussian, all results are represented by the median with first and third quartiles. The characteristics of the 12 patients studied are shown in Table 4.1. None of the patients had been previously filtered during their ICU admission. All patients received unfractionated heparin as an anticoagulant at a rate of 500-1000IU/hour. The lifespan of the filter was not related to prefiltration levels of any of the haemostatic parameters studied nor to plasma heparin levels. Monocyte, neutrophil and platelet counts as well as the haematocrit did not significantly change during haemofiltration. Unless otherwise stated, baseline levels of the variables studied were not significantly different between patients with a filter lifespan of <24 hours and >24 hours.

8/12 patients were septic and 8/12 had acute respiratory distress syndrome (ARDS). 8/12 patients were thrombocytopaenic prior to haemofiltration (Table 4.2). Only two patients showed a high INR (subjects 8&9, Table 4.2). Subject 9 had severely reduced levels of fibrinogen, clotting factors and inhibitors, thrombocytopenia and increased levels of D-dimers and markers of thrombin generation (table 4.2). These results suggest that patient 9 had acute disseminated intravascular coagulation (DIC). Patient 8 also had reduced levels of clotting factors and inhibitors, thrombocytopenia and increased levels of D-dimers and markers of thrombin generation (table 4.2). However, this patient had a normal level of fibrinogen, making a diagnosis of DIC.
unlikely. This patient had abnormal liver function tests [Bilirubin 172μM (NR = 3-17μM), aspartate transaminase 250 IU/l (NR = 11-55 IU/l)]. It is likely that liver dysfunction in this patient resulted in impaired synthesis of coagulation proteins.

<table>
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<th>Sex</th>
<th>Precipitating condition</th>
<th>ARDS</th>
<th>Vasopressor/Inotropes</th>
<th>Sepsis</th>
<th>Filter life (hrs)</th>
<th>Outcome</th>
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<td>+</td>
<td>38</td>
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<tr>
<td>2</td>
<td>36</td>
<td>F</td>
<td>Rhabdomyolysis 2° to epilepsy</td>
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<td>-</td>
<td>-</td>
<td>6</td>
<td>survived</td>
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<tr>
<td>3</td>
<td>54</td>
<td>M</td>
<td>Post CABG, sternal wound infection, pancreatitis</td>
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<td>+</td>
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<td>M</td>
<td>Sigmoid volvulus</td>
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<td>M</td>
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<td>+</td>
<td>30</td>
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<td>75</td>
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<td>61</td>
<td>F</td>
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<td>+</td>
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<td>+</td>
<td>48</td>
<td>survived</td>
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Abbreviations: CABG coronary artery bypass graft; ARDS acute respiratory distress syndrome

**Table 4.1 Patient characteristics**

**Thrombin generation**

TAT complexes were elevated (14.4, 11.7-20.9 μg/l) in all patients prior to haemofiltration (Table 4.2). After 15 minutes of filtration, TAT complexes tended to be lower (13.34, 11.2-21.9 μg/l), although this decrease did not achieve statistical significance (Table 4.3).
Table 4.2 Prefiltration levels of coagulation variables

<table>
<thead>
<tr>
<th>Subject</th>
<th>TAT</th>
<th>ProF1+2</th>
<th>D-dimer</th>
<th>FVII</th>
<th>F9</th>
<th>INR</th>
<th>FII</th>
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<td>500-1000</td>
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<td>500-1000</td>
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</tr>
<tr>
<td>6</td>
<td>12.75</td>
<td>1.94</td>
<td>1000-2000</td>
<td>0.70</td>
<td>3.98</td>
<td>1.30</td>
<td>0.54</td>
<td>48</td>
</tr>
<tr>
<td>7*</td>
<td>10.71</td>
<td>2.97</td>
<td>&gt;2000</td>
<td>1.09</td>
<td>3.98</td>
<td>0.96</td>
<td>0.71</td>
<td>321</td>
</tr>
<tr>
<td>8*</td>
<td>44.42</td>
<td>3.65</td>
<td>&gt;2000</td>
<td>0.10</td>
<td>2.41</td>
<td>2.12</td>
<td>0.43</td>
<td>21</td>
</tr>
<tr>
<td>9*</td>
<td>108.44</td>
<td>2.09</td>
<td>&gt;2000</td>
<td>0.11</td>
<td>0.44</td>
<td>3.02</td>
<td>0.14</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>18.95</td>
<td>3.75</td>
<td>250-500</td>
<td>0.48</td>
<td>1.14</td>
<td>1.13</td>
<td>0.43</td>
<td>174</td>
</tr>
<tr>
<td>11</td>
<td>15.78</td>
<td>1.26</td>
<td>&gt;2000</td>
<td>0.71</td>
<td>3.77</td>
<td>1.13</td>
<td>0.80</td>
<td>67</td>
</tr>
<tr>
<td>12</td>
<td>22.84</td>
<td>2.56</td>
<td>&gt;2000</td>
<td>0.81</td>
<td>3.14</td>
<td>1.31</td>
<td>0.54</td>
<td>73</td>
</tr>
</tbody>
</table>

NR 1.00- <250 0.45- 1.50- <1.3 0.50- 150- 0.40-
4.10 1.10 1.45 4.00 1.50 400 1.10 4.50 4000

Table 4.3 Plasma levels of coagulation variables over the lifespan (hours) of the haemofilter (median with first and third quartiles).

<table>
<thead>
<tr>
<th>n=</th>
<th>Pre</th>
<th>15 min</th>
<th>1 h</th>
<th>3-4 h</th>
<th>8-12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TAT</td>
<td>14.40</td>
<td>13.34</td>
<td>15.24</td>
<td>18.27</td>
<td>19.95</td>
<td>16.44</td>
<td>19.70</td>
<td>31.89</td>
</tr>
<tr>
<td></td>
<td>11.7-20.9</td>
<td>11.2-21.9</td>
<td>10.7-29.5</td>
<td>14.9-26.2</td>
<td>17.0-29.6</td>
<td>15.4-17.5</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>Pro</td>
<td>2.32</td>
<td>2.41</td>
<td>3.36</td>
<td>3.51</td>
<td>2.22</td>
<td>1.60</td>
<td>3.19</td>
<td>4.45</td>
</tr>
<tr>
<td>F9+2</td>
<td>1.77-3.58</td>
<td>1.62-3.86</td>
<td>1.77-5.11</td>
<td>1.45-3.51</td>
<td>1.25-4.85</td>
<td>1.18-2.23</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>FVII</td>
<td>0.69</td>
<td>0.63</td>
<td>0.56</td>
<td>0.59</td>
<td>0.63</td>
<td>0.89</td>
<td>0.77</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>0.4-0.9</td>
<td>0.4-0.8</td>
<td>0.4-0.9</td>
<td>0.4-0.9</td>
<td>0.4-0.9</td>
<td>0.4-0.9</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

In 8/12 patients there was a further increase in TAT complexes over the filter lifespan (13.93, 7.1-20.6 15 minutes v 26.20, 19.1-28.7 prior to clotting, p=0.018). In the 4 patients in which there was no further increase in TAT, the filter remained patent for longer than 24 hours. The increase in TAT complexes over the lifespan of the filter inversely correlated with filter running time (Figure 4.1).
Baseline levels of Pro Fl+2 were also elevated in all patients (2.32, 1.77-3.58 nM), but did not correlate with TAT complex levels (Table 4.2). In 4/12 patients there was an increase in Pro Fl+2 over the filter lifespan (from 2.53, 1.99-3.24 pre to 5.45, 4.60-6.21 nM prior to clotting). All 4 of these patients clotted their filters within 24 hours and also showed an increase in TAT during the period of haemofiltration. The correlation between % increase in Pro Fl+2 and filter lifespan did not achieve statistical significance. Levels of D-dimers were elevated in all patients at baseline (Table 4.2) but did not correlate with TAT complexes or Pro Fl+2 at this time point.

Pro Fl+2 could be detected in the ultrafiltrate of patients studied in chapter 3 and levels increased during haemofiltration (Table 4.4). When excess thrombin was present, the recognition of Pro Fl+2 by the ELISA was reduced (Table 4.5).
<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>15 mins</th>
<th>1 hour</th>
<th>3-4 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject 5 Ultrafiltrate</td>
<td>0.03</td>
<td>0.03</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>Subject 5 Plasma</td>
<td>2.02</td>
<td>1.99</td>
<td>2.04</td>
<td>3.16</td>
</tr>
<tr>
<td>Subject 8 Ultrafiltrate</td>
<td>0.05</td>
<td>0.41</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td>Subject 8 Plasma</td>
<td>3.28</td>
<td>3.21</td>
<td>2.93</td>
<td>4.06</td>
</tr>
</tbody>
</table>

Table 4.4 Levels of Prothrombin F1+2 (nM) in the plasma and ultrafiltrate of two subjects studied in chapter 3.

<table>
<thead>
<tr>
<th></th>
<th>Initial F1+2 (nM)</th>
<th>- thrombin</th>
<th>+ thrombin (100U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.25</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.83</td>
<td>4.64</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Levels of Prothrombin F1+2 measured by ELISA in two purified preparations of F1+2 in the presence or absence of bovine thrombin.

Naturally occurring anticoagulants

Prefiltration levels of AT-III were below the normal range in 11/12 patients, with HCl and PC levels following a similar pattern (Table 4.6). Free PS was reduced in 4/12 patients and at the lower end of the normal range in a further 5 patients with total PS following a similar pattern (Table 4.6). Free PS levels tended to be lower in patients occluding their filters early (0.51, 0.34-0.70 vs 0.76, 0.34-1.13 U/ml), although this did not achieve statistical significance.

As an indication of whether PC and PS levels were reduced because of impaired synthesis or due to specific consumption, the ratios PC:FVII and PS:FII were calculated. A reduced ratio of PC:FVII or PS:FII implies that the inhibitor levels are reduced due to specific consumption of the natural anticoagulants. In 8/11 patients with reduced PC levels at baseline, the ratio of PC:FVII was also reduced, whereas in 3/11 it was approximately normal (Table 4.6). Of these three patients one (9) had evidence of acute DIC which would result in the co-consumption of PC and FVII leading to a
normal PC: FVII ratio. Patient 8 had low levels of both FVII and PC, but had evidence of liver dysfunction which would effect the synthesis of both proteins. The ratio PS: FII only appeared to be reduced in one patient.

<table>
<thead>
<tr>
<th>Subject</th>
<th>ATIII</th>
<th>HCII</th>
<th>PC</th>
<th>PC: FVII</th>
<th>PS total</th>
<th>PS free</th>
<th>PS: FII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.83</td>
<td>0.56</td>
<td>0.98</td>
<td>0.97</td>
<td>0.98</td>
<td>1.13</td>
<td>1.76</td>
</tr>
<tr>
<td>2*</td>
<td>0.15</td>
<td>0.24</td>
<td>0.27</td>
<td>0.71</td>
<td>0.36</td>
<td>0.34</td>
<td>0.94</td>
</tr>
<tr>
<td>3*</td>
<td>0.46</td>
<td>0.46</td>
<td>0.60</td>
<td>0.87</td>
<td>0.68</td>
<td>0.72</td>
<td>0.91</td>
</tr>
<tr>
<td>4*</td>
<td>0.73</td>
<td>0.81</td>
<td>0.44</td>
<td>0.42</td>
<td>0.99</td>
<td>0.70</td>
<td>0.80</td>
</tr>
<tr>
<td>5</td>
<td>0.14</td>
<td>0.18</td>
<td>0.21</td>
<td>0.53</td>
<td>0.31</td>
<td>0.34</td>
<td>0.91</td>
</tr>
<tr>
<td>6</td>
<td>0.43</td>
<td>0.42</td>
<td>0.30</td>
<td>0.43</td>
<td>0.61</td>
<td>0.60</td>
<td>1.11</td>
</tr>
<tr>
<td>7*</td>
<td>0.45</td>
<td>0.67</td>
<td>0.53</td>
<td>0.49</td>
<td>0.71</td>
<td>0.64</td>
<td>0.91</td>
</tr>
<tr>
<td>8*</td>
<td>0.20</td>
<td>0.22</td>
<td>0.10</td>
<td>1.00</td>
<td>0.65</td>
<td>0.55</td>
<td>1.28</td>
</tr>
<tr>
<td>9*</td>
<td>0.18</td>
<td>0.16</td>
<td>0.12</td>
<td>1.09</td>
<td>0.11</td>
<td>0.11</td>
<td>0.79</td>
</tr>
<tr>
<td>10</td>
<td>0.20</td>
<td>0.23</td>
<td>0.27</td>
<td>0.56</td>
<td>0.34</td>
<td>0.25</td>
<td>0.58</td>
</tr>
<tr>
<td>11</td>
<td>0.38</td>
<td>0.46</td>
<td>0.38</td>
<td>0.54</td>
<td>1.25</td>
<td>1.21</td>
<td>1.51</td>
</tr>
<tr>
<td>12</td>
<td>0.36</td>
<td>0.27</td>
<td>0.30</td>
<td>0.36</td>
<td>1.25</td>
<td>1.03</td>
<td>1.89</td>
</tr>
</tbody>
</table>

NR 0.80- 0.65- 0.70- >0.76 0.55- 0.55- to establish

Units U/ml U/ml lU/ml - U/ml U/ml -

**Table 4.6 Prefiltration levels of naturally occurring anticoagulants**

Abbreviations: NR: normal range, others as in text. Filter life<24 hours denoted by *.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>15 min</th>
<th>1 h</th>
<th>3-4 h</th>
<th>8-12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>12</th>
<th>12</th>
<th>12</th>
<th>12</th>
<th>10</th>
<th>5</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS total</td>
<td></td>
<td>0.66</td>
<td>0.65</td>
<td>0.63</td>
<td>0.68</td>
<td>0.70</td>
<td>0.79</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.36-0.99</td>
<td>0.35-1.13</td>
<td>0.36-0.96</td>
<td>0.35-1.04</td>
<td>0.35-1.10</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>PS free</td>
<td></td>
<td>0.62</td>
<td>0.68</td>
<td>0.61</td>
<td>0.65</td>
<td>0.67</td>
<td>0.69</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.34-0.88</td>
<td>0.35-0.82</td>
<td>0.37-0.65</td>
<td>0.37-0.91</td>
<td>0.36-0.97</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td>0.30</td>
<td>0.30</td>
<td>0.31</td>
<td>0.36</td>
<td>0.30</td>
<td>0.40</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.24-0.48</td>
<td>0.25-0.45</td>
<td>0.24-0.54</td>
<td>0.22-0.54</td>
<td>(NA)</td>
<td>(NA)</td>
<td></td>
</tr>
<tr>
<td>AT-III</td>
<td></td>
<td>0.37</td>
<td>0.31</td>
<td>0.34</td>
<td>0.38</td>
<td>0.41</td>
<td>0.41</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.19-0.45</td>
<td>0.25-0.49</td>
<td>0.29-0.50</td>
<td>0.21-0.49</td>
<td>0.23-0.49</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>HCII</td>
<td></td>
<td>0.34</td>
<td>0.37</td>
<td>0.34</td>
<td>0.38</td>
<td>0.40</td>
<td>0.37</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.22-0.51</td>
<td>0.19-0.52</td>
<td>0.19-0.49</td>
<td>0.20-0.48</td>
<td>0.23-0.54</td>
<td>0.23-0.47</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

**Table 4.7 Plasma levels of naturally occurring anticoagulants over the lifespan (hours) of the haemofilter (median with interquartile range).**

NA = not applicable
The six patients who clotted their filters within 24 hours had low levels of all four inhibitors prior to commencement of haemofiltration; this was true for only two of the six patients that lasted longer than 24 hours. Plasma levels of AT-III, HCII, PC and PS did not alter significantly during the period of haemofiltration (Table 4.7).

**Markers of endothelial damage**

Prefiltration levels of soluble thrombomodulin, tissue factor and E-selectin were increased in most patients (Table 4.8) and correlated significantly with one another (Table 4.9). Baseline levels of endothelin-1 were also increased in most patients but did not correlate with any of the other markers of endothelial damage (Table 4.9). One patient had no detectable endothelin-1 or tissue factor prefiltration and also had the lowest levels of soluble thrombomodulin and E-selectin at this time point. This patient showed a marked increase in all four markers during the period of haemofiltration (Figures 4.3 and 4.4).

<table>
<thead>
<tr>
<th>Subject</th>
<th>TF</th>
<th>TM</th>
<th>E-selectin</th>
<th>ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>333</td>
<td>259</td>
<td>65</td>
<td>2.26</td>
</tr>
<tr>
<td>2*</td>
<td>208</td>
<td>71</td>
<td>93</td>
<td>3.51</td>
</tr>
<tr>
<td>3*</td>
<td>108</td>
<td>40</td>
<td>36</td>
<td>0.86</td>
</tr>
<tr>
<td>4*</td>
<td>380</td>
<td>240</td>
<td>141</td>
<td>1.41</td>
</tr>
<tr>
<td>5</td>
<td>968</td>
<td>112</td>
<td>113</td>
<td>2.11</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>192</td>
<td>88</td>
<td>1.19</td>
</tr>
<tr>
<td>7*</td>
<td>446</td>
<td>90</td>
<td>116</td>
<td>1.86</td>
</tr>
<tr>
<td>8*</td>
<td>598</td>
<td>284</td>
<td>&gt;400</td>
<td>12.60</td>
</tr>
<tr>
<td>9*</td>
<td>&lt;15</td>
<td>22</td>
<td>16</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>10</td>
<td>336</td>
<td>133</td>
<td>53</td>
<td>2.02</td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>73</td>
<td>231</td>
<td>0.89</td>
</tr>
<tr>
<td>12</td>
<td>1492</td>
<td>308</td>
<td>374</td>
<td>1.74</td>
</tr>
<tr>
<td>NR</td>
<td>64-129</td>
<td>5-55</td>
<td>29-64</td>
<td>0.30-0.90</td>
</tr>
<tr>
<td>Units</td>
<td>pg/ml</td>
<td>ng/ml</td>
<td>ng/ml</td>
<td>pg/ml</td>
</tr>
</tbody>
</table>

*Table 4.8 Prefiltration levels of markers of endothelial damage*

Abbreviations: NR: normal range, NA: not available, others as in text. Filter life<24 hrs denoted by *. 

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Figure 4.2 Change in a) tissue factor and b) endothelin-1 levels during haemofiltration (only patients showing a change are illustrated)

Time points: 1) prefiltration; 2) 15 mins; 3) 1hr; 4) 3-4hrs; 5) 8-12hrs; 6) 24hrs; 7) 48hrs; 8) 72hrs
Figure 4.3 Change in c) thrombomodulin and d) E-selectin levels during haemofiltration (only patients showing a change are illustrated)

Time points: 1) prefiltration; 2) 15 mins; 3) 1hr; 4) 3-4hrs; 5) 8-12hrs; 6) 24hrs; 7) 48hrs; 8) 72hrs
Table 4.9 Spearman's correlation coefficient between baseline levels of markers of endothelial damage (n=12, significance of association shown in brackets)

<table>
<thead>
<tr>
<th></th>
<th>TF</th>
<th>TM</th>
<th>E-selectin</th>
<th>ET-1</th>
<th>TFPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>0.77 (p=0.014)</td>
<td>0.62 (p=0.042)</td>
<td>0.49 (p=0.121)</td>
<td>0.66 (p=0.036)</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>0.77 (p=0.014)</td>
<td>0.59 (p=0.049)</td>
<td>0.48 (p=0.110)</td>
<td>0.48 (p=0.115)</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>0.62 (p=0.042)</td>
<td>0.59 (p=0.049)</td>
<td>0.36 (p=0.228)</td>
<td>0.45 (p=0.132)</td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>0.49 (p=0.121)</td>
<td>0.48 (p=0.110)</td>
<td>0.36 (p=0.228)</td>
<td>0.74 (p=0.014)</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>0.66 (p=0.036)</td>
<td>0.48 (p=0.115)</td>
<td>0.45 (p=0.132)</td>
<td>0.74 (p=0.014)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.4 Correlation between the % increase in TAT complex and tissue factor over the lifespan of the filter (n=10, one patient not studied, one had 5357% increase in TF and 227% increase in TAT complex not shown)
Only one patient (subject 3) had normal levels of all four markers prior to haemofiltration. Although when taken as a group there was no significant difference in levels of endothelial markers between pre-filtration and prior to clotting, in most patients there was an increase over the filter lifespan in one or other, but not all (with the exception of patient 9), of the markers of endothelial damage (Figures 4.2 and 4.3). The % increase in tissue factor during haemofiltration was significantly associated with thrombin generation (Figure 4.4).

**Activation and inhibition of the tissue factor pathway**

Baseline FVIIa and FVII levels were below the reference range in three and four subjects respectively, prior to commencement of haemofiltration (Table 4.10).

<table>
<thead>
<tr>
<th>Subject</th>
<th>FVII</th>
<th>FVIIa</th>
<th>TFPI am</th>
<th>TFPI ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>1.62</td>
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**Table 4.10 Prefiltration levels of tissue factor pathway proteins**

Abbreviations: NR: normal range, ag: antigenic method, am: amidolytic method, others as in text. Filter life<24 hours denoted by *.

Levels of FVIIa were not correlated with either TFPI or FVII prior to haemofiltration. After 15 minutes of filtration there was a significant decrease in FVIIa followed by an increase over the lifespan of the filter (Figure 4.5). The increase in FVIIa during haemofiltration did not correlate with either TAT complex generation, the % increase in
tissue factor nor filter lifespan. FVII levels did not change significantly during haemofiltration (Table 4.3).

![Boxplot showing FVIIa levels prior to haemofiltration, after 15 minutes of haemofiltration and prior to the haemofilter clotting (n=12).](image)

_Figure 4.5 FVIIa levels prior to haemofiltration, after 15 minutes of haemofiltration and prior to the haemofilter clotting (n=12)_

Horizontal line indicates median, box the interquartile range and bars the 95% confidence interval

Prefiltration total TFPI levels measured by both amidolytic and immunologic assay were above the reference range in 7/12 patients, 3 of whom (subjects 1, 5, 7) were receiving heparin therapy/prophylaxis prior to institution of haemofiltration (Table 4.10). However, one patient who was also receiving heparin therapy/prophylaxis prior to institution of haemofiltration had normal baseline levels of TFPI (subject 3). There was a good correlation between baseline total TFPI levels measured by chromogenic and ELISA methods ($r=0.80$, $p=0.0076$). Baseline total TFPI levels were correlated to soluble tissue factor and endothelin-1 levels (Table 4.9).

After 15 minutes of filtration TFPI levels were significantly increased (Figure 4.6) in most patients, except in two patients who had received heparin prior to haemofiltration (subjects 5 and 7).
**Figure 4.6** Total TFPI levels prior to haemofiltration, after 15 minutes of haemofiltration and prior to the haemofilter clotting (n=12)

Horizontal line indicates median, box the interquartile range and bars the 95% confidence interval. NS = not significant

**Figure 4.7** Correlation between the maximal increase in TFPI during haemofiltration and plasma heparin concentration at that time point (n=10, the two subjects without an increase in TFPI are not shown)
The % increase in TFPI was not significantly correlated with plasma heparin concentrations (Figure 4.7). TFPI levels then gradually decreased over the period of filtration such that prior to the filter clotting, plasma TFPI was not significantly different to prefiltration levels (Figure 4.6). This decrease in TFPI was significantly correlated to the % increase in FVIIa over the lifespan of the filter (Figure 4.8).

![Graph showing correlation between % increase in FVIIa and % decrease in TFPI](image)

*Figure 4.8 Correlation between the % increase in FVIIa with the % decrease in TFPI between 15 minutes of filtration and prior to the haemofilter clotting (n=12)*

### 4.4 Discussion

All of the patients studied in this chapter were critically ill and many had grossly deranged haemostasis prior to commencement of haemofiltration. None had previously been placed on haemofiltration and all were anticoagulated with unfractionated heparin. The lifespan of the haemofilter was not related to patient age, gender or to pre-filtration levels of any of the haemostatic variables studied.
**Thrombin generation**

TAT complex levels were increased in all patients prior to haemofiltration suggesting an underlying degree of coagulation activation. TAT complexes are known to be increased in patients with sepsis (Lorente *et al.*, 1993; Asakura *et al.*, 1994) and chronic renal failure (Kolb *et al.*, 1991; Yamazaki *et al.*, 1995). Initially there was a slight, though non-significant fall in TAT complexes during haemofiltration. This may reflect increased inhibition of the tissue factor pathway at this time point. There were further increases in TAT complexes over the lifespan of the filter in most patients; in those not showing a rise the filter remained patent for longer than 24 hours.

The degree of increase in TAT complex was inversely correlated with filter lifespan. This confirms the results of the previous study (chapter 3), namely that activation of coagulation with subsequent thrombin generation is occurring during haemofiltration and that this is related to filter occlusion. Interestingly, an increase in TAT complexes has been found during haemodialysis in patients with acute renal failure, but not with chronic renal failure (Kolb *et al.*, 1991). All patients in the present study were in acute renal failure.

Baseline levels of Pro F1+2 were also elevated in all patients prior to haemofiltration but did not correlate with TAT complexes. There may be several explanations for this. Firstly, the half-lives of TAT complexes and Pro F1+2 are different: 3 and 90 minutes respectively (Ofosu, 1995). Therefore, TAT complexes are more likely to represent ongoing thrombin generation. Secondly, Pro F1+2 is susceptible to proteolysis by thrombin and this may result in cleavage of Pro F1+2 into smaller fragments (Mann, 1994) which may not necessarily be detected by the ELISA (Pelzer *et al.*, 1991). This was confirmed by the addition of excess thrombin to purified Pro F1+2, which resulted in lower F1+2 levels measured by the ELISA, suggesting that thrombin cleavage of F1+2 may result in products not detected by this particular assay.

These differences between TAT complexes and Pro F1+2 may also explain why the increase in TAT complexes over the lifespan of the filter did not correlate with the increase in Pro F1+2. However, it is possible that Pro F1+2 may be cleared across the
membrane of the haemofilter since the molecular weight of Pro F1+2 is approximately 34 Kd (Aronson et al, 1977) which is close to the molecular weight cut-off of the haemofilter (30Kd). Examination of the ultrafiltrate collected from two patients who had shown an increase in plasma levels of TAT during haemofiltration in chapter 3 revealed that Pro F1+2 was cleared across the haemofilter membrane. Therefore as Pro F1+2 is cleared by the haemofilter, increasing plasma concentrations during haemofiltration will not necessarily be seen. D-dimer levels were also increased in all patients prior to haemofiltration, indicating that FXIIIa-dependent cross-linked fibrin had been formed.

**Naturally occurring anticoagulants**

A congenital deficiency of PC or PS is associated with an increased risk of thrombosis (Griffin et al, 1981; Comp et al, 1984). An acquired deficiency in PC and PS can occur in patients with liver disease, disseminated intravascular coagulation - DIC (D’Angelo et al, 1988; Heeb et al, 1989), sepsis (Hesselvik et al, 1991; Alcaraz et al, 1995), and critically ill patients (Hellgren et al, 1984; Sheth & Carvalho, 1991). In most patients in the present study, levels of PC antigen were low as was the ratio PC:FVII, prior to haemofiltration.

PC and FVII are both produced by the liver and have similar half lives of approximately 10 (Riess et al, 1985) and 4-6 hours (Mackie, 1996) respectively. The ratio PC:FVII has previously been used to aid in the diagnosis of PC deficiency in patients receiving oral anticoagulants. In warfarin-treated patients, synthesis of both proteins is reduced, resulting in a ratio of PC:FVII similar to that seen in normal subjects. In contrast, PC deficient individuals, even when treated with warfarin, have a reduced (less than 0.76) PC:FVII ratio (Jones et al, 1991).

These results may be extended to help assess whether reduced PC levels seen in the patients studied here are due to reduced synthesis or to enhanced consumption of the protein. If PC consumption is excessive compared to its synthesis, the ratio PC:FVII will be reduced. The cut-off value of 0.76 was established using normal subjects and warfarin-treated patients, and hence may not be applicable to the patient population.
under investigation here. Nonetheless, the reduced ratio seen in most patients prior to
haemofiltration provides an indication that PC levels may be reduced due to
consumption of PC. This suggests an underlying degree of activation of the PC pathway
in these patients. In the present study there was no change in PC levels during
haemofiltration. However, PC levels were determined immunologically, as, unfortunately,
the amidolytic assay for PC was influenced by other proteases in the plasma of the
subjects studied.

Patients with chronic renal failure undergoing maintenance haemodialysis have
plasma PC levels which are not significantly different to control subjects when measured
by immunological or amidolytic methods, but reduced levels when measured using a
functional (APTT) based assay (Sorensen et al, 1985; Knudsen et al, 1989; Lai et al,
1991; Faioni et al, 1991). Functional PC activity increases over the period of
haemodialysis (Knudsen et al, 1988; Lai et al, 1991), suggesting that the process of
haemodialysis removes an inhibitor of PC. This inhibitor has been partially
characterised (Faioni et al, 1991). One report also suggests that the haemodialysis
membrane may also functionally impair the function of PC (Knudsen et al, 1989).

PS exists in plasma as two forms: free and bound to C4b binding protein. However, it is only the free form of PS which has cofactor activity for the activated PC
degradation of factors Va and VIIIa. In this study, both total and free PS levels were
measured. Levels of free and total PS were at the lower end of the normal range or
below in most patients before haemofiltration. Although not established, the ratio PS:FII
may provide an estimation of whether plasma PS levels are reduced due to enhanced
consumption or to reduced synthesis in a similar manner to the ratio PC:FVII since they
have reasonably similar half-lives of 42 and 72 hours respectively (D'Angelo et al, 1988;
Mackie, 1996). It would appear that the low levels of PS seen in these patients at
baseline may be due to reduced synthesis of the protein. No further changes in PS levels
were observed during the course of haemofiltration.

Similar observations have been made in patients with chronic renal failure
receiving haemodialysis, where free protein S levels may be reduced prior to
haemodialysis (Lai et al, 1990, Lai et al, 1991; Mijares et al, 1996) with no further changes during the period of haemodialysis. Total and free PS concentrations may also be reduced in DIC (Heeb et al, 1989) and critical illness (Sheth & Carvalho, 1991).

ATIII and HCII levels were reduced in most patients prior to haemofiltration following a similar pattern to PC. The filter lifespan was not related to baseline levels of PC, PS, ATIII or HCII. This is in contrast to the results observed in Chapter 3 where levels of ATIII and HCII prior to haemofiltration were significantly lower in patients who occluded their filters within 24 hours of use. However, there are a number of differences between these studies. All patients in the latter study had not been previously on haemofiltration and were anticoagulated with unfractionated heparin, whereas in the earlier study, half the patients had been previously haemofiltered. Furthermore, the anticoagulant used varied between studies.

**Markers of endothelial damage**

Most patients studied had increased levels of soluble thrombomodulin prior to haemofiltration. Plasma levels of thrombomodulin are known to be increased in patients with DIC (Asakura et al, 1991; Amano et al, 1992), ARDS (Takano et al, 1990), and sepsis (Boldt et al, 1995b). In this study, during haemofiltration thrombomodulin levels increased in some, but not all patients. Patients receiving regular haemodialysis also have increased concentrations of plasma thrombomodulin with further increases during haemodialysis (Yamazaki et al, 1992; Takagi et al, 1994). There is one report that plasma thrombomodulin levels are increased in critically ill patients with ARF prior to haemofiltration and that after 4 days of CVVH levels are increased further (Boldt et al, 1995b).

A soluble form (lacking an intact cytoplasmic domain) of E-selectin is found in the serum of normal subjects. Increased levels are seen in patients with septic shock (Newman et al, 1993) and DIC (Okajima et al, 1997). E-selectin expression is only observed on endothelial cells and not on other cell types. *In vitro*, cultured endothelial cells may be induced to release soluble E-selectin in response to a variety of stimuli.
including IL-1 (Newman et al, 1993), TNF-α (Pigott et al, 1992) and endotoxin (Kneidinger et al, 1996), although the precise mechanisms by which this occurs are unknown. It would thus appear that E-selectin may be considered a specific marker of endothelial activation and injury. In this study, baseline levels of E-selectin were increased in most patients, but only a few showed a further increase over the lifespan of the filter. It is not clear if soluble E-selectin is influenced by renal function.

The function of plasma E-selectin remains uncertain. Some have speculated that since soluble E-selectin can bind to (Newman et al, 1993) and activate (Lo et al, 1991) neutrophils, it may play a role in either reducing neutrophil binding to the endothelium by blocking E-selectin binding sites, or enhancing neutrophil trapping at sites of inflammation (Smith et al, 1997). Higher levels of E-selectin found in patients with SIRS and sepsis are associated with MOF and a poor outcome (Cowley et al, 1994; Boldt et al, 1996).

ET-1 levels were also increased in most patients prior to haemofiltration, with further increases in some patients. ET-1 levels are known to be increased in uraemic patients and are further enhanced in those who receive maintenance haemodialysis (Koyama et al, 1989; Warrens et al, 1990; Deray et al, 1992). This would suggest that plasma ET-1 is influenced by renal function although the latter studies disagree as to whether ET-1 is correlated with serum creatinine or whether levels change during the period of dialysis. Levels of ET-1 are also increased in sepsis (Pittet et al, 1991; Weitzberg et al, 1991).

Plasma tissue factor levels were increased in most patients prior to haemofiltration commencement. Others have reported that plasma tissue factor levels are increased in DIC (Takahashi et al, 1994; Koyama et al, 1994, Francis et al, 1995, Shimura et al, 1997), malignancy (Francis et al, 1995) and vasculitis (Koyama et al, 1994). The latter authors also observed that plasma tissue factor is increased in chronic renal failure only if patients have been dialysed and, unlike plasma thrombomodulin, is not correlated with serum creatinine. This would suggest that plasma tissue factor, unlike thrombomodulin, is not influenced by renal function. However, others have noted
increased plasma levels of tissue factor in uraemic patients not receiving haemodialysis (Kario et al, 1992).

Currently, the clinical relevance of plasma tissue factor is not clearly understood. Since tissue factor is normally membrane-bound, and requires phospholipid for its activity, it is unclear what the source of plasma tissue factor is and whether this can support activation of coagulation. Tissue factor is known to be contained in plasma membrane-derived microvesicles shed by tumour cells and endotoxin-stimulated monocytes (Carr et al, 1985; Bona et al, 1987). There are two forms of tissue factor found in plasma: a water soluble form (lacking the transmembrane and cytoplasmic domains of the intact tissue factor protein) and an insoluble form, which is sedimentable by ultracentrifugation (Koyama et al, 1994). This suggests that at least part of the tissue factor in plasma could be derived from cellular sources.

It is possible that endothelial cell injury may result in an increase in plasma tissue factor (Koyama et al, 1997). Indeed, the latter authors noted a good correlation between plasma tissue factor and thrombomodulin in patients with vasculitis. Another attractive hypothesis is that plasma tissue factor may be derived from activated monocytes. However, sepsis, a syndrome known to increase monocyte tissue factor expression, is not associated with significantly increased plasma tissue factor (Koyama et al, 1994, Francis et al, 1995).

Some patients in this study showed a marked increase in plasma tissue factor during the period of haemofiltration. This increase was correlated with an increase in TAT complexes. This implies that either thrombin generation may be responsible for the endothelial dysfunction seen in these patients or, alternatively, plasma tissue factor may enhance the generation of thrombin. It is presently not understood whether plasma tissue factor has any potential for clotting activation. There is one report that plasma tissue factor can promote FVII-dependent procoagulant activity in vitro (Fukuda et al, 1989). Whether plasma tissue factor could be mediating the generation of thrombin remains speculative, but in this study it is unlikely given that the increase in tissue factor during haemofiltration was not related to the degree of FVII activation. Others have
reported that TAT complex and soluble thrombomodulin levels are correlated in haemodialysis patients (Kario et al, 1992).

Pre-filtration levels of all four markers were elevated in most patients suggesting a degree of endothelial dysfunction was already present. There was a good correlation between thrombomodulin, E-selectin and tissue factor at this time point, but not with ET-1. However, as ET-1 is synthesised by the renal collecting duct and glomerular epithelial cells (Kohan, 1991; Kasinath et al, 1992) it may thus be affected by renal dysfunction. In addition, as ET-1 is a small peptide (21aa) it is possible that it is cleared across the membrane of the haemofilter and removed in the filtrate. Since further increases in the markers of endothelial dysfunction during haemofiltration occurred in some, but not all patients, and in some but not all markers, it would appear that the haemofilter circuit itself is not solely responsible for increased endothelial damage in these patients, but more likely that this reflects underlying disease processes in certain patients which may or may not be augmented by haemofiltration.

It is surprising that for any one patient, an increase during haemofiltration in one endothelial dysfunction marker was not necessarily paralleled by an increase in the others. Presumably this reflects the differing clinical status of each patient, the influence of renal dysfunction and that the release of these soluble forms of thrombomodulin, tissue factor, endothelin and E-selectin may be mediated by different stimuli or differing sensitivities to the same stimuli. Increases in these markers occurring during haemofiltration were not related to the lifespan of the filter.

**Tissue factor pathway**

If increased baseline levels of TAT complexes seen in the subjects studied here were due to increased activation of the tissue factor pathway, one might expect to observe increased levels of FVIIa prior to haemofiltration. However, this was not the case. Indeed, some patients had low levels of FVIIa at baseline. In other clinical situations where there may be enhanced activation of the tissue factor pathway such as DIC and sepsis, FVIIa levels are not increased. In DIC, FVIIa levels were not elevated and...
negatively correlated with plasma tissue factor (Yamada et al, 1996). At the onset of sepsis, FVIIa levels are reduced and are significantly lower in patients that develop septic shock as opposed to severe sepsis (Mesters et al, 1996). The latter authors suggested that the low levels of FVIIa observed may be due to increased turnover, binding of FVIIa to cell associated tissue factor, impaired synthesis, or increased proteolytic degradation. The importance of the tissue factor pathway in sepsis may be illustrated however, by studies in animal models showing that coagulation activation induced by endotoxin may be attenuated by preadministration of antibodies to tissue factor or FVII/FVIIa (Taylor et al, 1991; Biemond et al, 1995).

After 15 minutes of haemofiltration plasma FVIIa levels were significantly decreased compared to baseline. This coincided with an increase in plasma TFPI at this time-point. However, after this initial decrease, FVIIa levels then increased over the lifespan of the haemofilter until it occluded. This would suggest that FVII is being activated during this period. The increase in FVIIa coincided with the generation of TAT complexes, although no significant correlation existed between the % increase in FVIIa and the % increase in TAT during haemofiltration. This would indicate that activation of FVII during this period does not sole mediate thrombin generation. However, FVII may also be activated on the surface of cells and this would not necessarily be detected in plasma samples as it would be retained at that site.

Likewise, there was no correlation between the % increase in FVIIa and the % increase in endothelial dysfunction markers during haemofiltration. This suggests that the activation of FVII is not mediated by endothelial dysfunction in these patients. The % increase in FVIIa correlated to the % decrease in total TFPI from 15 minutes of filtration to prior to the filter clotting. This may indicate that FVII activation can occur during haemofiltration in the face of falling TFPI levels.

The ratio of FVII:C : FVII antigen (a measure of FVIIa) is increased in patients with chronic renal failure receiving haemodialysis (Kario et al, 1992). In a further study, FVIIa measured using a direct technique was increased in uraemic patients and further enhanced in those who received maintenance haemodialysis (Kario et al, 1995). In the
latter study, in non-dialysed patients FVIIa was correlated to blood urea nitrogen, suggesting that it is dependent upon renal function, and thrombomodulin (even independently of renal function), but this was not true for the dialysed group. In both groups, plasma tissue factor did not correlate with thrombomodulin nor FVIIa. This would suggest that the increase in FVIIa seen in non-dialysed patients may be mediated by endothelial damage, but the further increase in FVIIa seen in the dialysed group is not related to endothelial cell injury. It would also appear that tissue factor and thrombomodulin are released by different mechanisms under these conditions.

Total TFPI levels were increased prior to haemofiltration in most patients. TFPI levels are known to be enhanced in sepsis (Brandtzaeg et al, 1989), DIC (Shimura et al, 1996) and ARDS (Sabharwal et al, 1995). This may reflect mobilisation of TFPI from the endothelium. However, a few of the patients were receiving heparin therapy prior to haemofiltration. Heparin induces a rapid and substantial release of TFPI from the endothelium (Sandset et al, 1988). Therefore, in some patients, increased levels of TFPI at baseline may be due to the influence of heparin. However, baseline TFPI levels correlated with soluble tissue factor and endothelin-1, suggesting that the observed increased levels of TFPI may be due to endothelial injury.

After 15 minutes of haemofiltration there was an increase in TFPI levels in most patients, presumably due to the release of TFPI by heparin. Of the four patients who received heparin therapy prior to haemofiltration, there were further increases in TFPI during haemofiltration in two, but not in two others. From 15 minutes of filtration to the filter occluding TFPI levels decreased. At this point they were not significantly different from baseline levels.

Repeated or continuous infusion of heparin is known to deplete intravascular pools of TFPI (Hansen et al, 1996), so that for a given dose of heparin the % increase in TFPI becomes less with repeated use. In septic patients it is possible that if the amount of TFPI bound to the endothelium is already reduced, a continuous infusion of heparin will quickly deplete this intravascular pool of TFPI. Without increased TFPI levels, if the stimulus for tissue factor-induced coagulation is enhanced, activation of FVII could
occur. Although the maximal % increase in TFPI generally mirrored plasma heparin concentrations, this correlation was not significant. However, TFPI released during haemofiltration may not be due entirely to heparin, as some TFPI may be released as a result of endothelial injury. Indeed, studies in haemodialysis patients suggest this may be true.

Plasma TFPI activity is increased in uraemic patients and further enhanced in uraemic patients who receive maintenance haemodialysis (Kario et al., 1994). In the latter study there were further increases in TFPI during the period of haemodialysis which correlated with heparin concentrations. However, increases in TFPI have been observed during haemodialysis when the patients are not anticoagulated (Cella et al., 1996); this indicates that some TFPI may be released due to endothelial or platelet modulation.

The role of TFPI in controlling coagulation in vivo is illustrated by animal models in which immunodepletion of TFPI in rabbits produces DIC induced by low doses of tissue factor or endotoxin which are without effect in normal animals (Sandset et al., 1991a&b). Normal levels of TFPI may thus be able to adequately control TF/FVIIa under basal conditions, but when the stimulus is overwhelming (e.g. severe trauma or sepsis) it is insufficient. Conversely, infusion of TFPI ameliorates DIC induced by endotoxin in rabbits (Bregengard et al., 1993) and decreases mortality in a baboon model of E.coli septic shock (Carr et al., 1995).

Although total plasma TFPI may be enhanced in sepsis, the free portion or that bound to the endothelium may be reduced, hence reducing its anticoagulant potential. In addition, TFPI released due to endothelial damage may be C-terminally truncated (which has less anticoagulant potential than the full, free form of TFPI). Thus, in the face of elevated TFPI levels in sepsis, administration of TFPI may still have potential benefits. No human studies have been completed, but Phase II clinical trials of rTFPI in sepsis are currently underway (Bajaj & Bajaj, 1997).

In summary, prior to haemofiltration, most of the patients in this study had (i) reduced levels of the naturally occurring anticoagulants PC, PS, ATIII and HCII, (ii)
increased levels of markers of endothelial cell injury, (iii) evidence of thrombin generation and (iv) increased levels of TFPI.

There were no further changes in PC, PS, ATIII or HCII during the period of haemofiltration. In most patients there was further generation of thrombin during haemofiltration which inversely correlated with the filter lifespan. During haemofiltration there was an increase in markers of endothelial injury but this was not related to filter lifespan. Initially, there was a fall in FVIIa levels, presumably due to the heparin-induced release of TFPI at this time point. As TFPI levels decreased over the lifespan of the filter, activation of FVII occurred but this was not related to endothelial dysfunction. The generation of thrombin was not related to baseline levels of any of the parameters studied. Although thrombin generation was co-incidental to activation of FVII during haemofiltration, there was no significant correlation between them.

The mechanism by which FVII becomes activated during haemofiltration is not clear from these results. It does not appear to be related to endothelial cell activation. It is possible that in septic patients increased expression of monocyte tissue factor could promote FVII activation. Alternatively, FVII may be activated by mechanisms independent of tissue factor. It has been shown that FVIIa can bind to the surface of platelets and initiate thrombin generation independently of tissue factor, even in the presence of ATIII and TFPI (Monroe et al, 1996). The mechanisms by which thrombin is generated during haemofiltration may be multifactorial. Decreased levels of naturally occurring anticoagulants may reduce the inhibitory capacity against thrombin. Increased activation and reduced inhibition of the tissue factor pathway may contribute towards thrombin generation, but it is possible that the primary stimulus may come from an alternative mechanism such as the activation of factor X by monocytes.

Monocytes are capable of activating FX by three mechanisms: i) the binding of FVa to the monocyte surface forms a receptor for FXa and assembly of the prothrombinase complex (Tracy et al, 1983) ii) the binding of FVII/FVIIa to monocyte expressed tissue factor results in cleavage of FX (Broze, 1982) and iii) the binding of FX to CD11b receptors can result in its activation (Altieri et al, 1988b). FX activation by
the intrinsic pathway protease complex assembled on monocytes is more efficient than activation via the extrinsic pathway protease complex (McGee & Li, 1991). Moreover, the activation of leucocytes may enhance coagulation activation since elastase or cathepsin G may mediate the activation of FV or FX by monocytes (Allen & Tracy, 1995; Plescia & Altieri, 1996). It is conceivable that activation of coagulation factors could occur on leucocyte surfaces during haemofiltration, and when this occurs in association with cell surfaces, may be relatively protected from inhibition by serpines.
5.1. Introduction

Traditionally, it has been considered that activation of the contact system of coagulation occurs during CPB and results in subsequent thrombin generation. However, the evidence for this is mainly indirect and is inconclusive. In the previous two chapters we have seen that thrombin generation occurs during haemofiltration, and is coincidental to activation of the tissue factor pathway rather than activation of the contact system. It was therefore postulated that during CPB, where there is gross surgical trauma with the possibility of tissue factor exposure, activation of the tissue factor pathway of coagulation may provide a potent stimulus for thrombin generation. The objective of this chapter was two-fold: firstly, to investigate systemic changes in proteins of the tissue factor pathway during CPB; secondly, to pay particular attention to coagulation activation that may be occurring locally in the static blood within the pulmonary vasculature during CPB, the rationale for this is described below.

Pulmonary injury may complicate recovery from operative procedures requiring the use of CPB. In some instances this may be due to cardiac dysfunction associated with high left ventricular end-diastolic pressures. However, a number of patients undergoing CPB develop pulmonary oedema with no evidence of left ventricular dysfunction and this is associated with an increase in mortality (Fowler et al, 1983). It has been suggested that this problem is due to the contact of blood with the extracorporeal circuit used during CPB (Westaby, 1987). It is well known that exposure of blood to the foreign surfaces of the extracorporeal circuit results in activation of complement, coagulation and fibrinolytic pathways as well as cellular

During CPB blood is taken from the right atrium and returned to the aorta with a cross clamp preventing back flow to the coronary vessels and left ventricle. Blood flow through the pulmonary vasculature during this period is effectively halted. For the duration of bypass heparinised blood remains static within the pulmonary vasculature. The surface of the endothelium is lined with molecules that determine many of its properties, such as vasoregulation, platelet aggregation, coagulation and fibrinolysis (Gordon & Pearson, 1987).

It is possible that changes in the static pulmonary blood during CPB could overwhelm these haemostatic control mechanisms and lead to endothelial damage. Intravenous heparin in humans induces a rapid increase in plasma xanthine oxidase which is located on the surface of endothelial cells and generates superoxide free-radical mediated tissue injury (Adachi et al, 1993). Heparin has also been shown to strip endothelial cells of superoxide dismutase type c, the presence of which is reported to reduce ischaemia reperfusion injury (Sjoquist et al, 1992). In addition leucocyte adherence to endothelium is enhanced under conditions of low flow and recent studies have emphasised the role of neutrophil mediated endothelial injury in the pathogenesis of post CPB pulmonary injury (Westaby et al, 1985; Johnson et al, 1994).

Thus with blood subjected to activation in the extracorporeal circuit, the pulmonary endothelium subjected to static heparinised blood, and the tissues subjected to surgical trauma, conditions would favour pulmonary endothelial dysfunction with the potential for development of post-operative acute pulmonary injury.

Attempts have been made to attenuate complement, coagulation and cellular activation occurring during CPB (Moat et al, 1992; Lu et al, 1991; Videm et al, 1991) but little attention has been paid to the degree of damage caused by the static heparinised blood in the pulmonary circulation. The aim of this study was to investigate changes in the haemostatic system occurring within the pulmonary vasculature during CPB. By taking paired samples from the pulmonary vein and from the central venous catheter
during bypass, it was possible to compare any changes within the pulmonary vein to those occurring due to the circulation of blood through the extracorporeal circuit.

5.2. Methods

5.2.1 Patient selection

10 patients undergoing first time aortic valve replacement surgery were studied. Patients receiving aprotinin were excluded from the study. Written informed consent was obtained from the patients at a pre-operative visit and ethical permission to undertake the study was obtained from the District Medical Ethics Committee. Patients' risk score for cardiac surgery was assessed according to Parsonnet et al., 1989. This scoring system takes into account numerous risk factors and categorises patients into five groups of increasing risk: good (0-4), fair (5-9), poor (10-14), high (15-19) and extremely high (≥20).

5.2.2 Cardiopulmonary bypass

All patients received an identical anaesthetic protocol and were treated by the same anaesthetic and surgical team. Patients underwent standard CPB procedure using hollow fibre oxygenators (Model D703 Compactflo system, Dideco, Mirandola, Italy). The extracorporeal circuit was primed with 1000ml of Hartmann's solution and 500ml of Gelofusine. After systemic heparinisation (300 IU/kg) the activated clotting time was maintained at greater than 450 seconds by the administration of additional heparin as required. All patients were cooled to 28°C. At the end of the procedure heparin was neutralised by the administration of an appropriate dose of protamine sulphate (3.0-4.5mg/kg).

5.2.3 Clinical outcome

A poor outcome was defined by one or more of the following criteria: death, delayed discharge from intensive care by greater than 24 hours, requirement for an intra-aortic balloon pump, requirement for resternotomy due to excessive bleeding with no obvious
surgical cause, major organ failure, delay in discharge from hospital by greater than two
days longer than anticipated.

5.2.4 Sample collection
Samples were collected from both the CVP line and the pulmonary vein at a total of seven time points during the peri-operative period as follows: a) baseline sample at anaesthesia; b) post sternotomy, pre heparin administration; c) post sternotomy, post heparin, pre bypass; d) 20 minutes on bypass; e) immediately prior to cross clamp removal; f) immediately after cross clamp removal; g) end of bypass, before protamine administration. As the pulmonary vein was not accessible at time point a), a sample was taken from an arterial line. Blood samples were collected into EDTA and citrate tubes and plasma was prepared as detailed in section 2.1.3. Full blood counts were determined using EDTA whole blood as outlined in section 2.2.

5.2.5 Measurement of haemostatic factors
Amidolytic substrate methods were used to assay FXII, prekallikrein, AT-III, HCII, heparin, C1-INH, TFPI and FVII as described in section 2.3. FVIIa was assayed according to the method of Morrissey et al, 1993 after heparin had been neutralised in all plasma samples (section 2.5). Neutrophil elastase: α₁-proteinase inhibitor complexes, TAT complexes, soluble thrombomodulin and endothelin-1 were assayed by solid phase ELISA as described in section 2.4.

Flow cytometry analysis of CD11b expression on the surface of monocytes and neutrophils was carried out as described in section 2.6. Citrated blood samples were processed immediately after collection as significant changes in CD11b expression with changes in temperature and handling have been observed. A whole blood method was used to avoid changes in CD11b with cell separation techniques. Positive control samples were prepared by stimulating citrated whole blood with 1μM f-Met-Leu-Phe (Sigma) for ten minutes at 37°C and then preparing samples as above.
5.2.6 Correction for haemodilution

All results, with the exception of flow cytometry data, were corrected to allow for haemodilution occurring during the use of extracorporeal circulation. This was achieved by measuring total protein in the plasma samples by conventional assay (Lowry et al, 1951), expressing each time point as a percentage of the baseline sample then multiplying parameters by the ratio of protein at baseline to protein at that time point.

5.2.7 Statistical analysis

The distribution of each parameter was non-gaussian with positive skew. Data was normalised by log transformation. A two-factor repeated measures analysis of variance (Statview II for Macintosh, Abacus Concepts Inc, Berkley, CA) was used to assess whether the parameters measured changed with sample type or over the study period. If a statistically significant result was obtained ($p<0.05$), Fisher’s protected least significant difference post hoc testing was used to assess changes in variables measured over the study period.

5.3 Results

Table 5.1 shows the characteristics of the ten patients undertaking the study. There were six males and four females, median age 64 years (range 35-77), median time on extracorporeal circulation 50 minutes (range 36-84). Patients received no platelets or fresh frozen plasma during the peri-operative period. Two patients died and one had a poor outcome due to a prolonged stay in hospital.

All parameters were within the normal range pre-operatively except TAT complexes which were slightly elevated. This was not unexpected given that the study population were chronically ill. No significant differences were found between samples taken from the pulmonary vein and the CVP line at any time point for all parameters studied unless otherwise indicated, therefore all results given are from the pulmonary vein. All results are expressed as median with first and third quartiles.
### Table 5.1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age</th>
<th>Sex</th>
<th>Outcome</th>
<th>Parsonnet Score</th>
<th>Bypass time (minutes)</th>
<th>Cross-clamp time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>F</td>
<td>poor</td>
<td>12</td>
<td>70</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>M</td>
<td>good</td>
<td>20</td>
<td>47</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>M</td>
<td>good</td>
<td>5</td>
<td>64</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>M</td>
<td>good</td>
<td>5</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>M</td>
<td>good</td>
<td>5</td>
<td>84</td>
<td>70</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>M</td>
<td>good</td>
<td>10</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>F</td>
<td>good</td>
<td>9</td>
<td>47</td>
<td>34</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>M</td>
<td>good</td>
<td>5</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>F</td>
<td>poor</td>
<td>12</td>
<td>53</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>F</td>
<td>poor</td>
<td>8</td>
<td>36</td>
<td>30</td>
</tr>
</tbody>
</table>

#### 5.3.1 Haemostatic factors

Table 5.2 indicates changes in plasma levels of haemostatic factors in the pulmonary vein during CPB for the study period. Plasma FXII did not differ significantly throughout the study period, but prekallikein and FVII levels decreased by 20 minutes on CPB. Alterations in levels of FVIIa and TFPI in the pulmonary vein and CVP line in nine out of ten patients are illustrated in Figure 5.1. In these nine patients the initial increase in FVIIa following sternotomy was not statistically significant but, following heparin administration plasma FVIIa levels fell (Table 5.2) which was coincidental to an increase in TFPI (Table 5.3). The increase in TFPI following heparin administration was significantly higher in the pulmonary vein than in the CVP line (1.21 (0.95-1.59) - 5.35 (3.87-6.06) CVP vs 1.09 (0.98-1.48) - 7.71 (3.93-11.18) U/ml PV, p< 0.05). In the last patient studied however, FVIIa levels increased dramatically during bypass and this was not accompanied by a marked heparin-induced increase in TFPI (Figure 5.2) despite heparin levels of greater than 3U/ml during bypass. There was also a rapid increase in TAT complexes during the bypass period in this patient (Figure 5.2).
<table>
<thead>
<tr>
<th>Time point</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXII (U/ml)</td>
<td>0.83</td>
<td>0.99</td>
<td>0.74</td>
<td>0.81</td>
<td>0.87</td>
<td>0.69</td>
<td>0.70</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td></td>
<td>(0.75-1.09)</td>
<td>(0.59-1.02)</td>
<td>(0.51-1.05)</td>
<td>(0.45-0.98)</td>
<td>(0.51-1.09)</td>
<td>(0.44-0.82)</td>
<td>(0.46-1.05)</td>
<td></td>
</tr>
<tr>
<td>PKK (U/ml)</td>
<td>0.94</td>
<td>0.90</td>
<td>0.74</td>
<td>0.69 †</td>
<td>0.68 †</td>
<td>0.67 †</td>
<td>0.65 † †</td>
<td>0.7-1.3</td>
</tr>
<tr>
<td></td>
<td>(0.75-1.03)</td>
<td>(0.77-1.00)</td>
<td>(0.66-0.81)</td>
<td>(0.53-0.93)</td>
<td>(0.56-1.06)</td>
<td>(0.6-1.09)</td>
<td>(0.43-0.88)</td>
<td></td>
</tr>
<tr>
<td>TM (ng/ml)</td>
<td>21.40</td>
<td>NT</td>
<td>20.38 †</td>
<td>24.15</td>
<td>15.80 †</td>
<td>19.11 †</td>
<td>19.65</td>
<td>5-55</td>
</tr>
<tr>
<td>ET-1 (pg/ml)</td>
<td>0.43</td>
<td>0.39</td>
<td>0.34</td>
<td>1.35 *</td>
<td>1.22 **</td>
<td>1.26 **</td>
<td>0.61</td>
<td>0.3-0.9</td>
</tr>
<tr>
<td></td>
<td>(0.29-0.53)</td>
<td>(0.29-0.49)</td>
<td>(0.22-0.44)</td>
<td>(1.15-2.02)</td>
<td>(0.86-1.65)</td>
<td>(0.99-1.68)</td>
<td>(0.46-0.73)</td>
<td></td>
</tr>
<tr>
<td>FVII (U/ml)</td>
<td>1.22</td>
<td>1.34</td>
<td>0.98 †</td>
<td>0.84 **</td>
<td>0.90 *</td>
<td>0.90 *</td>
<td>0.81 **</td>
<td>0.65-1.45</td>
</tr>
<tr>
<td></td>
<td>(0.99-1.48)</td>
<td>(1.17-0.38)</td>
<td>(0.81-1.12)</td>
<td>(0.66-1.00)</td>
<td>(0.75-1.16)</td>
<td>(0.86-1.35)</td>
<td>(0.57-1.01)</td>
<td></td>
</tr>
<tr>
<td>FVIIa (ng/ml)</td>
<td>2.98</td>
<td>3.36</td>
<td>1.62 **</td>
<td>1.03 **</td>
<td>1.24 **</td>
<td>1.45 **</td>
<td>1.32 **</td>
<td>0.8-5.9</td>
</tr>
<tr>
<td></td>
<td>(2.32-3.12)</td>
<td>(2.94-4.30)</td>
<td>(1.00-1.90)</td>
<td>(0.79-1.25)</td>
<td>(1.12-1.58)</td>
<td>(1.30-1.69)</td>
<td>(1.18-1.45)</td>
<td></td>
</tr>
<tr>
<td>TAT (µg/ml)</td>
<td>5.35</td>
<td>NT</td>
<td>10.55 †</td>
<td>30.71 **</td>
<td>39.71 **</td>
<td>51.03 **</td>
<td>NT</td>
<td>1.00-4.10</td>
</tr>
<tr>
<td></td>
<td>(3.43-6.42)</td>
<td>NT</td>
<td>(5.88-14.66)</td>
<td>(16.84-47.73)</td>
<td>(24.52-89.78)</td>
<td>(30.64-86.43)</td>
<td>NT</td>
<td></td>
</tr>
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</table>

Table 5.2. Changes in plasma levels of haemostatic factors in the pulmonary vein during cardiopulmonary bypass.

Results are expressed as median (with first and third quartiles), n =10 except for FVIIa where the last patient has been removed from the statistics. All results are corrected to allow for haemodilution. Abbreviations: NR; normal range, NT; sample not taken. Time points: a) pre-operative; b) post-sternotomy, pre-heparin dose; c) post-heparin dose, pre-bypass; d) 20 minutes on bypass e) pre-cross clamp removal; f) post-cross clamp removal; g) end of bypass. † denotes p < 0.05, † † p < 0.01, * p < 0.005, ** p < 0.001, compared to time point a).
<table>
<thead>
<tr>
<th>Time point</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U/ml)</td>
<td>(U/ml)</td>
<td>(U/ml)</td>
<td>(U/ml)</td>
<td>(U/ml)</td>
<td>(U/ml)</td>
<td>(U/ml)</td>
<td></td>
</tr>
<tr>
<td>C1-INH</td>
<td>0.77</td>
<td>0.76</td>
<td>0.73</td>
<td>0.69</td>
<td>0.75</td>
<td>0.86</td>
<td>0.68</td>
<td>0.7-1.3</td>
</tr>
<tr>
<td></td>
<td>(0.70-0.87)</td>
<td>(0.70-0.87)</td>
<td>(0.49-0.78)</td>
<td>(0.53-0.74)</td>
<td>(0.61-0.87)</td>
<td>(0.63-1.00)</td>
<td>(0.54-0.75)</td>
<td></td>
</tr>
<tr>
<td>AT-III</td>
<td>0.90</td>
<td>0.76</td>
<td>0.72</td>
<td>0.63 **</td>
<td>0.70 †</td>
<td>0.63 **</td>
<td>0.64 **</td>
<td>0.8-1.2</td>
</tr>
<tr>
<td>(IU/ml)</td>
<td>(0.79-1.00)</td>
<td>(0.66-0.91)</td>
<td>(0.62-0.89)</td>
<td>(0.39-0.74)</td>
<td>(0.56-0.91)</td>
<td>(0.47-0.70)</td>
<td>(0.42-0.81)</td>
<td></td>
</tr>
<tr>
<td>HCII</td>
<td>0.94</td>
<td>0.94</td>
<td>0.85</td>
<td>0.72 *</td>
<td>0.69 **</td>
<td>0.76 †</td>
<td>0.74 † †</td>
<td>0.65-1.45</td>
</tr>
<tr>
<td>(U/ml)</td>
<td>(0.80-1.20)</td>
<td>(0.87-1.06)</td>
<td>(0.77-1.01)</td>
<td>(0.44-0.91)</td>
<td>(0.48-0.78)</td>
<td>(0.47-1.07)</td>
<td>(0.44-0.87)</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>1.09</td>
<td>1.44</td>
<td>7.71 **</td>
<td>3.48 **</td>
<td>4.32 **</td>
<td>4.05 **</td>
<td>3.39 **</td>
<td>0.4-1.9</td>
</tr>
<tr>
<td>(U/ml)</td>
<td>(0.98-1.48)</td>
<td>(1.08-1.59)</td>
<td>(3.93-11.18)</td>
<td>(2.34-5.19)</td>
<td>(2.20-6.21)</td>
<td>(2.01-7.50)</td>
<td>(1.83-5.38)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3. Changes in plasma levels of serine protease inhibitors in the pulmonary vein during cardiopulmonary bypass.

Results are expressed as median (with first and third quartiles), \( n = 10 \). † denotes \( p < 0.05 \), † † \( p < 0.01 \), * \( p < 0.005 \), ** \( p < 0.001 \), compared to time point a). Abbreviations: NR; normal range. Time points as in table 5.2. Results are corrected to allow for haemodilution.
<table>
<thead>
<tr>
<th>Time point</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>monocyte CD11b</td>
<td>4.92</td>
<td>5.20</td>
<td>4.89</td>
<td>4.65</td>
<td>5.24</td>
<td>5.53</td>
<td>4.57</td>
<td>2.40-6.90</td>
</tr>
<tr>
<td>(MCF)</td>
<td>(4.48-6.18)</td>
<td>(4.20-5.78)</td>
<td>(3.78-5.75)</td>
<td>(3.96-5.25)</td>
<td>(4.17-5.94)</td>
<td>(3.71-6.08)</td>
<td>(3.38-5.34)</td>
<td></td>
</tr>
<tr>
<td>monocyte count</td>
<td>0.50</td>
<td>0.50</td>
<td>0.70</td>
<td>0.20 **</td>
<td>0.10 **</td>
<td>0.10 **</td>
<td>0.10 **</td>
<td>0.2-1.0</td>
</tr>
<tr>
<td>(10⁶/l)</td>
<td>(0.50-0.70)</td>
<td>(0.40-0.80)</td>
<td>(0.30-0.80)</td>
<td>(0.10-0.40)</td>
<td>(0.10-0.30)</td>
<td>(0.10-0.30)</td>
<td>(0-0.20)</td>
<td></td>
</tr>
<tr>
<td>neutrophil CD11b</td>
<td>4.09</td>
<td>4.26</td>
<td>4.47</td>
<td>5.58 **</td>
<td>7.62 **</td>
<td>8.19 **</td>
<td>7.97 **</td>
<td>1.80-5.90</td>
</tr>
<tr>
<td>(MCF)</td>
<td>(3.38-5.73)</td>
<td>(3.50-5.32)</td>
<td>(3.68-5.07)</td>
<td>(4.73-7.29)</td>
<td>(5.20-9.40)</td>
<td>(5.80-9.32)</td>
<td>(5.66-8.81)</td>
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</tr>
<tr>
<td>neutrophil count</td>
<td>4.20</td>
<td>3.35</td>
<td>4.20</td>
<td>0.90 ††</td>
<td>1.20 †</td>
<td>1.50</td>
<td>2.40</td>
<td>2.0-7.5</td>
</tr>
<tr>
<td>(10⁹/l)</td>
<td>(1.90-4.80)</td>
<td>(1.80-5.50)</td>
<td>(2.00-5.00)</td>
<td>(0.80-3.10)</td>
<td>(0.80-3.60)</td>
<td>(1.00-5.90)</td>
<td>(0.50-9.20)</td>
<td></td>
</tr>
<tr>
<td>elastase</td>
<td>85</td>
<td>115</td>
<td>128</td>
<td>142</td>
<td>275 **</td>
<td>368 **</td>
<td>498 **</td>
<td>30-172</td>
</tr>
<tr>
<td>(µg/l)</td>
<td>(45-144)</td>
<td>(59-189)</td>
<td>(65-233)</td>
<td>(63-244)</td>
<td>(143-539)</td>
<td>(230-458)</td>
<td>(384-732)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4. Changes in leucocyte characteristics in the pulmonary vein during cardiopulmonary bypass.

Results are expressed as median (with first and third quartiles), n = 10. † denotes p < 0.05, †† p < 0.01, * p < 0.005, ** p < 0.001, compared to time point a). Abbreviations: MCF; mean channel fluorescence, elastase; neutrophil elastase-α,-antitrypsin complexes, NR; normal range. Time points as in table 5.2. Cell counts and elastase results are corrected to allow for haemodilution.
Figure 5.1 Changes in plasma levels of FVIIa and TFPI in A) the pulmonary vein and B) the CVP line during CPB.

Results are shown as median with interquartile range, n=9. Horizontal bars indicate the time that ECC and heparin were present. Time points: a) pre-operative; b) post-sternotomy, pre-heparin dose; c) post-heparin dose, pre-bypass; d) 20 minutes on bypass e) pre-cross clamp removal; f) post-cross clamp removal; g) end of bypass. * denotes $p < 0.001$ compared to time point a).

Results are corrected to allow for haemodilution.
In this patient, AT-III was 0.70IU/ml pre-operatively falling to as low as 0.20IU/ml during bypass. All other variables studied on this patient showed a similar trend to the other nine patients.

Figure 5.2 Changes in plasma levels of FVIIa, TFPI and TAT in the CVP line during CPB in patient number 10.

Time points as in Figure 5.1. Horizontal bars indicate the time that ECC and heparin were present.
Figure 5.3 illustrates changes in soluble TM levels in the pulmonary vein and CVP line during the study period. Although TM levels tended to be higher in the pulmonary vein compared to CVP line mid-bypass, this difference did not achieve statistical significance. Endothelin-1 increased mid-bypass (Table 5.2) returning to normal levels by the end of the procedure. Table 5.3 shows changes in serine protease inhibitors in the pulmonary vein during CPB. There was no significant change in C1-esterase inhibitor levels for the duration of the study period (Table 5.3). Plasma AT-III and HCII levels decreased mid-bypass (Table 5.3), remaining low at the end of the procedure. TAT complexes showed a marked increase during the study period (Table 5.2).

5.3.2 Leucocyte characteristics

Changes in leucocyte characteristics in the pulmonary vein during CPB are given in Table 5.4. The monocyte count decreased mid-bypass in the pulmonary vein dropping further until the end of the procedure. The neutrophil count decreased mid-bypass in the pulmonary vein remaining low until the end of the procedure. However the neutrophil count 20 minutes on CPB was significantly lower in the pulmonary vein than that observed in the CVP line (0.90 (0.8-3.1) vs 2.10 (1.1-7.1) 10^9/l, p<0.01) and in the CVP line the neutrophil count returned to pre-operative levels by the end of the procedure. Neutrophil elastase: α1-antitrypsin inhibitor complexes increased during the study period (Table 5.4). Changes in the expression of neutrophil and monocyte expressed CD11b in the pulmonary vein are shown in Figure 5.4. There was no significant change in monocyte expressed CD11b for the study period in both the pulmonary vein and CVP line. Positive controls showed that monocyte expressed CD11b could be mobilised on stimulation (results not shown).
Figure 5.3. Changes in plasma levels of soluble thrombomodulin in A) the pulmonary vein and B) the CVP line during cardiopulmonary bypass.

Results shown as median with interquartile range, n=10. Horizontal bars indicate the time that ECC and heparin were present. Time points as in figure 5.1. † denotes p < 0.05 compared to time point a). Results are corrected to allow for haemodilution.
Figure 5.4. Changes in the expression of CD11b on the surface of A) neutrophils and B) monocytes in the pulmonary vein during cardiopulmonary bypass.

Results are expressed as mean channel fluorescence (median with interquartile range, n=10). Horizontal bars indicate the time that ECC and heparin were present. Time points as in figure 5.1. * denotes p <0.001 compared to time point a).
5.4 Discussion

Although the incidence of operative mortality during cardiopulmonary bypass has improved, postoperative organ dysfunction remains a persistent problem (Westaby, 1987) with some patients developing pathology indistinguishable from ARDS - the adult respiratory distress syndrome (Ratliff et al, 1973).

Despite the administration of high doses of anticoagulants during bypass, the use of heparin bonded circuits and improved biocompatibility of the circuit, generalised activation of the coagulation system still occurs. Thrombin is generated during CPB as demonstrated by a marked increase in TAT complexes and prothrombin fragment 1+2 (Boisclair et al, 1993). Traditionally activation of the contact system was considered to be the primary stimulus for this thrombin generation.

Activation of the contact system can be assessed by investigating the relationship between contact factor proteins, their inhibitors and cofactors. Conversion of FXII and PKK to their active forms may be assessed by depletion of functional FXII and PKK zymogen levels in plasma. The complexes formed between C1-INH and both KK and FXIIa can also be measured and increase during activation of the contact system. Levels of the inhibitors \( \alpha_2 \)-macroglobulin and C1-INH may also become depleted, but C1-INH levels may remain constant or even increase since it is a weak acute phase reactant and also interacts with the complement system (Colman, 1993).

In the pulmonary vein during CPB there was consumption of prekallikrein but little change in FXII or C1-INH, suggesting the contact system was not activated in the pulmonary vein during CPB, whereas these factors tended to fall (although not significantly) in blood that passed through the CPB circuit which may represent contact activation due to the CPB circuitry. Others have noted a reduction in plasma levels of PKK and FXII during simulated (Heller & Wendel, 1993) and clinical CPB (Heller et al, 1987; Mythen et al, 1993; Saatvedt et al, 1995) indicating conversion of these zymogens to their active forms (FXIIa and KK). Consistent with these findings is an increase in kallikrein activity measured by an amidolytic substrate method (which probably reflects KK: \( \alpha_2 \)-macroglobulin complexes) during simulated (Gallimore et al,

One drawback to the amidolytic assays for FXIIa and KK is that despite the inclusion of inhibitors in the assays to enhance specificity, the possibility remains that the substrates could be cleaved by enzymes other than FXIIa or KK. An assay has recently been developed to measure FXIIa immunologically, which relies on a monoclonal antibody specific for FXIIa, with no binding to native FXII (Ford et al, 1996) or FXIIa:inhibitor complexes (Esnouf et al, 1994). Using this assay during clinical CPB there was an increase in FXIIa antigen following sternotomy but no further increase following commencement of and during CPB (Boisclair et al, 1993b). However, since αFXIIa would be expected to remain surface bound on the CPB oxygenator circuit, and βFXIIa is rapidly complexed to its inhibitors, both of these forms would be unavailable to the plasma assay system and thus FXIIa generation cannot be excluded on the basis of these experiments. However, others have observed an increase in FXIIa antigen using this method in the latter stages of CPB as well as following sternotomy (Grossman et al, 1996).

Further indirect evidence for the generation of FXIIa and KK during CPB comes from the investigation of the formation of KK:C1-INH and FXIIa:C1-INH complexes. In an ex vivo CPB model KK:C1-INH (Wachtfogel et al, 1989; Wachtfogel et al, 1994; Sundaram et al, 1996) and FXIIa:C1-INH (Sundaram et al, 1996) complex levels were increased. In one study using clinical CPB, significant elevations in plasma KK:C1-INH complexes were only seen after and not during CPB (Wachtfogel et al, 1989). During the use of neonatal extracorporeal membrane oxygenation (a form of CPB) FXIIa:C1-INH complexes were increased significantly (Plotz et al, 1993). The generation of KK:C1-INH and FXIIa:C1-INH complexes during simulated extracorporeal circulation can be abolished by the administration of nafamostat mesilate, a serine protease inhibitor (Sundaram et al, 1996) or α1-antitrypsin Pittsburgh, a variant with enhanced inhibitory capacity for thrombin and KK (Wachtfogel et al, 1994).
In addition to the CPB circuit providing a surface capable of activating the contact system, the interaction of heparin with contact factors may also be important during CPB. *In vitro*, heparin can reduce βFXIIa inhibition and can have variable effects on αFXIIa inhibition depending upon the type used (Gallimore *et al.*, 1992b). In a model of CPB, the degree of contact activation observed is relative to the type of heparin used as an anticoagulant (Heller & Wendel, 1993). Although heparin does not activate FXII directly, under conditions such as those observed during CPB, it may interact to potentiate contact activation. As this effect is dependent upon the heparin preparation used, this has important clinical implications.

The evidence for contact activation during CPB is mainly indirect and has not been correlated to thrombin generation or to clinical outcome. Recent studies using activation peptide assays have suggested that activation of the contact factor pathway may not be the primary stimulus for thrombin generation during CPB. Levels of the peptide cleaved from FIX upon activation of this factor (FIXAP) are only significantly increased at the end of the CPB procedure, yet thrombin generation occurs during CPB (Boisclair *et al.*, 1993b). This would suggest that thrombin generation is not preceded by FIX activation, and hence intrinsic coagulation pathway activation. However, in the latter study the high degree of correlation between FIXAP and prothrombin fragment 1+2 may indicate a link between the activation of FIX and prothrombin. The development of an assay to measure the peptide cleaved from FX upon activation of this factor (FXAP) has added to these findings. Unlike FIXAP, there is an increase in FXAP within 5 minutes of establishing extracorporeal circulation and this correlates with thrombin generation (Philippou *et al.*, 1995). This would imply that FX activation is occurring before FIX activation and is thus presumably activated via the extrinsic pathway. There is an isolated case report that TAT complexes are also generated during CPB in a patient deficient in FXII (Burman *et al.*, 1994).

Taken together, the time course of appearance of FIX and FX activation peptides and the lack of apparent FXIIa formation, activation of the tissue factor pathway rather than the contact system may be the stimulus for thrombin generation during CPB. If this
was so, one might reasonably expect to see an increase in plasma levels of FVIIa during CPB.

In this study however, the majority of patients showed an initial rise in plasma FVIIa following sternotomy (although this was not significant), but following heparin administration plasma FVIIa levels decreased significantly and did not significantly change during the rest of the procedure. Since this thesis was undertaken there have been other reports on FVIIa levels during CPB. FVIIa levels are reported to decrease following heparin administration during CPB (Unsworth-White et al, 1994; Chung et al, 1996). However, FVIIa levels were significantly increased at the end of CPB and following protamine administration compared to immediately following heparin administration in these studies, which would suggest that activation of the tissue factor pathway is occurring during CPB. Futhermore, Chung et al demonstrated higher FVIIa, prothrombin fragment 1+2 and monocyte procagulant activity levels in blood sampled from the pericardial cavity during CPB compared to blood from the CPB circuit which may indicate that activation of FVII and thrombin generation is occurring locally at the wound site. With the reinfusion of blood aspirated from the surgical field into the CPB circuit, this could enhance levels of activated factors in the systemic circulation.

The decrease in FVIIa levels observed in this study was coincidental to an increase in plasma TFPI, which is known to be released rapidly and substantially from endothelial sites following administration of heparin (Sandset et al, 1988). Others have confirmed that there is an increase in TFPI following heparin administration during CPB (Ernofsson & Siegbahn, 1997). Interestingly, the heparin induced release of TFPI from the endothelium was greater in the pulmonary vein compared to CVP line. There are several reasons why this could occur. One possibility is that the local concentration of heparin is greater in the pulmonary vein compared to systemic circulation, as TFPI release by heparin is dose dependent, but we observed no evidence for this. Another possibility is that the endothelium in the pulmonary vasculature is a richer source of TFPI with a greater density of binding sites than in the systemic circulation. Indeed, the lung is known to contain large amounts of TFPI mRNA compared to other organs.
(Osterud et al, 1995b), other haemostatic properties of cultured endothelial cells are known to differ depending upon their anatomic site of origin (Grau et al, 1997) and it is reported that the lungs exert an anticoagulant effect on tissue factor induced DIC (Vad et al, 1997).

Increased activity in the extrinsic system may therefore be offset by heparin induced release of TFPI, provided that the patient’s haemostatic system is not otherwise compromised. In one patient however, FVIIa levels increased dramatically (greater than 5-fold) during bypass, followed by a marked increase in TAT complexes. In this patient TFPI levels did not increase following heparin administration, despite apparently adequate levels of heparin. The increase in FVIIa seen in this patient appeared to correlate with a dramatic fall in AT-III during bypass. Other ‘poor responders’ to heparin therapy in terms of the expected increase in TFPI have been reported (Maaoui et al, 1993).

Though there was little evidence of activation of FVII, plasma levels of FVII decreased during the study period. However it is known that elastase and cathepsin G released from activated neutrophils can inactivate FVII by proteolysis even in the presence of antiproteinases in blood, when these enzymes are associated with cell surfaces (Anderson et al, 1993). In this study there was a significant increase in neutrophil elastase: α₁-antitrypsin complexes towards the end of bypass, indicating neutrophil degranulation.

There was evidence of consumption of the naturally occurring serine protease inhibitors AT-III and HCII during CPB, which is not unexpected given that thrombin was generated as shown by an increase in TAT, and that AT-III also inhibits tissue factor:FVIIa complexes (Lawson et al, 1993) as well as other serine proteases. It is crucial that during CPB, AT-III levels remain at a level sufficient to achieve satisfactory anticoagulation with heparin. During clinical CPB others have reported a reduction (up to 50-60%) in plasma levels of AT-III in line with the effect of haemodilution (Tanaka et al, 1989; Feindt et al, 1991; Kern et al, 1992; Oba et al, 1995; Clark & Walker, 1995; Chan et al, 1997), although it is unclear from these studies if CPB has an effect on AT-
III in addition to haemodilution. It would appear that in both normal and AT-III deficient individuals, AT-III activity is sufficient for anticoagulation with heparin (Clark & Walker, 1995). Levels of HCII have been confirmed to be reduced during CPB (Chan et al, 1997). This decrease in plasma AT-III and HCII may result in a reduced inhibitory capacity towards thrombin.

A decrease in plasma soluble thrombomodulin levels was observed in blood that passed around the CPB circuit, as has been reported by other groups (Tanaka et al, 1989; Journois et al, 1994; Boldt et al, 1994b, Boldt et al, 1995) but, post-operatively there is an increase in this protein which may represent endothelial damage during CPB. During clinical CPB there is also a decrease in both functional and antigenic levels of protein C (Kobl et al, 1987; Tanaka et al, 1989; Feindt et al, 1991; Boldt et al, 1995) and both free and total protein S levels (Tanaka et al, 1989; Journois et al, 1994; Boldt et al, 1995). These findings are consistent with the hypothesis that thrombin generated during cardiac bypass can activate protein C in the presence of thrombomodulin, and protein S acts as a cofactor for the activated protein C inactivation of FVa and FVIIIa. This illustrates anticoagulant properties of thrombin and could have repercussions on post-CPB bleeding. If endothelial damage is occurring in the pulmonary vein during bypass it is reasonable to suggest that thrombomodulin and endothelin may be released into the pulmonary circulation (Charbrier, 1993; Blann & Taberner, 1995).

In this study there was an apparent increase in thrombomodulin levels in the pulmonary vein mid-bypass but this was not statistically significant. Interestingly, recombinant human soluble thrombomodulin has been reported to reduce endotoxin induced pulmonary vascular injury in an animal model and this is likely to be due to the inhibitory effect of activated protein C on leucocyte activation rather than its anticoagulant properties (Uchiba et al, 1995).

Plasma endothelin-1 levels increased in the pulmonary vein during the study period, but this is not meaningful given that the same changes were observed in samples taken from the CVP line, presumably due to systemic endothelial modulation. Heparin has been shown to have an inhibitory effect on thrombin and phorbol ester-induced
endothelin-1 release by endothelial cells in vitro (Imai et al, 1993) and others have speculated that endothelin-1 release during ischaemia may be involved in a protective mechanism that impedes thrombus formation (Leadly et al, 1995).

It is known that both the length of time on bypass and the sequestration of neutrophils in the pulmonary microvasculature are critical factors in the development of post CPB pulmonary insufficiency (Ratliff et al, 1973). Adherence of neutrophils to the endothelium is a crucial step in neutrophil transmigration and neutrophil mediated endothelial injury and is dependent upon leucocyte and endothelial adhesion molecules. Changes in cell deformability and expression of adhesion molecules are thought to create a micro-environment that favours microvascular injury (Donnelly & Haslett, 1992). This could occur directly by the release of cytotoxic substances or because agents such as neutrophil elastase are shielded from large molecular weight anti-proteinases such as α1-antitrypsin.

In this study neutrophil intracellular stores of CD11b, a β2 integrin involved in adherence to and migration through the endothelium, were mobilised during bypass which is in agreement with other studies in vitro (Kapplemayer et al, 1993) and in vivo (Rinder et al, 1992). Surprisingly, this increased expression of CD11b on neutrophils from the pulmonary vein was not different to that observed in the CVP line, but this may be due to the fact that the cells may be maximally stimulated as the changes seen were similar to those observed on stimulating cells in vitro. Consistent with these findings was a significant fall in neutrophil count during bypass which did not return to preoperative levels in the pulmonary vein by the end of the study period.

Although a significant decrease in monocyte count was observed there was not a significant change in monocyte expressed CD11b over the study period which contradicts the results of Rinder et al. who showed a significant increase in monocyte CD11b by the pre-warm stage of CPB, peaking 2-4 hours post-operatively, whereas neutrophil CD11b was significantly higher by 10 minutes on CPB peaking by the prewarm stage. The timecourse of CD11b mobilisation on monocytes is therefore different to that of neutrophils and as the mean bypass time in Rinder’s patients was
101±29 minutes compared to our median time of 50 minutes (range 36-84), any mobilisation of CD11b on monocytes may well occur beyond our study period. This discrepancy may be due to 1) differences in sample handling, 2) that the most adherent monocytes are either stuck to CPB circuitry or sequestered in the microvasculature and thus are unavailable for assay, 3) that the mobilisation of CD11b on monocytes and neutrophils may be mediated by different stimuli or that neutrophils and monocytes may have different sensitivities to the same stimuli, or 4) that other adhesion molecules may be important in monocyte sequestration.

Recent studies have emphasised the role of neutrophils in the pathogenesis of post CPB lung injury (Westaby et al, 1985; Johnson et al, 1994). Pre-administration of glucocorticoids attenuates the increase in neutrophil CD11b during CPB (Hill et al, 1994) and in a porcine model of CPB pre-treatment with a novel anti-inflammatory agent reduces the upregulation of neutrophil CD11b, myeloperoxidase content of the lung, free radical peroxidation and improves post-operative pulmonary function (Gillinov et al, 1994). In a porcine model, the therapeutic use of an antibody against CD18 (the common β chain of β₂ integrins) can improve endothelial function and water content of the lungs following CPB (Aoki et al, 1996).

The most striking finding in this study was that apart from the increase in TFPI following heparin administration and the neutrophil count, there was no significant difference in the parameters studied in the pulmonary vein compared to blood which had passed through the extracorporeal circuit. Considering the degree of chest wall trauma and ‘activation’ of blood components by artificial CPB circuits it is surprising that as few as 1.7% of patients undergoing CPB develop ARDS (Fowler et al, 1983). Heparin may exert a protective effect on post-CPB pulmonary injury due to the release of TFPI from endothelial sites and by modulating other endothelial proteins.

Heparin has many other effects in the vascular environment in addition to its anticoagulant role (Dawes, 1993). Heparin binds to monocytes and endothelial cells where it retains its ability to augment AT-III activity and can also bind to platelets. Heparin may confer protection from endothelial injury by a variety of mechanisms: it
has been shown to reduce free radical mediated endothelial damage (Hiebert & Liu, 1990), reduce stasis-induced endothelial injury (Trabucchi et al, 1991), enhance endothelial barrier function (Bannon et al, 1995), release superoxide dismutase which inhibits leucocyte adherence to the endothelium (Becker et al, 1993) and reverse the procoagulant activity of endothelial cells (Cadroy et al, 1996).

In conclusion, there was evidence of consumption of the haemostatic factors PKK and FVII as well as the serine protease inhibitors AT-III and HCII in the pulmonary vein during cardiopulmonary bypass and this was not significantly different to blood that had passed around the extracorporeal circuit. Any activation of the tissue factor pathway that occurred appeared to be offset by a marked heparin-induced release of TFPI.

Neutrophil expression of the β2 integrin CD11b increased during bypass which was coincidental to a decrease in neutrophil count. In the pulmonary vein the neutrophil count did not return to pre-operative levels by the end of the procedure, probably reflecting neutrophil sequestration in the pulmonary vasculature. There was a trend towards increased soluble thrombomodulin levels in the pulmonary vein during the study period which may reflect endothelial injury. Upregulation of neutrophil adhesion molecules results in neutrophil adhesion to and margination through the endothelium, where they can release their cytotoxic arsenal and create a local environment that favours endothelial injury. This is likely to be a fundamental factor in the development of postoperative pulmonary injury associated with the use of extracorporeal circulation.

It appears that the coagulation system is well controlled by its various plasma inhibitors during bypass, the mechanism by which thrombin is generated remaining an enigma. It is conceivable that activation of coagulation factors could occur on the surface of cells and lipoproteins, protected from plasma inhibitors and unavailable for assay using conventional methods. To this end, experiments were undertaken to investigate whether lipoprotein particles or peripheral blood cells could provide a surface capable of supporting activation of RXII. The rationale for these experiments and the data obtained are outlined in chapter 6.
6.1 Introduction

In Chapter 5 we have seen that during cardiopulmonary bypass there is activation of coagulation occurring as evidenced by an increase in markers of thrombin generation. However, the data provided no indication that this thrombin generation was related to either activation of the contact system or tissue factor pathway in systemic blood samples. This poses an interesting question: if there is no evidence for contact or tissue factor pathway activation, what are the mechanisms of thrombin generation during CPB?

One possibility is that thrombin is generated by activation of the contact factor pathway, but the methods used in the previous chapter do not allow detection of this. It is possible that activation of coagulation factors could occur on cell surfaces or the surface of the CPB circuit, for instance, and thus changes seen in plasma samples may not reflect activation occurring at these sites. In addition, activation of coagulation factors could occur on the surface of lipoproteins where they may be protected from inhibition and be unaccessible using some assays. The aim of this chapter, therefore, was to explore in vitro the possibility that peripheral blood cells and lipoprotein particles could provide a surface capable of supporting activation of the contact system. In addition, activation of FXII on the surface of CPB circuitry was explored in vitro with a view to examining CPB circuitry after use in vivo.

A relationship between lipids and coagulation activation has long been thought to exist. The Northwick Park Heart Study demonstrated that FVII coagulant activity (FVII:C), a function of both FVII zymogen and FVIIa, is an independent risk factor for fatal ischaemic heart disease in middle-aged men (Meade et al, 1986). There is also a positive association between FVII:C and triglyceride concentration in this subject group.
and this has been shown to reside mainly with triglyceride-rich lipoproteins (Mitropoulos et al, 1989). These observations led to the notion that haemostatic activation may be modulated by plasma lipids and that this could lead to the establishment of a hypercoagulable state in conditions in which plasma lipid concentrations are increased. Indeed, in primary hyperlipidaemia where plasma lipid concentrations are increased in a chronic manner it is reported that FVII:C is increased (Carvalho de Sousa et al, 1989a) and in the postprandial state, when triglyceride concentrations are increased transiently, it is reported that activated FVII (FVIIa) activity is increased (Miller et al, 1991 & 1996; Silviera et al, 1994a&b).

It has been suggested that lipoproteins may provide an endogenous surface capable of supporting activation of the FXII (Mitropoulos et al, 1989b). The lipolysis of triglyceride rich lipoproteins results in free fatty acids at the lipoprotein surface which are capable of supporting the activation of FXII with subsequent activation of FVII (Mitropoulos et al, 1993). This reaction is dependent upon the presence of lipoprotein lipase (Mitropoulos et al, 1992). In a purified system, lipase-dependent FXII activation on the surface of VLDL is reported to occur and is inhibited by β2GP1 (apolipoprotein-H) (McNally et al, 1996). β2Glycoprotein-I (β2GP1), has previously been shown to inhibit contact activation (Schousboe, 1985) and the auto activation of FXII (Henry et al, 1988). During CPB heparin is used as an anticoagulant. Lipoprotein lipase is released from the endothelium due to the action of heparin (Cryer, 1987). It is therefore feasible that lipolysis of lipoproteins during CPB could support activation of FXII and FVII.

It is established that lipoproteins influence other coagulation reactions in vitro: isolated lipoprotein fractions have been shown to support factor X and prothrombin activation in vitro (Bajaj et al, 1976; Sandgerg et al, 1976; Vijayagopal et al, 1978). In addition, FVII and factor X have been shown to be associated with triglyceride rich lipoproteins isolated from normolipidaemic subjects (Carvahlo de Sousa et al, 1988) and other studies have demonstrated the association of prothrombin with VLDL and also the binding of Factor X and Xa to VLDL (Bradley et al, 1988). β2GP1 has been demonstrated to be a constituent of all major lipoprotein classes (Poltz & Kosnter,
However, the direct association of FXIIa and FVIIa with isolated lipoproteins has not been previously demonstrated.

The aim of the first part of this chapter was to investigate the association of FXIIa and FVIIa with lipoprotein particles in normolipidaemic subjects. Initially, the most obvious route to isolating lipoproteins (VLDL, IDL, LDL, HDL) from plasma was chosen: lipoproteins were separated by preparative ultracentrifugation and assayed for FXIIa and FVIIa. However, lipoprotein separation by this method is a lengthy procedure and has to be carried out at 4°C, thus making 'cold activation' of plasma and exchange of apolipoproteins likely. The results obtained were deemed likely to be a result of artefactual changes and other ways of separating lipoproteins were therefore considered.

It is possible to separate lipoprotein classes by agarose gel electrophoresis, making it feasible to perform a western blot on the gel to detect FVIIa and FXIIa protein. However, there are several problems associated with this: (i) FXIIa and FVIIa are present in plasma at low protein concentrations (ng/ml) which is at the limit of the sensitivity for this technique, (ii) there are no monoclonal antibodies currently available that recognise FVIIa that do not cross react with FVII zymogen, and (iii) the actual technique may result in changes to the lipoprotein molecule which may mask or expose antigenic sites thus providing a potential for misleading results.

An alternative approach was to study coagulation factors associated with chylomicra. Chylomicra are triglyceride-rich lipoproteins synthesised by enterocytes from the products of fat digestion. They are very low-density lipoproteins and are separated from plasma by centrifugation easily, quickly and at ambient temperature. Since chylomicra are only present in plasma after food, I decided to investigate whether FXIIa, FVIIa and β3GP1 are associated with chylomicra isolated from the plasma of normolipidaemic subjects in the fasting (mainly small amount of VLDL) and postprandial state (VLDL + chylomicra). The effect of lipase (to release free fatty acids creating a negatively charged surface) or detergent treatment (to disrupt the lipoprotein

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structure) on the haemostatic factors was also studied. The results obtained using chylomicra were contrasted with FVII and FXII activation occurring in plasma.

The second part of this chapter is concerned with the possibility of peripheral blood cells supporting the activation of coagulation factors. During CPB red cells are traumatised resulting in haemolysis (Edmunds, 1995). Exposure of phospholipids from red cell membranes could provide a surface on which coagulation reactions could take place. Erythrocyte membranes and stroma were examined as potential surfaces on which the activation of FXII could occur.

During CPB neutrophils become activated. Neutrophils are not innocent bystanders as far as coagulation is concerned, they may be involved in the activation and regulation of the contact system. Neutrophils have been shown to contain and bind HK (Gustafson et al, 1989) and more recently all the contact factors have been shown to be present on the neutrophil surface (Henderson et al, 1994). It was postulated that neutrophils may participate in FXII activation, either through specific protein-receptor interactions or merely by providing a phospholipid surface on which FXII activation could take place. This was investigated in vitro using isolated neutrophils and purified FXII. The possibility of contact factors being present and functional on the neutrophil surface was investigated using confocal microscopy, however a number of technical difficulties made this approach unsuitable.

Finally, the activation of FXII on the surface of the CPB circuit was investigated in vitro. If FXII activation occurs on the CPB circuit, αFXIIa would be expected to remain surface-bound, while βFXIIa would rapidly be complexed to its major plasma inhibitor (Cl-INH). Therefore in plasma samples, this activation would not be detected. It was therefore decided to examine FXIIa bound to the CPB circuit using immunological and amidolytic substrate methods, as this could also be applied to CPB circuits after clinical use.

Aside from the main aims of this chapter, some further experiments on the ultrafiltrate obtained from patients undergoing haemofiltration that were studied in Chapter 3 were carried out. Following the observation in Chapter 4 that prothrombin
fragment 1+2 could be cleared across the membrane of the haemofilter, it was decided to
investigate more closely the clearance of other clotting factors across the membrane.
The molecular weight of most of the proteins studied would prohibit their clearance,
however, free βFXIIa has a molecular weight of approximately 28 Kd and could thus
possibly be removed by the haemofilter. Ultrafiltrate from some of these patients (4/12)
was assayed for FXIIa antigen previously, and found to contain no detectable amount of
FXIIa. Since βFXIIa rapidly complexes to C1-INH (molecular weight 105 Kd) in plasma,
this is not surprising. However, FXIIa antigen was assayed in ultrafiltrate samples from
all patients for completeness.

6.2 Methods

6.2.1 Association of FXIIa and FVIIa with lipoproteins

Blood was collected from four subjects after a 12 hour fast during which time only
water was taken. Postprandial samples were collected at 4 hours following the ingestion
of a large fatty meal. Citrated plasma was prepared as described in section 2.1. VLDL,
IDL, LDL and HDL were isolated from citrated plasma as described in section 2.7.3.
The isolated lipoprotein fractions were dialysed into 0.15M sodium chloride to remove
high salt concentrations. FVIIa was assayed by clotting assay as described in section
2.5.6. Samples were assayed for FXIIa, with and without the addition of 1% (v/v) Triton
X-100, by ELISA as described in section 2.4.10.

6.2.2 Activation of FXII and FVII on chylomicra-rich lipoprotein particles

Blood was collected from 6 apparently healthy normal subjects (non-smokers) after a 12
hour fast during which time only water was taken. Postprandial samples were collected
1, 2 and 4 hours following the ingestion of a standard milkshake drink containing 100g
fat prepared using 1 banana, 3g Duocal (Scientific Hospital Supplies, Liverpool) and
200ml of double cream. Citrated plasma and serum were prepared as described in
section 2.1.
Chylomicra were isolated by ultracentrifugation. An ultracentrifugation tube ('Ultra-Clear', Beckman Ltd) was filled with 10ml of citrated plasma and then overlaid with 3.5ml of 0.15 M sodium chloride (to reduce plasma protein contamination), the tubes sealed and spun at 100,000g in an 80Ti rotor for 30 minutes at 20°C in a Beckman L-80M ultracentrifuge. The chylomicra (most probably containing some VLDL, therefore referred to as chylomicra-rich lipoproteins) were removed and respun under the same conditions to reduce contamination with plasma proteins. The chylomicron fraction was made up to 2.5 ml with saline (chylomicra were therefore concentrated 4 fold during this process).

Chylomicra-rich lipoproteins and plasma were treated with detergent by the addition of 1% v/v (final concentrations given) of Triton X-100 (Sigma-Aldrich Chemical Co) and with lipase by the addition of 0.5U/ml bacterial lipase (Type XIII from Pseudomonas, Sigma) and incubation at 37°C for 60 minutes. The lipase treated chylomicra-rich lipoproteins or plasma were divided into two aliquots: in one, further lipase action was inhibited by the addition of 1% (v/v) Triton X-100; the other was not treated with detergent. Bacterial lipase was used since human lipoprotein lipase is inhibited by β2GP1. This allowed the specific investigation of the effect of lipolysis on the factors studied. FVIIa could not be measured on Triton X-100 treated samples due to the interference of this detergent on the FVIIa assay. Triton X-100 at a concentration of 1% v/v did not interfere with the detection of purified βFXIIa in the FXIIa ELISA.

The chylomicra-rich lipoproteins or plasma samples were assayed for FVIIa using a clotting assay as described in section 2.5.6, and FXIIa and β2GP1 by ELISA as described in section 2.4. Total cholesterol and triglycerides were measured using serum or chylomicra-rich lipoproteins that had been stored at 4°C as described in section 2.7.

In order to establish whether lipase treatment of chylomicra-rich lipoproteins resulted in generation of FXIIa or merely exposed FXIIa antigenic sites by disrupting the lipoprotein structure, specific inhibitors of FXIIa were added to chylomicra-rich lipoproteins before lipase treatment and also after lipase treatment. This was achieved by preincubating chylomicra-rich lipoproteins with 50μg/ml (final concentrations given)
corn trypsin inhibitor (Unicom Diagnostics Ltd) or 1.5 mM 3,4-dichloroisocoumarin (Sigma-Aldrich Chemical Co).

The inhibition of FXIIa generation on the chylomicra-rich lipoproteins by CI-INH and ATIII was studied by incubating chylomicra-rich lipoproteins with Cl-INH (CRTS, Lille) or ATIII (BPL Ltd) before or after treatment with lipase. Chylomicra-rich lipoproteins were diluted to a physiological concentration (1:4 in saline) and incubated with 0, 0.16, 0.32, 0.64, 1.28 U/ml Cl-INH or 0, 0.25, 0.50, 1.00, 2.00 U/ml ATIII.

The effect of the inhibitors used above on the FXIIa antigen ELISA was studied by adding Cl-INH (1.27 U/ml), ATIII (1.00 U/ml), CTI (50 μg/ml) and DCIC (1.5 mM) to the 0, 5 and 20 ng/ml purified βFXIIa standards and then assaying for FXIIa antigen.

Changes in parameters over time were assessed by repeated measures analysis of variance (Statview II for Macintosh, Abacus Concepts Inc, Berkley, CA). If a statistically significant result was obtained ($p<0.05$), Fisher’s protected least significant difference post hoc testing was used to assess changes in variables measured over the study period. Differences between treatments were assessed using the student t-test. A statistically significant result was defined as $p<0.05$. All results are expressed as mean ± SD.

### 6.2.3 Activation of FXII by red blood cells

Blood was collected from three healthy subjects and anticoagulated with sodium citrate (see section 2.1). Whole blood (20 ml) was centrifuged at 2000 g for 15 minutes and the plasma and buffy coat were removed. The remaining red cells were washed three times using PBS (0.01 M phosphate, 0.145 M sodium chloride, pH 7.2) and centrifugation at 2000 g for 5 minutes. The red cells were lysed by the addition of an equal volume of distilled water and centrifuged at 2000 g for 10 minutes and the stromal supernatant removed. The stroma was diluted 1/200 in PBS. The red cell membranes were resuspended in distilled water and centrifuged at 10000 g for 5 minutes and this step was repeated until there was no visible haemoglobin contamination of the supernatant, the red cell membranes finally being suspended in 400 μl of water.
The red cell membrane suspension or stroma was incubated with an equal volume of either autologous plasma or buffer (0.05M Tris, pH 7.9) for one hour at 37°C. As a control, autologous plasma in the absence of red cells or stroma was used at the appropriate dilution. The reaction mixture was then diluted 1/5 in buffer (0.05M Tris, pH 7.9) to bring the pH of the solution to that optimal for the substrate being used. Into the wells of a microtitre plate (F96 microplate, Alpha Laboratories Ltd, Eastleigh) diluted reaction mixture (150μl) was added to 150μl of FXII amidolytic substrate (Unicom Diagnostics Ltd, London), mixed and then incubated at 37°C. After 5, 10, 20, 30 and 60 minutes incubation the optical density at 405nm was read using an automated plate reader (Anthos 2001, Anthos Labtech Instruments Ltd.).

6.2.4 Activation of FXII by neutrophils

Blood was obtained from three healthy subjects and anticoagulated with 10IU/ml unfractionated heparin (Monoparin, CP Pharmaceuticals). Washed neutrophils were prepared as described in section 2.9 and finally suspended at a count of 5x10^6/ml in Dubellcco's PBS with magnesium and calcium containing 0.1% (w/v) ovalbumin (Sigma-Aldrich Chemical Co, A5378, Grade III) and 0.1% (w/v) glucose. Neutrophils were stimulated with 1μM f-Met-Leu-Phe (Sigma-Aldrich Chemical Co) for 10 minutes at 37°C or left unstimulated. Buffer (Dubellcco's PBS with magnesium and calcium containing 0.1% (w/v) ovalbumin and 0.1% (w/v) glucose), unstimulated neutrophils or stimulated neutrophils were incubated with purified human FXII (Enzyme Research Ltd) at a final concentration of 25μg/ml. To minimise the autoactivation of FXII due to contact with a negatively charged surface, this reaction was carried out in polypropylene ependorf tubes which had previously been washed overnight in PBS containing 0.5% ovalbumin, rinsed in distilled water and dried at 37°C. At time intervals of 0, 10, 20, 30, 40, 50 and 60 minutes, a subsample of the reaction mixture was removed (100μl) and added to 0.1M Tris pH 8.0 (100μl) and 1mM FXII substrate (100μl, Unicorn Diagnostics Ltd.) in the wells of a microtitre plate (F96 Microplate, Alpha Laboratories Ltd.). After an incubation period of 15 minutes at 37°C, the reaction was stopped by the
addition of 50μl of 50% (v/v) acetic acid. The absorbance at 405nm was read using an automated plate reader (Anthos 2001).

Inhibition of FXIIa generation by neutrophils was investigated by incubating the neutrophil suspension with 0.32, 0.64, and 1.27U/ml of purified C1-INH (CRTS Lille) before and after incubation with FXII as above. The effect of fixing the neutrophils was investigated by fixing in 1% paraformaldehyde prior to incubation with FXII as above. To determine whether the FXIIa generated by the neutrophils remained surface-bound or was released into the fluid phase, after incubation with FXII an aliquot of the neutrophil suspension was spun at 2000g for 5 minutes and the supernatant removed and added to the FXII substrate as above.

6.2.5 Activation of FXII by CPB circuitry
A 50mm piece of CPB tubing (William Harvey EEC pack, Baxter Ltd) was clamped at each end using tubing clips to give a 30mm length which would be exposed to solutions placed in it. 1ml of plasma (anticoagulated with 5IU/ml unfractionated heparin) was incubated in the tubing with mixing at room temperature for 2 hours. The plasma was removed, and the tubes washed three times with 2ml PBS (0.1M Phosphate, 0.145M sodium chloride, pH 7.4). 1ml of a mouse monoclonal antibody against FXIIa (α and β, a kind gift from Dr. R Ford, Shield Diagnostics Ltd) or an isotype matched control antibody (mouse IgG1, Dako Ltd) at a concentration of 10.5μg/ml was incubated in the tubing with mixing at room temperature for 2 hours. The antibody was removed, the tube washed three times with 2ml PBS and then 1ml of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma, diluted 1/500 in PBS) added and incubated with mixing for 2 hours at room temperature. The tube was washed three times with 2ml PBS and then 1ml of substrate added (1mg/ml phosphatase substrate (Sigma 104 tablets) in 1M diethanolamine, 0.5mM magnesium chloride hexahydrate, pH 9.8). The tubes were incubated with mixing at room temperature for 10 minutes and then the reaction stopped by the addition of 0.5ml 3M sodium hydroxide. The solution was removed from the tube and 300μl transferred to the wells of a microtitre plate. The absorbance at 405nm
was read using an automated plate reader (Anthos 2001).

6.2.6 Measurement of FXIIa antigen in ultrafiltrate samples

Ultrafiltrate samples obtained from patients undergoing haemofiltration described in chapter 3 were assayed for FXIIa antigen by ELISA (section 2.4).

6.3 Results

6.3.1 Association of FXIIa and FVIIa with lipoproteins

FXIIa and FVIIa were not detectable in any VLDL, IDL, or LDL fractions. However, the HDL fraction contained large amounts of FVIIa and FXIIa (Table 6.1). The FXIIa measured in the HDL fraction was higher after Triton X-100 treatment.

<table>
<thead>
<tr>
<th>Subject</th>
<th>FVIIa (ng/ml)</th>
<th>FXIIa (ng/ml)</th>
<th>FXIIa + triton (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 fasting</td>
<td>23.29</td>
<td>8.56</td>
<td>9.54</td>
</tr>
<tr>
<td>2 fasting</td>
<td>11.22</td>
<td>8.23</td>
<td>10.01</td>
</tr>
<tr>
<td>3 fasting</td>
<td>13.14</td>
<td>6.85</td>
<td>8.18</td>
</tr>
<tr>
<td>4 fasting</td>
<td>19.29</td>
<td>7.35</td>
<td>8.62</td>
</tr>
<tr>
<td>mean</td>
<td>16.74</td>
<td>7.75</td>
<td>9.09</td>
</tr>
<tr>
<td>SD</td>
<td>5.56</td>
<td>0.79</td>
<td>0.84</td>
</tr>
<tr>
<td>1 postprandial</td>
<td>18.77</td>
<td>8.63</td>
<td>9.92</td>
</tr>
<tr>
<td>2 postprandial</td>
<td>11.04</td>
<td>8.34</td>
<td>10.01</td>
</tr>
<tr>
<td>3 postprandial</td>
<td>16.85</td>
<td>7.13</td>
<td>8.15</td>
</tr>
<tr>
<td>4 postprandial</td>
<td>17.94</td>
<td>6.63</td>
<td>7.95</td>
</tr>
<tr>
<td>mean</td>
<td>16.15</td>
<td>7.68</td>
<td>9.01</td>
</tr>
<tr>
<td>SD</td>
<td>3.50</td>
<td>0.96</td>
<td>1.10</td>
</tr>
</tbody>
</table>

*Table 6.1* Levels of FVIIa and FXIIa (with and without the addition of 1% Triton X-100) in HDL isolated from normolipidaemic subjects (n=4) in the fasting and postprandial state.
Figure 6.1 Plasma levels of a) FXIIa, b) FVIIa and c) β₂GPI in the fasting state (0) and at 1, 2 and 4 hours postprandial in normolipidaemic subjects.

Results are expressed as mean ± SD (n=6 except FXIIa where n=5). Plasma was untreated or treated with Triton X-100 (1% v/v) or 0.5U/ml lipase for 60 minutes at 37°C. * denotes p<0.05, ** p<0.01, † p<0.005, †† p<0.001 compared to untreated plasma. Plasma FXIIa decreased (p=0.0001) and FVIIa increased (p=0.0001) over time.
6.3.2 Activation of FXII and FVII on TG-rich lipoprotein particles

Plasma proteins

The plasma levels of FXIIa, FVIIa and β2GP1 in the fasting samples were within the normal reference range for these assays. In 5/6 subjects FXIIa levels decreased (p=0.0001) during the postprandial period (Figure 6.1a) but increased in one subject (1.60 fasting and 2.00, 2.15, 2.23ng/ml at 1, 2 and 4 hours postprandial respectively), and this subject was therefore removed from the statistical analysis. The other parameters studied in this subject followed a similar pattern to the other five, and all six were therefore included in the rest of the analysis. Lipase or Triton X-100 treatment of plasma resulted in an increase (p<0.05) in plasma FXIIa at all time points (Figure 6.1a), and there was a significant increase in the % change in FXIIa with lipase (p=0.008) or Triton X-100 (p=0.049) treatment over time (Figure 6.2). In plasma the increase in FXIIa with Triton X-100 treatment was greater than with lipase at all time points (p<0.01).

![Graph showing percentage change in plasma FXIIa levels](image)

**Figure 6.2** Percentage change in the plasma level of FXIIa, in the fasting state (0) and at 1, 2 and 4 hours postprandial in normolipidaemic subjects treated with Triton X-100 (1% v/v) or 0.5U/ml lipase for 60 minutes at 37°C.

Results are expressed as mean ± SD (n=6).* denotes p<0.05, †† p<0.001 compared to 1 hour.
The addition of Triton X-100 after lipase treatment of plasma or chylomicra-rich lipoproteins to terminate the action of lipase produced no difference in FXIIa or FVIIa that could not be accounted for by the cumulative effect seen with lipase or Triton X-100 treatment alone. Therefore all results shown are plasma or chylomicra-rich lipoproteins either untreated, treated with Triton X-100 or treated with lipase only.

\( \beta_2 \)GP1 antigen levels did not change significantly with time, and were not significantly altered by treatment with Triton X-100 (Figure 6.1c). However, lipase treatment reduced \( \beta_2 \)GP1 levels in plasma at all time points \( (p<0.05) \) but the % reduction did not alter with time (Figure 6.1c).

FVIIa levels increased following the ingestion of fat \( (p<0.001) \) and in the postprandial period lipase treatment resulted in a small decrease in FVIIa \( (p<0.05) \) which did not significantly differ over the study period (Figure 6.1b). As expected, total serum triglyceride levels increased \( (p=0.001) \) in the postprandial period without any significant change in total serum cholesterol (Table 6.2).

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1hr</th>
<th>2hr</th>
<th>4hr</th>
<th>( p = )</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum triglyceride</td>
<td>0.74 ± 0.19</td>
<td>1.05 ± 0.32</td>
<td>1.33 ± 0.42</td>
<td>1.27 ± 0.48</td>
<td>0.0019</td>
</tr>
<tr>
<td>serum cholesterol</td>
<td>5.53 ± 0.38</td>
<td>5.50 ± 0.49</td>
<td>5.50 ± 0.55</td>
<td>5.35 ± 0.38</td>
<td>0.1852</td>
</tr>
<tr>
<td>chylo triglyceride</td>
<td>0.17 ± 0.09</td>
<td>0.55 ± 0.30</td>
<td>0.98 ± 0.50</td>
<td>0.91 ± 0.50</td>
<td>0.0011</td>
</tr>
<tr>
<td>chylo cholesterol</td>
<td>0.13 ± 0.05</td>
<td>0.28 ± 0.10</td>
<td>0.42 ± 0.20</td>
<td>0.46 ± 0.14</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

**Table 6.2** Fasting (0) serum and chylomicra-rich lipoprotein triglyceride and cholesterol levels and at 1, 2 and 4 hours following the ingestion of 100g fat in normolipidaemic subjects.

Results are given in mM and expressed as mean ± SD \( (n=6) \). \( P \) value refers to significance from repeated measures ANOVA (over time).

**Isolated chylomicra-rich lipoproteins**

There was a significant increase in both chylomicra-rich lipoprotein triglyceride \( (p=0.0011) \) and cholesterol \( (p=0.0003) \) levels during the postprandial period (Table 6.2), consistent with the postprandial production of chylomicra particles.
There was a significant ($p=0.004$) reduction in chylomicra-rich lipoprotein associated FXIIa with time (Figure 6.3) despite an increase in chylomicra-rich lipoprotein particle concentration of triglyceride and cholesterol. Treatment of chylomicra-rich lipoprotein with lipase ($p<0.01$) or Triton X-100 ($p<0.05$) resulted in an increase in FXIIa levels at all time points (Fig 6.3), but, interestingly in the fasting state the response to lipase varied widely between subjects. Chylomicra-rich lipoprotein associated FXIIa levels with lipase treatment increased over time ($p<0.003$, Figure 6.3).

![Figure 6.3](image)

**Figure 6.3** Levels of chylomicra-rich lipoproteins associated FXIIa in the fasting state (0) and at 1, 2 and 4 hours following the ingestion of 100g fat in normolipidaemic subjects.

Results are expressed as mean ± SD (n=6). Chylomicra-rich lipoproteins were untreated or treated with Triton X-100 (1% v/v) or 0.5U/ml lipase for 60 minutes at 37°C. * denotes $p<0.05$, ** $p<0.01$, † $p<0.005$, ‡ $p<0.001$ compared to untreated chylomicra-rich lipoproteins. There was a significant reduction in chylomicra-rich lipoproteins associated FXIIa ($p=0.004$) with time, with lipase treatment FXIIa increased over time ($p=0.003$).

The % change in chylomicra-rich lipoproteins FXIIa increased over time with lipase treatment ($p=0.04$) and approached statistical significance with Triton X-100 treatment ($p=0.0886$). The increase in chylomicra-rich lipoprotein associated FXIIa with lipase
treatment was greater than with Triton X-100 treatment at all postprandial time points (p<0.001).

| Buffer | 0.068 | 0.410 (4.28) | 0.879 (14.22) |
| Buffer | 0.073 | 0.085 (0.36) | 0.142 (0.80) |
| C1-INH (1.27 U/ml) | 0.088 | 0.275 (2.28) | 0.786 (11.92) |
| ATIII (1.00 U/ml) | 0.077 | 0.308 (2.38) | 0.794 (12.11) |
| DCIC (1.5mM) | - | 0.215 (1.56) | 0.582 (7.42) |

Table 6.3 The effect of various inhibitors on the detection of βFXIIa in the FXIIa antigen ELISA

Results given as OD at 540nm with observed level of FXIIa (ng/ml) in brackets

The effect of C1-INH, ATIII, DCIC and CTI on the detection of FXIIa in the FXIIa antigen ELISA is shown in Table 6.3. The addition of C1-INH to purified βFXIIa resulted in the abolition of its detection since FXIIa:C1-INH complexes are not recognised by the monoclonal antibody used in the ELISA. The presence of DCIC did not influence the detection of FXIIa at high levels, but did appear to have a slight effect at low levels of FXIIa. CTI appeared to partially mask FXIIa epitopes recognised by the ELISA. None of the inhibitors had an effect on the assay in the absence of FXIIa.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Treatment</th>
<th>FXIIa (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>none</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>lipase only</td>
<td>18.05</td>
</tr>
<tr>
<td>Dichloroisoumarin (DCIC)</td>
<td>DCIC added before lipase</td>
<td>10.41</td>
</tr>
<tr>
<td></td>
<td>DCIC added after lipase</td>
<td>18.21</td>
</tr>
<tr>
<td>Corn Trypsin Inhibitor (CTI)</td>
<td>CTI added before lipase</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>CTI added after lipase</td>
<td>14.48</td>
</tr>
</tbody>
</table>

Table 6.4 The effect of FXIIa inhibitors on chylomicra-rich lipoprotein associated FXIIa following lipase treatment

Chylomicra-rich lipoproteins from one subject 1 hr postprandially were treated with lipase (0.5U/ml) for 60 minutes at 37°C. Inhibitors were added either before or after lipase treatment.
The increase in chylomcra-associated FXIIa with lipase treatment could be partly attenuated by preincubation with dichloroisocoumarin and almost completely attenuated by preincubation with corn trypsin inhibitor (Table 6.4). The addition of physiological concentrations of C1-INH or ATIII prior to lipase treatment could not totally prevent the generation of FXIIa by chylomicra-rich lipoproteins (Table 6.5). When added after lipase treatment, the inhibition of FXIIa by C1-INH or ATIII was reduced.

<table>
<thead>
<tr>
<th>C1-INH (U/ml)</th>
<th>Added pre-lipase FXIIa (ng/ml)</th>
<th>Added post-lipase FXIIa (ng/ml)</th>
<th>ATIII (U/ml)</th>
<th>Added pre-lipase FXIIa (ng/ml)</th>
<th>Added post-lipase FXIIa (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.16</td>
<td>22.31</td>
<td>0</td>
<td>19.99</td>
<td>20.91</td>
</tr>
<tr>
<td>0.16</td>
<td>1.77</td>
<td>7.01</td>
<td>0.25</td>
<td>17.93</td>
<td>19.16</td>
</tr>
<tr>
<td>0.32</td>
<td>1.09</td>
<td>5.81</td>
<td>0.50</td>
<td>16.58</td>
<td>18.16</td>
</tr>
<tr>
<td>0.64</td>
<td>0.38</td>
<td>5.05</td>
<td>1.00</td>
<td>2.87</td>
<td>14.96</td>
</tr>
<tr>
<td>1.28</td>
<td>0.21</td>
<td>4.67</td>
<td>2.00</td>
<td>0.10</td>
<td>10.03</td>
</tr>
</tbody>
</table>

**Table 6.5** The effect of C1-INH and ATIII on chylomicra-rich lipoprotein associated FXIIa following lipase treatment

Chylomicra-rich lipoproteins from one subject 1 hr postprandially were treated with lipase (0.5U/ml) for 60 minutes at 37°C. Inhibitors were added either before or after lipase treatment.

The levels of chylomicra-rich lipoprotein associated FVIIa and β₂GP1 in the fasting and postprandial state are shown in Table 6.6.

<table>
<thead>
<tr>
<th>units</th>
<th>0</th>
<th>1hr</th>
<th>2hr</th>
<th>4hr</th>
<th>p =</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₂GP1</td>
<td>7±2.7</td>
<td>10±4.1</td>
<td>13±6.6</td>
<td>12±3.8</td>
<td>0.0179</td>
</tr>
<tr>
<td>β₂GP1 + lipase</td>
<td>5±1.5</td>
<td>7±1.9</td>
<td>10±5.7</td>
<td>7±1.1</td>
<td>0.0186</td>
</tr>
<tr>
<td>β₂GP1 + triton</td>
<td>6±2.1</td>
<td>8±3.0</td>
<td>11±4.5</td>
<td>11±3.0</td>
<td>0.0467</td>
</tr>
<tr>
<td>FVIIa + lipase</td>
<td>0.19±0.09</td>
<td>0.22±0.12</td>
<td>0.22±0.11</td>
<td>0.25±0.11</td>
<td>0.0701</td>
</tr>
</tbody>
</table>

**Table 6.6** Levels of chylomicra-rich lipoprotein associated FVIIa and β₂GP1 in the fasting state (0) and at 1, 2 and 4 hours following the ingestion of 100g fat in normolipidaemic subjects.

Results are expressed as mean ± SD (n=6). Plasma was untreated or treated with Triton X-100 (1% v/v) or 0.5U/ml lipase for 60 minutes at 37°C. P value refers to significance from repeated measures ANOVA (over time).
There was a significant ($p=0.0179$) increase in chylomicra-rich lipoprotein associated $\beta_2$-GPI during the postprandial period, which was not altered by treatment with Triton X-100 or lipase (Table 6.6). Chylomicra-rich lipoprotein associated FVIIa tended to increase during the postprandial period although this did not achieve statistical significance and lipase treatment did not significantly alter chylomicra-rich lipoprotein associated FVIIa.

6.3.3 Activation of FXII by red blood cells

In the absence of plasma, lysed red blood cell membranes (Fig. 6.4) or red cell stroma (Fig. 6.5) did not cleave the FXII amidolytic substrate. When autologous plasma (a source of FXII) was added to the red cell membranes or stroma, there was cleavage of the substrate but, this was not greater than plasma in the absence of these substances (Fig. 6.4 and 6.5).

![Figure 6.4 Cleavage of FXII amidolytic substrate by red blood cell membranes from normal subjects in the presence and absence of plasma (n=3, mean ± SEM).](image-url)
6.3.4 Activation of FXII by neutrophils

In the first series of experiments at time zero when either the neutrophil/FXII or buffer FXII mixture was subsampled, there was notable cleavage of the FXII chromogenic substrate (Figure 6.6a). This would indicate that either the purified FXII was contaminated with FXIIa or that activation of FXII was occurring due to an interaction with the microtitre plate. However, the cleavage of the substrate increased over time and appeared to be greater when neutrophils were present and even more so when the neutrophils had been preactivated (figure 6.6a), indicating that neutrophils may be able to enhance the activation of FXII.

To ascertain whether the cleavage of substrate was due to contamination of the purified FXII with FXIIa, a new batch of FXII was used. Surprisingly, using the new batch of FXII there was little cleavage of the substrate at time zero and this did not significantly change with time in either the absence or presence of neutrophils (Figure 6.6b).
Figure 6.6 Cleavage of FXII substrate by buffer neutrophils or activated neutrophils incubated with a) old FXII, b) new FXII, c) new:old FXII 9:1 (n=3, mean ± SEM)
This indicated that the previous batch of FXII was indeed contaminated with FXIIa, but more interestingly, it appeared that in order for neutrophils to enhance FXII activation, there was a prerequisite for FXIIa to be present in the first instance.

This was tested further by using a mixture of new FXII:old FXII in the test system at a ratio of 9:1, allowing only a small contamination of FXIIa in the reaction mixture. In the absence of neutrophils, the cleavage of the substrate over time was small, but was enhanced by the presence of resting neutrophils and further enhanced in the presence of activated neutrophils (Figure 6.6c).

The generation of FXIIa by neutrophils could be prevented by incubation with C1-INH or prefixation of the neutrophils (Table 6.7). After centrifugation of the neutrophils following incubation with FXII, the majority of the FXIIa generated was released into the supernatant (Table 6.7)

<table>
<thead>
<tr>
<th>Sample</th>
<th>A405nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>0.086</td>
</tr>
<tr>
<td>Buffer + FXII</td>
<td>0.210</td>
</tr>
<tr>
<td>Neuts only</td>
<td>0.200</td>
</tr>
<tr>
<td>Neuts + FXII</td>
<td>0.779</td>
</tr>
<tr>
<td>Neuts + FXII + C1-INH 0.32 U/ml</td>
<td>0.199</td>
</tr>
<tr>
<td>Neuts + FXII + C1-INH 0.64 U/ml</td>
<td>0.195</td>
</tr>
<tr>
<td>Neuts + FXII + C1-INH 1.27 U/ml</td>
<td>0.202</td>
</tr>
<tr>
<td>Fixed neuts + FXII</td>
<td>0.226</td>
</tr>
<tr>
<td>Neuts + FXII supernatant only</td>
<td>0.673</td>
</tr>
</tbody>
</table>

*Table 6.7 The effect of C1-INH and fixation on FXIIa generated by neutrophils*

Buffer or neutrophils were incubated with 25µg/ml of FXII for 1 hour at 37°C before the addition of FXII substrate. Neutrophils were pre-fixed with 1% paraformaldehyde or C1-INH added prior to incubation with FXII.

6.3.5 Activation of FXII by CPB circuitry

Initially the CPB tubing was incubated with either normal plasma or PBS as a control. However, in the PBS control there was greater binding of the antibodies than in normal plasma. This was presumably due to non-specific binding of the antibody to the plastic.
tubing in the absence of plasma proteins which may help to block this. Indeed, when the tubing was incubated with PBS, the binding of the secondary antibody was similar whether the primary antibody was present or not, suggesting that the secondary antibody bound non-specifically in the absence of plasma proteins.

A more appropriate control was therefore to incubate the tubing with normal plasma with either the primary antibody against FXIIa or an isotype matched control to estimate non-specific binding. In this case, the binding of the FXIIa antibody was much greater than the isotype matched control suggesting that FXIIa was bound to the CPB tubing and could be detected immunologically.

As the specificity of antibody binding remained questionable it was decided to incubate the tubing with immunodepleted FXII (and presumably FXIIa) deficient plasma or normal plasma to determine specificity. The FXII deficient plasma gave similar results to the normal plasma. This suggested that either there was a non-specific binding of the FXIIa antibody or that there was FXIIa present in the FXII deficient plasma. The FXII deficient plasma used and a number of commercially available immunodepleted or congenital FXII deficient plasmas were assayed for FXIIa antigen as described in section 2.4. Surprisingly, most commercial immunodepleted FXII deficient plasmas contained FXIIa (one as high as 8.0 ng/ml), but FXII deficient plasma derived from congenitally deficient patients did not contain detectable levels of FXIIa. Plasma from one of these patients was then selected for use in further experiments.

When repeated, similar results were obtained using congenitally deficient FXII plasma or normal plasma. It was deemed possible that even though the FXII deficient plasma did not have detectable levels of FXIIa, it may contain a small amount of FXIIa which could promote further FXIIa generation. To overcome this, the congenital deficient plasma was passed down a sepharose column which had been linked to a FXII polyclonal antibody (Sheep anti-human FXII, The Binding Site Ltd) to remove any FXIIa present in the plasma.

When repeated using congenitally deficient plasma which had been passed through the column, similar results to normal plasma were still obtained. The binding of
the FXIIa antibody to the CPB tubing could not be demonstrated as being specific, therefore this work was not pursued any further.

6.3.6 Measurement of FXIIa antigen in ultrafiltrate samples

FXIIa antigen was only found in the ultrafiltrate of 5/7 patients in which the haemofilter remained patent for longer than 24 hours (Table 6.8). Plasma levels of FXIIa antigen did not significantly change during haemofiltration in these patients (Chapter 3).

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Table 6.8 Levels of FXIIa antigen in the ultrafiltrate of patients undergoing haemofiltration (from chapter 3).

ND = not detectable; NA = not available

6.4 Discussion

Lipoproteins

In the first part of this chapter, postprandial plasma from healthy subjects was used to separate lipoproteins into VLDL, IDL, LDL and HDL by preparative ultracentrifugation. When these fractions were assayed for FVIIa or FXIIa, only HDL appeared to contain any of these proteins. However, this may be due to artefactual changes occurring during the isolation procedure.
HDL is the highest density lipoprotein and hence the last to be isolated by this procedure. If the association of FXIIa and FVIIa with lower density lipoproteins is weak, these proteins may dissociate from the lower density lipoproteins and end up in the higher density fraction. Also exchange of apolipoproteins during ultracentrifugation for long periods of time may occur. Normally lipoproteins are isolated using serum with EDTA added to it to prevent modification of the lipoprotein during the procedure. In this study, in order to assay FXIIa and FVIIa in the fractions, sodium citrate had to be used as an anticoagulant, which is not ideal for isolation purposes. In addition, the amount of FXIIa and FVIIa found in the HDL fraction was greater than their plasma concentrations, which would suggest contamination with plasma proteins that were increased due to 'cold activation' of the sample, or that HDL is a major source of circulating FXIIa and FVIIa. It was felt that the observed results were probably due to artefactual changes and therefore alternative methods of obtaining lipoproteins were considered.

Chylomicra were chosen to study the association and activation of FXIIa and FVIIa on triglyceride-rich lipoprotein as these could be isolated quickly from postprandial plasma, thus reducing artefactual changes occurring during the separation procedure itself. The action of lipase and detergent treatment of the chylomicra on FXIIa and FVIIa were studied and compared to changes occurring in plasma.

In normal subjects following the ingestion of fat there was an increase in plasma FVIIa as reported by other groups (Miller et al, 1991 & 1996b; Silveira et al, 1994 a&b). In the absence of tissue factor, an increase in plasma FVIIa may prove inconsequential, but the rupture of an atheromatous plaque, endothelial damage or monocyte activation may expose tissue factor and under these circumstances raised levels of FVIIa could promote coagulation with undesirable consequences. This increase in plasma FVIIa was not related to an increase in plasma FXIIa antigen, which is in agreement with the work of Miller et al 1996b. However, as with any studies measuring FXIIa antigen, care must be taken in interpreting these data as the molecular species detected by this assay are not fully understood.
There are two well characterised molecular forms of FXIIa, with different molecular weights, produced by sequential proteolysis (αFXIIa & βFXIIa). The initial activation of FXII produces αFXIIa, which remains surface bound, but further cleavage of αFXIIa produces βFXIIa, which lacks a surface binding region, and is released into the fluid phase (Griffin & Bouma, 1987). βFXIIa is rapidly inactivated by the formation of irreversible complexes with its natural inhibitors (predominantly C1-INH and antithrombin III).

The FXIIa antigen assay relies on a monoclonal antibody which recognises α- and βFXIIa, with no binding to native FXII zymogen (Ford et al, 1996) or FXIIa:inhibitor complexes (Esnouf et al, 1994). Therefore during FXII activation in vivo, αFXIIa will remain surface bound and βFXIIa can rapidly complex to its inhibitors and may be thus be unavailable to the plasma assay system. In addition, it is not known if this assay detects FXIIa associated with lipoproteins in plasma.

The results from this study indicate that FXIIa is associated with chylomicra-rich lipoproteins in normolipidaemic subjects. Moreover, the treatment of plasma or isolated chylomicra-rich lipoproteins with a detergent (Triton X-100) resulted in an increase in FXIIa levels which suggests that disruption of the lipoprotein structure may expose antigenic sites previously not ‘seen’ by the FXIIa monoclonal antibody. During the postprandial period the percentage increase in FXIIa in plasma produced by detergent treatment increased with time and yet we noted a decrease in both plasma and chylomicra-rich lipoprotein associated FXIIa which was not due to a reduction in chylomicra-rich lipoprotein particle concentration. Taken together these findings may indicate that there may be a redistribution of FXIIa from plasma and chylomicra-rich lipoproteins to other plasma lipoproteins or constituents in the postprandial period.

Lipase treatment of plasma and chylomicra-rich lipoproteins produced a significant increase in FXIIa levels. The % increase in FXIIa with lipase treatment increased over time, presumably reflecting an increase in lipase substrate (triglyceride). Mitropoulos et al, 1992 observed an increase in FXIIa activity on lipase treatment of plasma from a lipoprotein-lipase deficient patient and in standard plasma supplemented
with VLDL. They did not observe any changes in FXIIa with lipase treatment of standard plasma or the VLDL fraction in the absence of plasma. This discrepancy may result from the relative insensitivity of the amidolytic assay for FXIIa compared to the ELISA, or that we used chylomicra-rich lipoproteins not VLDL and that the preparation of lipoproteins by lengthy sequential ultracentrifugation techniques may result in loss or transfer of proteins between lipoproteins.

The dramatic increase in FXIIa observed with lipase treated isolated chylomicra-rich lipoproteins, may be augmented by the fact that plasma inhibitors such as C1-INH may not be present in the chylomicra-rich lipoprotein fraction. The increase in FXIIa with lipase treatment was greater than with Triton X-100 treatment in the chylomicra-rich fraction but not so in plasma. This would suggest that activation of FXII is occurring on the surface of chylomicra-rich lipoproteins following lipase treatment in the absence of plasma inhibitors and that the increase in FXIIa seen is not merely due to disruption of the lipoprotein structure with subsequent release of FXIIa. The data obtained using FXIIa inhibitors before lipase treatment supports this; FXIIa generation could be partially blocked by preincubation with dichloroisocoumarin (DCIC), and almost totally blocked by preincubation with corn trypsin inhibitor (CTI). DCIC is a low molecular weight serine protease inhibitor, whilst CTI is a protease inhibitor purified from sweet corn kernels which has relative specificity for FXIIa. Both inhibitors bind to the active site of βFXIIa.

When inhibitors of FXIIa were added to purified βFXIIa, recognition of FXIIa by the monoclonal antibody employed in the FXIIa antigen ELISA was totally masked by C1-INH, partially masked by CTI and virtually unaffected by DCIC. This is not surprising given the molecular weight of the inhibitors is approximately 104,000, 11,000 and 215 respectively. It is likely that the smaller inhibitors are able to bind to the active site of FXIIa and still allow antibody binding as well (i.e. they do not mask the epitope).

The generation of FXIIa on lipase treated chylomicra-rich lipoproteins was partially, but not completely inhibited by pre-incubation with physiological concentrations of C1-INH and, as expected, to a lesser extent ATIII (heparin was absent
from the system). It is possible that when associated with lipoprotein, some FXIIa may be protected from inhibition by plasma inhibitors. It has previously been shown that FXIIa may be protected from inhibition by C1-INH when it is surface-bound rather than in the fluid phase (Pixley et al, 1987). When either of these inhibitors were added following lipase treatment of chylomicra-rich lipoproteins the inhibition of FXIIa was reduced compared to when added prior to lipase treatment. This would suggest that either FXIIa formed in the absence of inhibitors is protected from inhibition or that, when added before lipase treatment, C1-INH and ATIII inhibit whatever is activating FXII. At sub-normal concentrations of both inhibitors (as would be seen during CPB or in critically ill patients) FXIIa generation may still occur even in the presence of these inhibitors.

There was no change in plasma \( \beta_2 \)GP1 levels between fasting and postprandial samples. This is consistent with previous data from our research group (McNally et al, 1993). However, another study reported an increase in serum \( \beta_2 \)GP1 five hours following the ingestion of a fatty meal (Polz & Kostner, 1979), although this was only based upon two subjects and used a less sensitive method of detecting \( \beta_2 \)GP1 antigen. Lipase treatment reduced plasma \( \beta_2 \)GP1 levels at all time points, indicating that lipase modification of lipoprotein or other plasma constituent structure may expose binding sites for \( \beta_2 \)GP1, possibly following the liberation of FXIIa.

Chylomicra-rich lipoprotein associated \( \beta_2 \)GP1 increased over time in the postprandial period. It is of interest that the postprandial increase in plasma \( \beta_2 \)GP1 seen by Polz & Kostner appeared to be due entirely to the increase in chylomicra associated \( \beta_2 \)GP1. The latter authors propose that \( \beta_2 \)GP1 might be newly formed and/or secreted during chylomicra synthesis or that ‘nascent’ chylomicra pick up some \( \beta_2 \)GP1 from the lymph. Our observations do not preclude the possibility that \( \beta_2 \)GP1 is transferred to chylomicra, possibly from other lipoproteins or lymph. Whether \( \beta_2 \)GP1 has a role in regulating haemostatic reactions occurring on lipoprotein surfaces is unclear. The observation that plasma \( \beta_2 \)GP1 levels are increased in primary hyperlipidaemia
(McNally et al, 1994) which is thought to be associated with increased activation of the contact system (Carvalho et al, 1976), may lend weight to this notion.

Chylomicra-rich lipoprotein associated FVIIa did not increase when samples were treated with lipase. Mitropoulos et al, 1993 report that treatment of large lipoprotein particles with lipase and their subsequent addition to dilute plasma produced an increase in FVII:C following incubation at low temperatures. In the plasma system used here, the presence of C1-INH may mean that the activation of FXII is not sufficient to activate FVII or that other plasma inhibitors prevent the activation of FVII by FXIIa.

An alternative explanation is that tissue factor-independent FVII activation can occur under certain circumstances without FXII activation. This view is supported by evidence that a postprandial increase in FVIIa is observed in patients with a deficiency of FXII or factor XI but not factor IX (Miller et al, 1996b). Factor IX may therefore play an important role in FVII activation under these conditions, and further support for this view comes from a study illustrating reduced FVIIa levels in haemophilia B patients (Wildgoose et al, 1992). There was a small decrease in plasma FVIIa levels on treatment with lipase. It is possible that on lipase treatment of plasma a surface capable of adsorbing FVIIa is created. Phospholipase treatment of plasma is known to result in a decrease in FVII levels (Hubbard & Parr, 1991).

In summary, the first part of this chapter has demonstrated that FXIIa, FVIIa and \( \beta_2 \)GP1 are associated with chylomicra-rich lipoproteins in normolipidaemic subjects and that lipase treatment of plasma and chylomicra-rich lipoproteins results in further generation of FXIIa without a concomitant increase in FVIIa. This generation of FXIIa occurred even in the presence of levels of plasma inhibitors likely to be seen during CPB or haemofiltration. The results obtained from detergent treatment of plasma and chylomicra-rich lipoproteins suggest that the FXIIa antigen assay does not fully recognise FXIIa when it is associated with lipoproteins, which may be important in interpreting data obtained with this assay.

Unfortunately, not all these techniques could be applied to samples from CPB as, patients will have been fasting and hence no chylomicra will be present, and
separation of lipoproteins by preparative ultra-centrifugation may result in artefactual changes to the proteins of interest.

However, these results do suggest that previous studies investigating FXIIa levels during CPB using the commercially available FXIIa ELISA may not give a true indication of the state of FXII activation, particularly if FXIIa is associated with lipoproteins. The application of this assay following Triton-X100 treatment of plasma from CPB patients may provide new insights in this area. During CPB triglyceride levels fall dramatically (Sgoutas et al, 1992), presumably reflecting the action of heparin-released lipoprotein lipase on triglyceride to release free fatty acids. Free fatty acids at the interface of large lipoproteins may create a surface which promotes the activation of FXII, particularly in the face of reduced plasma levels of inhibitors of FXIIa such as C1-INH and ATIII, and this may not be detected using conventional methods.

Lipids may also be important in the activation of coagulation during sepsis. Thrombin generation induced by endotoxin administration in humans may be potentiated by infusion of a triglyceride-rich lipid emulsion (van der Poll et al, 1996), but reduced by infusion of reconstitued HDL (Pajkrt et al, 1997).

**Peripheral blood cells**

The experiments performed using red cell membranes or stroma failed to show any ability to activate FXII. The generation of FXIIa from purified FXII (at a physiological concentration) by isolated neutrophils (at a physiological cell count) from healthy subjects could be demonstrated if a small amount of FXIIa was present initially. This activation of FXII was enhanced further if the neutrophils were themselves pre-activated. Unlike the generation of FXIIa on lipoprotein surfaces, the generation of FXIIa by neutrophils could be totally inhibited by C1-INH, even at sub-normal concentrations. Following incubation with FXII, if the neutrophil suspension was centrifuged, the majority of the FXIIa activity generated remained in the supernatant. It would thus appear that following activation on the neutrophil surface, FXIIa is released into the fluid phase and hence it is not surprising that this activity can be totally inhibited by C1-INH.
Whether neutrophils activate FXII by specific interactions with other contact factors and receptors or merely provide a phospholipid surface on which FXII activation can take place remains unclear. The latter explanation seems unlikely given that red blood cell membranes or platelets (unpublished data from others in our laboratory) do not appear to be able to promote FXII activation. In addition, fixation of neutrophils prevented the activation of FXII which suggests that the ability of the cells to activate FXII requires a functional cell surface and not merely the presence of phospholipid.

The results from this study support the notion that neutrophils can provide a surface on which FXII activation can take place and during CPB, when neutrophils are known to become activated, this may be augmented. Unfortunately the separation of neutrophils by density gradient centrifugation for these experiments requires a considerable volume of blood and it would be unethical to remove such large volumes of blood from a patient during CPB.

Others have shown that FXII, FXI, PKK, and HK are associated with the neutrophil surface (Henderson et al, 1994). Whether these proteins are synthesised by neutrophils or associated with neutrophils through other proteins and receptors remains unanswered. As part of this chapter it was also attempted to visualise the association of contact factors with neutrophils by confocal microscopy. This technique could also be applied to CPB patients as only a small blood sample would be required. Using the monoclonal antibody available to me against human FXIIa it should also be possible using this technique to establish not only the presence of contact factors on the neutrophil surface but also to gain some insight as to whether they have any functional significance. However, a number of technical difficulties were encountered using this method (mainly high non-specific labelling) which could not easily be overcome and therefore this approach was beyond the scope of this thesis.

**CPB circuitry**

The possibility that the CPB tubing itself could potentiate the activation of FXII has been explored by other authors using *in vitro* circuits and amidolytic substrates (Irvine
et al, 1991; Sundaram et al, 1993 & 1996; Matata et al, 1996). However, in my hands this technique results in extremely small changes in optical density and hence has poor sensitivity, and is also non-specific. I therefore tried to examine FXIIa bound to CPB tubing using immunological techniques. This would have the advantage of providing both sensitivity and specificity. Although it appeared that FXIIa could be detected on CPB tubing that had plasma circulated though it, this could not be shown to be totally specific and this was therefore deemed an unacceptable method to pursue.

**FXIIa in ultrafiltrate samples**

In chapter 3 it was demonstrated that plasma levels of FXIIa antigen did not change significantly during haemofiltration. To exclude the possibility that FXIIa may be generated during haemofiltration but is cleared by the haemofilter, the ultrafiltrate from four patients who clotted their filter within 24 hours of use and who showed an increase in plasma levels of TAT complexes during haemofiltration were assayed for FXIIa antigen. FXIIa antigen was not detectable in any of these samples. On subsequent examination, FXIIa antigen was detectable in the ultrafiltrate of patients in which the haemofilter remained patent for longer than 24 hours.

This was a surprising finding, but does not change the conclusions made in chapter 3. Firstly, even if the FXIIa found in the ultrafiltrate reflects clearance of this protein from plasma, the generation of FXIIa is not related to thrombin generation in these patients. Secondly, any FXIIa generated is not likely to reflect contact activation by the haemofilter circuit, since FXIIa only appears in the ultrafiltrate towards the end of haemofiltration, whereas activation of the FXII due to contact with the extracorporeal circuit would be expected to take place within minutes of haemofiltration commencing.

Indeed, it is intriguing that FXIIa is found in the ultrafiltrate at all. The molecular weight of both αFXIIa (approximately 80 Kd) and βFXIIa:C1-INH complexes (approximately 134 Kd) would suggest that these would not be cleared by the haemofilter. This implies that the FXIIa assayed in the ultrafiltrate samples is free βFXIIa which has a molecular weight of approximately 28 Kd. Why free βFXIIa should
be found in the ultrafiltrate is unclear. All the patients studied had normal or elevated plasma levels of C1-INH, and the molar excess of C1-INH over FXIIa in plasma would favour the formation of βFXIIa:C1-INH complexes on formation of βFXIIa. However, haemofiltration is carried out at room temperature at which the ability of C1-INH to inhibit FXIIa is markedly reduced compared to 37°C (Weiss et al, 1986). In addition, if the formation of βFXIIa occurred locally in the haemofilter itself, it is possible that it could be immediately cleared across the membrane of the haemofilter, avoiding inhibition by C1-INH in plasma. It is also interesting that FXIIa was only seen in the ultrafiltrate from patients whose filters remained patent for longer than 24 hours. Since βFXIIa can activate plasminogen directly (Goldsmith et al, 1978) it is conceivable that contact mediated fibrinolysis could help to maintain the patency of the haemofilter.

In conclusion, in this chapter it was demonstrated that the activation of FXII can occur on the surface of triglyceride-rich lipoproteins and under such circumstances may be partially protected from inhibition by plasma protease inhibitors. Furthermore, during CPB and haemofiltration the generation of FXIIa on lipoproteins may be enhanced since (i) C1-INH levels are sub-normal and (ii) the inhibitory capacity of C1-INH present is reduced, as both the extracorporeal circuits are used at temperatures between 25-32°C at which the ability of C1-INH to inhibit FXIIa is known to be reduced compared to 37°C. The generation of FXIIa from purified FXII by neutrophils isolated from healthy subjects was also demonstrated and this activity was enhanced if the neutrophils were 'activated'. This appeared to be a specific function of the neutrophil rather than the cell merely providing a phospholipid surface on which the activation of FXII could take place.

It is possible that FXIIa may be generated during CPB by these and other mechanisms (Figure 6.7) which may not be detectable using previously employed methods. The notion that activation of the contact system during CPB contributes, at least in part, to thrombin generation cannot be rejected.
Figure 6.7 Possible mechanisms of FXII activation during CPB
7.1. Introduction

In chapter 5 it was demonstrated that during CPB there is generation of thrombin without any evidence of systemic activation of either the tissue factor pathway or the contact system. The mechanisms responsible for this thrombin generation during CPB therefore remained unclear. Since that study was undertaken, several authors (Chung et al, 1996; Nieuwland et al, 1997) have suggested that activation of the tissue factor pathway occurs locally at the site of surgery, and that it is the reinfusion of ‘activated’ blood from the surgical field that is responsible for thrombin generation seen in the systemic circulation during CPB.

During cardiac surgery, once the patient has been anticoagulated systemically with heparin, blood shed in the surgical field (i.e. the pericardial cavity) is normally returned to the patient. Exactly what blood is recovered and when, and how it is processed before being returned to the patient varies depending upon the institution and CPB circuitry used. The policy at UCL Hospitals is that once the patient has been heparinised, blood in the surgical field is sucked up with the cardiotomy sucker, and infused directly into the venous reservoir of the CPB circuit. Thus from the initiation of CPB, blood from the pericardial cavity is reinfused into the patient.

Blood from the pericardial cavity is known to contain increased concentrations of TAT complexes, tPA antigen, fibrin degradation products (Tabuchi et al, 1993), Pro F1+2, FVIIIa, monocyte expressed tissue factor (Chung et al, 1996), β-TG, C3a (de Haan et al, 1996) and procoagulant cell derived microparticles (Nieuwland et al, 1997) compared to blood from the systemic circulation during CPB. Thus locally in the
pericardial cavity there may be enhanced activation of platelets, coagulation, fibrinolysis and complement.

The main objective of this study was to examine activation and control of the tissue factor pathway in blood from the pericardial cavity compared to systemic circulation during CPB. This was achieved by taking samples at eight time points during the peri-operative period from the systemic circulation and at four time-points from the pericardial cavity once the chest had been opened and the patient had been heparinised.

A second objective of this chapter was to further investigate the activation of FXII in the systemic circulation during CPB. Since there is disagreement between authors as to the course of FXIIa antigen levels during CPB (Boisclair et al, 1993; Grossman et al, 1996) and that FXIIa antigen may be masked when this protein is associated with lipoproteins (as demonstrated in Chapter 6), it was decided to measure FXIIa antigen during CPB in the presence and absence of a detergent to disrupt plasma lipoprotein structure. FXIIa antigen was also measured in plasma after treatment with lipase to release free fatty acids from lipoprotein particles.

The results presented here are from a small number of patients. This study was designed as a preliminary investigation before continuing with larger patient numbers, since the collection of samples and performance of assays are time consuming, and many of the assays expensive to carry out. This work will now form part of an ongoing study in which I will be collecting samples from many more patients. To include all patients is beyond the scope of this thesis.

7.2. Methods

7.2.1 Patient selection

3 patients undergoing first time coronary bypass grafting (CABG) surgery were studied. Patients receiving aprotinin were excluded from the study. Written informed consent was obtained from the patients at a pre-operative visit and ethical permission to undertake the study was obtained from the joint UCL/UCLH Committee on the Ethics of Human Research.
7.2.2 Cardiopulmonary bypass

All patients received an identical anaesthetic protocol and were treated by the same anaesthetic and surgical team. Patients underwent standard CPB procedure using hollow fibre oxygenators (‘Quantum’, Bard Ltd). The extracorporeal circuit was primed with 1000ml of Hartmann’s solution and 500ml of Gelofusine containing 5000 IU unfractionated heparin. After systemic heparinisation (300 IU/kg) the activated clotting time was maintained at greater than 450 seconds by the administration of additional heparin as required. All patients were cooled to 32°C. At the end of the procedure heparin was neutralised by the administration of an appropriate dose of protamine sulphate (3.0-4.5mg/kg).

7.2.3 Sample collection

Blood samples from the systemic circulation were collected at a total of eight time points during the peri-operative period as follows: 1) baseline sample at anaesthesia; 2) post sternotomy, pre heparin administration; 3) post sternotomy, post heparin, pre CPB; 4) 5 minutes on CPB; 5) 30 minutes on CPB; 6) 60 minutes on CPB; 7) 90 minutes on CPB; 8) end of CPB. Samples of blood from the pericardial cavity were collected at 4 time points after the chest had been opened (points 3-6). Blood samples were collected into EDTA and citrate tubes and plasma was prepared as detailed in section 2.1.3. Full blood counts were determined using EDTA whole blood as outlined in section 2.2.

7.2.4 Measurement of haemostatic factors

FVIIa was assayed according to the method of Morrissey et al, 1993 after heparin neutralisation (section 2.5). TAT complexes, prothrombin fragment F1+2 (Pro F1+2), TFPI, FXIIa ag, and soluble tissue factor (sTF) were assayed by solid phase ELISA as described in section 2.4. Heparin levels were quantitated using an amidolytic anti-Xa assay in the presence of excess AT-III (section 2.3).

Plasma samples from the systemic circulation were also treated with lipase or detergent before assaying for FXIIa antigen. Lipase treatment was performed by the
addition of 0.50 U/ml of lipase type XIII from Pseudomonas to plasma followed by incubation at 37°C for 60 minutes. Detergent treatment was performed by the addition of 1% (v/v) Triton X-100 to plasma followed by thorough mixing.

7.2.5 Correction for haemodilution

All results, including samples from the pericardial cavity, were corrected to allow for haemodilution occurring during the use of extracorporeal circulation. This was achieved by measuring the haematocrit and multiplying parameters at each time point by the ratio of haematocrit at baseline to haematocrit at that time point.

7.3 Results

Table 7.1 shows the characteristics of the three patients undertaking the study. None of the patients received platelets or fresh frozen plasma during the peri-operative period.

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*Table 7.1. Patient characteristics*

There was an increase in TAT complexes in the systemic circulation during CPB in all three patients (Figure 7.1). The level of TAT complexes in blood taken from the pericardial cavity at all time points was much higher than in the systemic circulation (an approximate 50-200 fold increase) and tended to increase during CPB (Figure 7.1). Similar results to TAT complexes were found with Pro F1+2 (Figure 7.2), although in two patients the rise in Pro F1+2 occurred earlier during the operation.
Figure 7.1 Plasma levels of TAT complexes in blood taken from a) the systemic circulation and b) the pericardial cavity during CPB

Time points: 1) baseline 2) post-sternotomy, pre-heparin 3) post-heparin, pre-CPB 4) 5 mins CPB 5) 30 mins CPB 6) 60 mins CPB 7) 90 mins CPB 8) end of CPB.

Results are corrected to allow for haemodilution.
Figure 7.2 Plasma levels of prothrombin fragment 1+2 in blood taken from a) the systemic circulation and b) the pericardial cavity during CPB

Time points: 1) baseline 2) post-sternotomy, pre-heparin 3) post-heparin, pre-CPB 4) 5 mins CPB 5) 30 mins CPB 6) 60 mins CPB 7) 90 mins CPB 8) end of CPB.

Results are corrected to allow for haemodilution.
Baseline systemic levels of plasma tissue factor were above the reference range (63-129 pg/ml) in all three patients prior to CPB (Figure 7.3). During CPB there was a decrease in tissue factor in the systemic circulation such that at the end of CPB there was no detectable tissue factor in all patients. Blood from the pericardial cavity contained higher levels of tissue factor compared to the systemic circulation (approximately 2-5 fold) which tended to increase during CPB (Figure 7.3).

In the systemic circulation FVIIa levels were within the reference range pre-operatively, decreased following heparin administration and did not alter greatly during CPB (Figure 7.4). Blood from the pericardial cavity contained higher levels of FVIIa compared to the systemic circulation (Approximately 8-70 fold, Figure 7.4).
Figure 7.4 Plasma levels of FVIIa in blood taken from a) the systemic circulation and b) the pericardial cavity during CPB

Time points: 1) baseline 2) post-sternotomy, pre-heparin 3) post-heparin, pre-CPB 4) 5 mins CPB 5) 30 mins CPB 6) 60 mins CPB 7) 90 mins CPB 8) end of CPB.

Results are corrected to allow for haemodilution.
Levels of unfractionated heparin were lower in blood from the pericardial cavity compared to the systemic circulation (Table 7.2). This was not due to endogenous proteases in blood from the pericardial cavity cleaving the FXa amidolytic resulting in falsely low heparin levels. When plasma heparin concentrations were corrected by haematocrit, the % difference between levels in the pericardial blood compared to systemic blood was reduced compared to uncorrected data.

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Table 7.2 Plasma level of unfractionated heparin (IU/ml) in blood from the systemic circulation and pericardial cavity during CPB.

Time points: C) post-heparin, pre-CPB D) 5 mins CPB E) 30 mins CPB F) 60 mins CPB G) 90 mins CPB H) end of CPB.

There was an increase in plasma TFPI following heparinisation in the systemic circulation (Figure 7.5). Blood from the pericardial cavity had lower TFPI levels compared to the systemic circulation (25-55%) at all time points and in all patients (Figure 7.5). The % reduction in TFPI in the pericardial blood compared to systemic was greater than the % reduction in heparin concentrations between these two sites.
Figure 7.5 Plasma levels of TFPI antigen in blood taken from the systemic circulation and the pericardial cavity during CPB

Time points as in figure 7.4. Results are corrected to allow for haemodilution.

Figure 7.6 Plasma level of FXIIa antigen in blood from the systemic circulation and pericardial cavity during CPB

Time points and legend as in figure 7.4. Results are corrected to allow for haemodilution.
Baseline levels of FXIIa antigen were within the reference range in all patients. There was an increase in FXIIa in the systemic circulation following heparin administration (Figure 7.6), but further changes in FXIIa levels on institution of and during CPB were small.

Treatment of plasma with Triton X-100 resulted in an increase in FXIIa antigen of approximately 140-190%. During the period of CPB there was an increase in the % change in FXIIa antigen on treatment with detergent (Figure 7.7). Plasma FXIIa increased during CPB when treated with detergent (Figure 7.8). Treatment of plasma with lipase resulted in small changes in FXIIa antigen (Table 7.3).

**Figure 7.7** The % increase in FXIIa antigen following treatment of plasma with detergent

Time points as in figure 7.4. Plasma was treated with 1% (v/v) Triton X-100.
Figure 7.8 Plasma levels of FXIIa antigen during CPB following treatment of plasma with detergent

Time points as in figure 7.4. Results are corrected to allow for haemodilution. Plasma was treated with 1% (v/v) Triton X-100.

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Table 7.3 FXIIa antigen levels (ng/ml) in plasma with and without lipase treatment from patients during CPB

Plasma was treated with 0.5U/ml lipase for 60 minutes at 37°C. Time points as in Figure 7.4.
7.4 Discussion

A significant amount of blood is lost during cardiac surgery, which originally led to a major blood transfusion requirement, although recent improvements in surgical techniques have progressively reduced this. The transfusion of homologous blood or blood products carries with it a risk of infection and immunologic reaction. To reduce the risk and cost associated with the use of homologous blood products, several methods for conserving blood during and after cardiac surgery have been established.

Firstly, during cardiac surgery blood from the surgical field is usually re-infused into the patient during the operation. Secondly, blood which is drained from the mediastinal chest tubes post-operatively may also be reinfused into the patient. In both cases, either whole blood is reinfused or whole blood is passed through a ‘cell saver’ system and only washed cells are returned to the patient. However, the safety and efficacy of these procedures remains questionable. Although early studies demonstrated that reinfusion of shed mediastinal blood post-operatively reduced homologous blood requirements (Shaff et al, 1978), others have suggested that in routine cases, at least, it may be unwarranted or even deleterious (Adan et al, 1989; Griffith et al, 1989; Roberts et al, 1991; Bouboulis et al, 1994).

Although many studies have focused on the nature of the post-operatively shed mediastinal blood, less attention has been paid to blood shed in the surgical field during cardiac surgery. Decades ago it was demonstrated that haemolysis of blood in the pericardial cavity was an important source of increased plasma haemoglobin seen during extracorporeal circulation (Morris et al, 1965) and that pericardial blood contained high levels of fibrin degradation products and had enhanced fibrinolytic capacity (Okies et al, 1977). In this study, the degree of activation and inhibition of the tissue factor pathway and thrombin generation was examined in blood from the pericardial cavity compared to blood in the systemic circulation during CPB.

As expected, in the systemic circulation there was an increase in markers of thrombin generation during CPB. Moreover, blood from the pericardial cavity had far higher levels (greater than 50 fold) of TAT complexes and Pro F1+2 compared to the
systemic circulation. This suggests that there is substantial thrombin generation occurring locally at the site of surgery during CPB, confirming the results of other studies which have shown increased levels of TAT complexes (Tabuchi et al, 1993) and Pro F1+2 (Chung et al, 1996) in pericardial compared to systemic blood during cardiac surgery. However, in the present study the degree of thrombin generation in the pericardial cavity is larger than in both the latter studies where a 2-3 fold increase was observed.

One possible mechanism by which large amounts of thrombin could be generated in the surgical field is due to the exposure of tissue factor by gross surgical trauma. Levels of soluble tissue factor were enhanced pre-operatively and decreased in the systemic circulation during CPB and by the end of CPB were undetectable. Since the assay recognises TF:FVII complexes, this fall cannot be explained by the binding of tissue factor to FVII. It is possible that soluble tissue factor may be rapidly cleared during CPB or that it becomes bound to CPB circuitry, cell surfaces or other proteins which prevent its detection.

There was an approximate 2-5 fold increase in soluble tissue factor levels in blood from the pericardial cavity compared to systemic circulation before the initiation of CPB, which increased further during CPB. This suggests that release of tissue factor is occurring in the surgical field during CPB. Consistent with this it has been demonstrated using the ELISA employed here that plasma tissue factor levels are above the reference range pre-operatively, but lower post-operatively in patients undergoing cardiac surgery (Davidson et al, 1997) and, approximately five fold higher in shed mediastinal blood compared to systemic blood post-operatively (Ernoffson et al, 1997c). The only other published study to examine soluble tissue factor during CPB showed a tendency for systemic levels to increase peri-operatively, although this was not significant (Grossman et al, 1996).

Plasma levels of FVIIa in the systemic circulation tended to increase after sternotomy, rapidly decrease after the administration of heparin and then remain fairly constant until the end of CPB, which confirms the observations made in Chapter 5.
Pericardial blood contained much higher levels of FVIIa compared to systemic blood (approximately 10-70 fold). Chung et al (1996) observed a two-fold increase in FVIIa levels in pericardial compared to systemic blood during CPB. They also observed a small increase in FVIIa in the systemic circulation between samples taken after heparin administration (0.38ng/ml) and immediately post-operatively (0.44ng/ml), suggesting that activation of FVII occurs during CPB. In this study, although there was large amounts of FVIIa in the pericardial blood, no increase in FVIIa was seen in the systemic circulation during CPB.

In the systemic circulation during CPB, there was a substantial increase in TFPI following heparin administration which remained elevated until the end of the procedure which confirms the observations made in Chapter 5. Presumably the suppression of FVIIa during the same period is due to the heparin-induced release of TFPI as suggested in Chapter 5. It is possible that Chung et al (1996) observed an increase in FVIIa in the systemic circulation during CPB because plasma TFPI levels were not maintained during this period. The latter authors did not measure TFPI in their study.

Pericardial blood contained lower levels of TFPI compared to systemic blood during CPB. This could be explained by either 1) a lower concentration of heparin in the pericardial blood, 2) consumption of TFPI or 3) that less TFPI is released in the pericardial cavity compared to systemic circulation.

Heparin concentrations were lower in pericardial blood compared to systemic. In two patients they were approximately 50% of systemic levels, and in one about 80%. If the heparin levels were corrected to allow for changes in haematocrit between the two sites, it could be seen that the reduction in heparin levels in the pericardial blood was partly, but not solely due to a slight increase in haemodilution of pericardial blood compared to systemic. Others have shown that heparin concentrations in pericardial blood are approximately 50% (Tabuchi et al, 1993) and 20% (de Haan et al, 1995) of systemic levels during CPB. In the latter two studies heparin levels in pericardial blood were reported to be approximately 1 and 0.75 IU/ml respectively, but in this study, the level of anticoagulation achieved in the pericardial blood was greater (approximately 2-3
IU/ml). This may have important consequences since Tabuchi et al showed that the ability of rabbit pericardium to generate thrombin and plasmin in vitro was markedly reduced in the presence of heparin at 2.5 IU/ml compared to 1.5 IU/ml.

Although it is likely that reduced heparin concentrations contributed to reduced levels of TFPI in the pericardial blood, this cannot be the only factor as one subject who had similar levels of heparin in pericardial and systemic blood had a lower ratio of pericardial:systemic TFPI than one with levels of heparin in pericardial blood 50% that of systemic blood. The increased activation of FVII seen in pericardial blood could be responsible for consumption of TFPI. However, since the TFPI assay employed here detects free TFPI, TFPI:FVIIa:TF and TFPI:FXa:FVIIa:TF complexes, reduced levels of TFPI are unlikely to be due to TFPI binding to TF:FVIIa complexes, but could be due to binding to cell or other surfaces. When larger patient numbers are available, the nature of relationships between TFPI, FVIIa and thrombin generation in pericardial blood will become more evident.

A recent study has also shown that pericardial blood contains higher levels of cell-derived microparticles than systemic blood during CPB (Nieuwland et al, 1997). In the systemic circulation these microparticles were derived mainly from platelets, whereas in pericardial blood they were derived mainly from erythrocytes but also from platelets, granulocytes and other unidentified cells. These microparticles, especially those isolated from pericardial blood, were able to support the generation of thrombin (using the endogenous thrombin potential) via the tissue factor pathway, and this reaction could be inhibited by the addition of recombinant TFPI. Strategies to enhance TFPI levels in the pericardial blood may therefore be beneficial in controlling activation of the tissue factor pathway associated with both microparticles and the fluid phase.

Reinfusion of this 'activated' pericardial blood during CPB has been shown to increase post-operative blood loss (de Haan et al, 1995). Moreover, two studies have shown an that there is an increase in systemic TAT complex levels during CPB only if retransfusion of pericardial blood occurs (Tabuchi et al, 1993; de Haan et al, 1996). However in the former study there was an increase in systemic TAT complex during
CPB in patients in which the retransfusion of pericardial blood was withheld, although this was not significant, probably due to small patient numbers. Since there is an increase in TAT complex levels during simulated extracorporeal circulation (Takano et al., 1992) where the patient is removed from the equation, surgical trauma and the reinfusion of pericardial blood are not the only mechanisms by which thrombin generation occurs during CPB.

In summary of the first part of this chapter, it was demonstrated that during CPB there was increased release of tissue factor, activation of FVII, and thrombin generation, but reduced levels of TFPI and heparin in blood taken from the pericardial cavity compared to systemic circulation. It is likely that reinfusion of this ‘activated’ blood could contribute to, but is not the sole mechanism by which systemic coagulation activation occurs during CPB. Strategies to reduce systemic coagulation activation due to the reinfusion of pericardial blood would include 1) the topical use of heparin in the pericardial cavity to increase local anticoagulation, 2) the topical use of aprotinin in the pericardial cavity to reduce coagulation and fibrinolytic activity, 3) the use of a cell saver to wash pericardial blood thus removing activated plasma components whilst permitting the autotransfusion of washed cells or 4) withholding reinfusion of pericardial blood and transfusing with predonated autologous blood.

The second part of this chapter was concerned with further experiments to investigate the activation of FXII during CPB. Some of these experiments were performed on systemic blood only. In the systemic circulation there was an increase in plasma FXIIa following heparin administration but prior to CPB. Heparin is known to reduce the ability of C1-INH to inhibit FXIIa (Gallimore et al., 1992b), therefore it is possible that the administration of heparin may enhance FXIIa levels by reducing inhibition of the contact system. In two patients FXIIa levels changed little during CPB, but in one patient further increases in FXIIa were seen during CPB. Therefore the course of FXIIa levels during CPB may vary depending on the patient. Boisclair et al. (1993) showed that FXIIa antigen levels did not change significantly during CPB, whereas Grossman et al. (1996) demonstrated an increase in FXIIa levels during CPB.
As already discussed in Chapters 5 and 6, during CPB on activation of FXII αFXIIa would be expected to remain surface bound and βFXIIa to rapidly complex to plasma inhibitors. Given that the FXIIa antigen assay does not recognise FXIIa:inhibitor complexes, it is not surprising that little change in FXIIa levels are observed during CPB. However, it will be interesting to see the results obtained from additional patients to see whether an increase in plasma FXIIa during CPB is exceptional or frequently observed. Pericardial blood contained higher levels (approximately two-fold) of FXIIa compared to systemic blood during CPB. This is not surprising as pericardial blood will contain fragments of bone, tissue, fat, and will have an increased blood-air interface. Activation of the contact system in the pericardial blood could result in the generation of free FXIIa due to the likely reduced levels of plasma inhibitors.

In Chapter 6 it was demonstrated that the FXIIa antigen assay did not fully recognise FXIIa when it is associated with lipoproteins and that detergent treatment of plasma could expose previously undetectable FXIIa antigen by disrupting lipoprotein and possibly other structures. In the present study, during CPB there was an increase in the amount of FXIIa liberated by treatment with a detergent. This suggests that FXII may become activated during CPB, but the association of FXIIa with lipoproteins and other surfaces mask any changes in FXIIa in untreated plasma.

The treatment of plasma with lipase produced very little change in FXIIa antigen during CPB. This is not surprising in the samples following heparin administration, since there will be substantial lipase activity in vivo during this period. However, at the time points before heparin administration one would expect to see an increase in FXIIa antigen with lipase treatment even though the patients will have been fasting pre-operatively. The lipase preparation was tested for its ability to produce free glycerol from triglyceride and found to be functional. It is possible that anaesthesia and stress are causing the plasma from these patients to behave differently from normal subjects in this respect.

The results from the second half of this chapter demonstrate that previous studies (Boisclair et al, 1993) measuring FXIIa antigen levels during CPB may have
drawn misleading conclusions from their data. Activation of the contact system may occur during CPB, but methods used previously have failed to detect this. The generation of FXIIa may occur on the surface of triglyceride-rich lipoproteins where it is not fully recognisable by the monoclonal antibody used in the FXIIa ELISA.

Obviously the number of patients studied in this chapter is very small which does not lend itself to firm conclusions. However, it has enabled some of the ideas which were demonstrated \textit{in vitro} in Chapter 6 to be applied to samples obtained from patients during CPB.
The aim of this thesis was to examine mechanisms of coagulation activation during extracorporeal circulation. What originally appeared to be a straightforward investigation proved to be far more complex than anticipated. The ambiguous nature of some of the results has required great reconsideration of some of the mechanisms and pathways leading to thrombin generation. In evaluating these, there have been considerable technical difficulties, and some of the problems that need further investigation will have to await technological advances and the provision of new biochemical and immunological tools. However, some of the results obtained were unexpected and raised further interesting questions. These avenues of thought led to the exploration of areas which I had not considered when this thesis was initiated. It was the investigation of these ‘other areas’ in several instances that provided some of the more interesting results in this thesis.

Two groups of patients were selected for study. The first group were patients undergoing elective heart surgery that required the use of CPB and were therefore relatively healthy (their haemostasis was not compromised) prior to their operation. The second group were critically ill patients in intensive care who had acute renal failure requiring the use of haemofiltration to function as their kidney. In contrast to the first group, the latter patients were more likely to have a compromised haemostatic system prior to extracorporeal circulation due to the underlying condition of the patient.

The desire to investigate activation of the coagulation system in the patients receiving haemofiltration was driven by observations made by clinicians on the Intensive Care Unit at UCL Hospitals. They noticed that instead of haemofilter circuits remaining patent for 48-72 hours as the manufacturer’s suggest, many patients clotted their filters within a few hours of use and this appeared to happen more frequently in sicker patients, even in those with concurrent thrombocytopenia and/or coagulopathies.
It was important to establish that these patients were being adequately anticoagulated during haemofiltration. Prior to this thesis, it was demonstrated by other workers in our research group that plasma heparin concentrations fell within the desired therapeutic range during haemofiltration using the local regime and that heparin was not cleared across the membrane of the haemofilter. A more detailed study of the haemostatic system during haemofiltration was thus embarked on.

Since during CPB exposure of blood to the foreign surface of the extracorporeal circuit was thought to result in activation of the contact system, it seemed reasonable to expect that during haemofiltration there may be a progressive activation of the contact system due to contact with the haemofilter circuit, which could lead to the generation of thrombin with consequent occlusion of the filter by fibrin and platelets. Activation of coagulation was also likely to be enhanced if patients had reduced levels of inhibitors of coagulation prior to haemofiltration. Activation of the contact system during haemofiltration was assessed by taking serial samples from patients during haemofiltration until their filter occluded, and measuring zymogen proteins and inhibitors of the contact system. A new immunoassay for FXIIa antigen was also employed to assess the activation of FXII. Changes in these proteins were then related to the generation of thrombin as evidenced by increasing levels of TAT complexes (Chapter 3). The lifespan of the haemofilter was not related to choice of anticoagulant, or the platelet count or INR prior to haemofiltration. Although levels of the contact factors FXII and PKK were reduced and FXIIa antigen increased prior to haemofiltration in most patients, suggesting an underlying degree of activation of the contact system, there were no further changes in the levels of these proteins during the period of haemofiltration.

Baseline levels of TAT complexes were increased in most patients, but only patients clotting their filters within 24 hours of use showed further increases in TAT during haemofiltration; these patients also had the lowest levels of the inhibitors ATIII and HCII prior to haemofiltration. There was an inverse relationship between the filter lifespan and degree of increase in TAT complexes. It would therefore appear that gross...
activation of the contact system was not occurring during haemofiltration, but rather that reduced levels of ATIII and HCII prior to haemofiltration may allow greater thrombin generation to take place when some trigger for coagulation activation is provided.

The stimulus for the generation of thrombin during haemofiltration remained unclear. It was postulated that in critically ill patients activation of the tissue factor pathway may occur during haemofiltration as a result of tissue factor exposure due to endothelial activation, or its expression by monocytes (many of these patients have sepsis). It was therefore decided to undertake another study to examine activation of the tissue factor pathway as well as to investigate whether the PC/PS system was compromised in during haemofiltration which might further enhance the generation of thrombin (Chapter 4).

In the second study on haemofiltration the results of the previous chapter were confirmed: activation of coagulation with subsequent generation of thrombin was occurring during haemofiltration and this was related to filter occlusion. Most patients had reduced levels of PC and both free and total PS prior to haemofiltration. The reduction in PC seen in these patients was most likely due to enhanced consumption of the protein. However, there were no further changes in the levels of PC or PS during haemofiltration suggesting that continuous activation and depletion of components of the PC/PS pathway was not occurring.

TFPI is the major regulator of the tissue factor pathway in vivo by binding to FXa and then forming an inactive quarternary complex with FVIIa:tissue factor. Levels of TFPI were increased in most patients prior to haemofiltration, probably as a result of endothelial modulation. After the initiation of haemofiltration TFPI levels increased, presumably due to the release of TFPI from the endothelium by the action of heparin administered as an anticoagulant. Over the lifespan of the haemofilter, TFPI levels then decreased, such that prior to clotting they were not significantly different to baseline levels. The release of TFPI by heparin in humans is tachyphylactic; after a period of time TFPI is no longer available to be released. Levels of FVIIa were normal or reduced in patients prior to haemofiltration and on institution of haemofiltration decreased
further, presumably due to the heparin-induced release of TFPI, as TFPI complexes with FVIIa:tissue factor complexes. However, during prolonged haemofiltration, as TFPI levels fell, levels of FVIIa increased. The correlation between the decrease in TFPI and increase in FVIIa indicates that activation of FVII occurred during haemofiltration in the face of falling TFPI levels.

To aid in the interpretation of the tissue factor pathway protein results, markers of endothelial activation were also measured during haemofiltration. Most patients had elevated levels (some grossly) of these markers (soluble plasma thrombomodulin, E-selectin, endothelin-1 and tissue factor) prior to haemofiltration. However, it appeared that haemofiltration itself did not induce further endothelial damage in these patients since there was an increase in some, but not all patients and in one, but not necessarily all markers during haemofiltration. These changes presumably reflect underlying disease processes occurring in certain patients and the release of these markers being mediated by differing stimuli.

The increase in FVIIa observed during haemofiltration was coincidental to the generation of thrombin, but there was no significant correlation between them, suggesting that the activation of FVII was not solely mediating thrombin generation under these circumstances. It is possible that in septic patients substances such as endotoxin and various cytokines may stimulate the expression of tissue factor by monocytes and this could provide a trigger for coagulation activation. It is unfortunate that, for logistical reasons, I could not collect suitable samples from these subjects for monocyte tissue factor analysis as this may have provided useful additional information. Alternatively other mechanisms may be responsible for the generation of thrombin during haemofiltration, such as the direct activation of FX by monocytes. Monocytes are capable of activating FX by three mechanisms: i) the binding of FVa to the monocyte surface forms a receptor for FXa and assembly of the prothrombinase complex; ii) the binding of FVII/FVIIa to monocyte expressed tissue factor results in cleavage of FX and, iii) the binding of FX to CD11b receptors can result in its activation. The last of these three mechanisms may involve a protease other than FVII.
The generation of FVIIa was not related to endothelial injury, implying that the activation of FVII was not mediated by endothelial damage. Others have made similar observations in patients undergoing haemodialysis. The mechanisms by which FVII is activated during haemofiltration cannot be fully elucidated from these results, but may well be multifactorial, involving tissue factor expression by monocytes and endothelium as a result of upregulation by cytokines caused by trauma, shock and sepsis, as well as direct effects of endotoxin on haemostatic proteins, and other factors related to the underlying condition and failure of one or more organs.

When this thesis was undertaken, it was known that despite large doses of heparin, thrombin generation still occurred during CPB, but the mechanisms by which this occurred was not known. The finding in Chapter 4 that FVII is activated during haemofiltration prompted me to investigate activation and inhibition of the tissue factor pathway during CPB as a possible mechanism by which thrombin is generated (Chapter 5). It seemed reasonable to suspect that the gross surgical trauma occurring during CPB may expose tissue factor and result in the systemic activation of FVII with the subsequent generation of thrombin. In addition to studying the tissue factor pathway in systemic blood samples (taken from the CVP line) during CPB, samples were also taken from the pulmonary vein, since it was postulated that activation of the coagulation system in the static pulmonary circulation during CPB could contribute to post-operative pulmonary dysfunction seen in some patients.

In the systemic circulation there was an initial, although non-significant increase in plasma FVIIa. However, following heparin administration there was a decrease in FVIIa levels which did not then significantly change throughout CPB. This decrease in FVIIa was coincidental to an increase in TFPI, an inhibitor of the TF:FVIIa complex which is rapidly released from the endothelium by heparin. It would thus appear that the heparin-induced release of TFPI suppressed FVIIa during CPB. Further evidence for this conclusion came from one patient who did not show an increase in TFPI following heparin administration yet showed a substantial increase in FVIIa during CPB. This was the first study to demonstrate the relationship between FVIIa and TFPI during CPB. I
am currently undertaking a study to investigate the phenomenon of non-responders to heparin in terms of TFPI release in cardiac surgery patients to see how common an occurrence this is and whether the clinical outcome of such patients is affected.

It was also interesting to compare the relationship between FVIIa and TFPI seen during CPB (Chapter 5) and haemofiltration (Chapter 4). During CPB following heparin administration TFPI levels were maintained until the end of ECC (average of 50 minutes on CPB), and there was no increase in FVIIa during this period. In contrast, during haemofiltration TFPI levels declined during the period of ECC and FVIIa levels increased. The decline in TFPI levels during haemofiltration is not surprising. Firstly, haemofiltration is a lengthy procedure compared to CPB, and the amount of TFPI bound to the endothelium is limited. Therefore, after a period of time, heparin cannot release TFPI from the endothelium because it has been depleted. Secondly, patients undergoing CPB are relatively well compared to those undergoing haemofiltration and critically ill patients may have reduced levels of endothelial-bound TFPI in the first instance. By comparing the results of these two studies, it is possible to see that the heparin-induced release of TFPI is important in controlling activation of the tissue factor pathway during ECC. The effect of alternative anticoagulants and heparin bonded circuits on the release of TFPI should therefore be carefully considered, as this fortuitous effect might be lost. These results also add support to the view that the anticoagulant actions of heparin are not solely mediated by antithrombin.

One of the aims of Chapter 5 was to compare haemostatic parameters between systemic blood and blood taken from the pulmonary vein during CPB. To my surprise, there was no significant difference in most parameters studied between the two sites, suggesting that the pulmonary circulation has a good capacity for controlling haemostasis. There was no evidence of activation of the contact system (a fall in the plasma FXII, PKK or C1-INH) in either the systemic circulation or pulmonary vein during CPB. During CPB there was a reduction in levels of the inhibitors ATIII and HCII, an increase in the generation of thrombin (TAT complexes), an increase in
neutrophil activation (neutrophil expressed CD11b, and elastase release), but to a similar extent in both sites.

However, the amount of TFPI released by heparin was greater (approximately 1.5-2 fold) in the pulmonary vein than in the systemic circulation. This may be due to the pulmonary vasculature having a greater TFPI binding density than the systemic circulation or that because the blood was static TFPI was not being diluted or removed from the circulation. There was evidence of increased endothelial injury (an increase in plasma thrombomodulin) occurring in the pulmonary vein during CPB, a finding that needs further clarification and investigation. There was also evidence of increased margination of neutrophils from the pulmonary vein into the microvasculature. Others have shown that this process is important in the development of post-CPB lung injury. This was the first study to examine haemostatic proteins in the pulmonary vein during CPB.

The results from Chapter 5 did not provide any indication as to the mechanisms by which the generation of thrombin occurs in the systemic circulation during CPB. There was no evidence of systemic activation of either the contact or tissue factor pathway during CPB. This prompted me to investigate other mechanisms of coagulation activation that may be relevant to extracorporeal circulation (Chapter 6) and which may have been overlooked in previous studies by only examining plasma samples.

Three areas were selected as likely candidates for study. Firstly, since CPB damages erythrocytes and activates leucocytes, the ability of peripheral blood cells to activate FXII was examined. Secondly, during CPB the administration of heparin results in the release of hepatic and lipoprotein lipase. Others have previously demonstrated in vitro that the action of lipase on triglyceride-rich lipoproteins results in the liberation of free fatty acids and the creation of a surface capable of supporting the activation of factors XII and VII. Therefore the activation of FXII and FVII on the surface of lipoproteins and the association of FVIIa and FXIIa with lipoproteins was investigated. Finally, since FXII is activated by contact with a negatively charged surface, the activation of FXII by contact with the CPB tubing itself was studied.
Using red blood cells isolated from healthy subjects, it was demonstrated that neither washed erythrocyte membranes nor erythrocyte stroma supported the activation of FXII. Since erythrocytes are the commonest peripheral blood cell, and that substantial haemolysis can occur during CPB, this was important to establish. However, it was demonstrated that neutrophils isolated from healthy subjects could support the activation of FXII. This was shown to require a small amount of FXIIa to be present initially and to be dependent upon a functional neutrophil surface. Moreover, the ability of neutrophils to activate FXII was enhanced if the neutrophils were pre-activated with f-MLP. These results are novel since the activation of FXII by neutrophils has not been previously studied. The generation of FXIIa on neutrophil surfaces may be one mechanism by which coagulation activation proceeds during CPB, especially as during CPB neutrophils become activated as demonstrated in Chapter 5.

The possibility that FXII is activated by contact with the CPB tubing, but, rather than being released into the fluid phase, remains bound to the surface of the tubing, was explored in vitro using a monoclonal antibody specific for FXIIa. After exposing CPB tubing to plasma, it appeared that FXIIa did become bound to the tubing. Unfortunately, the binding of the antibody to the tubing could not be shown to be totally specific and no other suitable antibodies were available, therefore these experiments were not pursued any further.

The experiments with lipoproteins demonstrated for the first time that FVIIa, FXIIa and β-glycoprotein-1 are associated with chylomicra-rich lipoproteins isolated from normal subjects. I wanted to examine these associations in all lipoprotein classes, but currently available methods for separation of lipoproteins have to be performed for long periods of time at 4°C, and result in transfer of apolipoproteins between classes. Lipase treatment of plasma or chylomicra resulted in further generation of FXIIa without a concomitant increase in FVIIa. This generation of FXIIa occurred even in the presence of physiological concentrations of the inhibitors C1-INH and ATIII. Both CPB (28-32°C) and haemofiltration (ambient) are carried out at temperatures which are less optimal for C1-INH activity compared to 37°C, and during CPB and in critical illness,
levels of C1-INH and AT-III may become depleted. Therefore activation of FXII on the surface of lipoproteins during haemofiltration and CPB may be enhanced by reduced levels of these inhibitors.

The treatment of plasma or chylomicra with the detergent Triton-X100, which disrupts cell membranes and lipids, resulted in an increase in FXIIa antigen. Thus, the FXIIa antigen ELISA may not fully recognise FXIIa when associated with lipoproteins, or any cell membrane or microparticles that may be present in plasma. This suggests that if an increase in FXIIa did occur during CPB, but the FXIIa was associated with lipoproteins, this increase may not be seen in untreated plasma samples. Whether this FXIIa is able to express functional activity on plasma proteins remains to be elucidated. Although I carried out these experiments to investigate possible mechanisms of coagulation activation during ECC, they obviously have wider implications in thrombosis and haemostasis. The interaction between lipoproteins and haemostasis is becoming increasingly studied. An increase in plasma FXIIa antigen is now known to be associated with the extent of coronary heart disease and myocardial infarction and may one day replace, or be used in conjunction with, plasma lipid determinations to assess the risk of cardiovascular disease. Attention is also focusing on remnant-like particles from chylomicra and triglyceride-rich lipoproteins (such as those studied in Chapter 6) which are now known to be atherogenic.

The demonstration in Chapter 6 that the FXIIa ELISA does not fully recognise FXIIa when it is associated with lipoproteins prompted me to examine FXIIa levels during CPB not only in untreated plasma, but also in plasma treated with detergent to disrupt lipoprotein structure. In the systemic circulation during CPB there was an increase in plasma FXIIa following heparin administration, but levels changed little during CPB. However, the amount of FXIIa exposed by treatment with detergent increased during CPB. Thus there may be an increase in FXIIa during CPB, but its association with lipoproteins may mask its detection in untreated plasma. This may help to explain in part the negative findings of Boisclair et al, (1993).
In Chapter 7, activation of coagulation occurring locally at the wound site during cardiac surgery was examined by comparing blood samples taken from the systemic circulation and pericardial cavity. It was demonstrated that blood from the pericardial cavity had higher levels of markers of thrombin generation, FVIIa, FXIIa, and soluble tissue factor, but reduced levels of TFPI and heparin. The reduction in TFPI in pericardial blood was not solely due to reduced local anticoagulation as suggested by other authors. Therefore, locally at the wound site during CPB there is enhanced activation and reduced inhibition of the tissue factor pathway. Grossly activated blood from the wound site is reinfused into the patient during CPB.

It must not be overlooked that activation of coagulation occurring at the wound site is crucial; otherwise the surgery would result in substantial blood loss. However, the reinfusion of this 'activated' blood to the systemic circulation may enhance systemic activation of coagulation, which may be deleterious to the patient. The reinfusion of blood from the pericardial cavity may contribute to, but is clearly not the only mechanism by which systemic coagulation occurs during CPB. I am currently extending the work carried out in Chapter 7 and also hope to undertake a study investigating systemic coagulation activation during CPB, comparing patients who receive retransfusion of pericardial blood and those for whom this blood is discarded. However, if blood in the pericardial cavity is not retransfused, this may necessitate the use of homologous blood products which would not otherwise be used and hence may be unethical. One possibility would be for these patients to predonate blood and then receive an autologous transfusion at the end of the procedure.

There is a current vogue in performing cardiac surgery using minimally invasive techniques, although currently these are only suitable for a small number of patients. Rather than performing a sternotomy with CPB and full cardiac arrest, these techniques may be performed using a reduced incision (thoracotomy or hemisternotomy), with or without CPB and some do not require cardiac arrest. If gross surgical trauma is responsible for some coagulation activation seen systemically during CPB, it would be
interesting to compare patients who undergo standard CABG against CABG with reduced surgical trauma.

Although I have not been able to totally address all of the questions I set out to answer at the start of this thesis, it is clear that the activation and control of the coagulation system during ECC is more complex than suggested by some authors. A role for the contact system in coagulation activation during CPB cannot be ruled out. Poorly characterised cell protease systems resulting from trauma and chronic exposure of blood to foreign surfaces may well contribute by providing atypical stimuli for thrombin generation. Undoubtedly, therapeutic strategies to counter some of the deleterious effects of ECC can only be achieved with a solid understanding of the mechanisms involved. The results obtained from this thesis go some way to a better understanding of these processes.


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### Appendix 1

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Address</th>
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<tbody>
<tr>
<td>Alpha Laboratories Ltd.</td>
<td>40 Parham Drive, Eastleigh, Hampshire SO5 4NU</td>
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<tr>
<td>American Diagnostica Inc.</td>
<td>222 Railroad Avenue, PO Box 1165, Greenwich, CT, USA. <em>(Distributor in UK: Alpha Laboratories, see above)</em></td>
</tr>
<tr>
<td>Beckman Instruments Ltd.</td>
<td>Oakley Court, Kingsmeade Business Park, London Road, High Wycombe, Bucks HP11 1JU</td>
</tr>
<tr>
<td>Becton Dickinson Ltd.</td>
<td>Between Towns Road, Cowley, Oxford OX4 3LY.</td>
</tr>
<tr>
<td>Behring Diagnostics Ltd.</td>
<td>Hoechst House, 50 Salisbury Road, Hounslow, Middlesex TW4 6JH.</td>
</tr>
<tr>
<td>Bio Products Laboratory</td>
<td>Elstree, Hertfordshire.</td>
</tr>
<tr>
<td>Chromogenix Ltd.</td>
<td>Mölndal, Sweden. <em>(Distributor in UK: Quadratex Ltd, PO Box 167, Epsom, Surrey, KT17 2SB)</em></td>
</tr>
<tr>
<td>Coulter Electronics Ltd.</td>
<td>Northwell Drive, Luton, Bedfordshire LU3 3RH.</td>
</tr>
<tr>
<td>CP Pharmaceuticals Ltd.</td>
<td>Ash Road North, Wrexham Industrial Estate, Wrexham, LL13 9UF.</td>
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<td>Dako Ltd.</td>
<td>16 Manor Courtyard, Hughenden Avenue, High Wycombe, Buckinghamshire HP13 5RE.</td>
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<tr>
<td>Diagnostic Reagents Ltd.</td>
<td>Chinnor Road, Thame, Oxon OX9 3NY.</td>
</tr>
<tr>
<td>Diagnostica Stago Ltd.</td>
<td>Asnieres-Sur-Seine, France. <em>(Distributor in UK: Shield Diagnostics Ltd.)</em></td>
</tr>
<tr>
<td>Enzyme Research Ltd.</td>
<td>Pen Yr Heol Drive, Sketty, Swansea, Wales SA2 9JT.</td>
</tr>
<tr>
<td>Epsilon Technology Ltd.</td>
<td>Station House, Manningtree, Essex CO11 2LH.</td>
</tr>
<tr>
<td>Gambro Ltd.</td>
<td>Lundia House, 124 Station Road, Sidcup, Kent DA15 7AS</td>
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Hospal Ltd.  Unit G, Forum Drive, Leicester Road, Rugby, Warwickshire CV21 1NT.

Immuno Ltd.  Artic House, Rye Lane, Dunton Green, Sevenoaks, Kent TN14 5HB.

Instumentation Laboratory Ltd.  Kevin Close, Birchwood, Warrington, Cheshire WA3 7PB


Labtech International Ltd.  Woodside, Easons Green, Uckfield, East Sussex TN22 5RE

Life Technologies Ltd.  3 Fountain Drive, Inchinnin Business Park, Paisley PA5 9RF.

Merck Ltd.  Hunter Boulevard, Magna Park, Lutterworth, Leicester LE17 4XN.

NIBSC  Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG

Novo Nordisk Ltd.  Novo Alle, 2880 Bagsvaerd, Denmark.

Pharmacia Ltd.  23 Grosvenor Road, St. Albans, Hertfordshire AL1 3AW

R&D Systems Ltd.  4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS.

Scientific Hospital Supplies Ltd.  100 Wavertree Boulevard, Liverpool, L7 9PT.

Serotec Ltd.  22 Bankside, Station Approach, Kidlington, Oxon OX5 1JE.

Shield Diagnostics Ltd.  The Technology Park, Dundee DD2 1SW.

Sigma-Aldrich Chemical Co.  Fancy Road, Poole, Dorset NH17 7NH.

Sysmex UK Ltd.  Sunrise Parkway, Linford Wood, Milton Keynes, Bucks, MK14 6QF.

Unicorn Diagnostics Ltd.  Rosslyn House, Latchmoor Drive, Gerrards Cross, Berkshire SL9 8LN.