The clinical utility of thrombin generation
Bevan, Shaun Patrick

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The Clinical Utility of Thrombin Generation

Shaun Patrick Bevan

PhD Thesis 2010
Abstract

The Calibrated Automated Thrombogram (CAT) thrombin generation assay is a technique that allows the characterisation of an individual’s capacity to produce thrombin in response to a procoagulant stimulus. As such it is a global screening test for both hyper and hypocoagulable bleeding disorders. This project focused on the introduction of the CAT assay into routine use in the haemostasis laboratory at the Royal London hospital and to assess its clinical utility.

Initial experimentation focused on determining the suitability of the assay for routine use, including ascertaining the levels of inter and intra assay variation, the effects of sample handling and storage, the limits of detection, variation in an individual with time and establishing a normal range. All of these experiments returned favourable results.

The assay was then used to characterise the thrombin generation profile of a cohort of women with pre-eclampsia. Women with pre-eclampsia were found to have higher thrombin generation parameters (Endogenous thrombin potential and peak height) when compared to normotensive pregnant women (p= <0.001). Re-analysis of the data showed the use of thrombin generation could detect pre-eclampsia with a specificity and sensitivity of 83% and 43% respectively when combined with the Pre-eclampsia community guideline screen. There were also statistically significant correlations between the measurement of thrombin generation and both birth weight and the length of stay in hospital at delivery.

Further analysis by flow cytometry and studies involving filtration of plasma revealed that the sensitivity of the assay to the detection of pre-eclampsia may be associated with the increased levels of microparticles present in the blood of pre-eclamptic women. There were statistically significant correlations (p= <0.05) between changes in thrombin generation parameters and the number of microparticles post filtration. This suggests that the CAT assay is sensitive to the number of microparticles in a patient’s plasma.
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Ethics Approval

Ethical approval for the experiments detailed in this thesis was obtained from the East London and the City HA Local Research Ethics Committee 3.
Acknowledgements

I would like to say a big thank you to my supervisor Professor Marion Macey for her advice, encouragement and support, without which I would never have been able to complete this thesis.

I would also like to thank Dr Peter MacCallum for his help and advice in improving this thesis.

I would also like to acknowledge all those who were involved in the pre-eclampsia study for all of their hard work, as well as all of my long suffering colleagues in the haemostasis laboratory for their support and for helping me understand the art of coagulation in the first place.

I would like to add a special thank you to everybody else who assisted in the smooth running of this project and who offered me help and support along the way, especially all of my friends, who proved themselves to be willing blood donors.

My final thank you goes to my wife Elizabeth, for all of her love and support throughout this project. I would be lost without you.

Thank you all
**Abbreviations**

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<td>AMC</td>
<td>Amino-Methyl-Coumarin</td>
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<td>APC</td>
<td>Activated Protein C</td>
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<td>Activated Partial Thromboplastin Time</td>
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<td>AUC</td>
<td>Area Under Curve</td>
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<td>CAT</td>
<td>Calibrated Automated Thrombogram</td>
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<td>CI</td>
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<td>CTI</td>
<td>Corn Trypsin Inhibitor</td>
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<td>CV</td>
<td>Coefficient of Variation</td>
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<td>Grams per Decilitre</td>
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<td>HELLP</td>
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<td>HIT</td>
<td>Heparin Induced Thrombocytopenia</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>HMWK</td>
<td>High Molecular Weight Kininogen</td>
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<td>IFE</td>
<td>Inner Filter Effect</td>
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<td>INR</td>
<td>International Normalised Ratio</td>
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<td>KIR</td>
<td>Killer Immunoglobulin Receptor</td>
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<td>Kilopascal</td>
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<td>LFT</td>
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<td>LMWH</td>
<td>Low Molecular Weight Heparin</td>
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<td>LSCS</td>
<td>Lower Segment Caesarean Section</td>
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<td>MFU</td>
<td>Microparticle Filtration Unit</td>
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<tr>
<td>mg</td>
<td>Miligram</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibilty Complex</td>
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<td>ml</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
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<td>MTHFR</td>
<td>Methylenetetrahydrofolate Reductase</td>
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<td>PIP</td>
<td>Phosphatidylinositol Phosphate</td>
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<td>Prekallikrein</td>
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<tr>
<td>PLGF</td>
<td>Placenta Derived Growth Factor</td>
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<td>pM</td>
<td>Picomolar</td>
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<td>PNH</td>
<td>Paroxysmal Nocturnal Haemoglobinuria</td>
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<td>Post Partum Haemorrhage</td>
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<td>PPP</td>
<td>Platelet Poor Plasma</td>
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<td>PRECOG</td>
<td>Pre-eclampsia Community Guideline</td>
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<td>PSGL-1</td>
<td>P-Selectin Ligand-1</td>
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<td>PT</td>
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<td>PTR</td>
<td>Prothrombin Time Ratio</td>
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<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
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<tr>
<td>rVIIa</td>
<td>Recombinant Activated Factor VII</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>sENG</td>
<td>Soluble Endoglin</td>
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<tr>
<td>sFlt1</td>
<td>Soluble Fms Like Tyrosine Kinase 1</td>
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<td>SHP</td>
<td>Standard Human Plasma</td>
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<td>Thrombin-Antithrombin</td>
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<td>Tissue Factor Pathway Inhibitor</td>
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<td>Transforming Growth Factor Beta</td>
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<td>Tumour Necrosis Factor</td>
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<td>Thrombin Time</td>
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<td>Thrombotic Thrombocytopenic Purpura</td>
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<td>Unfractionated Heparin</td>
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<td>µM</td>
<td>Micromolar</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>vWF</td>
<td>von Willebrand Factor</td>
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Chapter 1

Introduction
1.1 The Coagulation Cascade

The clotting of blood at the site of vascular injury involves a complex biological amplification process whereby a relatively small initiation signal can generate a large physiological response. The coagulation cascade is mediated via the sequential activation of serine protease enzymes which circulate in the blood as zymogens. The net result of the cascade is the formation of a haemostatic plug and the cessation of bleeding.

Coagulation *in vivo* is thought to be initiated via the binding of factor VII to exposed tissue factor at the sites of vascular injury. This binding causes the activation of factor VII. Activated factor VII then activates both factors IX and X via proteolytic cleavage. Activated factor X initiates the production of thrombin from prothrombin. The production of low levels of thrombin leads to the activation of factors V, VIII (cofactors for factor Xa and IXa respectively) and XI. Activated factor XI further increases the production of activated factor IX. Factors VIIIa and IXa form the intrinsic factor Xase complex on a membrane surface that can be provided by platelets, microparticles and endothelial cells. The Xase complex can activate factor X at a rate of 50-100 fold greater than factor VIIa and tissue factor (Butenas and Mann 2002). The production of the Xase complex therefore leads to the initiation of a positive feedback loop consolidating the production of activated factor X and thereby the conversion of prothrombin to thrombin.

The proteolytic activity of the clotting factors are dependent on the presence of both calcium ions and phospholipids, a number of the reactions are also surface-mediated, occurring on exposed collagen or platelets (Xase complex). The reactions are so dependent on the presence of cofactors and membrane binding sites that the absence of either is as significant as the absence of the clotting factor itself (Butenas and Mann 2002). Factors Xa and Va form the prothrombinase complex, this occurs on the phospholipid rich surface of activated platelets at the site of vascular injury. The thrombin generated hydrolyses soluble plasma fibrinogen to form fibrin monomers and yield the fibrinopeptides A and B. The fibrin monomers spontaneously form hydrogen bonds linking them together to create insoluble fibrin polymers. The fibrin polymers are
then covalently cross-linked via the activity of activated factor XIII. Factor XIII is itself activated by thrombin.

Coagulation in vitro can be stimulated by contact reactions between factor XII and high molecular weight kininogen (HMWK), this leads to the activation of factor XI and therefore factor IX, initiating the cascade. Individuals who demonstrate a deficiency of the contact factors do not however demonstrate abnormal bleeding, suggesting that these factors are not essential for in vivo coagulation. Factor XI is also non-essential in the initiation of coagulation in vivo although does play a role in the activation of factor IX and may have an important role at sites of major trauma.

The generation of thrombin via the clotting cascade can be divided into two distinct phases; the initiation phase and the propagation phase (Butenas and Mann 2002). The initiation phase begins following the addition of tissue factor and is characterised by the formation of femtomolar to picomolar levels of factors VIIa, IXa, Xa and XIa, partial platelet activation, the activation of cofactors Va and VIIIa and nanomolar levels of thrombin generated at a comparatively low rate. The initiation phase is roughly analogous to the clotting times measured in the laboratory. The propagation phase is characterised by the completion of platelet aggregation and the rapid generation of thrombin resulting in the formation of a solid clot.

In order to prevent the formation of pathogenic occlusions in blood vessels such as deep vein thrombosis (DVT) and pulmonary embolism (PE) the generation of thrombin and therefore the production of fibrin clots must be limited to the site of vascular injury. There are multiple negative regulators of the haemostatic system. The major negative regulator of the initiation phase is tissue factor pathway inhibitor (TFPI). TFPI is present in both plasma and platelets and accumulates at sites of injury due to local platelet activation. TFPI acts by inhibiting factors Xa and VIIa, the balance between the clot formation and inhibition therefore depends on the relative strength of the pro-coagulant and anti-coagulant signals. Once the cascade has reached the propagation phase the inhibitory effect of TFPI is diminished. Antithrombin is another potent inhibitor of the clotting cascade which circulates in the plasma. Antithrombin inactivates factors IXa, Xa, XIa and thrombin via irreversible binding to form stable high molecular weight complexes. In contrast to TFPI antithrombin has limited...
inhibitory effect during the initiation phase of the cascade and a more pronounced effect on the propagation phase. This is also true of protein C; a vitamin K dependent serine protease which is activated in response to thrombin binding to the endothelial cell surface receptor thrombomodulin. Activated protein C inactivates factors Va and VIIIa via proteolytic cleavage, its activity is enhanced by the presence of its cofactor protein S. An overview of the clotting cascade is shown in figure 1.1.

Figure 1.1

Figure 1.1 Diagrammatic representation of the clotting cascade and a number of the negative regulators involved. Inhibition is shown via red arrows. Figure adapted from: kuwaitmd.hsc.edu.kw/main/index.php?q=node/60

The inhibitors of the clotting cascade act in a synergistic manner; the presence of multiple inhibitors has a more pronounced effect than would be predicted by the presence of one alone (Butenas and Mann 2002).
1.2 Thrombin Generation and the Coagulation Cascade

Routine clotting screens include the prothrombin time (PT), which is converted to the international normalised ratio (INR), and the activated partial thromboplastin time (APTT). These tests effectively measure the time to clot formation when coagulation is stimulated via the extrinsic or intrinsic pathway respectively. As mentioned above this is roughly analogous to the duration of the initiation phase of the cascade. At this time only 10-20 nM of thrombin is formed, this constitutes less than 5% of the total thrombin that can be generated by the cascade (Hemker and Béguin 1995). This essentially means that the clotting time of a patient does not always correlate with their capacity to generate thrombin, for example a patient taking low molecular weight heparin has compromised thrombin generation but only a modestly prolonged APTT. A similar scenario is also posed by those patients with antithrombin, protein C or protein S deficiencies, factor V Leiden and activated protein C (APC) resistance. All of those conditions result in an increased thrombin generation capacity and hence a potentially pro-thrombotic state in the patient. Patients with these conditions all have a normal INR and APTT however, showing that these tests are insensitive to hypercoagulative states.

In contrast the endogenous thrombin potential (ETP) is a global assay that is sensitive to both hyper and hypocoagulant states and is a parameter that both increases and decreases in proportion to the thrombin forming capacity of a sample (Hemker and Béguin 1995). ETP was first described in 1993 as a way of quantifying the level of thrombin that a patient’s plasma was capable of generating (Hemker et al., 1993). The assay itself measures the production of thrombin in a patient’s plasma by continuously monitoring the amidolytic activity of thrombin on a substrate after the coagulation cascade is initiated via the addition of tissue factor. The substrate should be cleaved specifically by thrombin and cleaved at a rate slow enough to ensure it is not consumed before the process of thrombin generation is complete. When the substrate is cleaved by thrombin it yields a product that can be quantified (e.g. by the production of a fluorescent signal) thus the activity of thrombin can be measured over a specific time course yielding a bell shaped curve when plotted. The area under the curve is the patient’s ETP. The curve also has other parameters that can be interpreted these include the lag time (the time before thrombin production is initiated), the peak height which is representative of the maximum velocity of net thrombin production and the time to
peak, which is the time taken to reach the peak height. These parameters are shown graphically in figure 1.3.

1.3 The Use of Thrombin Generation in Hypocoagulable States

Since the measurement of the ETP provides a clear indication of an individual’s capacity to generate thrombin it should be sensitive to those with a hypocoagulable disorder, such as haemophilia. This was indeed shown to be the case in individuals who were deficient in factors V, VII, X, XI, XII and Prothrombin (Al Dieri et al., 2002). These experiments showed that there was no difference in the ETP of these patients when the clotting cascade was initiated via either the intrinsic or extrinsic pathway for patients deficient in factors VII, X and prothrombin. Patients who were deficient in factors XI and XII had ETPs which were only abnormal when examined following stimulation via the intrinsic pathway, although this was an anticipated result.

Interestingly it was shown that of the factor deficiencies examined only the level of prothrombin correlated with the ETP in a linear fashion. For factors V, VII, X and XI increasing concentration produced a hyperbolic curve which approached normal ETP the higher the concentration became. It was shown that relatively low levels of clotting factor could produce ETPs that approached a normal level. The levels of clotting factor, that could produce an ETP that was 50% of that of a pool of normal plasma were, 1% for factor V, 2% for factor VII, 5% for factor X and 1% for factor XI. As a general rule it was the peak height and the lag time that were affected by decreasing the clotting factor concentration. The levels of ETP remained relatively normal until the levels of clotting factor were markedly reduced. This implies that the bleeding tendency seen in patients with these factor deficiencies may be due to the speed that they produce thrombin rather than the level of thrombin production they are capable of. This also lends weight to the theory that the levels of clotting factors which circulate in the blood are in excess of that which is necessary to induce coagulation. The severity of bleeding was also found to correlate well with the patients ETP, it was discovered that the patients with a history of severe bleeding had an ETP which corresponded to less than 20% of normal. This neatly dovetails with the bleeding of anti-coagulated patients who have a marked increase in bleeding severity when their INR is in excess of 3.5 (Azar et
al., 1996), this was shown to correspond to an ETP of roughly 17% of normal (Hemker and Béguin 2000).

The correlation of bleeding severity of haemophilia patients to ETP is of great interest since bleeding severity does not always correlate with the level of clotting factor activity a patient demonstrates (Walsh et al., 1973). The correlation between ETP and bleeding severity was also demonstrated in patients with haemophilia A and B (factor VIII and IX deficiencies respectively) (Dargaud et al., 2005). Although in this study it was suggested that patients with a severe clinical bleeding tendency had an ETP of less than 50% normal, and that this roughly corresponded to a clotting factor activity of 10%. A number of exceptions to these rules were highlighted. Some of the patient cohort who were diagnosed as severe haemophiliacs (factor VIII activity <1%) demonstrated an ETP of greater than 50% normal and presented with mild bleeding tendencies. There were also a few cases of patients having a reduced ETP but no clinically significant bleeding. Over the course of the study 25% of the haemophilia patients had an ETP that did not correlate with the level of factor activity demonstrated by the patient and in the majority of these patients there was poor correlation between their clinical bleeding severity and the level of factor activity. It was also shown that patients who were treated with recombinant factor VIII, who had identical levels of residual factor VIII 24 hours post infusion could have markedly different ETPs. So perhaps the measurement of ETP could be used to help evaluate the efficacy of haemophilia treatment in a patient specific manner and therefore improve patient care and the cost effectiveness of the treatment regimen.

The above experiments were performed using platelet poor plasma (PPP), with the platelets having been removed by centrifugation. In these experiments the platelets were effectively replaced with phospholipid vesicles, which provide a platform for the surface mediated cascade reactions. It has been shown that the ETP of haemophilia A and B patients can be affected by decreasing platelet counts, when it is measured using platelet rich plasma (Siegemund et al., 2003). The ETP can be altered in a linear fashion with decreasing platelet count, when the count falls below 100 x10⁹/l. There is no effect on the ETP of a patient when the platelet count exceeds 100 x10⁹/l. It was also shown that the effects of reducing the platelet count were greater in patients with haemophilia B than those with haemophilia A; it is hypothesised that this may be due to the role in
platelets in the assembly of the Xase complex. The reduction of ETP caused by this thrombocytopenia could be overcome by increasing the levels of clotting factor in the patients’ samples thereby implying that the effect is not simply due to a decreasing surface area for the assembly of the Xase complex.

Some of the variation in these experiments may be due to the concentration of tissue factor used to initiate the coagulation cascade in these assays. Although thrombin generation assays performed in PPP are sensitive to the levels of both factor VIII and IX when a tissue factor concentration of 5 pM is used to initiate coagulation (Hemker et al., 2003) it has been shown that thrombin generation assays show greater sensitivity to the intrinsic pathway factors when the tissue factor concentration is reduced to 1-2 pM (Beltran-Miranda et al., 2005). The use of low concentrations of tissue factor is not without flaws however, since it can lead to increasing assay variability (van Veen et al., 2008) (Dargaud et al., 2007) and also an increase in contact factor activation (van Veen et al., 2008) (Dargaud et al., 2007) (Luddington and Baglin 2004) (auto activation of factor XII) leading to erroneous results. The use of corn trypsin inhibitor (CTI) can overcome these effects (Luddington and Baglin 2004), but significantly increases the costs of the assay. CTI is not required when 5 pM tissue factor is used (van Veen et al., 2008). A comparative study was carried out to determine the sensitivity of two different tissue factor concentrations (1 and 5 pM) when used to discriminate between patients with haemophilia A (of varying severity) and a healthy male population (van Veen et al., 2009a). It was shown that there was no practical advantage in using a tissue factor concentration of 1 pM to initiate thrombin generation rather than 5 pM. Both concentrations were able to distinguish between the patient and control groups, although interestingly the use of 1 pM tissue factor failed to significantly distinguish between mild and severe haemophiliacs. This was not the case with 5 pM tissue factor. This is highlighted by the authors as being at odds with other studies (Dargaud et al., 2005) (Beltran-Miranda et al., 2005) which have shown a difference between mild, moderate and severe haemophiliacs. It was postulated that this difference was due to the methodologies employed; where CTI was either not used or was added subsequently to the patient’s plasma, when it is most efficacious when the sample is taken directly into CTI. The analysis of patients with mild haemophilia using a tissue factor concentration of 1 pM in the absence of CTI can lead to an overestimation of thrombin generating capacity due to contact factor activation, thereby making the discrimination between
mild and severe haemophiliacs more pronounced. The study also highlighted that the main differences in the thrombin generation profile of haemophiliacs when compared to normal individuals is a decreased ETP, peak height and an increased time to peak. This is because the abnormalities in haemophilia primarily affect the propagation phase of thrombin generation and have a minor difference in the initiation phase. Interestingly this finding is contradictory to that of Al Dieri *et al.* (2002) above.

It has also been shown that thrombin generation assays can potentially be used to monitor haemophilia patients who are treated with factor eight inhibitor bypassing activity (FEIBA®) and recombinant activated factor VII (rVIIa) (van Veen *et al.*, 2009b). Both FEIBA® and rVIIa are used in the treatment of patients with haemophilia who have developed inhibitors (antibodies against factor VIII concentrate). FEIBA® is derived from human plasma and contains activated factor VII and non activated factors II, IX and X. Traditional coagulation tests are unsuitable in the monitoring of both rVIIa and FEIBA®. It was shown with *in vitro* spiking experiments of plasma samples taken from 10 severe haemophiliacs that there was a linear dose-response to FEIBA® with both the ETP and peak height and inverse hyperbolic relation of both the lag time and time to peak when the reaction was initiated with 1 pM of tissue factor. Similar results were shown for the use of rVIIa. Both agents normalised the thrombin generation parameters of the patients following spiking with therapeutic doses. Interestingly the use of 5 pM tissue factor led to substrate depletion for FEIBA® concentrations >0.5U/ml (therapeutic dose is believed to be 0.5-1.0U/ml) meaning it is impossible to calculate the ETP. Similarly the use of 5 pM of tissue factor was also unsuitable for the monitoring of rVIIa with no observable effect on either the ETP or peak height with increasing dose of rVIIa. This observation is similar in nature to a study which showed that a reduction in clotting time by a given concentration of rVIIa was inversely proportional to the tissue factor concentration (Butenas *et al.*, 2002). These observations mean that while the diagnosis of haemophilia may be performed using either 1 or 5 pM tissue factor as a reaction trigger, monitoring of either rVIIa or FEIBA® requires 1pM tissue factor. Spiking patients’ plasma with both rVIIa and FEIBA® showed that the agents were capable of acting synergistically. This is probably due to the increased level of prothrombin in the sample since synergy has been shown between the factor II (present in FEIBA®) and rVIIa (van Veen *et al.*, 2009 B)(Allen *et al.*, 2006), implying that the baseline level of factor II is important in the response of an individual to rVIIa. Again
this study suggests a potential role for thrombin generation assays in assessing the efficacy of treatment regimens, with the aim of both lowering the costs of the treatment, whilst optimizing both the effects and safety of the therapy.

ETP could potentially be used to great effect in the detection/treatment of factor deficiencies; however it does not distinguish which factor a patient is deficient in and so cannot completely replace the current screening methods. It does have benefits however, since an abnormality should be obvious immediately.

1.4 Thrombin Generation and Anticoagulation Therapy

Thrombin generation assays could potentially be used to monitor those patients undergoing anti-coagulant therapy, especially those undergoing combination therapy and those who are taking low molecular weight heparin, as these patients cannot be monitored using the standard clotting screen techniques. ETP has been shown to be sensitive to Warfarin and heparin (Hemker and Béguin 1995) (Hemker and Béguin 2000) (Nieuwenhuys et al., 2000) (Kakkar et al., 2002) and has been used as a method of comparing different heparin treatment regimens (Kakkar et al., 2002).

Heparins can be quantified in a patient’s plasma using assays to detect the anti-IIa and/or anti-Xa activity of that heparin. The detection of heparin activity does not always correlate with clinical effectiveness however since there is high variability in heparin pharmacodynamics between individuals (Dieri et al., 2004), with varying levels of heparin carrying proteins and anti/procoagulant factors. Measuring the effect of heparin on a patient’s plasma is believed to be a more effective way of monitoring a response to treatment. This is usually performed using the APTT ratio. A comparison between APTT and ETP parameters of a cohort of patients injected with a fixed dose of four heparins of varying molecular weight showed that the ETP was more sensitive to the presence of heparin than the APTT (Dieri et al., 2004). An anti-IIa activity of 0.1 IU of any heparin can cause an APTT ratio of 1.2-1.4 but an inhibition of ETP of between 50-80%. Thrombin generation assays therefore may be of use in clinically monitoring those patients in whom standard doses of heparin do not produce the desired effect, since dose adjustment may have little impact on the APTT ratio but a larger impact on the ETP, allowing more effective monitoring.
Further studies have not only shown that thrombin generation assays are more sensitive to the detection of different heparins/heparinoids, including unfractionated heparin (UFH), low molecular weight heparins (LMWH), Danaparoid and Fondaparinux than the traditional APTT and anti-Xa assays but they are also more sensitive to detecting their reversal by agents including protamine sulphate, NovoSeven®, FEIBA® and fresh frozen plasma (FFP) (Gatt et al., 2008), providing more potential uses for thrombin generation assays in the clinical setting.

Since thrombin generation assays are sensitive to the majority of anticoagulation agents it follows that they could be useful in determining the efficacy of new treatments in ex-vivo models. Indeed thrombin generation has been shown to have an advantage over clotting based assays in determining the effects of 6 different thrombin or factor Xa inhibitors specifically when regarding the total amount of thrombin generated and the time course of the generation as well as providing insights into the mechanisms of action of such compounds (Robert et al., 2009). Thrombin generation assays have also been used to evaluate the efficacy of new direct thrombin inhibitor drugs such as dabigatran etexilate (Wienen et al., 2007).

The above evidence shows multiple potential clinical roles for thrombin generation assays; from the development of new anticoagulant agents to patient specific monitoring of anticoagulant therapy, making it a valuable addition to existing laboratory tests.

1.5 The Use of Thrombin Generation in Hypercoagulable States

The measurement of ETP should be as sensitive to hypercoagulant states as it is to hypocoagulant states. Since there are a large number of conditions which can cause a pro-thrombotic state in a patient a high ETP can not be used to diagnose specific disorders but should give an indication as to the risks/likelihood of a patient developing a thrombosis in the future. Although the current laboratory investigations for thrombophilia are for specific disorders including Antithrombin, protein C and protein S deficiencies, APC resistance, factor V Leiden and prothrombin variant mutations and Lupus anticoagulant, the majority of patients screened have normal results, or transient abnormal results due to acute phase reactions or anticoagulation therapy. One of the largest risk factors involved in inherited thrombophilia is a family history; this indicates
that there are a number of predisposing factors which remain undiscovered; by the measurement of ETP in these patients it may be possible to elucidate an inherent risk, even if the cause remains to be found. There have been a number of recent arguments questioning the validity of ‘traditional’ thrombophilia screening (Dalen 2008). Thrombophilia screening is not usually performed to elucidate the risk of an initial episode of venous thromboembolism (VTE) but rather is primarily performed to determine the risk of recurrent events in patients with a history of VTE. However a meta analysis of studies found that only elevated levels of factor VIII, the presence of anti-phospholipid antibodies and the presence of multiple thrombophilic defects lead to a significant increase in the risk of recurrence of VTE. It also showed that there was a large cohort of patients with idiopathic VTE in which no heritable thrombophilia was found. These findings led the author to query whether thrombophilia screening was cost effective. It may be that screening thrombophilia patients via thrombin generation assays provides a more cost effective means of assessing risk.

Elevated levels of ETP are not associated with some pro-thrombotic states such as exhaustive exercise (Hilberg et al., 2002) (which causes activation of blood coagulation), hyperhomocysteinemia (Bos 1999) and pregnancy (Eichinger et al., 1999). It has also been reported that there was no difference in the ETP between pregnant women with and without the factor V Leiden mutation, despite their being a higher incidence of thrombotic events in pregnant women with factor V Leiden than in those without (Eichinger et al., 1999). This finding may be due to the set up of the assay however (see APC below). Interestingly none of the women in this study developed a thromboembolic event despite having levels of D-dimer, Thrombin- antithrombin (TAT) and F1+F2 comparable to those seen in DVT, PE and myocardial infarction. Thus the ETP in this cohort of patients may be of a diagnostic benefit, since all of them demonstrated normal ETP and no thrombotic events were recorded. ETP was also found to be insensitive when used in the diagnosis of DVT (Bucek et al., 2003) but has been previously reported to be elevated in patients with active deep vein thrombosis (Wielders et al., 1997). Thrombin generation has also been shown to be elevated following acute DVT (Ten Cate-Hoek et al., 2008), with a cohort of patients showing increased ETP and peak heights when compared to a normal population. This difference was shown to become more pronounced over the course of a year before stabilising in the patient population. The observed differences became more pronounced if 1pM tissue
factor was used as a reaction trigger rather than 5pM tissue factor. The authors also discovered that the addition of thrombomodulin to the patients’ plasma caused less inhibition of thrombin generation in the patient group than in the normal controls. Whether this apparent resistance to activated protein C was causative of the VTE in the patients demographic was not discussed. The mechanism for this APC resistance was thought to be partially explained by a negative correlation between levels of factor VIII and the thrombomodulin dependent thrombin generation reduction in the patient group. Elevated levels of factor VIII would lead to an enhanced feedback of thrombin mediated factor VIII activation which could counter-act the thrombomodulin mediated inhibition of factor VIII by APC.

A number of studies have also demonstrated a link between thrombin generation and the risk of recurrent VTE. Besser et al. (2008) showed in a prospective cohort of 188 patients with either unprovoked VTE or provoked VTE of a non-surgical nature that the patients with a high ETP had a significantly higher risk of an unprovoked recurrence than those with a low ETP. They also showed that individuals with an unprovoked VTE had a significantly higher risk of a subsequent unprovoked VTE than those who had an initial provoked VTE and that in this cohort an elevated ETP was associated with an increased risk of recurrent thrombosis. A similar experiment using a larger cohort of 861 patients found a similar result (Eichinger et al., 2008) i.e. an elevated ETP was associated with an increased risk of recurrent thrombosis. Interestingly this study also showed that an elevated D-dimer was also associated with recurrent risk of thrombosis and that there was a degree of synergy between the two parameters.

1.5.1 ETP and Prothrombin Variant

Elevated ETP has been demonstrated in association with the prothrombin variant (Kyrle et al., 1998). The prothrombin variant mutation is caused by a single G to A transition at position 20210 in the prothrombin gene. This causes carriers of the mutation to have an increased level of circulating prothrombin compared to normal individuals. The ETP of prothrombin variant heterozygotes is significantly elevated in comparison to normal individuals; prothrombin variant homozygotes were shown to have an ETP of almost twice that of a normal control. These results correlate well with the findings of Dieri et al. (2002) that there is a linear association between prothrombin concentration and the
magnitude of ETP. The carriers of this variant allele have an increased risk of venous thrombosis presumably due to over production of thrombin once the cascade is triggered.

**1.5.2 ETP and APC Resistance**

Extensive investigation has been performed into the capacity of ETP to elucidate a patient’s resistance to APC. An individual with APC resistance will not show an increased ETP *per se*. The defect does not cause pathogenesis via an overproduction of thrombin, as is the case for the prothrombin variant allele, but rather by a resistance to the negative regulation leading to prolonged thrombin production. The reason this is not reflected in the ETP assay is due to the mechanisms of protein C activation. Activation of protein C requires thrombin binding to thrombomodulin; a membrane bound protein that is therefore not present in the patients’ plasma. APC resistance can therefore be investigated using an ETP based assay by the addition of either activated protein C or recombinant thrombomodulin to the patient’s plasma.

The APC resistance is usually measured by a normalized APC ratio this is calculated by dividing the ETP of a patient following the addition of APC by the patients ETP without the addition of APC and further dividing this by the same ratio derived from a pool of normal plasma. See below.

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\text{Normalised APC Ratio} = \frac{(\text{Patient ETP with APC/ Patient ETP})}{(\text{Normal Plasma Pool ETP with APC/ Normal Plasma Pool ETP})}
\]

An increased normalised ratio when determined by ETP is indicative of APC resistance. The most common cause for APC resistance is the presence of the factor V Leiden allele. The allele is caused by a missense mutation at position 506 in the amino acid sequence of the factor V protein. This mutation removes one of the three specific arginine residues at which factor Va is cleaved by APC, thereby rendering the factor V Leiden protein more resistant to proteolytic cleavage than wild type factor V. APC resistance can also be acquired, this is most common in patients who are either undergoing hormone therapy (including oral contraceptives) or who are pregnant (Curvers *et al.*, 1999). It has been shown that the APC ratio (and therefore the incidence of APC resistance) when measured using the ETP is greater in women who take oral
contraceptives than in those who do not (Curvers et al., 1999). This has also been shown to be the case when the APC ratio was determined using the standard APTT based technique. It was however demonstrated that the ETP based method could distinguish a significant difference in the APC ratio between women on different types of oral contraception i.e. there is a significant difference between the APC ratio of those women taking second generation oral contraceptives and those taking third generation oral contraceptives. This difference was undetectable using the APTT based technique, implying that the ETP based method is more sensitive in detecting differences in the APC ratio. Retrospective studies however found no correlation between an increased APC ratio, measured via the ETP, caused by acquired APC resistance and an increased risk of venous thromboembolism in pregnancy (Zotz et al., 2004). This is in contrast to APC resistance caused by factor V Leiden, which was shown to correlate with the incidence of thrombotic events during pregnancy. The same results were shown for a second cohort of women who had a previous venous thromboembolism (Heinemann et al., 1998). Other studies however provide conflicting evidence and suggest that elevated APC ratios as determined via ETP do predict the risk of venous thrombosis in both male and female patients (Tans et al., 1999).

It has also been reported that the ETP based APC resistance test is sensitive to the prothrombin variant allele, hormone replacement therapy and protein S deficiency and insensitive to antithrombin and protein C deficiency (Curvers et al., 2002a). The sensitivity of this assay to the prothrombin variant allele is probably due to the inhibitory effect of prothrombin on APC dependent factor Va inactivation. The sensitivity of this assay to protein S is not unanticipated since free protein S is a cofactor for protein C. A deficiency of protein S would therefore reduce the effectiveness of any activated protein C used in the assay, whether endogenous or exogenous and therefore mimic APC resistance. The affects of protein S deficiency in this assay may explain the elevated APC ratios seen in patients who are pregnant and who are taking oral contraceptives since the protein S levels in these patients are reduced. The insensitivity of the assay to protein C deficiency is unsurprising only if exogenous APC is added in the assay. If the assay relies on activation of the patients own protein C a deficiency should again mimic APC resistance.
It was also shown that the effects of these variables on the APC ratio of a patient are additive, for example patients with factor V Leiden who take oral contraceptives have a higher APC ratio than those who are factor V Leiden carriers. The ETP based method of monitoring APC resistance is therefore a potentially powerful tool since despite its non-specific nature it can give a global assessment as to a patient’s risk of developing a venous thrombosis.

Some of the variation that is seen in the conflicting APC ratio results can potentially be explained by the protocols used in the different studies. It has been shown that there are a large numbers of pre-analytical variables that can affect the outcome of the assay. These include the concentration of the tissue factor, Calcium ions, and phospholipids as well as the concentration of anti-coagulant used in the vacutainer (Curvers et al., 2002b). The assay is unaffected by storage temperature, repeated thawing and centrifugation speed. It was shown that the majority of these variables could be overcome by using sufficient concentration of tissue factor, Calcium ions, and phospholipids since the effects of minor concentration changes on the assay were reduced when higher concentrations were used. The single largest factor which influenced the results was the concentration of the anti-coagulant used. Samples taken into 0.109M tri-sodium citrate showed higher APC sensitivity ratios than those samples taken into 0.130M tri-sodium citrate. There is also the possibility that the choice of method used can affect the result, although this variability can be overcome via the use of automated techniques (Lawrie et al., 2003).

ETP has also been shown to be sensitive to the level of antithrombin and the level of protein C assuming recombinant thrombomodulin is used in the assay. Measurement of the ETP shows a reduced sensitivity when investigating the patient for lupus anticoagulant, although the lag time of thrombin generation is characteristically increased (Regnault et al., 2004). The measurement of ETP is therefore sensitive to all of the conditions that are screened for in a patient that is investigated for a thrombophilic tendency, although it cannot replace the existing assays due to the fact it is unable to determine the exact nature of the patients condition. It has the advantage however, that it can give a global estimation of a patient’s risk of developing a venous thrombosis. This global estimation of risk can be even more potent when considering the fact that thrombin generation assays have been shown to be sensitive to some of the
more esoteric risk factors of thrombosis such as obesity (Cimenti et al., 2006) where the thrombin generation profile of a cohort of obese children, average body mass index (BMI) 33 had a higher ETP, peak height, lag time and time to peak than a cohort of children with a normal BMI (average 19). The elevated ETP and peak height is consistent with an increase thrombotic risk and gives support to the findings that some clotting factors such as factors VII and VIII are frequently raised in obesity (Rosito et al., 2004). The elevated lag time was more surprising but it was postulated by the authors that this is due to an elevation in the concentration of negative regulators of thrombosis, such as TFPI, which has been shown to correlate with increasing BMI (Vambergue et al., 2001). Thrombin generation assays have also been shown to correlate with smoking (Hioki et al., 2001) (Hjemdahl 2001) where it was demonstrated that platelet dependent thrombin generation is increased in smokers compared to non smokers and also increases immediately after smoking. Ex-vivo spiking of non smokers plasma with nicotine and cotinine was also found to increase levels of platelet dependent thrombin generation.

1.6 Project Aims

Since the measurement of thrombin generation has potentially great benefits when used clinically, it is necessary to evaluate the assay to determine if it is suitable for use in the routine diagnostic setting. This is of interest primarily in the detection of patients with thrombophilic disorders since the current screening methods are relatively expensive and do not always detect abnormalities in the patients that are tested. The measurement of thrombin generation in these patients who are apparently ‘normal’ may provide an indicator that they do have a thrombotic tendency even if the precise reason for this has yet to be elucidated. Success in the introduction of the thrombin generation assay for thrombophilia patients would lead to the introduction of the assay in the haemophilia setting; although this is slightly more problematic since the assay is sensitive to contact activation (auto activation of factor XII) when the capacity of a patients plasma to generate thrombin is low and a low concentration of tissue factor is used to initiate the reaction. The use of corn trypsin inhibitor can overcome this effect by inhibiting auto activation of factor XII (Luddington and Baglin 2004) but creates further problems in that it potentially increases the cost of the assay, ultimately making it less desirable for routine use.
The aim of this project was therefore to evaluate the clinical utility of the thrombin generation assay. In order to be effective when used in the diagnostic setting the assay should be relatively simple, quick and be cost effective. The assay should also produce reliable results, i.e. the assay should have a good level of reproducibility with low levels of both inter and intra assay variability. If it is to be used routinely in the diagnostic laboratory of a hospital the effects of sample handling, preparation and storage on the assay must also be ascertained. The assay must also have a normal range established as well as determining how this normal range is affected by both age and sex and how the results from an individual may vary over time. It is also necessary to ascertain the limits of detection for the assay. Above all the assay should produce results which are clinically useful.

1.7 The Calibrated Automated Thrombogram

The use of a primarily automated assay with commercially produced reagents should in theory overcome many of the issues encountered with the thrombin generation assay, especially those associated with varying levels of tissue factor, phospholipid and calcium ion concentration since these factors are known to affect the results generated by the assay (Regnault et al., 2004). To this end it was decided to use the Calibrated Automated Thrombogram (CAT) assay employing the reagents commercially produced by Diagnostic Stago. The CAT assay is essentially the same assay as described by Hemker et al. (1993) but relies on commercially produced reagents and a higher degree of automation.

The CAT assay functions by initiating the clotting cascade via the extrinsic pathway. This is achieved by the addition of patients plasma to a reagent which contains both tissue factor and phospholipids with a final concentration of 5pM and 4μM respectively (PPP reagent), followed by the addition of FluCa solution which contains a final concentration of 20mM calcium ions. This mixture is then incubated at 37°C. The initiation of the clotting cascade ultimately leads to the production of thrombin and the formation of a fibrin clot. The FluCa solution added to the patients’ plasma contains the fluorophore amino-methyl-coumarin (AMC). This is cleaved by the thrombin produced, and generates a fluorescent signal which can be measured at 390/460nm. In accordance with the specifications laid out above the AMC fluorophore is cleaved specifically by
thrombin and is cleaved at a rate which allows for the cessation of thrombin production before the consumption of the substrate. Importantly the use of a fluorogenic signal means the assay is unaffected by the production of a fibrin clot, this is not true of earlier methods which relied on chromogenic signals, which are susceptible to changes in plasma turbidity and therefore had to be performed on defibrinated plasma. The fluorescent signal generated in the assay is measured at regular intervals over a set time course; the data produced is displayed as a graph of fluorescent units against time. The curve produced by this measurement is not accurate however, there is variation due to the consumption of substrate, the inner filter effect (IFE) and even the colour of the patients plasma. To correct for this variation a calibrator is used.

1.7.1 The Use of a Calibrator

The calibrator used in the CAT assay from Diagnostica Stago is a compound with thrombin like activity but which is not inhibited by any of the plasma inhibitors of thrombin. Since the calibrator has a fixed level of amidolytic activity and is not inhibited by proteins in the patients plasma, when a graph of fluorogenic units against time is plotted it should be a straight line. This however is not the case, the graph begins to plateau to the right as the level of fluorescence increases (see figure 1.2A). This pattern is observed primarily due to the IFE and the effects of decreasing substrate concentration.

The inner filter effect essentially describes the non linear relationship between the level of fluorophore present and the degree of fluorescence produced by that fluorophore. At high concentrations of AMC (the fluorophore) not all of the fluorescence is detected by the analyser since some of the signal generated is absorbed inside the sample itself. This causes an underestimation of the amount of fluorescence generated at high concentrations of AMC and therefore ultimately leads to an underestimation of the amount of thrombin produced. The consumption of the fluorophore has a similar effect on the results as the IFE, i.e. an underestimation of the amount of thrombin as the levels of thrombin increase. This phenomenon is due to the kinetics of the enzymatic reaction. Even though the level of fluorogenic substrate is in excess, as the reaction progresses the rate of enzymatic conversion of fluorogenic substrate to AMC decreases due to a reduced rate of collision between the thrombin and the substrate. The correction
performed on the patients’ results is based on the assumption that the calibrator graph should be a straight line.

The analysis software transforms the curved calibrator graph to give a straight line and applies the calculations needed to achieve this to the patients results thereby correcting the patients results for both IFE and substrate consumption. This gives the graph seen in figure 1.2B, before the correction the graph would begin to plateau earlier. It is important to note that unlike the calibrator the patients graph should not be a straight line, this is due to the fact that the thrombin produced by the patient is inhibited by negative regulators such as Antithrombin. The graph shown in figure 1.2B is expressed as fluorescent units against time; in order to convert this to nanomolar (nM) thrombin the initial slope of the calibrator is used. The initial slope of the calibrator fluorescent units against time graph corresponds directly to the activity of the calibrator, i.e. the higher the activity the steeper the slope. An estimate can then be made as to the activity of the patients plasma by comparing the initial slope of the patients corrected fluorescent units/ time graph to that of the calibrator.

Importantly the initial slope of the calibrator is highly sensitive to the effects of donor to donor variability, such as the colour of the patient’s plasma, which can have an impact on the absorbance of fluorescent signal by the plasma. A calibrator is therefore run with each patient. So each patient that has their thrombin generation measured has a separate calibrator well where the fluorescent signal generated by the calibrator in their plasma is measured. Once the units are converted to nanomolar thrombin the first derivative of the curve is plotted, this converts the graph from showing the cumulative level of thrombin produced over time to showing the amount of thrombin produced at every time point. This produces a bell shaped curve shown in figure 1.2C. This curve represents the initiation of thrombin generation, followed by a peak of thrombin production and then a decrease in thrombin production over time due to inhibition.
Figure 1.2

A. Graph of fluorescent units against time (minutes) for thrombin calibrator performed on the plasma of a normal patient. B. Graph of fluorescent units against time (minutes) for PPP reagent performed on the same patient corrected for IEF and substrate consumption. C. First derivative of B, note graph axes are now fluorescent units/min against time. D. Graph C corrected for the activity of thrombin bound to α₂ macroglobulin. Note the units are now nM thrombin against time.

1.7.2 α₂ Macroglobulin

In a physiological setting the thrombin production would decrease to zero following inhibition, the graph in figure 1.2C shows that this is not true of the CAT assay. A low level of thrombin production continues at a fixed rate after the initial burst of productivity, this is also true of the fluorogenic units against time graph shown in figure 1.2B. If thrombin production had ceased the graph would reach a plateau instead the level of thrombin increases in a linear fashion. This phenomenon is observed due to the action of thrombin bound to α₂ macroglobulin. α₂ macroglobulin is a plasma inhibitor of thrombin and therefore plasma bound to α₂ macroglobulin has no activity in vivo but does have an in vitro effect on the CAT assay, i.e. thrombin bound to α₂ macroglobulin can still cleave the fluorogenic substrate to release AMC. This can lead to a large
overestimate in the level of thrombin produced, especially if the thrombin generation is measured over a long time course.

The amidolytic activity of the $\alpha_2$ macroglobulin thrombin complex must therefore be excluded in order to give a true value for the patient’s thrombin generation. This exclusion is performed by the analysis software, which uses a mathematical model to separate the part of the curve generated by free thrombin and the part due to the $\alpha_2$ macroglobulin thrombin complex. The basis of the mathematical model is the identification of when the production of free thrombin is finished in the sample and the thrombin generation seen is due solely to the action of thrombin bound to $\alpha_2$ macroglobulin. This is indicated by the persistent low level thrombin production seen after approximately 20 minutes in figure 1.2C. The analysis software can use this level to estimate the effect which thrombin bound to $\alpha_2$ macroglobulin has on every time point of the curve and ultimately exclude that activity. This gives the final curve which is seen in figure 1.2D. Note the level of thrombin production does now return to zero. The four primary data points which can be obtained from the graph are shown in figure 1.3 below.

Figure 1.3

A. B.

Figure 1.3 Diagrammatic representation of measured thrombin generation parameters. A: Peak Height and time to peak. B: Lag time and ETP. Note ETP is the area under the curve.
1.7.3 The CAT Assay and Phospholipid Concentration

The CAT assay can be performed using a number of different reagents which will have different effects on the results. Using reagents with varying concentrations of phospholipids will impact upon the results generated by the CAT assay. Since the coagulation cascade is dependent on the presence of phospholipids low concentrations would act to increase the lag time and potentially decrease the ETP. The use of a reagent with a low phospholipid concentration also has the potential to make the assay more sensitive to the presence of lupus anticoagulant and other antiphospholipid antibodies, since these antibodies will sequester the phospholipids in the reagent further decreasing the concentration and ultimately having a marked effect on the assay. Increasing the concentration of phospholipids will have the reverse effect; making the assay insensitive to the presence of lupus anticoagulant and decreasing the lag time as well as potentially increasing the ETP.

1.7.4 The CAT Assay and Platelet Rich Plasma

The CAT assay can also be performed using a platelet rich plasma (PRP) reagent. This reagent contains no phospholipids and the thrombin generation which occurs takes place on the surface of the patient’s platelets which remain in the plasma. This assay is therefore of greater relevance physiologically than the PPP reagent assay as in theory it is sensitive to defects in platelet function as well as those in the formal clotting cascade, although it will not be possible to ascertain the exact nature of the defect from the assay results. The PRP reagent assay is however more time consuming and more expensive than the PPP reagent assay, the difference in cost arises due to the fact that assays performed on platelet rich plasma must be performed within hours of the patient’s sample being taken. In a hospital setting this is undesirable as it means that patients must be analysed in a large number of small batches rather than smaller number of large batches. The time and labour involved in processing the patient’s plasma and running the assay a larger number of times will also ultimately increase the cost. The PPP reagent assay should prove more cost effective as the patients plasma can be frozen and stored thereby increasing the efficiency of the assay and ultimately lowering the cost. For these reasons in this project it was chosen to utilise the standard PPP reagent with a
final concentration of 4μM phospholipids to give an excellent indication of how robust and clinically useful the ‘basic’ CAT assay is in a hospital laboratory setting.

1.7.5 Tissue Factor Concentration

It was decided to use a tissue factor concentration of 5pM to initiate the coagulation cascade in this study. One of the deciding factors was that this was the only concentration commercially available at the start of the experiments and it was felt that the use of lower concentrations, which would be made ‘in house’, would introduce potential variables without adding much benefit. This was because any patient demographics used would be prothrombotic not haemophiliacs. A secondary consideration was that the use of 5pM tissue factor as a reaction trigger meant that it should not be necessary to use CTI, although this is discussed in more detail in chapter 2.
Chapter 2

Assay Validation
2.1 Introduction

The first stage of this project was the validation of the CAT assay for use in the routine diagnostic setting. The investigations which are the focus of this chapter include the determination of both the intra and inter assay variability, the effects of sample handling on the assay including the time taken between sample collection and sample processing and the temperature at which samples can be stored. This chapter also determines the assay limits of detection, the variability of a patient’s results over time and the normal range for the assay, including ascertaining the effects of age and sex on the results. This chapter also contains a comparison of thrombin generation data when patients were analysed with and without the use of CTI in order to establish whether or not there was a difference in the results produced when a 5 pM tissue factor trigger was used to initiate thrombin generation.

It is necessary that any parameters measured from a specific sample should be comparable if that sample is measured again using the same method at a different time (inter assay variation) or if the sample is analysed multiple times on the same run (intra assay variation). If an assay which is used to monitor patient results over time demonstrates high inter or intra assay variation the results generated are of little use clinically as it is uncertain as to whether any differences observed are due to variation caused by the assay or to physiological changes in the patient. In a similar fashion assays that are used in diagnosis which have high levels of inter and intra assay variability will be prone to the production of false positive and negative results; especially in those patients who have borderline results in reality.

To maximise the clinical utility of the assay it would be desirable if the results generated by the CAT assay were unaffected by the time between sample collection and the time the sample was received and processed by the laboratory. If the age of the sample did have an effect on the thrombin generation then it may dictate how the assay could be requested i.e. if it was found that samples must be separated and frozen within an hour of being taken then the patients who required the assay would have to make clinic appointments to have blood taken. This would have financial implications for the department. It would therefore be desirable if samples taken for thrombin generation
studies had a similar shelf life to those which are required for other haemostatic investigations, i.e. four hours.

Sample storage has important implications in laboratory diagnosis in terms of both the cost of the assay and the patient diagnosis. To minimise the cost it is preferable that samples can be stored in order that they can be analysed in batches, but it is important to ascertain that sample storage does not impact on the results generated by the assay, i.e. the results generated do not differ if a patients sample is analysed after storage for one day or analysed after storage for one week. Samples sent to the haemostasis laboratory for investigations are analysed within two weeks of receipt and are stored at -40°C for the duration of this time. It would therefore be beneficial if samples requiring analysis by the CAT assay could be treated in the same manner.

The limits of detection are necessary in order to determine whether an obtained result is valid or not. The limits of detection are defined as the upper and lower limits that an assay can produce an accurate answer. If for example the assay is inaccurate below a certain level all assay results which fall below that level are reported as lower than the cut off, rather than giving an actual numerical value. This is of importance since the assay may show an increased variability below the cut off which makes the monitoring of patients results unreliable.

The variability of a patient’s results over time can have a significant impact on the interpretation of any given result. This is similar to the inter assay variability but instead of variation in patients results being caused by the assay, the results are affected by physiological changes in the patients themselves. The effects however remain the same; a high variability across time will mean the assay is less useful in terms of both diagnosis and therapeutic monitoring. This chapter investigates the variation of results over time in a cohort of normal individuals.

The normal range of the assay is obviously essential in determining whether an obtained result is normal or abnormal. It is usually presented as a minimum and maximum level between which ~95% of all normal individuals fall. Patients with results which are outside the normal range are defined as abnormal. It is therefore extremely important that the normal range is accurately defined since it will be used diagnostically. In the
case of the CAT thrombin generation assay, levels of ETP and Peak height below the normal range will be indicative of a potential bleeding disorder whereas those above will be indicative of a potential prothrombotic condition. The reverse is true of the lag time and the time to peak parameters where a result above the normal range is indicative of a bleeding disorder and a result below the normal range potentially implies a tendency towards thrombosis. The normal range is often adjusted depending on a variety of different factors, most commonly age and sex. The experiments in this chapter are focused on establishing a normal range for the CAT assay and examining the effects of both age and sex when determining a normal thrombin generation.

2.2 Materials and Methods

2.2.1 PT/INR

The measurement of the PT/INR was an automated assay performed on the Ca-7000 analyser manufactured by Sysmex. Innovin (100µl) was added to 50µl of patient’s plasma in a plastic cuvette. This initiated the clotting cascade via the extrinsic pathway. The cuvette was then incubated at 37ºC. During the incubation the scattered light intensity of the sample was measured by a red light with a wavelength of 600nm. The conversion of fibrinogen to fibrin increases the turbidity of the sample and hence increases the scattered light intensity. The time in seconds at which the scattered light intensity changes is the prothrombin time. The PT is routinely converted to an INR. The INR is a calculated parameter and is derived using the formula below.

\[ \text{INR} = \left( \frac{\text{Patient PT}}{\text{mean normal PT}} \right)^{\text{ISI}} \]

The mean normal PT is determined by the geometric mean of the PT of 21 normal individuals who are representative of the local population. The ISI is effectively a calibrator value which indicates how tissue factors produced by different manufacturers compare to an internationally standardised sample (human brain thromboplastin held by the world health organisation).

The assay was controlled using Citrol 1 and Citrol 2 which have target values that are within the normal and therapeutic ranges respectively.
Innovin, Citrol 1 and Citrol 2 are lyophilised reagents and require reconstitution before use. 20ml of sterile distilled water was added to Innovin and 1ml of sterile distilled water was added to both Citrol 1 and 2. The reagents were then held at room temperature for 15 minutes before use.

2.2.2 APTT

The measurement of the APTT was an automated assay performed on the Ca-7000 analyser manufactured by Sysmex. Actin FS (100µl) was added to 50µl of patient’s plasma in a plastic cuvette. The cuvette was then incubated at 37°C for one minute. Following this time 50µl of 0.025M Calcium Chloride solution was added to the cuvette; this initiated the clotting cascade via the intrinsic pathway. The scattered light intensity of the sample was then measured using a red light with a wavelength of 600nm. The conversion of fibrinogen to fibrin increases the turbidity of the sample and hence increases the scattered light intensity. The time in seconds at which the scattered light intensity changes is the activated partial thromboplastin time.

The assay was controlled using Citrol 1 and Citrol 2 which have target values that are within the normal and therapeutic ranges respectively.

Citrol 1 and Citrol 2 are lyophilised reagents and require reconstitution before use. 1ml of sterile distilled water was added to both Citrol 1 and 2. The reagents were then held at room temperature for 15 minutes before use.

2.2.3 Thrombin Time

The measurement of the thrombin time was an automated assay performed on the Ca-7000 analyser manufactured by Sysmex. Thromboclotin reagent (100µl) was added to 50µl of patient plasma in a plastic cuvette. The cuvette was then incubated at 37°C. The scattered light intensity of the sample was measured using a red light with a wavelength of 600nm. The thromboclotin reagent contains activated factor II, which converts the fibrinogen in the patients sample into fibrin thereby increasing the turbidity of the sample and ultimately causing an increase in the scattered light intensity. The time in seconds at which the scattered light intensity changes is the thrombin time.
The assay was controlled using Citrol 1 and Citrol 2 which both have target values that are within the normal range.

Thromboclotin, Citrol 1 and Citrol 2 are lyophilised reagents and require reconstitution before use. 5ml of sterile distilled water was added to Thromboclotin and 1ml of sterile distilled water was added to both Citrol 1 and 2. The reagents were then held at room temperature for 15 minutes before use.

2.2.4 Preparation of Samples

Blood was collected into 0.109M tri-sodium citrate vacutainers. Samples were centrifuged at 1500 g for 10 minutes. Following this time the top two thirds of the patient’s plasma were removed and aliquoted into LP4 tubes. These aliquots were then centrifuged for a further 10 minutes at 1500 g. The top two thirds of the double spun plasma was then removed, placed in a corning tube and frozen.

2.2.5 Thrombin Generation

Thrombin generation was performed using the Fluoroscan FL plate reader manufactured by Thermoelectron. PPP and thrombin calibrator reagents were reconstituted with 1ml of sterile distilled water and left to equilibrate for 5 minutes at room temperature. During this time 10ml of sterile distilled water and an aliquot of fluoro buffer (120µl is required for each patient) were preheated to 37°C in a water bath. Following the 5 minute equilibration the PPP and thrombin calibrator reagents were shaken gently to ensure they were fully reconstituted. Each patient sample that was analysed required 6 wells on a 96 well 2HB Immulon round bottom plate. Into three of the wells 20µl of PPP reagent was added, 20µl of thrombin calibrator was added to the remaining three wells. Patient plasma (80µl) was then added to each of the six wells. The plate was then placed in the Fluoroscan FL plate reader to equilibrate to 37°C. The dispenser on the Fluoroscan FL was then cleaned and primed using the preheated sterile distilled water. The Fluca solution was then prepared by adding fluorochrome solution to the preheated fluoro buffer. The final ratio of fluorochrome solution to fluoro buffer was 1:40. The addition of the fluorochrome solution to the fluoro buffer caused the solution to become cloudy, the solution was then vortex mixed to yield a clear, colourless Fluca solution.
This solution was then primed into the dispenser and 20 µl was automatically dispensed into each well in use on the plate. The plate was then agitated for 10 seconds. The measurement of thrombin generation commenced following the agitation. Fluorescence was measured at 390/460nm every 30 seconds for up to one hour depending on the experimental procedure.

2.2.6 Reconstitution of Control Plasma P

1ml of sterile distilled water was added to each vial. The vials were then held for 15 minutes at room temperature. Following this time they were gently mixed.

2.2.7 Reconstitution of Standard Human Plasma

1ml of sterile distilled water was added to each vial. The vials were then held for 15 minutes at room temperature. Following this time they were gently mixed.

2.2.8 Heat Inactivation of Plasma

Blood was collected into 0.109M tri-sodium citrate vacutainers. Samples were centrifuged at 1500 g for 10 minutes. Following this time the patients’ plasma was removed and pooled. The pooled plasma was then aliquoted into LP4 tubes and placed in a water bath pre-heated to 58°C for 90 minutes. Following this incubation the plasma aliquots were again centrifuged at 1500 g for 10 minutes. This centrifugation separates any proteins precipitated during the heat inactivation from the plasma. Following the centrifugation the patients’ plasma was removed from the precipitated plasma proteins and pooled. The heat inactivated plasma was then separated into 5ml aliquots and frozen at -70°C.

2.2.9 Statistical Analysis

Statistical analyses were performed using Microsoft excel and PASW statistics 18. Comparisons between data sets were performed using either the student’s t-test (parametric data) or the Mann Whitney U test (non parametric data). Statistical significance was defined as a p value of ≤0.05. Normal distribution was determined
using the Kolmogorov-Smirnov test, where a p value of $\geq 0.05$ was indicative of a normal distribution.

### 2.2.10 Reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin FS</td>
<td>Dade Behring</td>
</tr>
<tr>
<td>Calcium Chloride solution (0.025M)</td>
<td>Dade Behring</td>
</tr>
<tr>
<td>Citrol 1</td>
<td>Dade Behring</td>
</tr>
<tr>
<td>Citrol 2</td>
<td>Dade Behring</td>
</tr>
<tr>
<td>Control P</td>
<td>Dade Behring</td>
</tr>
<tr>
<td>Corn Trypsin Inhibitor</td>
<td>Cambridge Bioscience</td>
</tr>
<tr>
<td>FluCa Kit</td>
<td>Diagnostica Stago</td>
</tr>
<tr>
<td>Innovin</td>
<td>Dade Behring</td>
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<tr>
<td>PPP Reagent</td>
<td>Diagnostica Stago</td>
</tr>
<tr>
<td>Standard Human Plasma</td>
<td>Dade Behring</td>
</tr>
<tr>
<td>Sterile Distilled Water</td>
<td>Baxter</td>
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<tr>
<td>Thrombin Calibrator</td>
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<td>Thromboclotin</td>
<td>Dade Behring</td>
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### 2.3 Results

#### 2.3.1 Inter and Intra Assay Variation

#### 2.3.1.1 Experimental Procedure

Using the thrombin generation method described in section 2.2.5 (materials and methods) 16 patients could be analysed on a single run. Due to potential problems with dead volume of reagents it was decided to run only a maximum of 15 patients per plate as this would mean any discrepancies in the results would be due solely to the workings of the assay and not any inconsistencies in the volumes of reagents used. To ascertain the intra and inter assay variability of the CAT assay the same plasma sample was run 15 times on each of 10 plates. Since this experiment required a large volume of plasma it was decided to use a lyophilised plasma product as it was easily obtainable. The plasma used in this experiment was Control plasma P produced by Dade Behring. A total of 80ml of the plasma was prepared; this plasma was then pooled and frozen at -70°C in 10 separate 8ml aliquots. The pooling of the plasma ensured that any differences observed were entirely due the technique and not due to the effects of using
plasma reconstituted at different times. It was decided that the aliquots of plasma should be frozen for a number of reasons. The first of which was to compensate for the time needed to analyse the plasma, since the thrombin generation was measured for 1 hour in this experiment, if the plasma was left at room temperature the aliquot which was analysed last would be at least 9 hours old. Since the half lives of some of the clotting factors in the coagulation cascade are shorter than 9 hours, indeed factor VII has a half life of only 4 hours, leaving the plasma at room temperature could have a huge impact on the thrombin production and ultimately the results generated by this analysis. It was decided to freeze the aliquots at the lowest temperature available in order to freeze the samples in the quickest time and thereby decrease the potential for any degradation of the plasma. The second reason for freezing the plasma was to follow the preparation of patient samples which are processed for thrombophilia investigations, these are frozen upon receipt. The aliquots of Control plasma P were defrosted by submersion in a 37°C water bath, again following the preparation of thrombophilia samples, and analysed using the CAT thrombin generation assay. The results for the intra assay variation are shown in tables 2.1-2.4 below.

2.3.1.2 Intra Assay Variation

When examining the results for intra assay variation each plate was considered separately for each of the four thrombin generation parameters. To be considered suitable for routine use the assay should have a coefficient of variation (CV) of approximately 10% or below and the closer this is the zero the lower the variation shown by the assay. All of the plates analysed demonstrate CV’s of below 10% for each of the four parameters. Some of the parameters seem to demonstrate more variation than others however. Both the peak height and the time to peak had CV’s below 5% for all the 10 plates analysed. The CV’s for the ETP were more widely spread ranging from 2.52% to 8.21%, the greatest difference was demonstrated by the lag time with values ranging from 0.00% to 7.5%. All these values did lie within the range of acceptability.
Table 2.1

<table>
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<tr>
<th>Plate Number</th>
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<th>SD</th>
<th>CV (%)</th>
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Table 2.1 showing the median, range, mean, standard deviation and coefficient of variation of the ETP of Control plasma P for each of the 10 plates analysed

Table 2.2

<table>
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<th>CV (%)</th>
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Table 2.2 showing the median, range, mean, standard deviation and coefficient of variation of the Lag time of Control plasma P for each of the 10 plates analysed.
Table 2.3 showing the median, range, mean, standard deviation and coefficient of variation of the peak height of Control plasma P for each of the 10 plates analysed.

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<td>211.50</td>
<td>7.58</td>
<td>3.69</td>
</tr>
<tr>
<td>9</td>
<td>203.67</td>
<td>22.53</td>
<td>204.62</td>
<td>6.75</td>
<td>3.30</td>
</tr>
<tr>
<td>10</td>
<td>206.82</td>
<td>25.69</td>
<td>205.67</td>
<td>7.25</td>
<td>3.52</td>
</tr>
</tbody>
</table>

Table 2.4 showing the mean, standard deviation and coefficient of variation of the time to peak of Control plasma P for each of the 10 plates analysed.

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.04</td>
<td>0.42</td>
<td>4.90</td>
<td>0.17</td>
<td>3.54</td>
</tr>
<tr>
<td>2</td>
<td>5.00</td>
<td>0.56</td>
<td>4.97</td>
<td>0.12</td>
<td>2.43</td>
</tr>
<tr>
<td>3</td>
<td>5.42</td>
<td>0.83</td>
<td>5.53</td>
<td>0.21</td>
<td>2.86</td>
</tr>
<tr>
<td>4</td>
<td>5.00</td>
<td>0.42</td>
<td>5.06</td>
<td>0.13</td>
<td>2.61</td>
</tr>
<tr>
<td>5</td>
<td>5.42</td>
<td>0.83</td>
<td>5.49</td>
<td>0.20</td>
<td>3.62</td>
</tr>
<tr>
<td>6</td>
<td>4.62</td>
<td>0.42</td>
<td>4.70</td>
<td>0.13</td>
<td>2.80</td>
</tr>
<tr>
<td>7</td>
<td>5.00</td>
<td>0.42</td>
<td>4.90</td>
<td>0.13</td>
<td>2.69</td>
</tr>
<tr>
<td>8</td>
<td>5.28</td>
<td>0.62</td>
<td>5.29</td>
<td>0.16</td>
<td>3.02</td>
</tr>
<tr>
<td>9</td>
<td>5.00</td>
<td>0.70</td>
<td>4.94</td>
<td>0.21</td>
<td>4.31</td>
</tr>
<tr>
<td>10</td>
<td>4.58</td>
<td>0.56</td>
<td>4.68</td>
<td>0.16</td>
<td>3.33</td>
</tr>
</tbody>
</table>
2.3.1.3 Inter Assay Variation

Tables 2.1-2.4 also show data relating to the inter assay variability, the means and the standard deviations from the 10 plates were expected to show similar values for each of the parameters if the inter assay variation was low. This information is displayed graphically in figure 2.1 below. The data shown in figure 2.1 shows that there was a marked difference between the assays for some of the parameters. Some fluctuation was expected but with the exception of the peak height (Fig 2.1C) where all the boxplots showed a degree of overlap there were distinct cases in each of the other graphs (Figures 2.1 A, B and D) where at least two of the boxplots showed no overlap at all implying that they were statistically distinct populations. E.g. Plates 3 and 10 on figure 2.1A. Indeed when analysed using the students t-test/Mann Whitney U test some of these comparisons did yield a p value of <0.05 proving this to be the case (Data not shown). This suggested a high inter assay variability making the assay unsuitable for routine use. However, when all 150 data points (15 replicates on each of the 10 plates) for each parameter were compared directly and the mean, standard deviation and CV were calculated the differences became less pronounced. The results of this analysis can be seen in table 2.5.

Figure 2.1

A.  B.
Figure 2.1. Boxplots showing the distribution of the 15 values for each parameter of each of the 10 analysed plates. Analysis performed using Control plasma P. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: Lag time, B: ETP, C: Peak height, D: Time to peak.

Table 2.5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP (nM Thrombin)</td>
<td>2695.91</td>
<td>217.55</td>
<td>8.07</td>
</tr>
<tr>
<td>Lag Time (Minutes)</td>
<td>2.54</td>
<td>0.29</td>
<td>11.44</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>213.54</td>
<td>9.13</td>
<td>4.28</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>5.05</td>
<td>0.33</td>
<td>6.49</td>
</tr>
</tbody>
</table>

Table 2.5. Meta analysis of all 150 Control plasma P data points.

The results from table 2.5 showed that as predicted the peak height had the lowest CV of the four parameters but the CV of the ETP and the time to peak also fell in the range of acceptability (<10%). The CV of the lag time however remained unacceptable. In summary the initial analysis of the assay produced results which were mixed; the assay showed reasonably low levels of intra assay variability but high levels of inter assay variation.
2.3.1.4 Intra Assay Trends

Although the levels of intra assay variation appeared low it was also important that the results across the plate did not show any trends, i.e. the results of the sample should not have been higher/lower at the 15th position on the plate than they are at position 1. This was of importance in the CAT assay as the bulk of the work was done manually and therefore relatively slowly. The PPP and the thrombin calibrator reagents were added to the plate first then the patients’ samples were added one at a time. The problem which arose from this was that by the time the 15th patient sample was added the first patients sample has been incubated in the reagent for a short while and although no thrombin generation can occur in this time (owing to lack of calcium ions) this incubation could lead to differences in the results. To investigate whether or not this sort of trend did occur in the assay it was decided to calculate the mean of each of the 15 possible patient positions across the 10 plates, and plot them on a scatter graph. The line of best fit and the $R^2$ value was then calculated using Microsoft excel. From the $R^2$ parameter a value of $t$ was obtained using the formula below.

$$t = r \sqrt{(n-2/1-r^2)}$$

The value of $t$ obtained was used to calculate a $p$ value using the distribution of $t$ table shown in the appendix (7.1), $n$-2 degrees of freedom was used. A $p$ value of $\leq 0.05$ was considered evidence of a significant trend. The results of this analysis are shown in figure 2.2 below. Figure 2.2 A, B and D all show relatively flat trend lines and values for $R^2$ which are comparatively low. An $R^2$ value approaching zero indicates that no trend is apparent whereas an $R^2$ value approaching one is a strong indication of the presence of a trend. The $p$ values calculated for the ETP, lag time and time to peak are all $>0.05$ implying there is no trend across the plate; this however is not the case for the peak height (figure 2.2C). The peak height graph shows a slight decrease across the plate positions i.e. the peak height at position one is higher than that of position 15. This trend has an $R^2$ value of 0.5434 which gives a highly significant $p$ value of $<0.01$. This is an undesirable result as it implies that the position a patient occupies on a plate can have an effect on the result generated. Interestingly although the trend appears highly significant the actual decrease in the peak height across the plate is only 13 nM thrombin (from 217nM thrombin at position 1 to 204nM thrombin at position 15), this is a difference of less that 10% which has already been predefined as acceptable when
considering the CV. It is also uncertain as to whether a difference of this magnitude is clinically significant or not. Regardless of these points a statistically significant trend across the plate is still highly undesirable. The reason for the difference can only be hypothesised; any degradation of the reagents or patients sample would have been likely to cause the opposite trend i.e. an increase in peak height across the plate. The increased incubation time of plasma with the reagents seems to be able to increase the maximum rate of thrombin generation possibly because of any calcium independent reactions have had more time to occur in the sample wells which are aliquoted first.

Figure 2.2

A.  

![Intra Assay Variation](image)

B.  

![Intra Assay Variation](image)

C.  

![Intra Assay Variation](image)

D.  

![Intra Assay Variation](image)

Figure 2.2. Graphs showing mean values for plate position across the 10 analysed plates, complete with trend lines and $R^2$ as an indication of correlation. A: ETP, B: Lag time, C: Peak height, D: Time to peak.

2.3.1.5 Initial Conclusions

The initial experimentation revealed some disappointing results. Although the intra assay variation was good, the assay did show a decreasing trend for one of the four parameters across the plate. The inter assay variation was poor with the lag time showing an unacceptable CV and some of the other parameters demonstrating marked differences between runs. One of the potential problems with these experiments may
have been the time differential of the addition of reagents and the patients plasma to the plate. In order to determine whether or not this was the case it was decided to use an automatic pipette to dispense the reagents and the patients plasma as this would be quicker and ultimately lead to lower CVs and eliminate any trends if they were indeed caused by the length of time taken to set up the plate.

2.3.1.6 Assay Reproducibility Using Electronic Pipetting

This experiment was set up in the same way as above, although only 5 plates were analysed as opposed to 10 in the original experiment. 40ml of Control P plasma was prepared and frozen at -70°C in five 8ml aliquots. These were defrosted in a 37°C water bath and analysed one by one. The intra assay variation results are shown in tables 2.6-2.9 below.

Table 2.6

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median ETP (nM Thrombin)</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2611.67</td>
<td>438.67</td>
<td>2649.07</td>
<td>126.32</td>
<td>4.77</td>
</tr>
<tr>
<td>2</td>
<td>2743</td>
<td>649.33</td>
<td>2708.29</td>
<td>170.53</td>
<td>6.30</td>
</tr>
<tr>
<td>3</td>
<td>2770</td>
<td>489.33</td>
<td>2788.46</td>
<td>141.07</td>
<td>5.06</td>
</tr>
<tr>
<td>4</td>
<td>2869.67</td>
<td>602.5</td>
<td>2825.28</td>
<td>164.50</td>
<td>5.82</td>
</tr>
<tr>
<td>5</td>
<td>2773.67</td>
<td>446.33</td>
<td>2742.28</td>
<td>126.61</td>
<td>4.62</td>
</tr>
</tbody>
</table>

Table 2.6 showing the median, range, mean, standard deviation and coefficient of variation of the ETP of Control plasma P for each of the 5 plates analysed. Each assay was set up using an electronic pipette.

Table 2.7

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median Lag Time (Minutes)</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.08</td>
<td>0.42</td>
<td>2.21</td>
<td>0.16</td>
<td>7.21</td>
</tr>
<tr>
<td>2</td>
<td>2.29</td>
<td>0.42</td>
<td>2.31</td>
<td>0.20</td>
<td>8.46</td>
</tr>
<tr>
<td>3</td>
<td>2.22</td>
<td>0.42</td>
<td>2.25</td>
<td>0.18</td>
<td>8.21</td>
</tr>
<tr>
<td>4</td>
<td>1.87</td>
<td>0.41</td>
<td>1.87</td>
<td>0.18</td>
<td>9.43</td>
</tr>
<tr>
<td>5</td>
<td>2.22</td>
<td>0.42</td>
<td>2.27</td>
<td>0.19</td>
<td>8.28</td>
</tr>
</tbody>
</table>

Table 2.7 showing the median, range, mean, standard deviation and coefficient of variation of the lag time of Control plasma P for each of the 5 plates analysed. Each assay was set up using an electronic pipette.
Table 2.8

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>218.10</td>
<td>9.03</td>
<td>217.88</td>
<td>2.72</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>215.03</td>
<td>20.14</td>
<td>215.93</td>
<td>6.08</td>
<td>2.82</td>
</tr>
<tr>
<td>3</td>
<td>217.51</td>
<td>20.76</td>
<td>217.09</td>
<td>5.68</td>
<td>2.62</td>
</tr>
<tr>
<td>4</td>
<td>218.74</td>
<td>18.27</td>
<td>219.87</td>
<td>4.72</td>
<td>2.15</td>
</tr>
<tr>
<td>5</td>
<td>221.63</td>
<td>20.01</td>
<td>221.13</td>
<td>5.11</td>
<td>2.31</td>
</tr>
</tbody>
</table>

Table 2.8 showing the median, range, mean, standard deviation and coefficient of variation of the peak height of Control plasma P for each of the 5 plates analysed. Each assay was set up using an electronic pipette.

Table 2.9

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.68</td>
<td>0.28</td>
<td>4.68</td>
<td>0.11</td>
<td>2.25</td>
</tr>
<tr>
<td>2</td>
<td>4.89</td>
<td>0.42</td>
<td>4.89</td>
<td>0.12</td>
<td>2.47</td>
</tr>
<tr>
<td>3</td>
<td>4.72</td>
<td>0.42</td>
<td>4.86</td>
<td>0.14</td>
<td>2.88</td>
</tr>
<tr>
<td>4</td>
<td>4.44</td>
<td>0.41</td>
<td>4.39</td>
<td>0.09</td>
<td>2.11</td>
</tr>
<tr>
<td>5</td>
<td>4.58</td>
<td>0.42</td>
<td>4.82</td>
<td>0.13</td>
<td>2.73</td>
</tr>
</tbody>
</table>

Table 2.9 showing the median, range, mean, standard deviation and coefficient of variation of the time to peak of Control plasma P for each of the 5 plates analysed. Each assay was set up using an electronic pipette.

2.3.1.7 Electronic Pipetting and Intra Assay Variation

The results in tables 2.6-2.9 were very similar to those obtained without the use of an electronic pipette. The CVs for each of the parameters on all of the plates are below the 10% cut off of acceptability. The best CVs were seen in the peak height and the time to peak parameters with the ETP and the lag time showing elevated CVs in comparison. Indeed the lag time still showed the highest degree of variation with CVs ranging between 7.21% and 9.43%.
2.3.1.8 Electronic Pipetting and Inter Assay Variation

The inter assay variation data is shown graphically in figure 2.3 below. The results for inter assay variation were also similar to those previously achieved without the use of electronic pipetting, although now it was the ETP and the peak height which demonstrated a high degree of overlap instead of the peak height alone. The lag time and the time to peak still showed a marked variation between runs some of which had a statistically significant p value when analysed by the student’s t-test (data not shown). Although the majority of the variation in these parameters seemed to come from a single run, that of plate 4. The meta analysis of the results obtained using electronic pipetting is shown in table 2.10 below. The CVs of all the parameters except that of the lag time fell below the 10% cut off and indeed all of them showed a degree of improvement when compared to the results obtained without the electronic pipette, although not by the magnitude that was anticipated. The assay still ultimately showed an unacceptably high level of inter assay variation.

Figure 2.3

A.  

B.
Figure 2.3. Boxplots showing the distribution of the 15 values for each parameter of each of the 5 analysed plates, each of which was set up using an electronic pipette. Analysis performed using Control plasma P. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: ETP, B: Lag Time, C: Peak height, D: Time to peak.

Table 2.10

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP (nM Thrombin)</td>
<td>2742.68</td>
<td>155.76</td>
<td>5.68</td>
</tr>
<tr>
<td>Lag Time (Minutes)</td>
<td>2.18</td>
<td>0.24</td>
<td>10.86</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>218.22</td>
<td>5.22</td>
<td>2.40</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>4.73</td>
<td>0.22</td>
<td>4.64</td>
</tr>
</tbody>
</table>

Table 2.10. Meta analysis of all 75 Control P data points, each set up using an electronic pipette.

2.3.1.9 Electronic Pipetting and Intra Assay Trends

The use of the electronic pipette also failed to correct the intra assay trends that were found in the initial experiment. The results of the trend analysis are shown in figure 2.4 below. Both the ETP and the time to peak showed no significant trends across the plate with non significant p values. The lag time and peak height both showed significant trends across the plate with p values of <0.01. The trends themselves were in opposite directions with the lag time increasing and the peak height decreasing with plate position. The magnitude of these trends was also different, with the changes in peak
height relatively small (<10%) and the changes in lag time much more pronounced, reaching approximately 20% across the plate.

Figure 2.4

A. B. C. D.

Figure 2.4. Graphs showing mean values for plate position across the 5 analysed plates each set up using an electronic pipette, complete with trend lines and R² as an indication of correlation. A: ETP, B: Lag time, C: Peak height, D: Time to peak.

The increasing lag time across the plate fitted well with the increased incubation time of plasma and reagents hypothesis, but this was highly unlikely since the same effects were not seen in the original experiment where the time differential in setting up the plate was larger.

The use of an electronic pipette therefore failed to correct any of the problems seen in the assay in the initial experiment, implying the underlying cause for the trends and poor inter assay variability observed were not due to the speed that the plate is set up. A potential reason for the differences is the lyophilised plasma itself. Upon analysis it became clear that the use of this plasma was associated with a high degree of ‘noise’ in
the assay i.e. the thrombin generation curve was not as smooth as was anticipated. An example of this is shown below in figure 2.5. The effects of this ‘noise’ can be most appreciated when considering the ETP. Since this is a parameter derived by calculating the area under the curve, the erratic curves obtained using Control plasma P may have interfered with the analysis software producing results which were ultimately less reliable. The background ‘noise’ may also have effects on the other parameters. It is therefore possible that the Control plasma P itself was not suitable for use in the CAT thrombin generation assay. The exact reason as to why Control P produces erroneous results was initially thought to be due to its composition, this however seems unlikely since the only chemical additive is Hepes buffer which is unlikely to have any effect on the generation of a fluorescent signal. It is likely that the disruption of the fluorescent signal could instead be due to the presence of particulate matter in the Control P plasma, possibly as a result of incomplete reconstitution of the reagent. To test this hypothesis this experiment was performed again using fresh plasma from volunteers.

Figure 2.5

![Thrombin Generation of Control P](image)

Figure 2.5. An example thrombin generation curve produced by analysing Control plasma P.

### 2.3.1.10 Assay Reproducibility Using Fresh Plasma

This experiment was performed in the same manner as those above but used plasma taken from healthy volunteers instead of a lyophilised plasma product. The electronic pipette was not used in this analysis. Since the concentration of the anti-coagulant used
can have large effects on the results of thrombin generation assays (Gatt et al., 2008) all blood was taken into 0.109M tri-sodium citrate vacutainers. Blood samples were taken and prepared as described in section 2.2.4 preparation of samples (materials and methods). This method of sample preparation was chosen as it was the method employed by the haemostasis laboratory for the preparation of samples for thrombophilia investigation. This method of separation should effectively remove platelets from the patient’s plasma and therefore limit the level of endogenous phospholipids in the patient’s sample. Since the double spinning procedure described yielded approximately 1ml of plasma from each vacutainer of whole blood, four 4.5ml vacutainers of whole blood were collect from 10 healthy volunteers. The criteria for selection of the volunteers were that they were consenting adults over 18 years of age with no current medical conditions who had no history of either bleeding disorders or thrombosis. A clotting screen including PT/INR, APTT and thrombin time was performed on the plasma collected from each volunteer to determine any unknown haemostatic disorders and to ascertain whether or not they were undergoing anticoagulation therapy. All volunteers screened for this experiment had normal results (data not shown). The plasma prepared from the 40 vacutainers was then pooled and frozen at -70°C in five 8ml aliquots. Each aliquot was defrosted by submersion in a 37°C waterbath and analysed separately. Thrombin generation was measured as described in section 2.2.5 for a total of 45 minutes in this experiment. The results for the intra assay variation are shown in tables 2.11-2.14 below.

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2123.00</td>
<td>256.67</td>
<td>2140.88</td>
<td>79.22</td>
<td>3.70</td>
</tr>
<tr>
<td>2</td>
<td>2028.33</td>
<td>239.66</td>
<td>2049.47</td>
<td>64.79</td>
<td>3.16</td>
</tr>
<tr>
<td>3</td>
<td>1973.67</td>
<td>274.67</td>
<td>1958.07</td>
<td>70.83</td>
<td>3.62</td>
</tr>
<tr>
<td>4</td>
<td>2072.00</td>
<td>341.33</td>
<td>2053.05</td>
<td>84.71</td>
<td>4.12</td>
</tr>
<tr>
<td>5</td>
<td>2103.67</td>
<td>329.00</td>
<td>2114.04</td>
<td>95.44</td>
<td>4.51</td>
</tr>
</tbody>
</table>

Table 2.11 showing the median, range, mean, standard deviation and coefficient of variation of the ETP of a pool of fresh human plasma for each of the 5 plates analysed.
Table 2.12

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median</th>
<th>Range (Minutes)</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.33</td>
<td>0.28</td>
<td>3.45</td>
<td>0.14</td>
<td>4.01</td>
</tr>
<tr>
<td>2</td>
<td>3.33</td>
<td>0.41</td>
<td>3.23</td>
<td>0.14</td>
<td>4.38</td>
</tr>
<tr>
<td>3</td>
<td>3.06</td>
<td>0.41</td>
<td>3.11</td>
<td>0.18</td>
<td>5.93</td>
</tr>
<tr>
<td>4</td>
<td>3.75</td>
<td>0.42</td>
<td>3.83</td>
<td>0.16</td>
<td>4.09</td>
</tr>
<tr>
<td>5</td>
<td>3.30</td>
<td>0.41</td>
<td>3.43</td>
<td>0.19</td>
<td>5.53</td>
</tr>
</tbody>
</table>

Table 2.12 showing the median, range, mean, standard deviation and coefficient of variation of the lag time of a pool of fresh human plasma for each of the 5 plates analysed.

Table 2.13

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median (nM Thrombin)</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>384.19</td>
<td>43.05</td>
<td>383.64</td>
<td>12.53</td>
<td>3.26</td>
</tr>
<tr>
<td>2</td>
<td>368.66</td>
<td>35.07</td>
<td>367.49</td>
<td>9.04</td>
<td>2.46</td>
</tr>
<tr>
<td>3</td>
<td>354.93</td>
<td>43.33</td>
<td>354.59</td>
<td>11.45</td>
<td>3.23</td>
</tr>
<tr>
<td>4</td>
<td>357.07</td>
<td>48.88</td>
<td>357.10</td>
<td>14.12</td>
<td>3.95</td>
</tr>
<tr>
<td>5</td>
<td>355.38</td>
<td>23.67</td>
<td>356.87</td>
<td>7.54</td>
<td>2.11</td>
</tr>
</tbody>
</table>

Table 2.13 showing the median, range, mean, standard deviation and coefficient of variation of the peak height of a pool of fresh human plasma for each of the 5 plates analysed.

Table 2.14

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Median (Minutes)</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.97</td>
<td>0.28</td>
<td>5.97</td>
<td>0.12</td>
<td>1.98</td>
</tr>
<tr>
<td>2</td>
<td>5.83</td>
<td>0.41</td>
<td>5.73</td>
<td>0.14</td>
<td>2.00</td>
</tr>
<tr>
<td>3</td>
<td>5.69</td>
<td>0.41</td>
<td>5.63</td>
<td>0.18</td>
<td>3.29</td>
</tr>
<tr>
<td>4</td>
<td>6.39</td>
<td>0.42</td>
<td>6.44</td>
<td>0.18</td>
<td>1.68</td>
</tr>
<tr>
<td>5</td>
<td>6.07</td>
<td>0.42</td>
<td>6.05</td>
<td>0.16</td>
<td>2.61</td>
</tr>
</tbody>
</table>

Table 2.14 showing the median, range, mean, standard deviation and coefficient of variation of the time to peak of a pool of fresh human plasma for each of the 5 plates analysed.
2.3.1.11 Fresh Plasma and Intra Assay Variation

The results in tables 2.11-2.14 show good levels of intra assay variation with the CVs of each of the four parameters on all of the 5 plates below 6%. This was an improvement on those obtained using Control plasma P. The reason for this apparent improvement can be elucidated by examining the thrombin generation curve obtained using the fresh pooled plasma seen in figure 2.6 below. The curve produced is much smoother than that obtained in analysis of Control plasma P eluding to a reduction of background ‘noise’ in the assay, implying that the ‘noise’ produced when analysing Control plasma P was responsible for some of the variation seen previously.

Figure 2.6

![Thrombin Generation of the Pooled Plasma](image)

Figure 2.6. An example of a thrombin generation curve produced by analysing a pool of fresh plasma

2.3.1.12 Fresh Plasma and Inter Assay Variation

The inter assay variation data is shown graphically in figure 2.7 below. The levels of inter assay variation showed a similar result to those achieved using Control plasma P. The results for ETP and peak height show a reasonable degree of overlap implying no statistical significance between the results which when analysed using the students t-test proved to be the case p= >0.05 (data not shown). The lag time and the time to peak however again showed marked variation between runs with statistically significant results when analysed using the students t-test p= <0.05 (data not shown). When a meta
analysis was performed using all 75 available data points the results showed a degree of improvement. See table 2.15. The CVs obtained on all four parameters fell below the 10% cut off of acceptability and were all better than those obtained using Control plasma P without electronic pipetting (table 2.5). The results obtained again demonstrate that the use of fresh pooled plasma decreased the level of variability in the assay compared to the use of lyophilised Control plasma P. The degree of inter assay variation was still a cause for concern however since there were still statistically significant differences between the plates when measuring the lag time and the time to peak. The magnitude of the observed difference was less than 10%, therefore the assay was technically suitable for routine analysis.

Figure 2.7

A.  

![Plasma Pool Inter Assay Variation: ETP](image)

B.  

![Plasma Pool Inter Assay Variation: Lag Time](image)

C.  

![Plasma Pool Inter Assay Variation: Peak Height](image)

D.  

![Plasma Pool Inter Assay Variation: Time to Peak](image)
Figure 2.7. Boxplots showing the distribution of the 15 values for each parameter of each of the 5 analysed plates. Analysis performed using a pool of fresh human plasma. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: ETP, B: Lag time, C: Peak height, D: Time to peak.

Table 2.15

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP (nM Thrombin)</td>
<td>2063.27</td>
<td>100.28</td>
<td>4.86</td>
</tr>
<tr>
<td>Lag Time (Minutes)</td>
<td>3.41</td>
<td>0.29</td>
<td>8.64</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>363.93</td>
<td>15.40</td>
<td>4.23</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>5.96</td>
<td>0.32</td>
<td>5.42</td>
</tr>
</tbody>
</table>

Table 2.15. Meta analysis of all 75 fresh pooled plasma data points.

2.3.1.13 Fresh Plasma and Intra Assay Trends

The use of fresh pooled plasma also eliminated the intra assay trends which were seen in the analysis of Control plasma P. The results are shown in figure 2.8 below. It can be seen that all of the R² values fell below 0.16 and therefore all yielded non significant p values of between 0.1-0.5 for ETP and the lag time and >0.5 for both peak height and time to peak.

Figure 2.8

A. B.
2.3.2 Sample Stability and Storage Assessment

2.3.2.1 Sample Stability and Thrombin Generation

To test the effects of sample age on the results produced by the CAT thrombin generation assay four 4.5ml 0.109M tri-sodium citrate samples were taken from a group of 10 volunteers. All of the volunteer samples had normal clotting screen results as determined by PT/INR, APTT and thrombin time (data not shown). The samples were then divided into four groups of 10 samples (consisting of one sample from each of the 10 volunteers). The first group of samples were prepared as described in section 2.2.4 and frozen immediately; the other 3 groups were then left at room temperature (22-24°C) for a predefined time course. The second group was processed after 4 hours, the third after 8 hours and the fourth after 24 hours. During the incubation the samples were left on their side to maximise the contact between the blood sample and the air in the vacutainer mimicking a worse case sample storage scenario. Each of the groups were left frozen at -70°C overnight in order to standardise any potential confounding variables. Each group of samples was then defrosted in a 37°C water bath and analysed on a separate plate. The results obtained from this analysis for each of the four thrombin generation parameters for each of the groups of samples can be seen graphically in figure 2.9 below.
Figure 2.9

A. 

Boxplots showing time between sample collection and storage and the distribution of the results obtained from a group of 10 volunteers for each of the four thrombin generation parameters. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: ETP, B: Lag time, C: Peak height, D: Time to peak.

B. 

Figures 2.9 A-D show that this was the case, with the populations for each of the four groups for each of the four thrombin generation parameters showing a high degree of overlap. Statistical comparisons of the populations using the student’s t-test confirmed this result. The p values obtained from this analysis are shown in tables 2.16-2.19 below. None of the groups showed any
statistical significant difference from the group separated at time zero or indeed each other, implying that the samples were stable for at least 24 hours between collection and storage.

Table 2.16

<table>
<thead>
<tr>
<th>Time to Separation (Hours) and ETP</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A</td>
<td>0.64</td>
<td>0.67</td>
<td>0.64</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>N/A</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.95</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.16. Matrix of p values comparing each of the times before sample separation and the values of ETP produced following analysis.

Table 2.17

<table>
<thead>
<tr>
<th>Time to Separation (Hours) and Lag Time</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A</td>
<td>0.87</td>
<td>0.90</td>
<td>0.66</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>N/A</td>
<td>0.77</td>
<td>0.55</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.74</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.17. Matrix of p values comparing each of the times before sample separation and the lag time produced following analysis.

Table 2.18

<table>
<thead>
<tr>
<th>Time to Separation (Hours) and Peak Height</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A</td>
<td>0.77</td>
<td>0.65</td>
<td>0.95</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>N/A</td>
<td>0.86</td>
<td>0.58</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.58</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.18. Matrix of p values comparing each of the times before sample separation and the values of peak height produced following analysis.
Table 2.19

<table>
<thead>
<tr>
<th>Time to Separation (Hours) and Time to Peak</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A</td>
<td>0.83</td>
<td>0.90</td>
<td>0.95</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>N/A</td>
<td>0.73</td>
<td>0.86</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.85</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.19. Matrix of p values comparing each of the times before sample separation and the time to peak produced following analysis.

### 2.3.2.2 Sample Stability and Assay Trends

Although there were no statistical differences detected using the student’s t-test, the results were then analysed to confirm that there were no obvious trends across the four groups. This was performed by drawing a linear regression trendline across all of the values obtained for each of the four groups and calculating a value for $R^2$ for that trend using Microsoft excel. The results of this analysis are shown in figure 2.10 below.

**Figure 2.10**

A. **Sample Stability: ETP**

B. **Sample Stability: Lag Time**

C. **Sample Stability: Peak Height**

D. **Sample Stability: Time to Peak**
Figure 2.10 Graphs showing time between sample collection and storage and the results obtained from a group of 10 volunteers for each of the four thrombin generation parameters. Graphs show a trendline and a value for $R^2$ as an indicator of correlation. A: ETP, B: Lag Time, C: Peak Height and D: Time to Peak

The p values obtained using the distribution of t table shown in 7.1 (appendix) were >0.5 for all the of the four thrombin generation parameters, showing that there was no trend across the groups for any of the four parameters. This result confirms the fact that the storage of samples at room temperature for up to 24 hours did not have an effect on the thrombin generation as measured by the CAT assay. An example of the thrombin generation curves of one of the volunteers across the four groups is shown in figure 2.11. This gives a visual indication of what the above data shows. The four curves show a high degree of overlap, with no obvious shift between any of the time points.

Figure 2.11

![Sample Stability](image)

Figure 2.11. Thrombin generation curves produced from four different samples taken from the same donor and stored at room temperature for varying lengths of time.

### 2.3.2.3 Storage Temperature and Thrombin Generation

In order to ascertain the effects of storage temperature on the results produced by the CAT thrombin generation assay four 4.5ml 0.109M tri-sodium citrate samples were taken from a group of 10 volunteers. All of the volunteers’ samples had normal clotting screen results as determined by PT/INR, APTT and thrombin time (data not shown).
These samples were prepared as detailed in section 2.2.4 and separated into four groups of 10 (consisting of one sample from each of the 10 volunteers). Each of the groups were then placed at a different storage temperature, one at 4°C, one at -20°C, one at -40°C and one at -70°C. Following 24 hours storage one of the aliquots from each volunteer stored in the -70°C freezer was thawed in a 37°C water bath and analysed using the CAT assay. This group was designated as ‘fresh’ and was used as a base line to which the other groups would be compared. It was decided to freeze and thaw the fresh group to eliminate any confounding variables which may arise from the freeze/thawing process.

Following four weeks incubation (longer than the required turn-around time for routine haemostatic investigations) at the various storage temperatures the four remaining groups were defrosted one at a time in a 37°C water bath and analysed on separate plates. Following the analysis the means and standard deviations of the four groups and the fresh group were calculated and plotted on the graphs shown in figure 2.12 below. If the four week storage at the varying temperatures had no effect on the results the median values and the distribution of data of the various groups should be analogous to that of the fresh group. It was anticipated that the storage of samples at 4°C would have a detrimental effect on the results since the levels of clotting factors would begin to decrease. It was also anticipated that the samples frozen at lower temperatures (-70°C) would show a greater degree of stability than those frozen at higher temperatures (-20°C). From the graphs it can be seen that the groups stored at -20°C, -40°C and -70°C show similar results to the fresh groups for each of the different parameters. The samples stored at 4°C however did show marked differences to the fresh groups for all four of the measured parameters and also showed a difference to the groups stored at -20°C, -40°C and -70°C. Statistical analysis of these results using the student’s t-test is shown in tables 2.20-2.23 below.
Figure 2.12

Boxplots showing storage temperature of samples and the distribution of the results obtained from a group of 10 volunteers for each of the four thrombin generation parameters following a four week incubation at that temperature. The fresh group was stored at -70°C for 24 hours before analysis. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: ETP, B: Lag time, C: Peak height, D: Time to peak.
Table 2.20

<table>
<thead>
<tr>
<th>Sample Storage and ETP</th>
<th>Fresh</th>
<th>4°C</th>
<th>-20°C</th>
<th>-40°C</th>
<th>-70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>N/A</td>
<td>0.12</td>
<td>0.50</td>
<td>0.34</td>
<td>0.35</td>
</tr>
<tr>
<td>4°C</td>
<td>-</td>
<td>N/A</td>
<td>0.04</td>
<td>1.2x10^{-4}</td>
<td>0.02</td>
</tr>
<tr>
<td>-20°C</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.78</td>
<td>0.80</td>
</tr>
<tr>
<td>-40°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.98</td>
</tr>
<tr>
<td>-70°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.20. Matrix of p values comparing each of the storage temperatures and the value of ETP produced following analysis. Results highlighted in red are statistically significant.

Table 2.21

<table>
<thead>
<tr>
<th>Sample Storage and Lag Time</th>
<th>Fresh</th>
<th>4°C</th>
<th>-20°C</th>
<th>-40°C</th>
<th>-70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>N/A</td>
<td>6.6x10^{-6}</td>
<td>0.82</td>
<td>0.66</td>
<td>0.40</td>
</tr>
<tr>
<td>4°C</td>
<td>-</td>
<td>N/A</td>
<td>2.1x10^{-6}</td>
<td>1.1x10^{-3}</td>
<td>4.3x10^{-3}</td>
</tr>
<tr>
<td>-20°C</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.81</td>
<td>0.49</td>
</tr>
<tr>
<td>-40°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.66</td>
</tr>
<tr>
<td>-70°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.21. Matrix of p values comparing each of the storage temperatures and the lag time produced following analysis. Results highlighted in red are statistically significant.

Table 2.22

<table>
<thead>
<tr>
<th>Sample Storage and Peak Height</th>
<th>Fresh</th>
<th>4°C</th>
<th>-20°C</th>
<th>-40°C</th>
<th>-70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>N/A</td>
<td>1.7x10^{-3}</td>
<td>0.99</td>
<td>0.85</td>
<td>0.75</td>
</tr>
<tr>
<td>4°C</td>
<td>-</td>
<td>N/A</td>
<td>1.3x10^{-3}</td>
<td>8.6x10^{-4}</td>
<td>7.4x10^{-4}</td>
</tr>
<tr>
<td>-20°C</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.85</td>
<td>0.75</td>
</tr>
<tr>
<td>-40°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.89</td>
</tr>
<tr>
<td>-70°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.22. Matrix of p values comparing each of the storage temperatures and the peak height produced following analysis. Results highlighted in red are statistically significant.
Table 2.23

<table>
<thead>
<tr>
<th>Sample Storage and Time to Peak</th>
<th>Fresh</th>
<th>4°C</th>
<th>-20°C</th>
<th>-40°C</th>
<th>-70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>N/A</td>
<td>4.1x10⁶</td>
<td>0.66</td>
<td>0.63</td>
<td>0.52</td>
</tr>
<tr>
<td>4°C</td>
<td>-</td>
<td>N/A</td>
<td>3.9x10⁶</td>
<td>5.2x10⁵</td>
<td>1.8x10⁻⁵</td>
</tr>
<tr>
<td>-20°C</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.95</td>
<td>0.79</td>
</tr>
<tr>
<td>-40°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.84</td>
</tr>
<tr>
<td>-70°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.23. Matrix of p values comparing each of the storage temperatures and the time to peak produced following analysis. Results highlighted in red are statistically significant.

The results in tables 2.20-2.23 show that there is no difference in the results generated for any of the four thrombin generation parameters by the CAT assay in samples that have been frozen at -20°C, -40°C and -70°C for four weeks compared to samples taken from the same pool of donors which have been frozen overnight at -70°C. Samples which are stored at 4°C for the same time period show marked differences in the results however, with statistically significant differences between the lag time, peak height and time to peak when compared to the fresh group and in all four parameters when compared to the groups frozen at -20°C, -40°C and -70°C. This result implies that samples for thrombin generation can be stored at any temperature between -20°C and -70°C for longer than the required turn-around time for other routine diagnostic test samples. Since there is no stability advantage to be gained by storing the samples at -70°C rather than -40°C patients requiring both thrombin generation and other haemostatic investigations could have their samples stored together. Samples stored at 4°C produce results which are statistically significantly different when compared to those of samples which have been frozen, indicating samples stored in this manner cannot be analysed using the CAT assay. The impact of 4°C storage on a patients results can be seen in the thrombin generation curves of one of the volunteers across each of the five groups in figure 2.13 below.
Figure 2.13. Thrombin generation curves produced from five different sample aliquots taken from the same donor and stored at varying temperatures for 4 weeks. The fresh group was stored at -70°C for 24 hours.

2.3.3 Limits of Detection

When considering the upper and lower limits of detection for the thrombin generation assay it is important to note that there are four parameters which are measured: the ETP, the peak height, the lag time and the time to peak. The lag time and the time to peak are the time taken for the initiation of thrombin generation and the time until the maximum rate of thrombin generation is achieved, since these parameters are both times in minutes it is feasible that they may take any value (within reason) from 0 onwards. The lower limit of detection is therefore 0 and the upper limit of detection for any given run is the pre-specified time course for the assay. In order to calculate the results of a thrombin generation assay the computer software detects an initial thrombin burst, followed by a cessation of thrombin production and then a consistent low level of thrombin production which characterises the activity of thrombin bound to α II macroglobulin. The point at which this commences is defined as the ‘start tail’ by the software. If the measurement time is reached before the thrombin curve is complete or the ‘start tail’ is found the software will not calculate the results. A reaction time which is too short will therefore yield no results. Since the lag time and the time to peak have
clearly defined upper and lower limits of detection this section focuses on the
determination of the limits of detection for both ETP and peak height.

The upper and lower limits of detection for assays in the haemostasis department are
frequently determined via different methods. The lower limit of detection is often
defined as the mean of a cohort of 10 ‘blank’ samples plus three standard deviations of
the results of that cohort. This is the minimum result that the assay in question can
reliably detect. The upper limit can be difficult to determine, this is because many
assays are dependent on the action of an enzyme (which is being measured) on a
substrate which is in excess over a given time course. The actual limit of detection is
therefore the amount of enzyme which can diminish the substrate quicker than the
specified time course of the assay. This is unlikely to be a value obtainable in human
plasma, pathological or otherwise, since the substrate is in excess. A large number of
assays have results which are interpolated from stored standard curves or at the
extremes derived from extrapolated standard curves. The limit at which it is deemed the
extrapolation is no longer valid is the upper limit at which results are no longer
reported. Most curves in the laboratory are extrapolated 1.5 times their original length.
This is more correctly an upper limit of quantification rather than detection. The CAT
assay poses difficulties when considering the upper limit of quantification rather than detection. The results are not derived from stored standard curves making defining an
upper limit of quantification via the above method impossible. An upper limit of
detection is challenging since it requires a plasma sample which has a thrombin
generation capacity so high as to consume the substrate before the computer software is
capable of calculating the results. A titration of this sample until a valid result is
achieved would give a respectable upper limit of detection. Unfortunately obtaining
such a plasma sample is difficult. van Veen et al. (2009b), managed to achieve substrate
depletion by spiking plasma with >0.5U/ml FEIBA®, but obtaining samples of FEIBA®
was not possible in this study. The upper limit of detection for the CAT assay was not
therefore determined in this study.

The lower limit of detection for both the ETP and the peak height could be determined
by the above method if a suitable blank could be found. Finding a ‘true’ blank can be
challenging, since materials known to have a thrombin generation of zero, such as saline
or water have a different matrix to that of plasma and therefore introduce other
confounding variables. Suitable blanks for the thrombin generation assay could either be serum or heat inactivated plasma. It was chosen to use heat inactivated plasma in this experiment since it was felt that the use of serum could still yield a low thrombin generation if all the clotting factors were not adequately consumed. Plasma subjected to heat inactivation as described in section 2.2.8 should have all plasma proteins denatured and therefore provide a true blank.

Samples for heat inactivation were obtained from patients at the Royal London Hospital, who had normal clotting screens as defined by PT/INR, APTT and thrombin time and were therefore not undergoing any anti-coagulant therapy which could interfere with the analysis. The heat inactivated plasma was then analysed 10 times on a single plate using the CAT assay as described in section 2.2.5. The measurement time was set for 1 hour. Although inspection of the thrombin generation curves as the analysis was progressing revealed no obvious thrombin generation, the computer software was unable to interpret the results and instead flagged the 10 groups with a ‘flat curve’ error. This was an undesirable result as it implies that the CAT assay cannot be used to measure a true blank sample i.e. the CAT analysis software cannot interpret a flat generation curve as a zero result. This meant that the lower limit of detection could not be determined in the normal manner.

2.3.3.1 Standard Human Plasma Dilutions

In order to discern what level of result could be accurately calculated by the CAT analysis software it was decided to dilute a normal human plasma with heat inactivated plasma to see what levels of clotting factors the CAT assay could accurately measure. The lowest dilution with an accurate result could then be used as a ‘blank’ for the lower limit of detection. These dilution experiments would also provide an insight into what effects decreasing the levels of clotting factors in a patients sample has on the assay results.

The normal human plasma that was chosen to form the basis of the dilutions was standard human plasma (SHP) produced by Dade Behring. This was chosen because it was known to contain normal levels of each clotting factor and naturally occurring anticoagulants. SHP is a lyophilised plasma product and was chosen despite the
observed difficulties in the measurement of lyophilised plasma by the CAT assay because it was felt that dilution of SHP in heat inactivated plasma would also dilute out the particulate matter, which was thought to cause the observed variation.

The first stage in this experiment was to produce a series of 11 different dilutions of SHP in heat inactivated plasma. These initial dilutions varied from 100% SHP to 5% SHP. Each dilution was analysed once on a single plate as described in section 2.2.5. The thrombin generation curves for each of the dilutions are shown in figure 2.14 below.

Figure 2.14

![Dilution of SHP](image)

Figure 2.14. A graph showing the thrombin generation curves of a series of dilutions of SHP in heat inactivated plasma.

From figure 2.14 it can be seen that as anticipated the lower the concentration of SHP the smaller the thrombin generation curves become. This correlates with a decreasing ETP and peak height. From the shape of the curves it also appears that the lag time and time to peak parameters are increasing as the concentration of SHP is decreasing. In order to better determine the relationship between the concentration of SHP and the individual assay parameters the results of the above experiment were plotted on separate graphs as shown in figure 2.15 below.
From figure 2.15 A and B it can be seen that the ETP and peak height demonstrate a striking linear relationship between the result and the concentration of SHP (and therefore the levels of clotting factor present) with $R^2$ values exceeding 0.95 in both cases, indicating statistically significant trends as calculated in section 2.3.1.4 ($p < 0.001$). This pattern holds best with clotting factors levels between ~10% and ~90%. It would appear that the results for both peak height and ETP begin to plateau when the clotting factor concentration reaches $\geq 90\%$ of normal. Levels of clotting factor below 10% of normal are suggestive of a hyperbolic trend, although it can only be hypothesised from the above graphs. The linear relationship demonstrated here is similar to that shown by Al Dieri et al. (2002), who showed a linear relationship between the levels of prothrombin and the ETP. Although that experiment used a plasma containing normal levels of other clotting factors and a varying level of factor II.
Deficiencies of other clotting factors showed a hyperbolic relationship with ETP when varied individually. SHP contains ~100 U/dL of all clotting factors, it is interesting therefore that the relationship continues to be linear and not hyperbolic, which was the result most commonly observed by Dieri et al, when all of the clotting factors are reduced simultaneously. This implies that deficiency of factor II is a rate limiting step in the CAT assay. This is not an unanticipated result since a normal level of thrombin generation cannot occur if there is not a normal level of prothrombin present.

Figure 2.15 C and D show that the relationship between clotting factor concentration and the lag time and time to peak is different to that of ETP and the peak height which both show a proportional increase as the concentration of clotting factors increase. The relationship between the lag time and the time to peak with the concentration of clotting factors is less dramatic as indicated by the $R^2$ values, which are lower than both the ETP and peak height but the p values still indicate statistically significant trends ($p < 0.01$). Demonstrating that there is an inversely proportional relationship between both the lag time and time to peak and the concentration of clotting factors, i.e. the lag time and time to peak increase as the levels of clotting factor decrease. This relationship however is non-linear with near normal clotting times once the concentration of clotting factors reaches 30% of normal. This result implies that plasma with only 30% levels of all clotting factors can produce clotting times which are ~90% of normal. Due to the exponential shape of the curves in figure 2.15 it was decided to plot the graphs again on a logarithmic scale in order to better establish the relationships between the concentration of clotting factors in a sample and the thrombin generation capacity of that sample. The results of this are shown in figure 2.16 below.

**Figure 2.16**

A. B.
Figure 2.16 Graphs showing the results for each of the thrombin generation parameters against the concentration of SHP for a series of dilutions of SHP in heat inactivated plasma, complete with trend lines and \( R^2 \) value as an indication of correlation. Plotted on a log-log scale. A: ETP, B: Peak Height, C: Lag Time, D: Time to Peak

From figure 2.16 it can be seen that plotting the data on a logarithmic scale enhances the correlation between clotting factor concentration (percentage SHP) and all four of the thrombin generation parameters. In the case of the ETP and peak height parameters this change is relatively modest, whereas for the lag time and time to peak parameters the increase is profound rising to 0.96 and 0.95 respectively from 0.61 and 0.63. This change increases the significance of the correlation to a p value of <0.001 for both parameters.

Importantly it can be observed that all of the 11 dilutions of SHP have produced valid results by the CAT assay. In order to ascertain the limits of detection it is necessary to continue the titration of SHP until the assay fails to produce a valid result. In the next step of the experiment dilutions of between 1% and 4% of SHP in heat inactivated plasma were made. These dilutions were measured in duplicate on a single plate as described in section 2.2.5. The thrombin generation curves of one of each of the dilutions from this analysis are shown in figure 2.17 below. The CAT assay produced valid curves for the dilutions between 2-4% of SHP in heat inactivated plasma, but was unable to find an appropriate start tail for the curve of the 1% SHP dilution. This can be set manually and the curve is therefore shown on figure 2.17 for comparative purposes.
It can be seen from figure 2.17 that the trends described above still hold for the low dilutions of SHP. Although the curves appear to show high levels of ‘noise’ this is more an artefact of the magnitude of the graph axes than any increased variability. As noted above the CAT assay was unable to calculate any results for the 1% SHP dilution and although the start tail can be set manually to produce results this is subjective, and can lead to an increased level of variation especially when the levels of thrombin generation are low. For this reason it was chosen to use a dilution of 2% SHP to represent the limit of detection. The above analysis was performed for 50 Minutes. In order to ascertain whether the failure of the software to detect a start tail was due to the analysis time, the experiment was repeated with an analysis time of 70 minutes with identical results (data not shown).

The lower limit of detection for the peak height and the endogenous thrombin potential was therefore determined by analysing a 2% dilution of SHP in heat inactivated plasma 10 times on a single plate as described in section 2.2.5 with a measurement time of 70 minutes. The results are shown in table 2.24 below. From the results it can seen that the CVs of the 10 replicates for the four parameters were all good ranging from 1.59-5.62%, well below the 10% target as discussed previously. This is a good demonstration of precision, proving that the CAT assay has a low level of variability even at the lower
limit of detection. The lower limit of detection itself was calculated by the mean +3SD. This is used in laboratory terms as a cut off, below which it is assumed that any results generated are inaccurate. In this respect it is more correctly a lower limit of quantification, although the lower limit of detection and quantification are effectively the same in this assay. Any numerical result for the CAT assay falling below 325 nM thrombin for ETP and below 18.6 nM Thrombin for peak height will be reported as <325 nM thrombin and <18.6 nM thrombin respectively.

Table 2.24

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
<th>Lower Limit of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP (nM Thrombin)</td>
<td>278.23</td>
<td>15.65</td>
<td>5.62</td>
<td>325.18</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>16.99</td>
<td>0.54</td>
<td>3.16</td>
<td>18.60</td>
</tr>
<tr>
<td>Lag Time (Minutes)</td>
<td>12.58</td>
<td>0.36</td>
<td>2.85</td>
<td>N/A</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>17.94</td>
<td>0.28</td>
<td>1.59</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.24 A Table showing the mean, standard deviation, coefficient of variation and lower limit of detection of each of the four thrombin generation parameters calculated from the analysis of 10 replicates of a 2% dilution of SHP in heat inactivated plasma.

2.3.4 Variation Across Time

In order to investigate the variation of a patient’s capacity to generate thrombin over time it was decided to take blood samples from a cohort of healthy individuals across a predefined time course, to establish whether or not there was any significant change in the results generated by the CAT thrombin generation assay.

A cohort of 10 healthy volunteers (5 male and 5 female) were chosen for this experiment, all of these individuals had no prior history of bleeding or thrombosis and had normal clotting screens as defined by PT/INR, APTT and thrombin time. Blood
samples were collected from each individual every week for four weeks (giving a total of four samples for each participant). These samples were prepared as described in section 2.2.4 and frozen at -70°C. Once all four samples were collected on a single individual and the final sample had been frozen for 24 hours, all four stored samples were defrosted in a 37°C waterbath and analysed via the CAT thrombin generation assay as described in section 2.2.5. The analysis time for these experiments was 45 minutes. The analysis of samples was performed in this manner to ensure that no patients sample was stored for longer than the maximum four week storage time that was validated in section 2.3.3.3. This procedure also ensured that all of the samples obtained on a single patient were analysed on a single run, thereby minimising any confounding variables associated with inter assay variation.

Following the analysis the results obtained from the cohort for each of the thrombin generation parameters across the four weeks were compared using a paired student’s t-test. The results of this statistical analysis are shown in tables 2.25-2.28 below. A p value of <0.05 was considered statistically significant. It can be seen from the tables that there were no statistically significant differences between the results obtained from the cohort from any of the four weeks. This result implies that there was no statistically significant difference between the thrombin generation results of the individuals in the cohort across a four week time course.

Table 2.25

<table>
<thead>
<tr>
<th>Week</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>N/A</td>
<td>0.18</td>
<td>0.60</td>
<td>0.54</td>
</tr>
<tr>
<td>Week 2</td>
<td>-</td>
<td>N/A</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>Week 3</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>0.67</td>
</tr>
<tr>
<td>Week 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.25. Matrix of p-values comparing the lag time results of a cohort of individuals across a four week time course
Table 2.26

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>0.12</td>
<td>0.47</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.50</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.26. Matrix of p-values comparing the ETP results of a cohort of individuals across a four week time course

Table 2.27

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>0.90</td>
<td>0.93</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.96</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.27. Matrix of p-values comparing the peak height results of a cohort of individuals across a four week time course

Table 2.28

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>0.62</td>
<td>0.40</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.42</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.28. Matrix of p-values comparing the time to peak results of a cohort of individuals across a four week time course

In order to try and accurately assess the level of variation seen between the results of each patient over the four weeks the CVs were determined for each of the thrombin generation parameters for each of the 10 patients. The median CV and the range of results obtained for each parameter are shown in table 2.29 below.
<table>
<thead>
<tr>
<th>Thrombin Generation Parameter</th>
<th>Range of CVs Obtained (%)</th>
<th>Median CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time</td>
<td>0.0 - 12.7</td>
<td>6.3</td>
</tr>
<tr>
<td>ETP</td>
<td>1.0 - 6.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Peak Height</td>
<td>1.4 - 6.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Time to Peak</td>
<td>0.0 - 5.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 2.29. Range and median CVs obtained for each thrombin generation parameter following the analysis of samples taken weekly for four weeks from a cohort of 10 individuals.

It can be seen from table 2.29 that the CVs obtained from the analysis of the individual patient’s results were relatively low. Only 2 of the 40 calculated values exceeded the 10% cut-off for acceptable variation discussed in previously. These values were both 12.7% and were both obtained from measurement of the lag time, which was previously highlighted as the parameter with the highest levels of variation. The median CVs obtained from this analysis are of similar magnitude to those obtained when measuring the intra assay variability of pooled fresh plasma (tables 2.11-2.14). This implies that the variation seen between the results of the individuals in the cohort across the four week time course is consistent with intra assay variation and is probably not a result of physiological changes in the patients themselves. Importantly this finding is consistent with the analysis of the results using the student’s t-test (above). Together these analyses are suggestive of minimum variation in the thrombin generation potential of an individual across time. An example of the thrombin generation curves produced from one of the individuals in the cohort over the four week time course is shown in figure 2.18 below.
2.3.5 Normal Range

In order to establish a normal range for the CAT assay it was necessary to take blood samples from a cohort of individuals who had no history of bleeding or thrombosis and who were healthy at the time of sampling. The normal control cohort was composed of 40 males and 40 females of between 20 and 60 years of age. Both the male and female cohort were composed of 10 individuals aged between 20-29, 10 aged between 30-39, 10 aged between 40-49 and 10 aged between 50-59. The individuals chosen were composed of healthy volunteers and outpatients samples from individuals attending surgical clinics (such as potential kidney donors). All volunteers had normal clotting screen results as determined by PT/INR, APTT and thrombin time, thus ensuring the patients were not undergoing any anticoagulant therapy, which may affect the results. A normal clotting screen should also exclude most inherited bleeding disorders, which may have been previously undiagnosed. An even distribution of age and sex was chosen to ensure any differences due to these variables could be detected.

Although it would have been interesting to also determine a paediatric normal range it was decided, due to a lack of suitable patients and ethical concerns over consent, that it would not be attempted in this study. All samples were prepared as described in section
2.2.4. The samples were analysed as described in section 2.2.5. The measurement time was 45 minutes.

Ideally to facilitate the ease of use in the laboratory the CAT assay would have a single normal range, although this is by no means essential, as it is not uncommon to have different normal ranges depending on factors such as age, sex or ethnicity. Before any meta analysis of all of the 8 groups could be performed it was necessary to determine whether there were any differences between any of the groups for any of the four parameters analysed. This was important as it was unknown whether age, sex or both were confounding variables. Initially a comparison of the differences due to age in both the male and female groups was performed. Comparisons were performed using the student’s t-test and the results of the analysis are shown in tables 2.30-2.37 below. A p value of ≤0.05 was considered significant. From the results it can be seen that there are no significant differences between any of the comparisons performed. This implies that age has no effect on any of the thrombin generation parameters when males and females are considered separately.

Table 2.30

<table>
<thead>
<tr>
<th>Male: ETP</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Group 20-29</td>
<td>N/A</td>
<td>0.91</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>Age Group 30-39</td>
<td>.</td>
<td>N/A</td>
<td>0.60</td>
<td>0.40</td>
</tr>
<tr>
<td>Age Group 40-49</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td>0.73</td>
</tr>
<tr>
<td>Age Group 50-59</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.30. Matrix of p values comparing the ETP results of each of the 4 age groups in the male cohort.

Table 2.31

<table>
<thead>
<tr>
<th>Male: Lag Time</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Group 20-29</td>
<td>N/A</td>
<td>0.82</td>
<td>0.63</td>
<td>0.86</td>
</tr>
<tr>
<td>Age Group 30-39</td>
<td>.</td>
<td>N/A</td>
<td>0.72</td>
<td>0.98</td>
</tr>
<tr>
<td>Age Group 40-49</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td>0.78</td>
</tr>
<tr>
<td>Age Group 50-59</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.31. Matrix of p values comparing the Lag Time results of each of the 4 age groups in the male cohort.
Table 2.32

<table>
<thead>
<tr>
<th>Male: Peak Height</th>
<th>Age Group</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>N/A</td>
<td>0.65</td>
<td>0.76</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>.</td>
<td>N/A</td>
<td>0.47</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.32. Matrix of p values comparing the Peak Height results of each of the 4 age groups in the male cohort.

Table 2.33

<table>
<thead>
<tr>
<th>Male: Time to Peak</th>
<th>Age Group</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>N/A</td>
<td>0.87</td>
<td>0.58</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>.</td>
<td>N/A</td>
<td>0.67</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.33. Matrix of p values comparing the Time to Peak results of each of the 4 age groups in the male cohort.

Table 2.34

<table>
<thead>
<tr>
<th>Female: ETP</th>
<th>Age Group</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>N/A</td>
<td>0.73</td>
<td>0.70</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>.</td>
<td>N/A</td>
<td>0.48</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.34. Matrix of p values comparing the ETP results of each of the 4 age groups in the female cohort.

Table 2.35

<table>
<thead>
<tr>
<th>Female: Lag Time</th>
<th>Age Group</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>N/A</td>
<td>0.26</td>
<td>0.09</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>.</td>
<td>N/A</td>
<td>0.57</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.35. Matrix of p values comparing the Lag Time results of each of the 4 age groups in the female cohort.
Table 2.36

<table>
<thead>
<tr>
<th>Female: Peak Height</th>
<th>Age Group</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>N/A</td>
<td>0.65</td>
<td>0.91</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td></td>
<td>N/A</td>
<td>0.74</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td></td>
<td></td>
<td>N/A</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.36. Matrix of p values comparing the Peak Height results of each of the 4 age groups in the female cohort.

Table 2.37

<table>
<thead>
<tr>
<th>Female: Time to Peak</th>
<th>Age Group</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>N/A</td>
<td>0.36</td>
<td>0.23</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td></td>
<td>N/A</td>
<td>0.64</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td></td>
<td></td>
<td>N/A</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.37. Matrix of p values comparing the Time to Peak results of each of the 4 age groups in the female cohort.

The next stage of the analysis was to compare the results of each of the four thrombin generation parameters for each of the four age groups from the male and female cohorts with each other. Comparisons were again made using the student’s t-test. A p value of \( \leq 0.05 \) was considered a statistically significant result. The results of the comparisons are shown in tables 2.38-2.41 below.

Table 2.38

<table>
<thead>
<tr>
<th>ETP</th>
<th>Male</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-29</td>
<td>0.29</td>
<td>0.16</td>
<td>0.53</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>30-39</td>
<td>0.32</td>
<td>0.18</td>
<td>0.59</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>40-49</td>
<td>0.66</td>
<td>0.44</td>
<td>0.97</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>50-59</td>
<td>0.95</td>
<td>0.71</td>
<td>0.76</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 2.38. Matrix of p values comparing the ETP results for each of the four age groups of the male and female cohorts.
Table 2.39

<table>
<thead>
<tr>
<th>Female</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>0.28</td>
<td>0.97</td>
<td>0.56</td>
<td>0.68</td>
</tr>
<tr>
<td>30-39</td>
<td>0.09</td>
<td>0.85</td>
<td>0.62</td>
<td>0.80</td>
</tr>
<tr>
<td>40-49</td>
<td>0.07</td>
<td>0.65</td>
<td>0.85</td>
<td>0.88</td>
</tr>
<tr>
<td>50-59</td>
<td>0.21</td>
<td>0.89</td>
<td>0.68</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 2.39. Matrix of p values comparing the Lag Time results for each of the four age groups of the male and female cohorts.

Table 2.40

<table>
<thead>
<tr>
<th>Female</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>0.67</td>
<td>0.41</td>
<td>0.61</td>
<td>0.32</td>
</tr>
<tr>
<td>30-39</td>
<td>0.31</td>
<td>0.13</td>
<td>0.27</td>
<td>0.06</td>
</tr>
<tr>
<td>40-49</td>
<td>0.98</td>
<td>0.72</td>
<td>0.91</td>
<td>0.66</td>
</tr>
<tr>
<td>50-59</td>
<td>0.40</td>
<td>0.18</td>
<td>0.35</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 2.40. Matrix of p values comparing the Peak Height results for each of the four age groups of the male and female cohorts.

Table 2.41

<table>
<thead>
<tr>
<th>Female</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>0.76</td>
<td>0.65</td>
<td>0.42</td>
<td>0.40</td>
</tr>
<tr>
<td>30-39</td>
<td>0.60</td>
<td>0.76</td>
<td>0.49</td>
<td>0.47</td>
</tr>
<tr>
<td>40-49</td>
<td>0.34</td>
<td>0.87</td>
<td>0.77</td>
<td>0.87</td>
</tr>
<tr>
<td>50-59</td>
<td>0.23</td>
<td>0.72</td>
<td>0.88</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Table 2.41. Matrix of p values comparing the Time to Peak results for each of the four age groups of the male and female cohorts.

From the results it can be seen that there were no statistically significant differences between any of the four age groups in the male and female cohorts for any of the four parameters analysed. This result suggests that age does not affect the results for any of the thrombin generation parameters when grouped as shown above. This comparison also alludes to the fact there is potentially no differences between the male and female cohorts. To confirm this finding a further analysis was performed. Since there were no differences between any of the age groups in either of the two cohorts a t-test comparison was performed between all 40 of the data points for each parameter in the
male cohort and all 40 data points for each parameter in the female cohort. This would distinguish definitively whether or not there were any differences in any of the four thrombin generation parameters between the two cohorts. The p values for the comparisons ranged between 0.10 and 0.87 indicating that there were no statistically significant differences due to sex in any of the thrombin generation parameters analysed. This result was desirable since it meant that a single normal range could be derived from the available data for both male and female patients. Before that analysis was performed a final comparison was made to definitively exclude any effect of age on any of the thrombin generation parameters. Although the t-test comparisons performed above showed no differences between the different age groups both within and between the different sexes, it is possible that the results could be skewed due to the effects of grouping the data rather than considering age as a continuous variable. To properly ascertain the impact of age on thrombin generation a correlation was performed for each parameter using all 80 of the available data points. The graphs depicting these analyses are shown in figure 2.19 below.

Figure 2.19

A. ETP

B. Lag time
From the graphs it can be seen that there was no clear correlation of age with any of the four thrombin generation parameters, this was also indicated in the low values for $R^2$. Calculation of the p value using the distribution of t as described in section 2.3.1.4 confirmed that there was no general trend between any of the thrombin generation parameters and age (p values >0.05 for all 4 parameters).

Since neither age nor sex had any statistically significant effect on any of the parameters analysed, a single normal range could be defined for all patients analysed via the CAT assay using the 80 available data points for each parameter. Frequency charts for each parameter were then drawn to determine the distribution of the population, as this would determine how the normal range was calculated. The frequency charts are shown in figure 2.20 below.

The frequency charts in figure 2.20 imply that the data for the ETP, peak height and time to peak parameters are normally distributed and that the data for the lag time may be skewed. Analysis using the Kolmogorov-Smirnov test revealed this was indeed the case. For each of the normally distributed parameters the normal range was defined by the mean ±1.96 Standard deviations. This provides a range into which 95% of all normal individuals should fall. The normal range for the lag time was determined by calculating the 2.5 and 97.5 percentiles of the data as this provides the equivalent normal range. The results of this analysis are shown in table 2.42.
Figure 2.20

A. Distribution of Lag Time

B. Distribution of ETP

C. Distribution of Peak Height

D. Distribution of Time to Peak

Figure 2.20. Frequency charts showing the distribution of the four thrombin generation parameters of the 80 normal individuals analysed. A: Lag Time, B: ETP, C: Peak Height, D: Time to Peak.

Table 2.42

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP (nM Thrombin)</td>
<td>1336 - 2886</td>
</tr>
<tr>
<td>Lag Time (Minutes)</td>
<td>2.00 - 4.01</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>258 - 454</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>4.38 - 7.65</td>
</tr>
</tbody>
</table>

Table 2.42. Table showing the derived normal range for the four thrombin generation parameters. ETP and Peak Height shown to 4 and 3 significant figures respectively. Lag Time and Time to Peak shown to 2 decimal places.
2.3.6 Thrombin Generation and CTI

The final stage of the evaluation was to ascertain the effects of the addition of CTI on the CAT thrombin generation assay. CTI inhibits contact activation in thrombin generation assays and therefore prevents an overestimation of a patient’s thrombin generation capacity. There is evidence in the literature to suggest that the addition of CTI is not required when a 5pM concentration of tissue factor is used as a reaction trigger, as in this study (van Veen et al., 2008). To ascertain whether or not this was the case blood samples were taken from a cohort of 6 healthy volunteers. Two samples were taken from each individual, one sample was taken into a standard 4.5ml 0.109M tri-sodium citrate sample, the second was taken into a 4.5ml 0.109M tri-sodium citrate sample containing 77µl of 1.3mg/ml CTI. This gave a final concentration of 20µg/ml CTI, which is similar to the concentration employed in a paper by van Veen et. al., (2009a), who used a final concentration of 18.3µg/ml. The CTI was added to the vacutainer before the blood sample was collected as described previously (van Veen et. al., 2009a). The samples were processed as described in section 2.2.4 and then frozen at -70°C overnight. The samples were thawed in a 37°C waterbath before being analysed on a single plate as described in section 2.2.5. The analysis time for this experiment was 45 minutes. The results obtained from the samples with and without CTI were then compared using a paired t-test. The data obtained from this experiment as well as the results of the paired t-tests are shown in figure 2.21 below.

From the figure it is clear that the addition of CTI to an individual’s blood sample has no effect on the lag time, ETP or time to peak parameters as evidenced by the non-significant t-test results (p ≤0.05 was considered significant). This was not true however for the peak height results which show a statistically significant reduction (p= 0.007) in the samples to which CTI was added when compared to those without the addition of CTI. This suggests that the addition of CTI can lead to a reduction in the maximum rate of thrombin production and that contact activation may play a part in the maximum rate of thrombin generation when CTI is not added into the blood sample. It may be noted that although there is a statistically significant decrease in the peak height of a sample when CTI is added the magnitude of this difference is relatively small, with the mean decrease across the cohort being ~20nM Thrombin. This equates to a decrease of 6% which is inside the 10% for variation deemed acceptable earlier in this chapter.

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Figure 2.21

A. **CTI Addition: Lag Time**

B. **CTI Addition: ETP**

C. **CTI Addition: Peak Height**

D. **CTI Addition: Time to Peak**

Figure 2.21. Boxplots showing the effect of the addition of CTI to the samples of a cohort of 6 healthy individuals when compared to samples without the addition of CTI. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: Lag Time, B: ETP, C: Peak height, D: Time to peak. P values calculated using a paired t-test.

2.4 Discussion

The overall aim of this project was to introduce the CAT thrombin generation assay into routine use in the haemostasis laboratory and ascertain the clinical utility of the results which the assay generates. The primary starting point was to determine how reliable the results generated by the CAT thrombin generation assay are in terms of both the inter and intra assay variation. If the assay demonstrated poor levels of variability its clinical
utility would be questioned as the results produced would be of little use in either a diagnostic or therapeutic monitoring scenario.

The initial experimentation for the assay variability yielded poor results, with levels of inter assay variability exceeding the 10% cut off of acceptability of the coefficient of variation. All other diagnostic assays currently in use in the haemostasis laboratory demonstrate a lower level of inter assay variability than this. The assay also revealed a trend of decreasing peak height across the plate. Although initially it was felt that these results could be due to the effects of the time taken to prepare the plate for analysis, decreasing the preparation time by using an electronic pipette did not eliminate these effects. Although the intra and inter assay variability did decrease, the levels of inter assay variability remained unacceptably high. The intra assay trends were also unaffected by the use of the electronic pipette indeed the problem was accentuated with an increase in lag time across the plate in combination with the decreasing peak height. Since it seemed preparation time was not responsible for these effects, assuming the assay itself was not at fault the only other constant across the experiments was the use of the lyophilised Control plasma P.

The Control plasma P was chosen for use in this assay for a number of reasons; the most obvious of which was that it was available in large enough quantities to enable the generation of a large number of results. The second reason it was chosen was down to the unavailability of control plasma for the CAT thrombin generation assay. Control plasma P is used as an abnormal control in a large number of routine assays in the haemostasis laboratory including a number of thrombophilia investigations. It contains ‘pathologically’ low levels of most of the proteins involved in the coagulation process. It was felt that if the plasma yielded acceptable results it could potentially be used as an abnormal control in the CAT assay. The results from this analysis revealed that Control plasma P would make a poor abnormal (low) control for the CAT assay due in part to its elevated ETP, decreased lag time and time to peak in comparison with a pool of fresh human plasma. The assay also produced a high level of background noise when analysing Control plasma P. The level of interference in the assay was unprecedented and is most likely to be caused by particular matter in the reconstituted plasma which could interfere with the generation of the fluorescent signal. This particular matter could
be generated by inefficient reconstitution of the reagent. This hypothesis is given weight by the elimination of the assay ‘noise’ when a fresh pool of plasma was analysed.

The analysis of the pooled plasma eliminated the intra assay trends and decreased the inter and intra assay variability to acceptable levels. The CAT thrombin generation assay therefore is suitable for routine diagnostic use in the haemostasis laboratory. It is worth noting that the repeatability of both the lag time and the time to peak parameters were still subject to a high variation between runs. The reasons for this may have been due to sample preparation. The time to peak measurement was highly dependent on the lag time since the maximal rate of thrombin production cannot occur until thrombin generation has begun, therefore the variability in the time to peak measurement may have been exclusively due to the variation in the lag time. The lag time itself is dependent on the phospholipid concentration in the sample, although the concentration of phospholipids in the reagent is standardised the concentration of phospholipid particles in the patient’s plasma may be subject to high variability. The double spinning preparation of patient’s plasma is designed to remove endogenous phospholipids in the form of platelets but does not remove platelet microparticles. These can only be removed by filtration of the patient’s plasma and may be the cause of some of the inter assay variation witnessed in the assay.

The sample handling and storage experiments were highly successful with the sample stability proving surprisingly robust. It was hoped that the time between collection and storage would have no impact on the results up until at least four hours; it was actually proved that the samples could be left at room temperature for up to 24 hours before they were separated and frozen without any effect on any of the four thrombin generation parameters. This result is perhaps less surprising when considering that the levels of clotting factors can be markedly decreased and still produce near normal levels of ETP (Al Dieri et al., 2002). Factor VII has the shortest half life of all the clotting factors, with a half life of just four hours. It is quoted that a level of 2% factor VII can produce an ETP approaching normality. Since the normal range of factor VII extends from 50-150% a patient with a factor VII level at the top end of this range who has their plasma stored at room temperature for 24 hours (6 half lives) would have a factor VII level of 2.3% after this time. It was anticipated that decreasing clotting factor concentration would have a more pronounced effect on the lag time and peak height. This trend was
not observed in the course of this experiment. This was possibly due to the fact that the
decrease of clotting factors may not have been as dramatic as demonstrated above. This
is due to the incubation temperature; factor VII has a half life of 4 hours at 37°C, which
may well be prolonged at room temperature, yielding higher levels of clotting factor
following a 24 incubation and ultimately a reduced effect on the thrombin generation
parameters. Indeed the in vitro half life of factor VII (and other clotting factors) may
well be elevated in comparison to that observed in vivo since they are unlikely to be
consumed due to physiological processes.

The sample storage experiment yielded the anticipated results with a good level of
stability following 4 weeks storage provided the samples remained frozen. Storage at
4°C was unsuitable due to a statistically significant decrease in ETP and peak height
and a statistically significant increase in lag time and time to peak when compared to
the other storage temperatures. This pattern i.e. decreased total thrombin generation and
maximum rate of production coupled with prolonged time to initiation of thrombin
generation and time to maximum rate of generation is consistent with a decrease in the
levels of clotting factors associated with sample degradation as described above. This
shows that at an incubation temperature of 4°C the levels of clotting factors are
diminished markedly after 4 weeks.

These results indicate that the CAT thrombin generation assay sample handling
requirements are suitable for routine laboratory use, with the samples stable for up to 24
hours post collection (longer than any sample should realistically be in transit) and once
frozen the samples remained stable longer than the required turn-around time of any
routine haemostatic investigation. The fact that the samples can be stored at any
temperature ≤-20°C means that there is also a great flexibility of where the samples can
be kept.

The limit of detection experiments initially produced undesirable results, with the
analysis software unable to produce valid thrombin generation curves on a ‘true blank’
sample, indicating that a flat generation curve cannot be interpreted as a zero result.
This is a potential limitation of the assay since it is unlikely that the CAT analysis
software will produce valid results on patients with an extremely low thrombin
generation. Patients with a flat curve error therefore will have their results reported as
below the lower limit of quantification. It is worth noting however, that patients with thrombin generation levels below the limit of detection will have an extremely pathological condition, if not undergoing anticoagulation therapy, since the lower limit of detection was defined using a concentration of ~2% for all clotting factors. This limitation is also unlikely to have any effect on this study since it is primarily focused on patients with thrombotic tendencies rather than haemophilia and therefore the thrombin generation parameters are likely to be elevated rather than decreased. Unfortunately, for the above reason, it would be desirable therefore to also establish an upper limit of detection, which proved impossible in this study. Although no formal limit was established, no patient in this study had a level of thrombin generation so great as to cause substrate depletion over the time course of the reaction. Therefore the upper limit of detection is obviously a value of ETP and Peak Height that are highly pathological since they must be in excess of 3965 and 611 respectively (the highest values seen in this study), which are 37% and 34% above the upper limit of the normal range.

Although the initial experiments to define the lower limit of detection using a thrombin generation blank were unsuccessful the titration experiments performed with SHP in heat inactivated plasma provided an adequate substitute, proving that the analysis software cannot produce valid answers when the clotting factor levels in a patient’s plasma are below ~2 u/dL. A 2% dilution of SHP in heat inactivated plasma was used to successfully define the lower limit of detection for both the ETP and the Peak Height. The CVs generated in these analyses were also surprisingly good, suggesting that the assay is capable of good precision even at very low levels of thrombin generation.

The SHP titration experiments also provided a valuable insight into the effects of decreasing clotting factor concentration on the results generated, with statistically significant linear correlations between clotting factor concentration and both ETP and peak height. The majority of which was probably caused by the concentration of prothrombin in the sample. Interestingly the effect was not so dramatic when considering the impact of clotting factor concentration on both the lag time and the time to peak parameters. Although there was a significant negative correlation (decreasing times with increasing clotting factor concentration) which could be significantly increased in magnitude when plotting the data on a logarithmic scale, most of the
variation was caused when the clotting factor levels are <30% of normal. This means that when the clotting factor concentration levels are greater than this there are sufficient levels of prothrombin and other factors to initiate thrombin generation and reach the maximum rate of thrombin generation in a time that is approximately 90% of normal. This is the case even if the subsequent level of thrombin generation and the rate of maximum thrombin generation are markedly reduced. The discovery that a clotting factor concentration exceeding 30% of normal (>30 u/dL) can lead to relatively normal clotting times as defined by the lag time and time to peak parameters in the thrombin generation assay fits neatly with the levels of clotting factors needed to prevent bleeding episodes in patients with factor deficiencies. Patients with mild haemophilia are classified as those who have factor levels of between 5-30 u/dL. Those with levels between 30-50 u/dL are technically deficient but are rarely symptomatic.

The measurement of a patient’s thrombin generation over time yielded positive results. When performing an assay for routine diagnostic testing it is important to be confident that the result generated reflects the pathological condition of the patient i.e. a decreased thrombin generation is indicative of a bleeding tendency. If there was a high level of variance in a patients thrombin generation from one day to the next this would reduce the diagnostic power of the assay. Although it would be fair to suggest the variation would have to be large in order to misdiagnose a patient with a severe bleeding tendency or predisposition to thrombosis, this would not be the case for patients with mild disorders. If the level of variation was high then it would be likely patients with borderline results would have to be repeated several times in order to be confident of a correct diagnosis. A similar scenario is adopted in the screening of patients for von Willebrand disease as the level of vWF can vary quite markedly due to stress and acute phase. Repeat testing of patients is ultimately undesirable since it has negative implications for assay cost and places additional pressures on the clinics to which the patients are referred. A high level of assay variation in individual patients across time would also impact upon the use of the assay for therapeutic monitoring as it would be difficult to discern whether any changes observed in a patient’s thrombin generation profile were due to variation in the patients themselves or a physiological response to the patient’s therapy.
The levels of variation observed in the cohort of normal individuals in this study over the four week time course were consistent with those achieved in the intra assay variability experiments. The majority of the participants displayed levels of variation below the 10% cut-off previously discussed and the differences observed between the weeks were non-significant as defined by a paired t-test. This result implies that a patient’s baseline level of thrombin generation is relatively stable and therefore abnormal results obtained via the CAT thrombin generation assay can be interpreted with a degree of confidence. This result also suggests that the CAT assay is suitable for use in the monitoring of patients undergoing treatment.

Although the results in this experiment are suggestive of minimal variance in thrombin generation capacity over time, this experiment was only carried out over the course of four weeks. It would be beneficial to perform this experiment over a longer time course in order to more accurately determine the variability of an individual’s thrombin generation capacity. It would also be of interest to study the degree of variance in thrombin generation over several years in order to ascertain the effects of aging on thrombin generation although such an experiment is outside the scope of this study. It may be noted that although the variance observed in this experiment was low and therefore suggestive of the fact that the assay should be sensitive to the diagnosis of congenital conditions, the individuals measured in this study were all healthy. One would expect the thrombin generation profile of an individual, with an acquired condition (acquired haemophilia or DIC for example), to show marked variation from their resting thrombin generation profile. Variation in patients results over and above that which is seen in normal inter assay variation may therefore be consistent with a pathological change in the patient.

The experiments to define a normal range for the thrombin generation assay provided good results, with no statistically significant differences between any of the groups analysed, suggesting that neither age nor sex have any effect on the thrombin generation of an individual. The fact that no significant differences were found meant that a single normal range could be calculated for use in all patients. This was a desirable result since it simplifies assay interpretation, and therefore makes routine use of the thrombin generation assay easier. The fact that all of the available data could be pooled to establish the normal range also means that any other potential confounding factors are
likely to have a smaller impact. The greater the level of subdivision of the available data the greater the impact that anomalous results can have on the normal range. Although every effort has been made to choose individuals who should have ‘normal’ levels of thrombin generation it is impossible to exclude patients who have underlying but currently asymptomatic clotting disorders. The impact that such an individual has on the overall normal range is reduced if the total number of patients in the cohort is larger.

There were no statistically significant differences between any of the groups for either age or sex however some of the p values displayed in tables 2.30 to 2.37 were approaching significance. In particular the comparison between the lag times of the 20-29 year old women and the 50-59 year old women (p= 0.06) and to a lesser extent the comparison of the time to peak parameter between the same cohorts (p= 0.14). The mean lag time and time to peak of the 50-59 year old women was elevated in comparison to the mean times of the 20-29 year old women. Although the magnitude of these differences were not statistically significant it is suggestive of an increase in lag time and time to peak parameters with age in female patients. Interestingly the age of the cohorts would imply that any potential trend may be due to changes in hormonal expression associated with the menopause. If this were to be the case it would imply that post menopausal women exhibit changes in hormonal expression which directly impact upon their levels of thrombin generation. It is possible that if cohorts of 60-69 year olds were compared with 20-29 year old women there would be a significant difference between them. Alternatively it may be that the sample size of the groups in this study were too small to adequately detect the differences between the two cohorts. It is also possible that due to the relatively small sample size of the cohorts the statistical analysis is suggestive of a difference between the two groups when in fact there is no difference between them. It is clear that further analysis is required to ascertain whether or not there is a difference between the two cohorts or indeed between older cohorts of women.

The normal range defined in this study is not without limitations however, since technically it is only valid for patients between the ages of 20-60. Although the analyses revealed no variation in the thrombin generation parameters with age it is potentially unsafe to assume that this is the case for patients outside the age range analysed. This is especially true with paediatric patients who are known to have reduced levels of clotting
factors and therefore potentially reduced levels of thrombin generation. The use of the assay is therefore limited to adult patients and any analysis performed on paediatric patients must be interpreted with caution. It would have been desirable to include patients over the age of 60 in the normal range but finding appropriate patients was difficult since very few individuals who work in the laboratory are above 60 years of age and the number of patients available without medical conditions which might affect the results was also very small. It was therefore decided to exclude any patients above 60 from the normal range experiments.

The addition of CTI to samples prior to analysis by the CAT thrombin generation assay showed a statistically significant decrease in the peak height compared to samples without the addition of CTI. This conflicts with a report in the literature which states that the use of CTI is not required when a 5pM tissue factor reaction trigger is used to initiate thrombin generation (van Veen et al., 2008). There was no statistically significant difference in any of the other thrombin generation parameters analysed following the addition of CTI. A potential explanation for this result could be the concentration of tissue factor in the PPP reagent manufactured by Diagnostica Stago. No attempt was made in this study to verify the manufacturer’s claim that the reagent contained 5pM tissue factor (or indeed 4µM phospholipid). Variations in the concentration of tissue factor (specifically reductions) in the PPP reagent could, potentially, make the assay more sensitive to contact activation. Whether this was the case is unknown. There is evidence to the contrary. The manufacturer’s data was used (ie concentration of tissue factor, phospholipid and composition of phospholipids in the PPP reagent) in all studies. If there were fluctuations in the concentration of the PPP reagent this would have resulted in fluctuations of the results across the course of this study. This means that samples taken from the same individuals that were analysed in several different experiments using different batches of PPP reagent would show discordant results. The same would be true of the experiments using Control Plasma P. This was not the case. It may be concluded that the composition of the PPP reagent did not have significant batch to batch variation.

The change in peak height following the addition of CTI was small, with an average decrease of ~6%, which was within the 10% cut off as previously discussed. Indeed, the difference is similar to the average intra assay variation for peak height attained
following the analysis of a pool of fresh human plasma, which was 3% (table 2.13). The decrease in peak height may also be due to a dilution effect of the blood samples to which CTI was added, since the volume of liquid in the vacutainer was increased from 500µl to 577µl. This equated to a decrease of ~1.5%, so that the actual average decrease was ~4.5% (6-1.5). The change in peak height following the addition of CTI is not likely to be of clinical significance in the demographic of patients analysed in this study (patients with elevated thrombin generation), because a small variation in a sample with an elevated peak height would have a minimal impact on the result generated compared to the same change seen in a patient with a relatively low peak height. A final consideration is the cost implication to the study. If CTI was used at the concentration specified above the cost of the assay would rise from ~£3 to ~£13 per sample. An increase of 433%. In conclusion, it was decided to forgo the addition of CTI in this study as it would not increase the clinical utility of the experiments performed but would substantially increase the cost.
Chapter 3

Pre-eclampsia
3.1 Introduction

With the completion of the validation experiments covered in chapter 2, it was decided that the CAT thrombin generation assay was suitable for routine diagnostic use in the haemostasis laboratory. The first group that was analysed by the new technique was a cohort of pregnant women with and without pre-eclampsia.

3.1.1 Thrombophilia and Pregnancy

Pregnancy itself is associated with marked changes in haemostasis that lead to a prothrombotic state in the mother. These changes include elevated levels of factor VII, factor VIII, von Willebrand factor and fibrinogen, combined with decreased levels of natural anticoagulants such as free Protein S and an increased incidence of resistance to activated protein C (Stirling et al., 1984), (Clark et al., 1998). These changes are thought to have arisen at an evolutionary level in order to provide potential protection against post partum haemorrhage and thereby confer a selective advantage. These natural physiological changes may be aggravated by either acquired or inherited thrombophilia however, giving rise to a number of serious obstetric complications such as recurrent pregnancy loss, intrauterine growth retardation (IUGR), placental abruption, pre-eclampsia and recurrent thromboembolism (Pabinger 2009).

Evidence linking recurrent pregnancy loss, as defined as three consecutive pregnancy losses before the 20th week of gestation, and inherited thrombophilia is often conflicting although some meta-analyses have shown a correlation; most notably Rey et al. (2003) who showed factor V Leiden, APC resistance, Prothrombin variant and free protein S deficiency were all associated with early recurrent pregnancy loss with odds ratios ranging from 2.01 to 14.72. The meta analysis demonstrated no correlation between Antithrombin and protein C deficiencies as well as the Methylenetetrahydrofolate reductase (MTHFR) enzyme mutation and recurrent foetal loss. The evidence linking acquired thrombophilia and foetal loss is more striking with an estimated 7-25% of all recurrent spontaneous abortions having antiphospholipid syndrome (APS) as the main risk factor (Vinatier et al., 2001).
The association between thrombophilia and pre-eclampsia is also not clearly defined with a number of studies providing conflicting evidence. A meta-analysis by Kosmas et al. (2003) examined the incidence of the factor V Leiden allele in women with pregnancy related hypertension and a cohort of normotensive controls and found an odds ratio of 2.3 (95% CI: 1.5-3.4). Interestingly not all of the studies examined in the analysis demonstrated an association between factor V Leiden and hypertension. A further meta-analysis by the same authors (Kosmas et al., 2004) showed a weak correlation between the MTHFR gene mutation and pre-eclampsia but other studies have shown no link between hyperhomocysteinemia and pre-eclampsia (Pabinger 2009).

Some studies such as that by van Pampus et al. (1999) have shown a striking relationship between pre-eclampsia and thrombophilia, with 40% of a cohort of women with severe pre-eclampsia having a diagnosed thrombophilia, approximately four times the incidence compared to a cohort of normotensive pregnant women. Although this study included a broader range of tests including APC resistance, hyperhomocysteinemia, anticardiolipin antibodies as well as a genetic test for factor V Leiden. The incidence of thrombophilic conditions also showed an increase of between 1.5-2 times more in the women who delivered before 28 weeks gestation when compared to those who delivered after 28 weeks gestation.

The apparent conflicting evidence in the literature linking thrombophilia with pregnancy loss and pre-eclampsia may be partially explained by the focused nature of some of the studies. Most papers focus on a single test for thrombophilia whereas there are several known causes and therefore multiple assays to be performed to get an accurate idea of any association. It is no surprise therefore that the study by Pampus et al. (1999) showed the greatest level of correlation since their diagnosis of thrombophilia was the most broad i.e. they performed the highest number of analyses. However as discussed in section 1.5 a large number of patients who come to the Royal London Hospital for thrombophilia screening have normal results for the tests performed. This does not mean that they do not have an inherited thrombophilia however, but merely confirms that they do not have one of the conditions for which they were screened. The use of a global method such as the CAT thrombin generation assay may therefore be a powerful
tool in detecting the true association between any prothrombotic conditions and pregnancy loss/pre-eclampsia.

3.1.2 Pre-eclampsia

Pre-eclampsia is a complication of pregnancy, which is characterised as proteinuria (the presence of protein in urine) and new-onset gestational hypertension. It is important to distinguish between pre-eclampsia and other hypertensive disorders of pregnancy since pre-eclampsia carries the greatest risk to both mother and foetus. The hypertensive disorders of pregnancy include hypertension which pre-dates pregnancy (either acute or chronic) and transient gestational hypertension. Women who have chronic hypertension who develop proteinuria during pregnancy are said to have chronic hypertension with superimposed pre-eclampsia. (Roberts et al., 2005). Pre-eclampsia is a disorder which has a large impact on both mother and foetus and is one of the major causes of maternal/foetal morbidity and mortality worldwide. Hypertensive diseases of pregnancy were the second leading cause of direct maternal death in the UK in 2001 and hypertension and/or proteinuria are associated with 20% of all still births, of which 7% are directly caused by Pre-eclampsia (PRECOG 2004).

Once diagnosed pre-eclampsia can only be cured by delivery, in the developed world where antenatal care is readily available this is the most frequent intervention, indeed it is estimated that 15% of all preterm births are due to delivery of women in order to halt the progression of pre-eclampsia to eclampsia (convulsions often followed by the onset of coma) which can be fatal. In the third world where antenatal care is less prevalent progression of pre-eclampsia accounts for approximately 50000 deaths per year (Duley 1992).

Although the diagnosis of pre-eclampsia is primarily based around the development of proteinuria and gestational hypertension, the condition itself is far more wide ranging causing multiple systemic changes in patients. These include endothelial dysfunction, activation of the clotting cascade, an increased inflammatory response, vasoconstriction and numerous changes to metabolic pathways (Roberts et al., 2005). Models detailing the natural history of pre-eclampsia commonly describe a two stage process, the first stage of which is reduced placental perfusion (reduction of blood flow in the placenta).
This then leads to the second stage which is multisystemic pre-eclampsia in the mother. It is important to note that although there is a myriad of evidence for such a model, much still remains to be discovered. For example although it is clear that reduced placental perfusion is prevalent in women with pre-eclampsia it is by no means certain that reduced perfusion will lead to the development of the condition itself. The molecular mechanism of the progression from stage one to stage two also remains to be elucidated, although this is discussed in more depth below.

### 3.1.3 Stage 1: Reduced Placental Perfusion

The first links between pre-eclampsia and reduced placental perfusion were based on clinical findings and epidemiological evidence which linked pre-eclampsia to disorders where the placenta was enlarged. Such conditions include molar pregnancies (a tumour arising from the abnormal development of the embryo) and multiple pregnancies where it was postulated that the enlarged placenta could not be adequately perfused given its size. Striking evidence for reduced placental perfusion in pre-eclampsia was provided by Starzyk et al. (1999) who demonstrated that the uterine spiral arteries of women with pre-eclampsia either failed to undergo the vascular remodelling process which is characteristic of normal pregnancy or the vascular remodelling was only partially complete. The uterine spiral arteries would normally be remodelled in order to offer less resistance to blood flow to the placenta. This remodelling typically includes an increase of vessel diameter, the loss of smooth muscle surrounding the vessel, as well as a loss of the internal elastic lamina. Importantly the loss of the internal lamina and therefore the loss of many endothelial receptors renders the vessels immune to vasoactive stimuli. These observations are confirmed by Doppler velocimetry of uterine vessels in women with pre-eclampsia which show increased resistance to blood flow in those vessels which supply the intravillous space. Interestingly this increased resistance can also be detected in women early in gestation who subsequently go on to develop pre-eclampsia (Papageorghiou et al., 2002).

In normal pregnancy embryo implantation is mediated via the eroding of the uterine surface endothelium by embryonic trophoblastic cells. Trophoblasts can either retain their cellularity (cytotrophoblasts) or fuse with other trophoblasts to form syncytiotrophoblasts. The cytotrophoblasts proliferate and are responsible for invasion
into the maternal decidual tissue. The decidual tissue into which the embryo invades is effectively ‘digested’ to form a yolk reservoir. Remodelling of the uterine spiral arteries is thought to be a result of trophoblast invasion; this invasion is mediated by both changes in the local oxygen concentration and via various immune mechanisms. The oxygen tension of the intervillous space remains relatively low for the first 12 weeks of pregnancy, following this time perfusion of the intervillous space begins to increase and as a result the oxygen tension rises. This conversion from anaerobic to aerobic metabolism is accompanied by a dramatic rise in the formation of reactive oxygen species such as free radicals. Such free radicals can rapidly cause indiscriminate damage to cellular proteins, lipids and DNA. This cellular damage is limited by various antioxidant defences. Syncytiotrophoblasts are particularly vulnerable to free radical damage since they exist adjacent to the intervillous space and therefore undergo the conversion to aerobic metabolism early but also because they have reduced levels of antioxidant enzymes when compared to other villous tissues early in pregnancy (Jauniaux et al., 2000). It is hypothesised that this burst of oxidative stress is required for normal placental development, such as the initiation of a variety of differentiation pathways. This potentially includes the conversion of cytotrophoblasts from a proliferative phenotype to an invasive phenotype. Endovascular invasion of the cytotrophoblasts leads to invasion of the endometrial arteries and therefore allows adequate perfusion of the intervillous space and subsequently adequate placental exchange.

A potential cause of pre-eclampsia could therefore be a disruption of the normal interaction between the maternal and foetal antioxidants leading to inefficient endovascular invasion of cytotrophoblasts and therefore a failure of the uterine spiral arteries to undergo vascular remodelling. A second school of thought is associated with increased damage to the syncytiotrophoblast layer due to inadequate antioxidant defences leading to apoptosis in these cells. Syncytiotrophoblast fragments can then pass directly into the maternal circulation and can lead to the activation of peripheral maternal endothelial cells and hence the second stage of pre-eclampsia (Hung et al., 2002).

Invasion by cytotrophoblasts into the uterine decidual tissue is also mediated via immune interactions. The most important of which is thought to be between the MCH
class I molecule HLA-C present on the cytotrophoblasts and the killer inhibitory receptors (KIR) found on uterine natural killer cells. Uterine natural killer cells are thought to be functionally and phenotypically distinct from the natural killer cells found in peripheral blood. Once activated they release cytokines and chemokines which are thought to play a role in remodelling the uterine spiral arteries (Hiby et al., 2004). It was hypothesised by the authors that certain combinations of KIR and HLA-C result in an increased risk of pre-eclampsia. Specifically the combinations responsible were uterine killer cells with KIR AA haplotypes (which lack functional activator receptors but have functional inhibitory receptors) and trophoblasts displaying HLA-C2. It is thought that this combination leads to increased suppression of uterine killer cell activity and ultimately a suppression of cytotrophoblast invasion and vascular remodelling.

3.1.4 Stage 2: The Maternal Syndrome

Although pre-eclampsia is thought to arise from reduced placental perfusion, reduced perfusion is by no means limited to the uterus in women with the systemic condition. Indeed evidence of decreased perfusion is present in virtually all organs in a woman with pre-eclampsia. The reduced perfusion is thought in part to be due to increased vasoconstriction caused by an increased sensitivity of vascular endothelial cells to vasopressors (Roberts et al., 2002). Reduced perfusion can ultimately lead to both infarction and necrosis of affected tissues. Reduction in blood flow can further reduce the level of placental perfusion leading to an exacerbation of the condition via a positive feedback mechanism, which can only be cured by delivery. Systemic vasoconstriction is accompanied in women with pre-eclampsia by an activation of the clotting cascade and subsequently the formation of occlusive microthrombi (with can lead to further infarction) as well as a decrease in the volume of fluid in the intravascular space. Since endothelial function is linked with all the pathological changes described above it is no surprise that studying endothelial changes has become a central focus of research into pre-eclampsia. This research has yielded a great deal of results showing increased levels of circulating markers of endothelial activation such as von Willebrand factor, cellular fibronectin, thrombomodulin and endothelin as well as an increased level of thromboxane released from platelets in the blood of women with pre-eclampsia (Roberts et al., 2002). It has also been shown that many of these endothelial markers are
elevated before the clinical symptoms are manifest which implies a causal role for these markers in disease development (Roberts et al., 2005).

Many of the above markers of endothelial activation are associated with haemostasis; it is thought that elevated levels of thrombomodulin, tissue factor activity and circulating procoagulant phospholipids may reflect the level of vascular endothelial damage in patients with pre-eclampsia. These parameters were all significantly elevated in women with pre-eclampsia compared to normotensive pregnant women and non-pregnant women (Rousseau et al., 2009). Elevated levels of von Willebrand factor, fibrinogen, D-dimer and thrombomodulin were also reported in the circulation of women with severe pre-eclampsia by Heilmann et al. (2007), who also reported a decreased platelet count in the same cohort. These findings are consistent with activated coagulation in women with pre-eclampsia. Further evidence of the activation of the haemostatic system was provided by Peracoli et al. (2008) who reported reduced platelet function and reduced numbers of platelets in conjunction with increased levels of transforming growth factor β (TGF-β) in the circulation of women with pre-eclampsia. These findings were consistent with platelet activation in vivo since the reduced number of platelets was likely to be caused by consumption and the reduced function was likely to be the result of partial granule release in the platelets which remained. The high circulating levels of TGF-β were also consistent with platelet granule release since TGF-β is stored in the alpha granules of platelets. These findings provide compelling evidence for a significant shift towards a prothrombotic state in women with pre-eclampsia.

There is also evidence to implicate other metabolic changes with having a role in the genesis of pre-eclampsia. These include elevated levels of uric acid, triglycerides, free fatty acids and LDL cholesterol, insulin resistance and reduced levels of HDL cholesterol. Interestingly elevated levels of uric acid have been shown to be evident early in pregnancy in women who develop pre-eclampsia. Elevated levels of uric acid have also been shown to cause increased blood pressure in a rat model. Subsequent reduction in uric acid levels in the rat model alleviated the hypertension (Mazzali et al., 2001). Despite this evidence the mechanism for a causal role in pre-eclampsia remains to be elucidated.
3.1.5 What Links Stage 1 and Stage 2?

The link between the initial reduction of placental perfusion and the subsequent systemic condition of pre-eclampsia is not clear cut. In an ideal scenario the reduced perfusion would lead to the production of a unique substance by the placenta which would enter the maternal circulation and trigger the maternal condition, in reality the link is much more subtle. Increased apoptosis of syncytiotrophoblasts due to oxidative stress as described above can lead to an increased level of microvillus particles in the blood of women with pre-eclampsia. It is hypothesised that these fragments can interact with and activate circulating inflammatory cells or even directly activate the vascular endothelium leading to the maternal condition (Redman and Sargent, 2000). Although this theory fits neatly with the ideal scenario outlined above it is not the only suggested hypothesis.

3.1.5.1 Angiogenic Factors and Pre-eclampsia

A large body of evidence links the levels of a number of angiogenic factors to the pathogenesis of pre-eclampsia. Specifically patients with pre-eclampsia demonstrate an imbalance of circulating angiogenic factors, with reduced levels of the pro-angiogenic factors placenta-derived growth factor (PLGR) and vascular endothelial growth factor (VEGF) and elevated levels of the anti-angiogenic factors soluble Fms like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng). This imbalance in angiogenic factors which results in a primarily anti-angiogenic phenotype is thought to trigger vascular endothelial cell injury in multiple organs including the placenta, liver, kidney and brain. This systemic vascular injury then results in the systemic maternal syndrome (Steinberg et al., 2009). sFlt1 is a splice variant of Flt-1 which lacks both the transmembrane and cytoplasmic domain of the Flt-1 protein. Flt-1 is the receptor for VEGF and ordinarily the binding of VEGF to Flt-1 would result in activation of intracellular signalling pathways yielding a pro-angiogenic response. sFlt1 is an antagonist of both VEGF and PLGF and acts by binding to these proteins in the circulation. Once sequestered by sFlt1 VEGF and PLGF cannot bind to Flt-1 and therefore their pro-angiogenic action is inhibited. sFlt1 is synthesised in elevated amounts in the placentas of women with pre-eclampsia where it is subsequently exported into maternal circulation. The levels of sFlt1 have been reported to be elevated in pregnant women who subsequently develop
pre-eclampsia compared with those who remain normotensive (Levine et al., 2004) and have also been shown to correlate with the severity of the condition where patients with severe pre-eclampsia had statistically higher levels of sFlt1 than those patients who had mild pre-eclampsia (Chaiworapongs et al., 2004). Although this evidence suggests that sFlt1 is increased in pre-eclampsia it is not evidence that this increase can lead to the condition itself. Evidence is however provided in rat models, where pregnant rats given exogenous sFlt1 developed hypertension, proteinuria and other symptoms found in pre-eclampsia in humans (Maynard et al., 2003). Similar findings have also been reported in non pregnant mice treated with antibodies against VEGF (Sugimoto et al., 2003) as well as in oncology patients treated with VEGF antagonists (Patel et al., 2008).

Multiple isoforms of the sFlt1 protein have been reported. One of these isoforms has a unique C-terminus structure when compared to sFlt 1 and is referred to as sFlt1-14 (by virtue of the fact it is composed of the first 14 N-terminal exons of the Flt 1 receptor rather than the first 13 like sFlt 1). This difference in structure between sFlt 1 and sFlt1-14 is due to the utilisation of a previously unknown slice acceptor site present in intron 14 to produce the extended sFlt1-14 mRNA. Despite the differences in structure sFlt1-14 still functions as a potent inhibitor of VEGF and the authors propose its function is to protect vascular smooth muscle cells (where it is primarily expressed) from the pro-angiogenic signals of VEGF (Sela et al., 2008). Interestingly the authors also proposed a role for sFlt1-14 in the development of pre-eclampsia, they suggest that the increased expression of soluble VEGF receptors seen in pregnancy is entirely due to increased expression of sFlt1-14 after the first trimester and that expression of sFlt1-14 is increased in women with pre-eclampsia. It was demonstrated that sFlt1-14 is the primary isoform produced by the placenta in women with pre-eclampsia and that the majority of this expression was found in the syncytial knots of the placenta. Syncytial knots are localized aggregations of syncytiotrophoblastic nuclei in the villi of the placenta, these structures are thought to be induced by placental hypoxia and are a histological hallmark of pre-eclampsia. More anecdotal evidence linking sFlt1-14 to pre-eclampsia comes from the fact that the C-terminus structure of sFlt1 which is replaced in sFlt1-14 is conserved on an evolutionary level and the sFlt1-14 isoform is only found in humans and primates which are also the only species to exhibit pre-eclampsia.
The fact that sFlt1-14 expression is primarily found in syncytial knots which themselves are produced in response to hypoxia provides further evidence linking reduced oxygen tension and pre-eclampsia. This is given further weight by the fact that VEGF and Flt1 (and by extension sFlt 1) gene expression are controlled by the transcription factor hypoxia-inducible factor 1α (HIF-1α) (Gerber et al., 1997) and that HIF-1α levels are significantly elevated in the placentas of women with pre-eclampsia (Rajakumar et al., 2003).

Despite the compelling evidence for the involvement of sFlt 1 in pre-eclampsia it has been observed that animals treated with sFlt 1 in order to provoke pre-eclampsia do not develop symptoms associated with complications of severe pre-eclampsia such as the HELLP syndrome (Haemolysis, elevated liver enzymes and low platelets). It was therefore hypothesised that other soluble placenta derived factors may act in synergy with sFlt 1 to cause sufficient endothelial dysfunction and result in severe pre-eclampsia. It was hypothesised by Venkatesha et al. (2006) that sEng, a truncated form of the cell surface co-receptor for TGF-β, was responsible. Endoglin (CD105) is a co-receptor for TGF-β. Binding of TGF-β to Endoglin results in intracellular signalling which influences the activity of endothelial nitric oxide synthase (eNOS). Endothelium-derived nitric oxide (NO) is a potent vasorelaxant that contributes to the regulation of systemic blood pressure, vascular permeability and angiogenesis. sEng can sequester TGF-β in a similar way to the interaction of sFlt 1 and VEGF resulting in an amelioration of the vasorelaxant effect of TGF-β. Expression of sEng was shown to be increased four-fold in the placentas of women with pre-eclampsia compared to normotensive pregnant women. sEng was also shown to inhibit capillary tube formation in vitro and induce vascular permeability and hypertension in vivo. In rat models co-administration of both sFlt 1 and sEng exacerbated the effects seen with sEng alone and led to severe pre-eclamptic like symptoms including the HELLP syndrome and restricted foetal growth (Venkatesha et al., 2006).

Despite all the evidence linking an over-expression of sFlt 1 with pre-eclampsia, there still remains a sub set of pre-eclampsia cases including some women with severe pre-eclampsia where the patients demonstrate low levels of sFlt 1 and high levels of PLGF (Levine et al., 2004). Anti angiogenic action of a different mechanism cannot be excluded however. In studies of pregnant rats treated with the angiogenesis inhibitor
Suramin (which can bind to basic fibroblast growth factor, platelet derived growth factor, VEGF receptor 2 and fibroblast growth factor receptors) the animals displayed symptoms of pre-eclampsia including a failure of vascular remodelling, but the serum levels of sFlt are known to decrease in Suramin treated rats (Jankowski 2009). It is possible in this study that the Suramin used initiated the pre-eclamptic symptoms via inhibition of the synthesis or release of vasodilators such as nitric oxide, in a similar way to the over-expression of sEng. This potentially highlights a role for the over-expression of sEng as causative of pre-eclampsia in those women with low levels of circulating sFlt 1.

### 3.1.5.2 The Renin-Angiotensin System and Pre-eclampsia

Other biological systems have also been implicated in the natural history of pre-eclampsia. One of these is the renin-angiotensin system, which is a signalling cascade responsible for the regulation of blood pressure and the balance of electrolytes. The renin-angiotensin system is regulated by proteins made in multiple organs around the body but is primarily controlled by the production of the enzyme renin which is synthesised by the juxtaglomerular cells of the kidney. Renin release is stimulated by either a decrease in blood pressure or low circulating levels of sodium chloride. The primary function of renin is to cleave the functionally inactive protein angiotensinogen, which is produced in the liver, to produce angiotensin-I. Angiotensin-I is still biologically inactive however and requires further enzymatic cleavage by angiotensin converting enzyme (ACE), which is primarily synthesised in the lung endothelium, in order to produce the functionally active angiotensin-II. This conversion is mediated via proteolytic cleavage to convert angiotensin-I from a 10 amino acid protein to the 8 amino acid protein angiotensin-II (Irani and Xia 2008).

There are two primary receptors for angiotensin-II these are AT$_1$ and AT$_2$. The two receptors share 34% sequence homology and have similar affinities for the binding of angiotensin-II. The biological effects that the differential binding mediates are markedly different however. Activation of AT$_1$ results in the activation of signalling pathways which lead to elevated levels of intracellular calcium, which ultimately leads to vasoconstriction, sympathetic activity and aldosterone release (aldosterone increases the reabsorption of sodium and water and the release of potassium in the kidneys
subsequently increasing blood volume and pressure). Activation of AT₂ results in an inhibition of cell growth, an increase in the level of apoptosis and causes vasodilation. The AT₁ receptor is primarily expressed on the surface of vascular smooth muscle cells and the adrenal glands where as the AT₂ receptor is highly expressed in the foetal kidney and its expression decreases after birth. AT₁ receptor expression is more prevalent in the adult kidney than the AT₂ receptor (Irani and Xia 2008).

The renin angiotensin system which is classically described in the kidneys has been increasingly shown to be present in other tissues; most notably components of the renin angiotensin system including angiotensinogen, renin, ACE and the AT₁ receptor have been described in both the foetal placental tissues (Irani and Xia 2008) and the maternal decidua (Morgan et al., 1998). As these findings would suggest, the regulation of the renin-angiotensin system is altered in normal pregnancy. The primary changes are increased levels of renin, angiotensin-I and angiotensin-II (and therefore increased levels of aldosterone) and decreased levels of ACE (Langer et al., 1998). Intuitively these changes would facilitate an increase in blood pressure in pregnant women; this potential effect is however repressed by a decrease in sensitivity of pregnant women to angiotensin-II (Gant et al., 1980). Since the maternal sensitivity to angiotensin-II is decreased it is thought that the ‘intended target’ of the increased levels of angiotensin-II is foetal tissue and that of the placenta. This theory is given weight by the fact that trophoblastic cells are rich in AT₁ receptors. It is thought that the renin-angiotensin system is responsible for the regulation of foetal-placental circulation and ultimately ensuring there is adequate blood flow for both nutrient supply and oxygen exchange (Irani and Xia 2008).

Interestingly the changes observed in the renin-angiotensin system during normal pregnancy are subverted in patients with pre-eclampsia to give a new phenotype. The circulating maternal levels of renin, angiotensin-I, angiotensin-II and aldosterone in a patient with pre-eclampsia are all significantly lower than those of a normotensive pregnant woman, although they are all increased when compared to a non pregnant woman. The levels of ACE in the circulation of women with pre-eclampsia can be equivalent to a non pregnant woman and therefore can be elevated when compared to a normotensive pregnant woman (Langer et al., 1998). The most striking difference however is that pre-eclampsia patients typically do not demonstrate the reduced
sensitivity to angiotensin-II that is the norm in pregnancy. This reduction in sensitivity is thought to be due to the structure of the AT1 receptors in the two groups. In normotensive pregnant women the AT1 receptors are primarily monomers. These receptors are sensitive to inactivation by reactive oxygen species. In patients with pre-eclampsia however the AT1 receptor is often present in a heterodimer with the bradykinin receptor, these heterodimers show increased resistance to deactivation by reactive oxygen species (AbdAlla et al., 2001) and therefore predispose women with pre-eclampsia to increased blood pressure when compared to normotensive pregnant women and especially when compared to non pregnant women since they have elevated levels of angiotensin-II. Another possible route to subvert the normal pregnancy related insensitivity to angiotensin II is thought to be associated with the expression of haemopexin. Plasma haemopexin activity is increased in normal pregnancy in contrast to pre-eclampsia and increased haemopexin activity has been shown to down-regulate expression of the AT1 receptor (Bakker et al., 2009). Therefore decreased haemopexin activity in pre-eclamptic patients leads to increased levels of AT1 receptor expression when compared to normotensive pregnant women and ultimately an increase in blood pressure.

The elevated sensitivity of pre-eclamptic women to angiotensin-II when compared to normotensive women is further exacerbated by the fact that the expression of the AT1 receptor itself is upregulated in the maternal decidua of women with pre-eclampsia (Herse et al., 2007). In addition to this the placentas of women with pre-eclampsia demonstrate increased levels of chymase when compared with the placentas of normotensive pregnant women (Wang et al., 2007). Chymase is a serine protease enzyme released by mast and smooth muscle cells which is capable of producing angiotensin-II in a similar way to ACE. Transgenic mice engineered to over express human chymase have been shown to demonstrate several symptoms of pre-eclampsia including hypertension and vasoconstriction (Ju et al., 2001).

Despite the changes in the renin-angiotensin system seen in pre-eclampsia described above, one of the most profound findings was the discovery that women with pre-eclampsia produced an agonistic AT1 receptor autoantibody. This antibody has been shown to bind to a seven amino acid sequence present on the extracellular loop of the AT1 receptor and ultimately cause the activation of the associated cell signalling
pathways resulting in vasoconstriction and elevated blood pressure (Wallukat et al., 1999). Interestingly one of the regulatory genes controlled by the AT\(_1\) receptor is that of sFlt1. The production of a AT\(_1\) receptor activating autoantibody coupled with a loss of sensitivity of the receptor to deactivation via reactive oxygen species would therefore provide a secondary mechanism for the over expression of sFlt1. This is significant as it indicates that hypoxia and hence activation of the transcription factor HIF-1\(\alpha\) is not the only route to high circulating levels of sFlt1. Indeed it is possible that over expression of sFlt1 via the production of an agonistic AT\(_1\) receptor autoantibody or in combination with hypoxia in the placenta could lead to a positive feedback loop where increased levels of sFlt1 lead to decreased angiogenesis and therefore increase the severity of the hypoxia. This pattern is indicative of pre-eclampsia progression where the symptoms only show an increase in severity until such time as the foetus is delivered.

Aside from its role in enhancing the expression of sFlt1 the AT\(_1\) autoantibody found in the circulation of pregnant women with pre-eclampsia has been implicated in disease progression in a number of other ways. The first of these is the fact that the AT\(_1\) receptor autoantibody can lead to the production of free radicals and other reactive oxygen species in placental trophoblastic cells and smooth muscle cells. This reaction was mediated by activation of NADPH oxidase (Dechend et al., 2003). This finding provides a direct link with the experiments of Hung et al. (2002) discussed above, who hypothesised that a link between placental disease and the maternal pre-eclampsia syndrome may be caused by syncytiotrophoblast fragments passing into maternal circulation following excessive free radical damage. These fragments can then systemically activate the maternal endothelium.

The AT\(_1\) autoantibody has also been shown to regulate the levels of plasminogen activating inhibitor 1 (PAI-1). PAI-1 has a role in the regulation of fibrinolysis (the breakdown of fibrin clots). It mediates this effect by the inactivation of urokinase-like plasminogen activator and tissue plasminogen activator. Urokinase-like/tissue plasminogen activators convert the zymogen plasminogen to the biologically active plasmin, which is directly involved in the breakdown of fibrin. Elevated levels of PAI-1 therefore result in a decreased level of fibrinolysis. PAI-1 can also indirectly inhibit extracellular matrix degradation via matrix metalloproteinases. This impacts on pre-eclampsia progression in two ways, the first of which is an impairment of trophoblast
invasion in the developing placenta. Effective fibrinolysis and extracellular matrix digestion is thought to be necessary in efficient trophoblast invasion. AT$_1$ receptor activation via the AT$_1$ receptor autoantibody has been shown to both increase PAI-1 expression in trophoblasts and ultimately decrease trophoblastic invasion (Bobst et al., 2005). This shows a potential role for the AT$_1$ receptor autoantibody in the development of pre-eclampsia via decreased placental perfusion. Over expression of PAI-1 is also thought to contribute to some of the symptoms of the maternal condition, particularly those involving renal impairment. It is thought that an excess of fibrin deposits in the kidney could lead to increased tissue damage and that fibrin deposits in the glomeruli may decrease renal filtration rates and therefore contribute to proteinuria (Irani and Xia 2008).

Expression of the AT$_1$ receptor autoantibody may also cause further disruption in the haemostatic pathways since activation of the AT$_1$ receptor causes an over expression of tissue factor in both vascular smooth muscle cells (Dechend et al., 2000) and monocytes (Dorffel et al., 2003). Since tissue factor is a potent stimulator of coagulation these findings may explain the hypercoagulability seen in patients with pre-eclampsia. Indeed this prothrombotic state in patients with pre-eclampsia is only likely to be exacerbated by the dysregulation of the fibrinolytic system caused by the over expression of PAI-1. This imbalance of haemostasis is likely to result in the formation of systemic microthrombosis and ultimately further organ damage.

The above evidence shows that the AT$_1$ receptor autoantibody can produce a wide variety of pathological effects. Evidence that the antibody alone can induce pre-eclampsia is provided by animal models where Zhou et al. (2008) injected pregnant mice with the autoantibody obtained from pregnant women with pre-eclampsia. The mice subsequently demonstrated pre-eclamptic symptoms including hypertension, proteinuria and elevated levels of sFlt-1. Importantly the use of an antibody neutralising peptide ameliorated these effects proving the autoantibody played a part in disease development. The origin of the pre-eclamptic AT$_1$ receptor agonistic antibody remains unclear however. The fact that the Antibody retains homology in terms of the 7 amino acid peptide to which it binds across patients with pre-eclampsia implies a common immunological origin (Irani and Xia 2008). Whether or not this ‘antigen’ is found in the
placenta due to vascular damage associated with reduced perfusion remains to be elucidated.

3.1.5.3 Other Possible Links

Numerous other molecules and biological systems have also been proposed as either being possible causes of or contributing factors to pre-eclampsia. The first of these is the expression of the tumour necrosis factor (TNF) gene variant 308A. This is a polymorphism of the wild type TNF gene where there is a G to A transition at position 308. This results in a mutated promoter region which leads to elevated levels of transcription of the TNF gene. TNF is a pro-inflammatory cytokine which is thought to be involved with endothelial activation associated with systemic pre-eclampsia. The TNF 308A variant is therefore thought to contribute to disease progression via increased levels of TNF. Studies investigating the association were however conflicting. In a large meta analysis involving 1919 patients with pre-eclampsia and eclampsia the expression of TNF 308A was found to have a pooled relative risk of 1.02 (95% CI 0.86-1.20). This indicates that there was no correlation between the TNF 308A isoform and pre-eclampsia (Bombell and McGuire 2008).

Other studies have attempted to characterise genetic changes in pre-eclampsia using microRNA analysis. MicroRNAs are short (21-24 nucleotides in length) non-coding RNAs which function as negative regulators of gene expression via binding to specific mRNAs and therefore preventing translation. A study by Zhu et al. (2009) showed differential expression of 34 microRNAs in the placentas of women with pre-eclampsia when compared with a control group. They found 11 of the microRNAs were over-expressed in the patient group and 24 were under-expressed. Although this study provides clear evidence for changes in gene expression in pre-eclampsia the targets for the differentially expressed microRNAs were not clearly defined.

Immunological activation is also thought to play a part in the development of pre-eclampsia and is also a possible route to explain the systemic maternal condition. Specifically the up regulation of CD40 and the CD40 ligand have been described in pre-eclampsia (Alacacioglu et al., 2007), (Lukanov et al., 2009). CD40 is a member of the TNF receptor family and is primarily expressed on the surface of antigen presenting
cells such as macrophages. CD40 ligand was first described on T-cells and has since been found to be expressed on other leukocytes as well as platelets, smooth muscle cells, endothelial cells and epithelial cells. The binding of CD40 ligand to CD40 causes activation of macrophages and the release of pro-inflammatory cytokines as well as reactive oxygen species. Alacacioglu et al. (2007) reported a significant increase in the level of soluble CD40 ligand, thought to be released by activated platelets, in the blood of pre-eclamptic women when compared to healthy pregnant women. Lukanov et al. (2009) reported elevated surface expression of CD40 ligand on platelets and CD40 expression on the surface of macrophages in women with pre-eclampsia when compared to normotensive pregnant women. The authors also reported increased platelet expression of P-selectin (CD62P), a cell adhesion molecule, in pre-eclamptic women. Elevated numbers of platelet derived microparticles expressing P-selectin were also reported in the blood of women with pre-eclampsia compared to that of healthy pregnant controls by Lok et al. (2007). Expression of P-selectin on the surface of platelets and the production of platelet microparticles are consistent with platelet activation in pre-eclampsia. Platelets expressing P-selectin can bind to neutrophils and monocytes via leukocyte P-selectin ligand-1 (PSGL-1). The resultant intracellular signalling causes leukocyte surface expression of tissue factor (Barnard et al., 2005). Tissue factor is a potent trigger of the coagulation cascade and may ultimately yield the production of thrombin, which itself is a potent agonist of platelet activation. It is possible therefore that platelet activation can lead to a positive feedback mechanism where P-selectin expression yields increased levels of tissue factor, and hence thrombin production, leading to further platelet activation. Increased platelet activation can lead to increased CD40 ligand production (both soluble and surface expressed) which could potentially sustain systemic leukocyte activation and ultimately the resultant pro-inflammatory cytokine response. Although this mechanism fits with the gradual exacerbation of the maternal condition it is not known what the initial driving force may be.

What is clear when considering the link between the two stages of pre-eclampsia is that there are many potential antecedents of the condition and there is conflicting evidence in the literature surrounding the subject. One possibility is that there are simply multiple ways of getting from reduced placental perfusion to the systemic maternal condition and that there is no one causal factor responsible for disease progression. There are a great
many molecular pathways reported as disrupted in the development of pre-eclampsia some of which can be subverted in numerous ways. For example when considering over-expression of sFlt-1, this can be caused via hypoxia or activation of the AT₁ receptor. Activation of the receptor itself can be caused by increased levels of angiotensin II coupled with impaired down-regulation of the receptor or via the expression of the AT₁ receptor agonistic autoantibody. The anti-angiogenic response mediated by sFlt-1 may itself be unnecessary in disease development if sEng is sufficiently over-expressed. This hypothesis would provide reasons as to why there always appears to be a sub group of women with pre-eclampsia who develop the disease despite having normal levels of the protein under investigation.

3.1.6 Predicting Pre-eclampsia

Whatever the cause of the condition, predicting the onset of disease and the severity of the condition is of direct benefit to both the mother and foetus. Pre-eclampsia can be characterised as either mild or severe. Mild pre-eclampsia is most commonly classified as blood pressure exceeding 140/90 mmHg with proteinuria of 0.3-3g/24 h. Severe pre-eclampsia has diagnostic criterion of blood pressure exceeding 160-170/100-110 mmHg, heavy proteinuria of between 3-5g/24 h or the occurrence of symptoms such as headache or visual disturbance. It is of benefit to identify those women with the greatest risk (women with severe pre-eclampsia) so as they can be closely monitored and receive intervention when necessary, either in the form of premature delivery or the use of MgSO₄. Interestingly since these interventions are associated with foetal and maternal morbidity respectively it is of benefit to identify women who are at low risk of developing complications (mild pre-eclampsia) and therefore avoid the use of potentially harmful interventions (von Dadelszen et al., 2009).

Numerous studies have tried to either predict the onset of pre-eclampsia or classify the disease severity based on the measurement of biological markers. Decreased 24 hour urinary calcium and elevated proteinuria between 20-28 weeks gestation were shown to be risk factors for the development of pre-eclampsia in one study (Sirohiwal et al., 2009). Although the study highlighted potential useful markers in the prediction of pre-eclampsia, in an ideal scenario any potential biological predictors of pre-eclampsia would be able to detect the onset of disease early in pregnancy.
Measurement of the angiogenic factors sFlt-1 and PLGF was shown to predict the early onset of pre-eclampsia with a sensitivity of 83% and specificity of 95% (Stepan and Jank 2009). This predictive value was increased to a sensitivity of 100% and specificity of 93.3% if the measurement of sEng was also included. Although the predictive values of this screening test are impressive it is important to note that these values were obtained via the measurement of angiogenic factors in women who had already been defined as high risk second trimester pregnancies characterised by abnormal placental perfusion. It is unlikely that this measurement will be introduced as a routine screening test due to a prohibitive cost.

Since the measurement of proteinuria is diagnostic in pre-eclampsia some effort has been made to determine whether the degree of proteinuria correlates with the severity of the disease. A meta analysis of 16 articles with a total cohort of 6749 women with pre-eclampsia found proteinuria to be a poor predictor for either maternal or foetal complications in pre-eclamptic women (Thangaratinam et al., 2009) (Hofmeyr and Belfort 2009).

It has been suggested that a high dietary intake of antioxidants such as vitamins C and E may prove protective against pre-eclampsia, although two trials investigating this hypothesis have shown no correlation between the incidence of pre-eclampsia and dietary intake of a combination of vitamins C and E (Poston et al., 2006) (Klemmensen et al., 2009). Interestingly the trial by Klemmensen et al. did report a statistically significant decreasing trend in the numbers of women with severe pre-eclampsia, eclampsia or HELLP syndrome with increased vitamin C intake. This suggests that antioxidants may be protective against disease progression even if they do not prevent the onset of pre-eclampsia itself.

Many predictors of pre-eclampsia rely on epidemiological evidence as a means of ascertaining whether or not an individual is at risk of developing pre-eclampsia. Conditions which are known to increase the risk of pre-eclampsia include obesity (Sibai et al., 1995), hypertension and diabetes (Caritis et al., 1998), hyperhomocysteinemia (Powers et al., 2001) and being of Black ethnic origin (Eskenazi et al., 1991), although the latter is only a risk in first time pregnancies. There is also compelling evidence for a
hereditary predisposition to pre-eclampsia. Familial inheritance studies have shown the incidence of pre-eclampsia to be 2-5 times greater in blood relatives in families than between genetically unrelated women in the same families (Cooper et al., 1993). Given the number of potential molecular causes of pre-eclampsia a genetic link is of no great surprise, the pattern of inheritance is however difficult to define. The reasons for this stem from the fact that pre-eclampsia is not a condition which is easily observed. It is prevalent only in women, even if the condition itself is not sex linked, does not manifest until an individual is of reproductive age and then only if the individual actually falls pregnant. Since there are no specific genetic tests for pre-eclampsia as the molecular cause is unclear, tracking its inheritance through a family is problematical. Despite this, efforts have been made to characterise the pattern of inheritance. Data accumulated by Arngrimsson et al. (1995) was consistent with a dominant pattern of expression coupled with variable penetration.

The pre-eclampsia community guideline (PRECOG) was first published in the UK in 2004 and includes guidance on how to identify women at risk of developing pre-eclampsia, so they can receive specialist referral early in pregnancy. The PRECOG assessment is based on both epidemiological evidence taken as a history during an antenatal visit and physiological measurements taken from the patient, including blood pressure and a measure of proteinuria. Patients receive specialist referral early in pregnancy if they meet any of the following criteria:

- Multiple Pregnancy
- Underlying medical conditions
  - Pre-existing hypertension or booking diastolic blood pressure ≥90mmHg
  - Pre-existing renal disease or booking proteinuria (>1+ on more than one occasion or quantified at ≥0.3g/24 hour)
  - Pre-existing diabetes
  - Presence of antiphospholipid antibodies
- Pre-eclampsia in any previous pregnancy
- Any two other factors from:
  - First pregnancy
  - Age 40 years or more
- Body Mass Index of 35 or more
- Family history of pre-eclampsia (in mother or sister)
- Booking diastolic blood pressure ≥ 80 mmHg

It was the aim of this chapter to investigate the thrombin generation profile of women with pre-eclampsia with a view to using thrombin generation to enable the detection of pre-eclampsia either in conjunction with the PRECOG assessment or as a stand-alone test. Little is currently known about the thrombin generation capacity of women with pre-eclampsia; although it was anticipated that they may show an elevated level of thrombin generation due to systemic activation of the coagulation cascade. The thrombin generation data of this cohort of women would also be used to try and predict other outcomes of pregnancy associated with pre-eclampsia such as birth weight, mode of delivery, length of stay in hospital and postpartum haemorrhage (PPH).

3.2 Materials and Methods

3.2.1 Selection of Patients and Controls

All patients and controls were recruited from the obstetrics unit at the Royal London Hospital, Whitechapel, London. The study was approved by the local research ethics committee and all participants gave informed consent. In total 104 participants were recruited to the study, 52 patients with pre-eclampsia and 52 controls. All women presenting with pre-eclampsia during weekdays were invited to participate. Pre-eclampsia was defined as new onset hypertension and proteinuria on or after 20 weeks gestation. New onset hypertension was defined as a blood pressure of ≥ 140 mm Hg (systolic) or ≥ 90 mm Hg (diastolic) on at least two occasions and at least 4-6 hours apart at or after the 20th week of pregnancy in women previously shown to be normotensive. New proteinuria was defined as protein excretion ≥ 0.3 g per 24 h or ≥ 1 + on dipstick in at least 2 random urine samples taken at least 4-6 hours apart. Samples for thrombin generation analysis were taken when the women presented with pre-eclampsia.

The control group was chosen from pregnant women attending the same antenatal clinics as the women presenting with pre-eclampsia and were women with no reported
medical or pregnancy related complications. Any women with known medical or pregnancy related complications were excluded from the study. The controls were matched to the patient group to try and minimise potential confounding factors including age, parity, ethnicity and gestational age.

### 3.2.2 Defining Disease Severity

Mild pre-eclampsia: greater than 20% increase in blood pressure from booking or a diastolic blood pressure of at least 90 mmHg with a trace of protein in urine. Aspartate transaminase (AST)/ Alanine transaminase (ALT) may be slightly abnormal with raised urate. May require medication.

Moderate Pre-eclampsia: Diastolic blood pressure of between 90-100 mmHg, Proteinuria 1+ or +2 on dipstick and abnormal Liver function tests (LFT). Will require medication/observation

Severe Pre-eclampsia: Diastolic blood pressure >100 mmHg, proteinuria 2+ or 3+ on dipstick with raised LFTs and urate. Patient is symptomatic and requires HDU admission and medication/delivery

### 3.2.3 Preparation of Samples

Please refer to section 2.2.4 for details

### 3.2.4 Thrombin Generation

Please refer to section 2.2.5 for details

### 3.2.5 Statistical Analysis

Please refer to section 2.2.9 for details

### 3.2.6 Reagents

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<tr>
<td>PPP Reagent</td>
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<tr>
<td>Sterile Distilled Water</td>
<td>Baxter</td>
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<tr>
<td>Thrombin Calibrator</td>
<td>Diagnostica Stago</td>
</tr>
</tbody>
</table>
3.3 Results

Following collection of the patient’s samples they were prepared as described in section 3.2.3 and frozen at -70°C. The samples were stored for no longer than 4 weeks. Thrombin generation was performed on the samples as described in section 3.2.4 with a measurement time of 60 minutes. Samples were analysed in groups of 15 where possible. All analyses were performed blind. A total of 4 samples were unsuitable for analysis: 3 samples were underfilled and 1 was clotted. As a result, of the 104 patients recruited for the trial thrombin generation data was available on 100 patients (51 controls and 49 cases).

3.3.1 Pregnancy and Thrombin Generation

The first stage of the analysis was to ascertain the affects of pregnancy on thrombin generation. Since pregnancy is known to be associated with a shift towards a pro-thrombotic state (Stirling et al., 1984) (Clark et al., 1998) it was anticipated that the thrombin generation profile of the normotensive pregnant patients in this experiment would reflect this. A comparison of the 51 normotensive pregnant women in this experiment with the normal range described in chapter 2 using the students t-test shows this was indeed the case. The pregnant women showed a statistically significant increase of both ETP and peak height and statistically significant decrease of both the lag time and time to peak (all P values were <0.01) when compared to normal non-pregnant individuals. This reflects the fact that the pregnant women started thrombin production earlier than normal individuals, their maximum rate of thrombin generation was higher and reached more quickly than the normal individuals and their maximum level of thrombin production exceeds that of a normal individual. These results are shown graphically in figures 3.1 and 3.2 below.
Figure 3.1

A. B. C. D.

Figure 3.1. Boxplots showing a comparison between the four thrombin generation parameters of pregnant women and a cohort of normal individuals. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. P values were calculated using the student’s t-test. A: Lag Time, B: ETP, C: Peak Height and D: Time to Peak.
Figure 3.2. Thrombin generation curves showing one normal control and one pregnant woman who are most representative of the average parameters for each of the two groups.

3.3.2 Pre-eclampsia Severity and Thrombin Generation

Since it is clear that the thrombin generation profile of a pregnant woman is markedly different from a non-pregnant individual, no further comparisons were made between the women with pre-eclampsia and the normal range. This was because it would not be possible to ascertain whether any observed difference was due to pre-eclampsia or due to pregnancy. The women with pre-eclampsia in this study were further divided into women with mild, moderate and severe pre-eclampsia depending on their degree of proteinuria and severity of their hypertension as well as the development of further symptoms (section 3.2.2). In order to ascertain the effects of pre-eclampsia on thrombin generation the results of the three groups of pre-eclampsia patients were compared with each other and the normotensive pregnant control group using the student’s t-test. Boxplots showing the results for each of the thrombin generation parameters for each group can be seen in figure 3.3 below. P values for the comparison between the four groups are shown in tables 3.1-3.4 below. A value of $\leq 0.05$ was considered statistically significant.
Figure 3.3

A. B. C. D.

Boxplots showing a comparison between the four thrombin generation parameters of women with pre-eclampsia of varying severity and normotensive pregnant controls. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *.

A: Lag Time, B: ETP, C: Peak Height and D: Time to Peak
Table 3.1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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Table 3.1. Matrix of p values comparing the severity of pre-eclampsia and the lag time. Statistically significant values are shown in red.

Table 3.2

<table>
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<tr>
<td>Severe</td>
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<td>-</td>
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Table 3.2. Matrix of p values comparing the severity of pre-eclampsia and the ETP. Statistically significant values are shown in red.

Table 3.3

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
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<tr>
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<td>-</td>
<td>-</td>
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Table 3.3. Matrix of p values comparing the severity of pre-eclampsia and the peak height. Statistically significant values are shown in red.
Table 3.4

<table>
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<tr>
<td>Severe</td>
<td>-</td>
<td>-</td>
<td>-</td>
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Table 3.4. Matrix of p values comparing the severity of pre-eclampsia and the time to peak. Statistically significant values are shown in red.

These results clearly show that there is a difference between the thrombin generation profile of a normotensive pregnant woman and a woman with pre-eclampsia. All three of the pre-eclampsia groups show a statistically significant elevation in ETP compared to the cohort of normotensive controls with P values ≤0.01. Interestingly the mean ETP seems to increase in line with severity (figure 3.3 B) although the differences between the three pre-eclampsia groups were all non significant. Trend analysis revealed that there was no significant correlation between ETP and disease severity (data not shown). The women with mild and moderate pre-eclampsia also demonstrate an elevated peak height when compared to the normotensive controls with p values of <0.01 and 0.01 respectively. The difference between the peak height of the women with severe pre-eclampsia and the normotensive controls is non-significant. This result is marginal however and with an increased number of patients may have yielded a positive result. From the analysis it can be seen that there are no differences between the three pre-eclampsia groups and the normotensive pregnant controls when comparing either the lag time or the time to peak parameters. It can also be seen from the comparisons that there are no significant differences between any of the three pre-eclamptic groups of patients for any of the four thrombin generation parameters. This would suggest that pre-eclampsia of any severity has the same impact on a patients capacity to generate thrombin. The changes seen in pre-eclamptic women seem to be consistent with an elevated level of thrombin generation and an increased rate of maximum thrombin production with no decrease in the time taken for initiation of thrombin generation or time taken to reach the maximum rate of thrombin production. It is worth noting however this is a comparison with pregnant controls so the pre-eclamptic women demonstrate the same changes as the pregnant women (decreased lag time and time to
peak) when compared with normal individuals with a further enhancement of their ETP and peak height parameters over and above those levels consistent with normal pregnancy.

The subdivision of the pre-eclampsia patients into three groups seemed to be of little benefit as there were no differences between them. It should be noted that the subdivision of the pre-eclamptic patients also resulted in a decrease in the statistical power of the analysis, since the groups were all smaller in number. There were a total of 24 patients with mild pre-eclampsia, 12 patients with moderate pre-eclampsia and 13 patients with severe pre-eclampsia. The small sample size could have led to the non significant difference between the peak height of the severe pre-eclamptic women and the normotensive controls as mentioned above. This is because a small number of aberrant results can have a large impact on any comparison if the sample size is small.

3.3.3 Pre-eclampsia Cases Verses Controls

Since there were no differences between the mild, moderate and severe pre-eclamptic women it was decided to group them into a single cohort. This group was then compared with the normotensive pregnant controls in order to ascertain if the differences observed above still held. Since this comparison was between 49 pre-eclamptic women and 51 normotensive controls the statistical power of the analysis should also be increased. The results of this comparison can be seen in figure 3.4 below. This analysis clearly shows the same results as the above comparison. The pre-eclamptic women (cases) show a statistically significant elevation in both the ETP and peak height parameters when compared to the normotensive pregnant controls (p= 0.0007 and 0.0009 respectively). No difference was observed between the two groups for either the lag time or time to peak parameters. This finding is consistent with elevated thrombin production and increased maximum rate of thrombin generation in pre-eclamptic women compared to normotensive pregnant controls as described above. Average thrombin generation curves for the two groups as well as a comparison with a normal non-pregnant individual are shown in figure 3.5 below.
Figure 3.4

A. 

Boxplots showing a comparison between the four thrombin generation parameters of a cohort of women with pre-eclampsia and a cohort of normotensive pregnant controls. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. P values were calculated using the student’s t-test. A: Lag Time, B: ETP, C: Peak Height and D: Time to Peak.
3.3.4 Confounding Factors

The preliminary analysis seemed to show an apparent difference between the thrombin generation profile of pregnant women with and without pre-eclampsia. The next stage of the analysis was to examine the effects that any confounding variables may have had on the results of this analysis. In order to minimise the effects of confounding variables the cases and controls were matched for a number of parameters including age, parity, ethnicity and gestational age. A comparison of the two groups is shown in table 3.5 below.

From the table it appears that the two groups are very closely matched for both age and parity and hence these parameters are not likely to influence the results of the above comparison. Indeed age was shown to have no effect when considering thrombin generation in chapter 2. Both the gestational age and ethnicity were matched less closely between the two cohorts.
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<tr>
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<td>(0 – 6)</td>
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</tr>
<tr>
<td>Range</td>
<td>(9 – 42)</td>
<td>(23 – 40)</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>62</td>
<td>38</td>
</tr>
<tr>
<td>Asian</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>Black</td>
<td>44</td>
<td>56</td>
</tr>
</tbody>
</table>

Table 3.5. A comparison of the potential confounding factors between the cohort of pre-eclampsia cases and the normotensive pregnant controls.

3.3.4.1 Gestational Age

The median gestational ages of the two cohorts match relatively closely but the mean gestational ages are 32.8 and 36.1 for the controls and cases respectively. This is because the control group contains a number of individuals with a gestational age of below 20 weeks. This is not possible in the pre-eclampsia cohort since the diagnosis of pre-eclampsia relies on new onset proteinuria and hypertension after 20 weeks gestation. It is therefore possible to infer that the reason for the observed difference between the ETP and the peak height of the control and case groups is because of differences in gestational age and not due to pre-eclampsia. In order to disprove this hypothesis all of the data was grouped together and re-analysed to show the effects of gestational age on thrombin generation. A clear trend showing an increase in thrombin generation throughout pregnancy would be indicative of the differences between the two groups being due to gestational age and not pre-eclampsia. The results of this analysis are shown in figure 3.6 below.
It is clear from this analysis that there is little evidence for either increasing ETP or peak height with increasing gestational age. Using the value of $R^2$ to calculate a value for $p$ as described in section 2.3.1.4 shows that both trends are non-significant with a $p$ value of $>0.5$ for ETP and a $p$ value of 0.1-0.5 for the peak height. The lack of any significant trend throughout pregnancy effectively eliminates gestational age as a confounding factor in the above analysis. Indeed if the data is again split into cases and controls and the same analysis is performed the result still holds true. See figure 3.7 below.

**Figure 3.7**

![Graphs showing the impact of gestational age on thrombin generation in a cohort of pregnant women with pre-eclampsia (cases) and a cohort of normotensive pregnant women (controls), complete with trend lines and $R^2$ values as an indication of any correlations. A: ETP and B: Peak Height](image)

Calculation of $p$ values as described above reveals that all of the trends shown in figure 3.7 are non-significant, with $p$ values of 0.1-0.5. Indeed if anything figure 3.7 shows a decreasing trend of both ETP and peak height throughout pregnancy in both the cases.
and controls. This would imply the actual differences between the cases and controls may have been even more pronounced if the two cohorts had been more closely matched for gestational age.

3.3.4.2 Ethnicity

The ethnicity data presented in table 3.5 shows the distribution of the three ethnic groups involved in this study between the cases and controls. An ideal scenario would be to have 50% of the total number of each ethnic group in both the case and control cohorts. Table 3.5 shows that this is not the case, all three ethnic groups show some level of disparity. The biggest difference is seen between the Caucasian patients with 62% in the control cohort and 38% in the cohort of women with pre-eclampsia. In absolute terms this is only a difference of 8 patients between the two groups however. The actual numbers of patients in each group is shown in table 3.6 below.

Table 3.6

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Control</th>
<th>Case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Asian</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Black</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3.6. A table showing the absolute number of patients of each ethnic group in the two cohorts.

In order to establish what effect, if any, ethnicity had on the obtained results it was decided to compare the case and control data of the three ethnic groups separately. If there was no observable difference between them, this would imply that ethnicity had no impact on the trends described above. The results of this comparison for the endogenous thrombin potential are shown in figure 3.8 and tables 3.7-3.8 below. The results for the comparison of peak height are shown in figure 3.9 and tables 3.9-3.10 below.
Figure 3.8

A. Boxplots showing the ETP for Caucasian, Black and Asian women in a cohort of women with pre-eclampsia (A) and in a cohort of normotensive pregnant women (B). Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by ⋆.

B.

Table 3.7

<table>
<thead>
<tr>
<th>ETP: Cases</th>
<th>Caucasian</th>
<th>Black</th>
<th>Asian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>N/A</td>
<td>0.39</td>
<td>0.99</td>
</tr>
<tr>
<td>Black</td>
<td>-</td>
<td>N/A</td>
<td>0.28</td>
</tr>
<tr>
<td>Asian</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.7. A matrix of p values comparing the ETP and the ethnicity of a group of pregnant women with pre-eclampsia. Significant values are shown in red.

Table 3.8

<table>
<thead>
<tr>
<th>ETP: Controls</th>
<th>Caucasian</th>
<th>Black</th>
<th>Asian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>N/A</td>
<td>0.005</td>
<td>0.49</td>
</tr>
<tr>
<td>Black</td>
<td>-</td>
<td>N/A</td>
<td>0.008</td>
</tr>
<tr>
<td>Asian</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.8. A matrix of p values comparing the ETP and the ethnicity of a group of normotensive pregnant women. Significant values are shown in red.
Figure 3.9
A.

Figure 5.9. Boxplots showing the peak height for Caucasian, Black and Asian women in a cohort of women with pre-eclampsia (A) and in a cohort of normotensive pregnant women (B). Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *.

Table 3.9

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Peak Height: Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caucasian</td>
</tr>
<tr>
<td>Caucasian</td>
<td>N/A</td>
</tr>
<tr>
<td>Black</td>
<td>-</td>
</tr>
<tr>
<td>Asian</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.9. A matrix of p values comparing the peak height and the ethnicity of a group of women with pre-eclampsia. Significant values are shown in red.

Table 3.10

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Peak Height: Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caucasian</td>
</tr>
<tr>
<td>Caucasian</td>
<td>N/A</td>
</tr>
<tr>
<td>Black</td>
<td>-</td>
</tr>
<tr>
<td>Asian</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.10. A matrix of p values comparing the peak height and the ethnicity of a group of normotensive pregnant women. Significant values are shown in red.
These analyses showed that there were no differences between any of the ethnic groups for either the ETP or peak height when considering the cohort of women with pre-eclampsia. This however was not the case when examining the results for the cohort of normotensive women. Whilst there were no observed differences between the Caucasian and Asian women in either their ETP or peak height results, the Black women showed a significantly higher ETP than both the Asian (P=0.008) and Caucasian (P=0.005) groups. The Black women in the control cohort also showed a significantly higher peak height when compared to the Asian sub group (P=0.002) and a difference approaching statistical significance when compared to the Caucasian group. There were no significant differences found between any of the ethnic groups in either cohort for either the lag time or time to peak parameters. These findings indicate that normotensive pregnant Black women have a level of thrombin generation which is significantly elevated when compared to normotensive pregnant Caucasian or Asian women.

To further investigate this finding the case and control data for each ethnic group was then compared directly to ascertain whether or not the findings of the preliminary analysis still held i.e. there is a difference between the ETP and peak height parameters in women with pre-eclampsia when compared with normotensive pregnant women. The results of this analysis are shown in figure 3.10 below.

Figure 3.10
A. B.
Figure 3.10. A series of boxplots showing a comparison between the ETP and peak height for both cases and controls for each of the three different ethnic groups in the study. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by * . P values derived via student’s t-test. A and B: Black women, C and D: Caucasian women, E and F: Asian women.

This data shows that whilst the Caucasian and Asian women demonstrate a clear difference between the controls and cases for both the ETP and peak height parameters, the same trend is not present in the Black women, who show strikingly similar results in both the control and case groups. This is a highly interesting result as the implications are potentially quite large. What the data appears to show is that while Asian and Caucasian women show abnormal thrombin generation patterns when they develop pre-eclampsia, Black women do not. What is most interesting is that the apparent lack of
difference between the Black women with and without pre-eclampsia does not manifest in the way one might expect (i.e. a failure of the pre-eclamptics to demonstrate elevated thrombin generation parameters) but rather the opposite way around. The normotensive black women demonstrate a level of thrombin generation which is equal to the pathological level seen in the Asian and Caucasian women with pre-eclampsia. The implications for this unexpected result in terms of a confounding factor for this analysis would probably be to reduce the size of the observed difference between the cases and controls. This is because the control values of the Black women would skew the mean and standard deviation of the control group (making them higher) and ultimately increasing the p value of the comparisons. The actual difference however is likely to be small given the size of the cohort of Black women in the study is only 13 of the 100 patients analysed. The small number of Black women analysed obviously increases the likelihood of obtaining a non-significant difference between the cases and controls by chance. This is because one or two aberrant values can have a large impact on the analysis. In some ways this is true of the control group of Black women, which contains the woman with the highest value of ETP in this entire study. Excluding this individual does not eliminate all the observed differences between the groups however (data not shown). Since both the Asian and Caucasian groups show the same pattern as each other and as the preliminary analysis when considering the differences between the cases and controls it can be assumed that the imbalance between the number of Caucasian and to a lesser extent Asian women between the control and case cohorts did not directly impact on the findings of the study. As mentioned above, the impact of the Black women is most likely to reduce the statistical significance of the study findings. Since this impact is likely to be small, based on the sample size of the Black cohort, the Black women were not excluded from further analysis.

3.3.5 Prediction of Pre-eclampsia

As stated the primary aims of this chapter were three fold, the first aim was to investigate the thrombin generation profile of women with pre-eclampsia, the second was to establish whether or not any observed differences in the thrombin generation profile of women with pre-eclampsia could be used in order to potentially predict which patients would develop pre-eclampsia. The third aim was to try and correlate the
thrombin generation data with some end points of pregnancy associated with pre-eclampsia

It is clear from the above analysis that there is a significant difference between the thrombin generation profiles of women with pre-eclampsia and normotensive pregnant women. The next stage of this study was to ascertain whether the observed differences were of sufficient magnitude that they could be potentially used as a prospective screening test for pre-eclampsia in the future. In an ideal scenario the magnitude of the difference between the ETP and peak height of women with pre-eclampsia and normotensive pregnant women would be so great that there would be no overlap between the two populations. An upper limit of normal could then be established for normotensive women and any women presenting with thrombin generation parameters over that limit could be flagged as at risk of pre-eclampsia. As mentioned above, any measure of thrombin generation could be considered in conjunction with the PRECOG score to potentially increase its diagnostic power.

Before any analysis was undertaken to establish how good the thrombin generation assay was at discriminating between normotensive women and pre-eclampsia patients, the data for the PRECOG score was analysed. This would establish how effective the PRECOG screen was at detecting the women at risk of pre-eclampsia as a stand-alone assay. Before the women were enrolled on the study each of them was assessed for risk using the PRECOG scoring system described in section 3.1.6. This system is designed to offer early referral for specialist care to those women who are at risk of pre-eclampsia. Often such specialist referral includes tests such as uterine Doppler investigation to detect the changes (or lack thereof) in the uterine spiral arteries which are indicative of pre-eclampsia. Such assays are both time consuming and expensive to the NHS and as a result it is important the right patients are referred for screening. Having been assessed using the PRECOG scoring system each woman in the study was flagged as either high or low risk of developing pre-eclampsia. Since the diagnosis of each individual was known it was possible to retrospectively analyse the data to establish how good the PRECOG assessment was at detecting the women who went on to develop pre-eclampsia. In order to ascertain the effectiveness of the PRECOG score the sensitivity, specificity, positive predictive value and negative predictive value were established as shown in table 3.11 below. The definitions of these terms are as follows:
Sensitivity: The proportion of ‘positive’ patients (pre-eclampsia cases) correctly identified as being positive (high risk)

Specificity: The proportion of ‘negative’ patients (normotensive controls) correctly identified as being negative (low risk)

Positive predictive value: The proportion of high risk individuals who are correctly diagnosed (pre-eclampsia cases)

Negative predictive value: The proportion of low risk individuals who are correctly diagnosed (normotensive controls)

Table 3.11

<table>
<thead>
<tr>
<th>Prediction</th>
<th>Diagnosis</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Risk</td>
<td>True Positive</td>
<td>TP/(TP+FP)</td>
<td>TN/(TN+FN)</td>
</tr>
<tr>
<td></td>
<td>False Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Risk</td>
<td>False Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>True Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.11. A table showing the calculation of the sensitivity, specificity, positive predictive value and negative predictive value. Derived answers were reported as percentages by multiplying by 100.

Data were available for all 104 women recruited to the trial for both the PRECOG score and the diagnosis. The analysis of the predictive power of the PRECOG score provided surprisingly low values for both sensitivity and specificity, 58% and 69% respectively, with similar values for both the positive predictive value (65%) and negative predictive value (62%). Whilst it is common for screening tests to often sacrifice specificity in order to enhance their sensitivity or vice versa, this analysis suggests that the PRECOG score is neither very effective as detecting the high risk patients or excluding those who
are low risk. In absolute terms, from the cohort of women in this study 16 (35% of those scored as high risk) were falsely scored as high risk and would therefore go on receive unnecessary specialist referral, whereas 22 (38% of those scored as low risk) of those women flagged as low risk would receive no early specialist care even though they would subsequently develop pre-eclampsia. This shows the PRECOG score is not an effective stand alone screening test as it can neither successfully predict which women will develop disease nor detect those who are not at risk of developing disease. In real terms this means the NHS will suffer from elevated costs due to unnecessary referrals and a number of patients will fail to receive the care they actually need.

In order to ascertain whether the measurement of thrombin generation was any more effective than the PRECOG score at discriminating between normotensive and pre-eclamptic women, the women in the trial were flagged as either high or low risk depending on their thrombin generation parameters and this risk assessment was compared to their actual diagnosis as described for the PRECOG assessment above. Before this could be done however it was necessary to determine a cut off for the ETP and/or peak height over which an individual would be classified high risk. As stipulated above, an ideal scenario would be little or no degree of overlap between the thrombin generation parameters of the case and control cohorts. Examining the results as shown in figures 3.4 B and C, this does not appear to be the case, despite the highly significant p values of the comparisons, since there is a relatively large degree of overlap between the two populations. In order to better assess the overlap of the results between the two populations, the obtained results for ETP and peak height from each cohort were plotted as frequency charts on the same axis. These graphs are shown in figure 3.11 below. It is clear from these graphs that although the data appears roughly normal in distribution, there is no clear delineation point between the cases and controls for either parameter. Selecting an appropriate cut off in order to establish a patient as either high or low risk was therefore problematical.

Selecting cut off values for high and low risk using the mean ETP of either the control cohort +1 SD or case cohorts -1SD respectively yielded results which were not compelling. The selection of these cut-off values immediately poses a problem since any population which is normally distributed would by definition have ~16% of values above/below 1 standard deviation from the mean. Hence these cut-off values would
result in approximately one sixth of the populations being incorrectly assigned as high/low risk.

Figure 3.11

A. 

![Distribution of Case and Control Results: ETP](image)

B. 

![Distribution of Case and Control Results: Peak Height](image)

Figure 3.11. Graphs showing the distribution of the ETP (A) and peak height (B) results for both the case and control cohorts.
Choosing more extreme cut off values for characterising patients as high and low risk such as high risk being defined as values of ETP exceeding the mean ETP of the control cohort +2 SD or low risk being defined as values of ETP below the mean ETP of the case cohort -2SD improved the specificity and sensitivity of the individual screens respectively dramatically to values exceeding 90%. These improvements were however at the expense of the sensitivity and specificity respectively. This was as a result of classifying the vast majority of patients as either low risk or high risk (depending on the cut off used). The negative and positive predictive values for these cut off values were ~50% showing that the use of such cut off values were highly impractical since nearly half of the women in this study would have been falsely identified as low or high risk respectively using the cut off values described above. In NHS terms this would result in either specialist referral of only a handful of women who needed further treatment or the referral of virtually every woman screened.

A number of potential different cut-off values were examined using either the ETP, the peak height or both parameters in combination in an attempt to optimise the use of thrombin generation as a screening test for pre-eclampsia. The cut off values which resulted in the greatest number of correctly categorised patients (64 out of 100 patients) of any of the cut-off values or combinations thereof used was the categorisation of patients as high risk if they had an ETP and peak height exceeding the mean of the case cohort minus one standard deviation (ETP >2263nM Thrombin and a peak height of >445nM Thrombin). Patients were categorised as low risk if only one parameter was elevated or if both parameters were below the high risk cut-off. This resulted in a specificity of 43% and a sensitivity of 86%. The positive predictive value was 59% and the negative predictive value was 76%.

It is clear from the above analyses that the use of thrombin generation as a stand-alone screening test for the detection of pre-eclampsia is flawed. This is primarily due to the high overlap of the thrombin generation parameters between the case and control cohorts, which ultimately makes the selection of an appropriate cut-off difficult. The next stage of analysis was therefore to use the thrombin generation parameters in conjunction with the PRECOG score in order to try and improve its predictive power. In order to combine the tests it was necessary to first consider how the women would be classified as high risk and which high risk cut-off value for the thrombin generation
parameters would be used in the analysis. Since neither the PRECOG score nor the thrombin generation results could be relied upon to classify women as high/low risk independently it was decided to only classify a woman as high risk when both the thrombin generation parameters were elevated and the PRECOG score also suggested they were high risk. If only one of the screening tests suggested the women were high risk they were classified as low risk. The thrombin generation cut-off used in this analysis to classify an individual as potentially high risk, was the one which had produced the most promising results so far, namely an ETP and peak height exceeding one standard deviation below the mean ETP and peak height of the case cohort.

The results of the predictive power of this combination of screening tests did not initially appear positive. The sensitivity and specificity were 37% and 75% respectively with a positive predictive value of 58% and a negative predictive value of 55%. The real power of this combination of tests however lies in those individuals who had both low risk thrombin generation parameters (ETP $\leq$ 2263nM Thrombin and a peak height of $\leq$ 445nM Thrombin) and a low risk result via the PRECOG risk assessment. The negative predictive value of these individuals was 94%. This result was of great interest since it meant that in combination thrombin generation analysis and the PRECOG score could be used to successfully detect a subgroup of women in whom pre-eclampsia was highly unlikely to occur. When considering this subgroup of women the negative predictive value of the two assays in combination exceeds that of either assay in isolation, (62% and 76% for PRECOG and thrombin generation respectively).

Whilst this finding was encouraging it would have been better if the positive predictive value or sensitivity was also elevated when the screening tests were combined, as this would increase the clinical utility of the screen. One of the primary reasons for the poor sensitivity of the combined screen was the high number of false negative patients. It was felt that classifying women as high risk only when they were designated high risk by both screening tests was excluding too many positives. Classifying women as high risk when either screen was positive was likely to make the assay too sensitive however. As a compromise it was decided to give one screening test more weight in the classification of risk than the other. In the following analysis an individuals risk was decided using the rules below:
Women with high risk PRECOG score and elevated thrombin generation parameters (ETP >2263nM Thrombin and a peak height of >445nM Thrombin) = HIGH RISK
Women with high risk PRECOG score and low risk thrombin generation parameters (ETP ≤2263nM Thrombin and a peak height of ≤445nM Thrombin) = HIGH RISK
Women with low risk PRECOG score and elevated thrombin generation parameters (ETP >2263nM Thrombin and a peak height of >445nM Thrombin) = LOW RISK
Women with low risk PRECOG score and low risk thrombin generation parameters (ETP ≤2263nM Thrombin and a peak height of ≤445nM Thrombin) = LOW RISK

This analysis seemed to provide no additional benefit over the original screen, although the ‘double negatives’ retained their screening power, the sensitivity and specificity results for the screen as a whole were poor (49% and 61% respectively). The positive and negative predictive values were 55%.

Subsequent to this analysis the process was repeated again, this time giving more weight to the thrombin generation results rather than the PRECOG score, so patients with a low risk PRECOG score and elevated thrombin generation parameters were categorised as high risk and patients with a high risk PRECOG score and low risk thrombin generation parameters were categorised as low risk. Again this analysis conserved the predictive power of the low risk patients but also reduced the number of false negative patients, increasing the sensitivity to 86%. The specificity (43%) was decreased when compared to the original combination analysis however. The positive and negative predictive values of this analysis were 59% and 76%. On the whole this combination of tests was the most clinically effective described to this point. This is due to the fact that it is able to detect the majority of cases in a population and therefore allows effective referral and treatment but also can effectively exclude a subgroup of patients from referral testing. This is not true of some of the other methods examined which demonstrate improved sensitivity when compared to this screen.

If this screen is modified so that women are classified as high risk if either the PRECOG score or thrombin generation parameters are high risk, the sensitivity is increased from 86% to 98%, but the specificity is reduced to 29%. The positive predictive value is similar at 57% and the negative predictive value rises to 94% (since the only negative patients are those who are low risk via both screening tests).
These predictive algorithms are not without flaws however, in that there are a relatively large number of false positives. In order to combat this, it was decided to investigate the possibility of introducing a more effective 'double positive' screening result i.e. looking for a higher cut-off above which the positive predictive value would be enhanced. To investigate the feasibility of this a separate comparison was performed using a combination of the PRECOG score and a thrombin generation cut-off of greater than one standard deviation above the mean of the control cohort. This cut off was chosen since it had subsequently shown an elevated positive predictive value when compared to the other cut-off values (72%). In this analysis a patient was categorised as high risk only if they had a high risk PRECOG assessment and high risk thrombin generation parameters (ETP >2920 nM Thrombin and peak height >519 nM Thrombin). This combination of cut-off values with the PRECOG score did not enhance the positive predictive value however. Indeed the positive predictive value fell to 60%, this comparison highlights that although low risk thrombin generation and PRECOG scores might be used to exclude pre-eclampsia, these tests cannot be used in unity to predict pre-eclampsia with the same degree of success.

In a final attempt to choose a cut-off for the thrombin generation parameters which would enhance the predictive value of thrombin generation in the detection of pre-eclampsia it was decided to use a cut-off value for the ETP that was mid way between the two values employed most successfully so far i.e. between one standard deviation below the mean of the case cohort (2263 nM Thrombin) and one standard deviation above the mean of the control cohort (2920 nM Thrombin). The mid way point between the two values is 2591 nM Thrombin. Classifying patients with ETP values >2591 nM Thrombin as high risk yielded sensitivity and specificity values of 71% and 61% respectively and positive and negative predictive values of 64% and 69% respectively. In retrospect these values could have been predicted, since the cut-off is mid range the sensitivity and specificity values fall between those of the higher and lower cut-off values. The use of the lower cut-off increases the sensitivity, whereas the use of the high cut-off increases the specificity. The use of a mid range cut-off for the peak height (482 nM Thrombin) in conjunction with the mid range cut-off for ETP, with high risk patients defined as those who show both elevated ETP and peak height, does little to improve the predictive values with a sensitivity of 59% and a specificity of 71%.
Interestingly when the mid range ETP cut-off is used in conjunction with the PRECOG score, with high risk individuals defined as those with either a high risk PRECOG score, elevated ETP (>2591 nm thrombin) or both, the predictive values of the screen are very similar to those of the modified combination of thrombin generation (low cut-off) and PRECOG described above. The sensitivity and specificity values were 84% and 43% respectively. A major disadvantage of this screen however is that the ‘double negative’ result using the mid range cut-off has a lower negative predictive value of 73% when compared to the 94% of the lower cut-off (1SD below the mean case value).

Although the methods employed above demonstrate that the measurement of thrombin generation could be potentially used as a predictor of pre-eclampsia or used to effectively characterise a subgroup of women who will not develop pre-eclampsia when used in conjunction with the PRECOG score, the selection of appropriate cut off values proved problematical due to the high degree of overlap between the case and control cohorts. To further characterise the predictive value of the measurement of thrombin generation in pre-eclampsia it was decided to plot receiver operating characteristic (ROC) curves of both ETP and peak height in relation to disease state (case or control). A ROC curve is effectively a graphical plot of the sensitivity (true positives), against 1 – specificity (false positives). The area under the curve is used to determine the effectiveness of a parameter as a predictive characteristic. A value of 1 is indicative of a perfect screening test whereas values approaching 0.5 indicate poor predictive parameters. The ROC curves for both ETP and peak height are shown in figure 3.12 below.

Figure 3.12 shows that both ETP and peak height have very similar areas under the curve of ~0.7, indicating that the two parameters have very similar predictive values in determining whether a patient has pre-eclampsia or not. A value of 0.7 suggests that if a random individual was selected from both the case and control cohorts the individual from the case cohort would have a higher ETP/peak height in 70% of cases. This finding is supportive of the analysis above, that the measurement of thrombin generation is potentially of moderate predictive value in pre-eclampsia.
Figure 3.12

A.

![ROC Curve: ETP and Pre-eclampsia](image1)

Area under curve: 0.702

B.

![ROC Curve: Peak Height and Pre-eclampsia](image2)

Area under curve: 0.691

Figure 3.12. ROC curves of ETP (A) and Peak Height (B) against disease status. The blue line indicates the ROC curve of the parameter. The green line indicates an area under the curve of 0.5. Area under the curve is shown as an indicator of the predictive value of the parameter.
Further to the analysis discussed in this project, samples were taken from all the subjects enrolled on this study for analysis by flow cytometry. The parameters investigated included, platelets expressing P-selectin, P-selectin expressing microparticles, platelet-monocyte aggregates and platelet-neutrophil aggregates. In comparison to the normotensive pregnant women the women with pre-eclampsia demonstrated significantly elevated levels of P-selectin expressing platelets and platelet microparticles as well as platelet-monocyte aggregates (p = <0.05) (Macey et al., 2010a) (Paper in the appendix, section 7.2). These parameters were then used in conjunction with the ETP to try and distinguish between the cases and the controls. Women were classified as high risk if they had an elevated level of one or more of the selected parameters. The cut-off for high risk patients was defined as greater than two standard deviations above the mean of a cohort of normal non-pregnant controls. The ETP value used to define high risk in this study was >2579 nM Thrombin, which is roughly analogous to the mid range cut-off employed above. The sensitivity and specificity values for this analysis were 83% and 80% respectively. The positive and negative predictive values were 81% and 82% respectively.

3.3.6 Thrombin Generation and Pregnancy Endpoints

It is clear from the above analyses that both the peak height and ETP are elevated in a cohort of women with pre-eclampsia when compared to a cohort of normotensive pregnant women and that these parameters can potentially be used as predictive indicators in pre-eclampsia, however this study was a retrospective analysis. To further elucidate the clinical utility of the CAT thrombin generation assay it was decided to use the thrombin generation data obtained in this investigation in a small number of prospective studies. The parameters measured in these studies were either associated with pre-eclampsia or linked to a patient’s capacity to generate thrombin. Importantly they were all events which were recorded following the measurement of the patients’ thrombin generation capacity. These parameters were birth weight, length of stay in hospital, mode of delivery and post partum haemorrhage (PPH)
3.3.6.1 Thrombin Generation and Birth Weight

The birth weights of the control cohort were significantly higher than those of the case cohort (Macey et al., 2010a), this was not an unexpected finding since patients with pre-eclampsia often have pre-term deliveries in order to prevent the progression of pre-eclampsia to eclampsia and as a result the babies of pre-eclamptic women have lower birth weights. The data in this study is supportive of this theory. The birth weights of the babies from the women enrolled on this study were correlated with both the ETP and peak height. Data was available for 42 of the pre-eclamptic women and 44 of the normotensive pregnant women for this analysis. The results of this correlation are shown in figure 3.13 below.

Figure 3.13
A.  
B.  
C.  
D.  

Figure 3.13. A series of graphs showing the correlation between birth weight and ETP in the control (A) and case (B) cohorts and the correlation between birth weight and peak height in the control (C) and case (D) cohorts. Trend lines and $R^2$ are shown as an indication of correlation.

Since the ETP and peak heights were significantly elevated in the case cohort in comparison to the normotensive control cohort and the birth weights were significantly
lower in the case cohort when compared to the controls, it was hypothesised that there would be a significant correlation between the two parameters. This was especially true of the ETP which seems to show a stepwise increase with disease severity in figure 3.3B (although this is not statistically significant) and it was felt that those patients with the most severe disease would have the infants with the lowest birth weight. Figure 3.13 however does not support this hypothesis for any of the correlations shown. All the correlations were non-significant (p= >0.05). Figure 3.13 B, C and D show that the modest correlations present were in accord with the hypothesis as indicated by the trend lines (decreasing birth weight with increasing ETP/peak height). To increase the statistical power of the analysis the case and control cohorts were combined into a single group and the correlations performed again. The results of this analysis are shown in figure 3.14.

Figure 3.14
A.  

Figure 3.14. Graphs showing the correlation between birth weight and ETP (A) and peak height (B) in a cohort of pregnant women. Trend lines and R² are shown as an indication of correlation.

Although the correlations initially do not appear enhanced when comparing the values of R² in figures 3.14 A and B with those in figure 3.13, the p values are significant (p=<0.05) for both ETP and peak height. This is probably as a result of the initial correlations having insufficient numbers of data points to detect what is a weak statistical trend (R values of 0.26 and 0.27 for ETP and peak height respectively). In conclusion there is a weak association between decreasing birth weight and both an increasing ETP and peak height.
3.3.6.2 Thrombin Generation and Length of Stay in Hospital

The association between thrombin generation parameters and the length of stay in hospital were also investigated for similar reasons to those outlined above. Women with the most severe cases of pre-eclampsia may be expected to be the ones who had the longest stays in hospital at the time of delivery. Data for this comparison was available for 46 of the women in the control cohort and 47 of those in the case cohort. A comparison of the length of stay in hospital can be seen in figure 3.15 below. From the figure it may be seen that the women in the case cohort had a longer stay in hospital at delivery when compared to the women in the control cohort. When comparing the two populations using a Mann Whitney U test this is proved to be statistically significant (p=<0.001).

Figure 3.15

Figure 3.15. Boxplots showing the length of time spent in hospital at the time of delivery in a cohort of normotensive pregnant women (Controls) and a cohort of women with pre-eclampsia (cases). Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. P value derived using the Mann Whitney U test.
Correlations of ETP/peak height with the length of stay in hospital for the case and control groups respectively are shown in figure 3.16.

Figure 3.16

A.  

B.  

C.  

D.  

Figure 3.16. A series of graphs showing the correlation between length of stay in hospital at delivery and ETP in the control (A) and case (B) cohorts and the correlation between length of stay in hospital at delivery and peak height in the control (C) and case (D) cohorts. Trend lines and $R^2$ are shown as an indication of correlation.

Figure 3.16 shows that the length of stay in hospital does correlate with the measurement of ETP but not with the peak height. There was a significant p value of <0.02 for the correlations of length of stay with ETP in both the case and control cohorts and non significant p values of between 0.1 and 0.5 for the two peak height correlations. It appears from figure 3.16 B that the statistically significant correlation between the length of stay and the ETP in the case cohort is largely due to the outlying data point at 24 days. Excluding this point from the analysis causes the $R^2$ value to decrease to 0.06 which in turn increases the p value of the correlation to between 0.05 and 0.1. The case and control cohorts were then combined to increase the statistical power of the correlations. The results of this analysis are shown in figure 3.17.
Figure 3.3.6.3 Thrombin Generation and Mode of Delivery

In this section it was decided to examine whether or not the measurement of thrombin generation could be used to potentially predict the mode of delivery in the cohort of women analysed in this study. Data were available on the mode of delivery for 85 of the women enrolled on the study, 43 in the control cohort and 42 in the case cohort. To simplify the analysis it was decided to try to ascertain whether or not the measurement
of either the ETP or peak height could be used to predict whether or not the women in the study would have a lower segment caesarean section (LSCS) instead of a standard vaginal delivery. Women who had other methods of delivery were excluded from the analysis; this included 2 women who had forceps deliveries and one woman who had a ventouse delivery. Data was available on 41 women in each of the control and case cohorts. The incidence of LSCS in the two cohorts was very similar (13/41 in the control cohort and 16/41 in the case cohort). It was anticipated that the incidence of LSCS would be higher in the pre-eclamptic women since premature delivery is often necessary in these women to prevent progression to eclampsia. Figure 3.18 shows ROC curves of ETP/peak height predicting the incidence of LSCS in the control and case cohorts respectively.

From figure 3.18 it can be seen that the ETP and peak height are poor indicators of the mode of delivery in this cohort of women, this is especially true in the case cohort as the area under the curve is 0.502 for both parameters. The ETP and peak height have slightly more predictive value in the control cohort with areas of 0.629 and 0.566 for the ETP and peak height parameters respectively, although these values are far from compelling. Combining the two cohorts and re-plotting the curves does not enhance the predictive value with areas of 0.571 and 0.554 for the ETP and peak height respectively. This analysis is shown in figure 3.19 and is consistent with the fact that the measurement of thrombin generation cannot be used to accurately determine the mode of delivery in this cohort of women.
Figure 3.18

A series of ROC curves showing the predictive value of the ETP in determining whether or not women have LSCS deliveries in the control (A) and case (B) cohorts and the predictive value of the peak height in determining whether or not women have LSCS deliveries in the control (C) and case (D) cohorts. The blue line indicates the ROC curve of the parameter. The green line indicates an area under the curve of 0.5. Area under the curve (AUC) is shown as an indicator of the predictive value of the parameter.
3.3.6.4 Thrombin Generation and Post Partum Haemorrhage

This section focuses on the use of the measurement of the ETP and the peak height to predict whether or not women are at risk of PPH. In order to establish the degree of blood loss during delivery the change in haemoglobin of each of the women was calculated by subtracting the haemoglobin measurement pre delivery from that obtained post delivery. Women who had a loss of ≥2.0 g/dL haemoglobin during delivery were assumed to have had a PPH. Those women who did not have their haemoglobin measured post delivery were assumed not to have had a PPH (Definitions devised following discussion with an obstetric consultant at The Royal London Hospital).

Although PPH is not associated with pre-eclampsia it was thought that there may be an association between an individual’s capacity to generate thrombin and their risk of bleeding at delivery. It was thought that the individuals with the highest ETP and peak height parameters would be those with the lowest risk of having a PPH. If this hypothesis was correct it would be reasonable to assume that the incidence of PPH would be reduced in the case cohort when compared to the controls since women with pre-eclampsia have a higher ETP and peak height when compared to normotensive
pregnant women as discussed above. Interestingly this does not appear to be the case; data were available on 46 women in the control cohort and 47 women in the case cohort and of these 10 in the control cohort and 9 in the case cohort were classified as having had a PPH using the definition above. Figure 3.20 shows ROC curves of ETP/peak height predicting the incidence of PPH in the control and case cohorts respectively.

Figure 3.20

A. B. C. D.

Figure 3.20. A series of ROC curves showing the predictive value of the ETP in determining whether or not women have a PPH in the control (A) and case (B) cohorts and the predictive value of the peak height in determining whether or not women have a PPH in the control (C) and case (D) cohorts. The blue line indicates the ROC curve of the parameter. The green line indicates an area under the curve of 0.5. Area under the curve (AUC) is shown as an indicator of the predictive value of the parameter.
Figure 3.20 clearly shows that the ROC curves for the case and control cohorts appear contradictory. The curves for the control cohort show a modest predictive value in using ETP and peak height in the prediction of PPH (AUC values are 0.567 for both the ETP and peak height). This association is contrary to the above hypothesis in which higher ETP and peak height values are protective against PPH and implies a relationship between increasing ETP and peak height and increasing risk of PPH. The AUC values for the case cohort are in support of the initial hypothesis (values below 0.5 are indicative of an association in opposition to that entered into the analysis program) indeed the attained values are equivalent to AUC values of 0.55 (1-0.45) and 0.529 (1-0.471) for increasing values of ETP and peak height being associated with not having a PPH. These findings and the relatively low AUC values suggest that the measurement of thrombin generation is not a good predictive parameter for the detection of PPH in this cohort of women. Combining the two cohorts into a single group and recalculating the curves yields unsurprising results, as the two contradictory results effectively cancel each other out, yielding AUC values close to 0.5 as shown in figure 3.21 below. In conclusion the measurement of thrombin generation is a poor predictor of the incidence of PPH in this cohort of women.

Figure 3.21
A.  
B.  

Figure 3.21. ROC curves showing the predictive value of the ETP (A) and peak height (B) in determining whether or not women have a PPH in a cohort of pregnant women. The blue line indicates the ROC curve of the parameter. The green line indicates an area under the curve of 0.5. Area under the curve (AUC) is shown as an indicator of the predictive value of the parameter.
To ascertain whether there was any association between the measurement of thrombin generation and the degree of blood loss at delivery, the change in haemoglobin following delivery was correlated with both the ETP and peak height. All of the correlations yielded non significant p values regardless of whether the case and control groups were analysed separately or combined into a single cohort. Data not shown.

3.4 Discussion

The primary aims of this chapter were to investigate the effects of pre-eclampsia on thrombin generation and attempt to utilise any observed differences to devise a predictive screen for pre-eclampsia. The measurement of thrombin generation was also correlated with several end points of pregnancy in order to better assess its clinical utility in a prospective study.

The initial investigation of the normotensive pregnant women yielded the anticipated results, in that the thrombin generation profile of the cohort of normotensive pregnant women was profoundly altered when compared to the normal range described in chapter 2. The normotensive pregnant women demonstrated a significantly elevated ETP and peak height and a significantly reduced lag time and time to peak when compared to non-pregnant individuals. This result is consistent with a shift towards a pro-thrombotic state during pregnancy, as characterised by an increase in several clotting factors throughout pregnancy and a decrease in the levels of or sensitivity towards some of the negative regulators of haemostasis (Stirling et al., 1984), (Clark et al., 1998). Although this result was unsurprising, elevated levels of ETP were not described as characteristic of pregnancy by Eichinger et al. (1999).

The next stage of the analysis provided compelling evidence for further pro-thrombotic changes in women with pre-eclampsia when compared to the cohort of normotensive pregnant women. The thrombin generation profile of women with pre-eclampsia of varying severity showed a statistically significant increase in both ETP and peak height when compared to the cohort of normotensive pregnant women. Whist the changes in the ETP were conserved regardless of the severity of a patients pre-eclampsia the observed differences for peak height were only seen between the normotensive controls and the women with either mild or moderate the pre-eclampsia. The difference between
the peak height of the normotensive cohort of pregnant women and the women with severe pre-eclampsia was non-significant, although the small number of patients in this group (n = 13) could have contributed to the lack of a significant difference between the two groups. What was disappointing concerning this analysis was that there were no differences in any of the four thrombin generation parameters between any of the three classes of pre-eclampsia severity. As highlighted by von Dadelszen et al. (2009), it would be beneficial to diagnose those most in need of intervention (those with severe pre-eclampsia) and also to avoid potentially harmful interventions in those patients who are unlikely to develop further complications (those with mild pre-eclampsia). The data available in this study however does not support the use of any of the thrombin generation parameters in the diagnosis of pre-eclampsia severity. It is quite likely however that due to the small sample size used in this analysis the comparisons were of insufficient power to distinguish between the different levels of pre-eclampsia severity. It would be beneficial to investigate the differences regarding the thrombin generation profiles of women with differing severity levels of pre-eclampsia in a separate study in order to establish whether or not there is actually a difference between the disease states. This is especially true of the ETP since it seems to show an increasing trend with disease severity in figure 3.3B.

What is clear from the data is that when combined into a single cohort the pre-eclampsia cases show the same pattern when compared to the normotensive pregnant controls as when they were considered as three distinct groups i.e. a significantly elevated ETP (p = 0.0007) and peak height (p = 0.0009). This difference is consistent with the further activation of the haemostatic system, over and above that which is expected in normal pregnancy, as reported in patients with pre-eclampsia (Roberts et al., 2005) (Roberts et al., 2002) (Heilmann et al., 2007). The discovery of this difference is profound, since it potentially highlights a role for the use of thrombin generation in the diagnosis of pre-eclampsia.

The analysis of the data for potential confounding factors revealed that it was unlikely that any of the parameters for which the case and control groups were matched (age, parity, gestational age and ethnicity) impacted on the observed difference between the two cohorts. This analysis did flag up an interesting result however. The Black women recruited to the study seemed to demonstrate no difference in any of the thrombin
generation parameters between those in the case and control cohorts. The same is not true of the Caucasian and Asian women who demonstrate the same pattern between the case and control cohorts as that which is described above. Interestingly the source of this difference is that the normotensive Black controls seem to have a level of thrombin generation which is consistent with the pathological level seen in the Caucasian and Asian pre-eclampsia cohorts. This result would imply that the baseline level of thrombin generation in a normotensive pregnant black woman is higher than that of a Caucasian or Asian woman. Assuming that this finding is correct it highlights several potential implications, not least of which is the implication for the thrombin generation profile of non-pregnant Black individuals. The normal range as described in chapter 2 did not make any concession towards the ethnicity of any of the individuals used in the analysis. In the light of the potential difference exhibited by the normotensive Black controls in this study, this may have been an oversight. It would be worthwhile comparing the thrombin generation profile of normal non-pregnant individuals of different ethnicities to see if the observed differences between Black and Caucasian/Asian normotensive controls still holds true. If this was to be the case a separate normal range would have to be established for individuals of Black ethnic origin.

The reason as to why the thrombin generation profile of a Black individual should be different to that of an individual of a different ethnic origin remains unclear. None of the thrombophilia parameters to which thrombin generation assays have been shown to be sensitive have separate normal ranges for different ethnic groups when analysed at the Royal London Hospital. Indeed the primary difference between individuals of Black ethnic origin and other ethnic backgrounds is if anything likely to result in an individual of Black ethnic origin having a thrombin generation which is lower than that of a Caucasian or Asian individual, the incidence of factor V Leiden is practically zero in Black populations in comparison to the incidence of ~6% in Caucasian populations. The incidence of factor V Leiden had no impact upon the results produced in this study however since the thrombin generation assay was not specifically set up to be sensitive to its presence.

It was reported by Eskenazi et al. (1991), that being of Black ethnic origin was a risk factor for pre-eclampsia. It is possible that part of the reason for this is that Black
women have higher baseline levels of thrombin generation than women of other ethnic backgrounds and are therefore naturally more pro-thrombotic. This theory is given weight by the findings of Montagnana et al. (2009) who reported that the global incidence of venous thromboembolism is highest in individuals of Black origin. It is possible that this ethnic predisposition to thrombosis contributes to the development of pre-eclampsia, for example by increasing the level of microthrombi formation and exacerbating placental infarction following disease onset. A natural prothrombotic tendency may also lead to an increase of disease severity; a study by Mulla et al. (2007) found that being of Black race was a risk factor for prolonged length of stay in hospital among patients with pre-eclampsia.

It is worth considering that the overall number of Black women in this study is quite low, which pre-disposes the statistical analysis to errors. This study was not specifically set up to analyse the differences in thrombin generation between patients of different ethnic groups and as such any conclusions drawn from the data should be subject to intense scrutiny. It is possible that the lack of difference between the Black case and control cohorts in this study is entirely down to a play of chance during the selection of patients. Although all of the individuals in the control cohorts were women with uncomplicated pregnancies, it is worth noting that pre-eclampsia is not the only factor which can impact upon the thrombin generation capacity of a patient. It is perfectly possible that some of women in the Black control cohort have underlying and to this point non-symptomatic pro-thrombotic mutations, which have led to higher than expected thrombin generation parameters. Since the sample size is small a small number of patients of this type would be sufficient to skew the analysis. It is also worthy of note that since the control patients were not followed up after the samples were taken there is the possibility that some of these women may have subsequently developed pre-eclampsia later in pregnancy. This is especially true of the small number of women who were recruited before 20 weeks gestational age as the diagnosis of pre-eclampsia is not possible until this point. It may be that some of the women in the black control cohort were therefore classified as controls when they should have been cases thereby reducing the statistical difference between the case and control cohorts. This is also theoretically possible of the Asian and Caucasian groups although this scenario is likely to impact most upon the cohort of Black women due to the relatively small sample size in this cohort. The elevated parameters in the Black control cohort are by no means down to a
single patient however, the entire cohort have ETP and peak height values in excess of the mean of the Caucasian and Asian control groups. It is unlikely (but not impossible) that the entire cohort have an undiscovered pro-thrombotic tendency/went on to develop pre-eclampsia but since none of the women were screened for thrombophilia before analysis or followed up subsequent to analysis this possibility cannot be excluded. Further studies therefore must be carried out to elucidate the reasons for the differences between the thrombin generation profiles of the different ethnic groups observed in this study.

The discovery of a difference in the thrombin generation capacity between women with pre-eclampsia and normotensive pregnant women (at least in Caucasian and Asian women) led to the possibility of the CAT thrombin generation assay being used to predict the onset of pre-eclampsia. In order to ascertain whether this would be possible the data in this study was extensively analysed to try and choose an appropriate cut-off for the thrombin generation parameters to enable the detection of those patients with pre-eclampsia.

What was surprising in this analysis was the relatively low predictive value of the PRECOG screen (sensitivity and specificity 58% and 69% respectively). The advantages that this screen offers are that it is relatively quick and obviously has a low cost to the NHS. It can also be performed in the community, effectively a point of care pre-eclampsia screen. What is obvious is that although cheap and convenient, it is not particularly effective at excluding low risk patients or accurately defining which patients are high risk.

It is debateable what predictive power a ‘good’ screening test for pre-eclampsia should have. Obviously in an ideal world any screening tests would have sensitivities and specificities of 100%, this is often not the case. In terms of pre-eclampsia a test of high sensitivity is likely to result in a higher degree of false positives ultimately leading to the specialist referral of a number of patients who do not require further treatment and therefore using valuable NHS resources. A high sensitivity assay would however result in the specialist referral of those patients who do require further treatment and specialist care. A high sensitivity assay is most useful in cases where intervention can have a positive effect on the outcome i.e. a curative treatment. This is not always the case for
pre-eclampsia where there is no cure available and early intervention may have no impact on the clinical outcome. The most effective treatment is premature delivery and that is not always desirable.

An assay with high specificity would in the case of pre-eclampsia result in the referral of a low number of patients for specialist care, ultimately saving money, but would result in a large number of false negatives and may impact on the morbidity and mortality of those patients who were not referred for specialist care early in pregnancy. The difficulty of devising a ‘good’ screening assay is therefore tempered by trying to balance the necessity of saving money verses the clinical impact of failing to refer the patients who may develop pre-eclampsia.

The choice of a suitable cut-off for the thrombin generation parameters proved problematical in this analysis. Despite the magnitude of the statistical difference between the control and case cohorts the two populations showed a high degree of overlap as shown in figures 3.11 A and B, with a number of the control patients showing very high levels of thrombin generation and some of the pre-eclamptic patients demonstrating low levels of thrombin generation. Indeed the highest ETP value found in both cohorts was one of the normotensive controls and the lowest value for ETP in either cohort was found to be one the severe pre-eclampsia cases. The reason for this overlap can only be postulated, it is possible that a number of women in the control cohort had underlying pro-thrombotic conditions/or were misclassified as controls (as discussed above), this is a likely scenario for those women in the normotensive control group who showed extreme values for both ETP and peak height. It may also be the case that the pro-thrombotic shift seen in normal pregnancy is more pronounced in some women when compared to others. This is reflected in the fact that some normotensive pregnancies are associated with thrombosis where as others have post partum haemorrhage. The pre-eclampsia patients also showed a high level of variation and this is consistent with the heterogeneous nature of the disease state itself. As discussed above it is possible that there are many pathways all leading to the same net result of the systemic pre-eclampsia condition. Some but by no means all of these involve activation of the coagulation cascade, which results in the elevated thrombin generation parameters seen in this analysis. It may be that the measurement of thrombin generation may be more sensitive to the development of some ‘types’ of pre-eclampsia rather than
others. It is also worth noting that this is not a longitudinal study and that the subjects in this analysis were analysed only once. It may be that the pre-eclamptic patients with low thrombin generation parameters have naturally low levels all of the time and that the level of thrombin generation demonstrated in this analysis represents a significant jump from their base line levels before the start of their pregnancy, and therefore the onset of the disease. It may be that the most effective screen for pre-eclampsia is the monitoring of an individuals thrombin generation before and during pregnancy and that the magnitude of any change is the best indicator of disease onset. It is impossible to either prove or disprove this theory using the data obtained in this study, further experimentation is necessary in order to prove whether or not this is indeed the case.

Based on the available information, the choice of cut-off for ETP and peak height in order to establish whether an individual is high risk or not can greatly affect the sensitivity and specificity of the assay. Choosing a low cut-off value (such as 1SD below the mean of the case cohort) increases the sensitivity of the assay, whereas increasing the cut-off value leads to an increase of the specificity of the assay (at the expense of the sensitivity). Using extreme cut-off values, such as ±2 SD from the mean of either the case or control cohorts can result in very high sensitivity or specificity values but this is achieved by characterising practically all women as high risk or low risk and is therefore unpractical. The measurement of thrombin generation is unnecessary when the cut off will classify all women as high risk. The use of a cut-off which is ±1SD from the mean of either group appears therefore to be more clinically relevant.

The combined use of both ETP and peak height in the same analysis (where both parameters must be elevated in order for an individual to be classified as high risk) appears more effective than using just the ETP alone as the small loss in sensitivity (from 92% to 86%) is more than compensated for by the increase in specificity (22% to 43%). These statistics refer to the use of a cut-off of 1SD below the mean values of the case cohort. When considering the cut-off of 1SD above the mean values of the control cohort the impact of using both parameters together is much less pronounced. What is clear from these analyses is that the measurement of thrombin generation is far from perfect as a stand-alone assay for the detection of pre-eclampsia in this cohort of
women. Evidence in support of this can also be seen in the modest area under the curve values (~0.70) calculated from the ROC curves in figure 3.12.

Since it is clear that the use of thrombin generation cannot adequately separate those women who have pre-eclampsia from those who have not, it is perhaps better to focus on cut-off values that will result in higher sensitivity values. This remains the case even if the positive predictive value is low (high degree of false positives) since this will result in the separation of a cohort without disease from a cohort who may have disease. Specialist referral of those women will then reveal which have disease. If a high specificity cut-off was chosen there would be a high number of false negatives with no means of picking out the women at risk until the onset of pre-eclampsia.

When considering the above statement the use of both thrombin generation and the PRECOG score in tandem can provide more useful clinical information than either screening test can on its own (thrombin generation parameter cut-off greater than 1SD below the mean of the case cohort). The primary clinical utility of combining the two assays is to enhance the negative predictive value of the screens. From this study 94% of the women classified as low risk by both thrombin generation and the PRECOG score did not have pre-eclampsia. The use of this ‘double negative’ approach is analogous to the use of a low risk clinical score in conjunction with a normal D-dimer in the exclusion of DVT (Wells et al., 2003). The assays can be combined in a number of ways in order to classify women as high risk or not, which can progressively increase the sensitivity of the assay but decrease the specificity as a result. This is shown in table 3.12 below.

All of the combinations in the table retain the 94% negative predictive value for the ‘double negative’ results. Which combination is judged ‘best’ is a difficult decision. Based on the earlier assumptions classifying patients as high risk if they have either high risk thrombin generation parameters (ETP > 2263nM Thrombin and a peak height of >445nM Thrombin) or a high risk PRECOG score (or both), gives the highest sensitivity value. This would ensure that 98% of women with pre-eclampsia received specialist referral, although it would mean that 71% of women who did not have pre-eclampsia would also receive further specialist testing. Since the incidence of pre-eclampsia in the UK is approximately 2-7%, this would mean specialist referral for
about two thirds of all normotensive pregnancies. So although this is the most effective screen for the detection of pre-eclampsia which can be devised using a combination of thrombin generation and the PRECOG screen, it is not the most cost effective. It is perhaps better to give the thrombin generation results more weight than the PRECOG score in determining high risk status, whilst it is true that this reduces the sensitivity it would in absolute terms reduce the number of specialist referrals considerably. Whether this cost benefit outweighs the cost in terms of morbidity in the cohort of women who would not receive specialist care as a result is another question. Whichever classification is used, the women characterised as low risk by both assays can be 94% certain that they will not develop disease. Practically the measurement of thrombin generation combined with the PRECOG score would not be too difficult, even if the patient was assessed in the community. The sample handling procedures for the measurement of thrombin generation are not too stringent as discussed in chapter 2, indeed as long as the sample reaches the referral laboratory in less than 24 hours the analysis should not be affected. The thrombin generation assay is also relatively cheap with a cost of approximately £3 per assay.

Table 3.12

<table>
<thead>
<tr>
<th>High Risk Classification</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated Thrombin generation and high risk PRECOG only.</td>
<td>37</td>
<td>75</td>
</tr>
<tr>
<td>High risk PRECOG and Thrombin generation and high risk PRECOG only</td>
<td>49</td>
<td>61</td>
</tr>
<tr>
<td>High Risk PRECOG and Thrombin generation and Thrombin generation only</td>
<td>86</td>
<td>43</td>
</tr>
<tr>
<td>High Risk PRECOG or High risk Thrombin generation</td>
<td>98</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 3.12. Sensitivity and specificity values obtained when different strategies are employed in order to determine whether an individual is high risk of developing pre-eclampsia, using a combination of the PRECOG score and a cut off for thrombin generation of greater than 1SD below the mean values of the case cohort.
A final consideration as to the classification of women as either high or low risk is whether the Black women should be excluded from the analysis. In actual fact when considering the scoring system for high risk as described above the exclusion of the Black women makes very little difference to the figures obtained. This is due to the fact that the cohort of Black individuals is evenly distributed between the cases and the controls and removing them from the analysis removes as many actual positives as it does false positives.

Overall this study has shown that the thrombin generation profile of a woman with pre-eclampsia is distinct from that of a normotensive pregnant woman (in the Caucasian and Asian cohorts) and that this difference when used in conjunction with the PRECOG score can be used to effectively distinguish a cohort of women from this study who do not have disease from a cohort of women in which pre-eclampsia is prevalent. This potentially highlights a new clinical application of the CAT thrombin generation assay.

One of the most profound findings of this study as a whole is the successful combination of the thrombin generation analysis (the ETP in particular) with the other parameters analysed by flow cytometry in the same cohort of patients. These include platelets expressing P-selectin, P-selectin expressing microparticles, platelet-monocyte aggregates and platelet-neutrophil aggregates. Although combined data was only available in 92 of the 104 women recruited to the study the predictive values far outstrip any of those obtained when either considering thrombin generation alone or in combination with the PRECOG score. The sensitivities and specificities of the analysis were 83% and 80% respectively. The positive and negative predictive values were 81% and 82% respectively (Macey et al., 2010a). As a screening test the cost of performing the combined analysis would exceed the cost of just thrombin generation and the PRECOG screen but the benefits of reduced specialist referral rates would more than compensate for this. What was interesting regarding this analysis was the comparison between healthy normal controls, normotensive pregnant women and women with pre-eclampsia when considering the different parameters. The ETP and peak height showed a step wise progression throughout those three groups. The normotensive pregnant women showed significantly elevated ETP and peak height values when compared to the normal range and the pre-eclamptic women had significantly higher values still. This pattern however was not conserved when considering the parameters measured by
flow cytometry. There were no differences in the levels of platelets expressing P-selectin, P-selectin expressing microparticles and platelet-monocyte aggregates between the normotensive pregnant women and a cohort of healthy normal individuals. The women with pre-eclampsia however demonstrated significantly elevated levels of all three of these parameters when compared to the normotensive pregnant women (p<0.03 in all cases). What this result could imply is that whereas the difference in thrombin generation seen between the normal range and the normotensive pregnant women could be due to changes in the plasma levels of some clotting factors. The further elevation in both peak height and ETP seen in women with pre-eclampsia over and above that which is normal in pregnancy may be due to the elevated levels of platelets expressing P-selectin, P-selectin expressing microparticles and platelet-monocyte aggregates. To be more precise the changes could be due to the elevated levels of microparticles found in the blood of patients with pre-eclampsia. The preparation of patient’s plasma for thrombin generation should both exclude platelets and platelet monocyte aggregates but is unlikely to remove the microparticles.

The presence of P-selectin expressing microparticles could impact upon thrombin generation in a number of ways. This is because the measurement of thrombin generation relies on the addition of a fixed quantity of both tissue factor and phospholipid. Microparticles themselves are effectively phospholipid vesicles and can induce the expression of tissue factor from leukocytes (Barnard et al., 2005); it is quite likely therefore that they can elevate the level of thrombin generation in a patient’s plasma by elevating the level of tissue factor and or phospholipids in the reaction. This possibility is discussed in more depth in chapter 4. It is important to note that this is another potential explanation as to why all of the women with pre-eclampsia did not demonstrate an elevated level of thrombin generation. Although elevated levels of platelets expressing P-selectin, P-selectin expressing microparticles and platelet monocyte aggregates are elevated in pre-eclampsia (Lukanov et al., 2009) (Lok et al., 2007) (Macey et al., 2010a) they are by no means elevated or elevated to the same extent in all the patients analysed.

A further point of note is that the parameters analysed via flow cytometry described above are also elevated in association with cardiovascular disease. Indeed there is a great deal of evidence showing a link between pre-eclampsia and subsequent risk of
cardiovascular disease in later life, with pre-eclamptic women approximately twice as likely to develop cardiovascular disease when compared to normotensive pregnant women (Craici et al., 2008). Assuming the CAT thrombin generation assay is sensitive to the presence of microparticles in the blood of women with pre-eclampsia it may also be sensitive to the changes in microparticle number seen in patients with cardiovascular disease. If this is the case then perhaps thrombin generation analysis may also be of use in the detection of cardiovascular disease.

Despite the many compelling findings of this investigation, it is apparent that as mentioned above, this study is not longitudinal i.e. the individuals recruited to the study, whether cases or controls, were only analysed once. In some cases the analysis occurred quite late in pregnancy and although this helped in determining the effect of gestational age on the parameters analysed, it does mean that it is unknown when during pregnancy the parameters investigated begin to manifest their increased magnitude in pre-eclampsia. In other words it is currently unknown whether the parameters flagged as raised in this study in the pre-eclamptic patients are precursors to disease onset or are manifested only in late stage disease. The most effective screening test would involve the use of those parameters which demonstrated the pathological changes associated with pre-eclampsia earliest in pregnancy. Without analysis of these parameters in a prospective manner it will be impossible to determine whether the measurement of these parameters will make an effective screen for the detection of pre-eclampsia or not.

What this study was intended to show then, is that these parameters are elevated in association with pre-eclampsia and that suitable cut-off values can be determined for these parameters which enable the case and control cohorts to be distinguished. It was necessary to perform this study retrospectively in order to ensure that a sufficient number of cases were present to allow a more accurate determination of any cut-off values. If this were attempted as a prospective study upwards of 700 patients would need to be recruited to obtain the same number of cases.

A further point to note in this study is that these experiments were intended to highlight the clinical utility of what could be considered the ‘basic’ thrombin generation assay i.e. the use of commercially produced reagents apparently containing known levels of tissue factor and phospholipids (5pM and 4µM respectively). No attempt was made to
optimise the sensitivity of this assay for the detection of disease in pre-eclamptic women. It is possible that adjusting the concentration of tissue factor and/or the concentration of phospholipids in the reagents may make the assay more sensitive in the detection of pre-eclampsia. One area that this may be of use is in determining whether or not there is a difference in thrombin generation with disease severity. As highlighted above there was no difference between any of the four thrombin generation parameters in any of the levels of disease severity in this study using reagents with 5pM tissue factor and 4µM phospholipids, although figure 3.3B is suggestive of the fact that the ETP is increasing in-line with disease severity. It is possible that the relatively high concentrations of tissue factor and phospholipids used in this study are creating an artificial plateau for the results (although they are not leading to substrate depletion) and that this is restricting the magnitude of difference that can be measured between patients with mild, moderate and severe pre-eclampsia. It may be that the use of a reagent with lower levels of tissue factor or indeed phospholipids may remove this plateau and therefore allow the determination of disease severity in women with pre-eclampsia using the thrombin generation assay. If the elevated level of thrombin generation in women with pre-eclampsia is indeed due to elevated levels of circulating microparticles (which are effectively phospholipid vesicles) then the use of a reagent with a lower concentration of phospholipids would certainly make the assay more sensitive to the detection of pre-eclampsia. Optimisation of the thrombin generation assay in this manner may also allow for a much more effective cut-off to be calculated in order to predict disease onset in pregnant women. Further work is required to elucidate whether or not this is the case.

Although assay optimisation may improve the results presented it is important to note that at the time this study was undertaken the only commercial reagents available were those which contained 5pM tissue factor and 4µM phospholipids, reagents of different strengths would have had to have been manufactured in-house, leading to potential problems with batch to batch variation.

In order to better highlight the clinical utility of the commercially produced reagents it was decided to correlate the thrombin generation data measured in the case and control cohorts with some end points of pregnancy in order to elucidate the effectiveness of the measurement of thrombin generation in a true prospective study.
The measurement of thrombin generation as characterised by the ETP and peak height parameters was shown to have little predictive value in determining either the mode of delivery or the risk of PPH in this cohort of women. The failure of the measurement of thrombin generation to correlate with either blood loss during delivery defined as a continuous variable or the incidence of PPH defined as a discreet variable was unanticipated. Intuitively blood loss and thrombogenicity seem intrinsically linked although the poor AUC values attained when the case and control cohorts are combined (0.482 and 0.514 for the ETP and peak height respectively) shown in figure 3.21 imply this is not the case. On reflection this result is not surprising as blood loss during pregnancy is not always spontaneous and can be due to a number of medical/surgical complications and is not therefore solely dependent on an individual’s capacity to generate thrombin. There is also the potential for a large number of confounding variables in this analysis, not least of which is the incidence of LSCS and other surgical procedures which may exacerbate blood loss in patients. There is also the possibility that some of the patients in this study may have had red cell transfusions which could have masked the magnitude of blood loss and therefore skewed the results of this analysis. In real terms the impact of this is likely to be small as the number of patients requiring a transfusion would be low and haemoglobin levels are frequently monitored before transfusing patients. In order to better gauge the relationship between thrombin generation and blood loss during delivery a much larger study must be undertaken as this will reduce the impact which confounding variables can have on the analysis and also allow the determination of relationships between thrombin generation and much rarer outcomes of delivery such as fatal PPH.

The apparent lack of correlation between the ETP or peak height with the mode of delivery as evidenced by the low AUC values, especially in the case cohort (0.502 for both ETP and peak height) was also perhaps unsurprising. Initially it was felt that the incidence of LSCS would be higher in the case cohort when compared to the normotensive controls because women with pre-eclampsia often have emergency deliveries to prevent disease progression. Since the peak height and ETP are typically raised in the case cohort when compared to the controls it was felt that the two parameters might correlate quite well. This was not the case; and is probably because the incidence of LSCS was quite similar in both the case and control cohorts highlighting that pre-eclampsia is not the only reason for women to undergo LSCS.
What is interesting regarding this analysis is that the predictive value of the ETP in whether or not a pregnant woman will have an LSCS is higher in the control cohort when compared to the case cohort (figure 3.18), although the AUC is far from compelling at only 0.629.

The measurement of both the ETP and peak height were found to have statistically significant correlations with both the birth weight of the baby and the length of time spent in hospital at delivery. The correlation between birth weight and thrombin generation was also a parameter in which an association was thought to be likely, since those women with pre-eclampsia often have pre-term babies (which have a lower birth weight) in order to prevent disease progression. The correlations between ETP/peak height and birth weight were only apparent when the data from the case and control cohorts were combined. This is probably an artefact of the relatively weak statistical trend (R values of 0.26 and 0.27 for ETP and peak height respectively) but nevertheless this finding highlights a statistically significant association between both increasing ETP and peak height with decreasing birth weight in this cohort of women. This finding is pleasing since it suggests a new clinical application of the measurement of thrombin generation, since it may be able to highlight which pregnancies may be at risk at delivery.

The correlation between ETP and peak height with the length of stay in hospital at delivery was even more compelling with R values of 0.44 and 0.29 for the ETP and peak height respectively. Although these values did decrease when the outlying data was removed, this did not markedly reduce the statistical significance of the correlation (p=<0.001 and <0.02 for the ETP and peak height respectively). One of the most interesting findings in this analysis is that the correlation of the ETP with the length of stay in hospital was the only correlation from this phase of analysis which demonstrated a statistically significant trend in both the case and control cohorts prior to them being combined. This implies that this trend may be independent of pre-eclampsia.

This analysis would benefit from the recruitment of more pregnant women in order to fill in the gap between the outlying value (seen in figure 3.17 A and B) and the rest of the cohort of women. If this further analysis confirms the trend suggested here then it provides a very useful clinical application of the measurement of thrombin generation in
pregnancy, since the magnitude of the ETP can potentially highlight which women will need the most postnatal care.

In conclusion this phase of analysis has yielded some very promising results, since it highlights two new potential roles for the measurement of thrombin generation using the ‘basic’ CAT assay during pregnancy; including whether or not the baby will have a low birth weight and providing an indication of the level of maternal care required post delivery. It may be possible to optimise the CAT assay as discussed above to enhance the correlations described and therefore further enhance the clinical utility of the measurement of thrombin generation.
Chapter 4

Microparticle Analysis
4.1 Introduction

Following the discovery that there were no differences in the levels of P-selectin expressing microparticles between normotensive pregnant women and a cohort of non-pregnant individuals and that the numbers of P-selectin expressing microparticles were significantly elevated in a cohort of women with pre-eclampsia in comparison (Macey et al., 2010a), it was hypothesised that the increase in thrombin generation observed between a cohort of women with pre-eclampsia and a cohort of normotensive pregnant women was due, in part, to the increase in the number of circulating microparticles. The primary aim of this chapter was to investigate the impact of microparticles on thrombin generation.

4.1.1 Microparticles

Microparticles are vesicles derived from the budding of cell membranes of multiple cell types including: platelets, endothelial cells, monocytes, granulocytes and erythrocytes. Typically they range in size between 0.2-2µm in diameter, although those derived from erythrocytes are generally smaller, being approximately 0.15 µm in diameter (Piccin et al., 2007). Platelet derived microparticles are by far the most abundant in the circulation, constituting between 70-90% of the total number of microparticles (Flaumenhaft, 2006). Indeed these were the first to be described, experiments dating as far back as 1949 were able to identify a precipitable factor which could be isolated from platelet free plasma and was capable of inducing thrombin generation (Chargaff and West, 1949). Further experimentation (Wolf, 1967) revealed that there was a linear correlation between the platelet count of an individual and the level of ‘Platelet dust’ (platelet microparticles) in a sample of platelet free plasma from the same individual. The use of electron microscopy conclusively proved that the microparticles originated from the surface of activated platelets. It was not until more recently that microparticles of different cellular origins such as monocytes (Satta et al., 1994) and endothelial cells (Combes et al., 1999) were described. Typically the different microparticle subtypes can be differentiated via flow cytometry due to the differential expression of surface proteins. Platelet microparticles express CD42b (GpIb), GpIIb-IIIa, CD62P (P-selectin), CD31, CD63, CD41a and CD61 whereas endothelial microparticles express CD31,
CD34, CD51, CD52, CD62E (E-selectin), CD62P P-selectin, CD105 (endoglin) and CD146 (Piccin et al., 2007). Although these lists are by no means exhaustive.

4.1.2 Formation of Microparticles

Regardless of their cellular origin it is believed that microparticle formation is dependent on the disruption of the asymmetric distribution of phospholipids in the cell membrane. The expression of phospholipids is conserved and maintained throughout all cell types. Phosphatidylcholine and sphingomyelin are expressed externally whereas phosphatidylserine and phosphatidyl-ethanolamine are found on the internal cell membrane. This asymmetrical distribution is maintained by a number of cellular proteins including Aminophospholipid translocase, floppase, scramblase, calpain and gelsolin, although the latter is only found in platelets.

Aminophospholipid translocase is an enzyme whose activity is dependent on the presence of ATP. Its function is specific for the transport of aminophospholipids (phosphatidylserine and phosphatidyl-ethanolamine) from the outer layer of the cell membrane to the inner layer. Floppase is similar to aminophospholipid translocase in that it too is dependent on ATP for its activity but its function is to export lipids from inside of the cell to the outside of the cell. Interestingly its function is not limited to specific lipids. Scramblase is usually inactive, but when activated facilitates the passage of lipids across the cell membrane. Whereas aminophospholipid translocase, floppase and scramblase are all cell membrane bound proteins both calpain and gelsolin are cytoplasmic enzymes. Typically microparticle formation is triggered by cellular activation, apoptosis or necrosis; these events are generally accompanied by an influx of calcium ions released by the endoplasmic reticulum. The influx of calcium ions leads to the inhibition of aminophospholipid translocase activity and the activation of scramblase. This ultimately leads to the loss of lipid asymmetry across the cell membrane, with the expression of phosphatidylserine on the external membrane. Calpain is also activated in the presence of calcium ions. Calpain is a cysteine proteinase of the papainase family and can cleave long actin filaments thereby disrupting the cytoskeleton (Piccin et al., 2007). It has also been shown that calpain can cleave both type I and type II phosphatidylinositol phosphate (PIP) kinases (O’Connell et al., 2005). PIP kinases mediate the conversion of PIP to phosphatidylinositol 4,5-
biphosphate (PIP$_2$). Enrichment of PIP$_2$ in cell membranes increases the membrane-cytoskeleton adhesion energy (Raucher et al., 2000) and incubation of platelets with PIP$_2$ inhibits microparticle formation when stimulated by agonists such as thrombin and collagen (O’Connell et al., 2005). The action of calpain is therefore two fold in that it can both destabilise the cellular cytoskeleton directly and prevent its reinforcement via the inhibition of PIP$_2$ formation. In platelets actin microfilaments are further protected by proteins such as Adductin and Cap Z which cap the actin filaments and prevent the interaction of actin with other proteins and ultimately therefore maintain the structure of the cellular cytoskeleton. These capping proteins are themselves the target of proteolytic cleavage mediated by the enzyme gelsolin. Gelsolin is activated by calcium ion influx following platelet activation (Piccin et al., 2007). The disruption of the cytoskeleton mediated by calpain and gelsolin (in platelets) is central to microparticle formation. With the cell membrane no longer anchored to the cytoskeleton it begins to bud from the cell surface, eventually leading to detachment of the bud from the cell and to the production of a microparticle which has exposed phosphatidylserine on its external surface.

It is known that individuals maintain a constant baseline level of microparticle expression (Brogan et al., 2004) but given that platelet microparticles are cleared rapidly from the circulation following infusion into mice (Flaumenhaft, 2006) it is uncertain whether the level of platelet activation seen in healthy individuals is sufficient to maintain these levels of microparticles. This would imply that platelet activation is not the only mechanism by which platelet microparticles are produced. Other potential routes of production include the shedding of microparticles directly by megakaryocytes or production of microparticles from ‘resting’ platelets. Evidence that both of these routes of microparticle production are possible has been shown by *in vitro* experimentation. Megakaryocytes cultured for 11-14 days show evidence of microparticle production (Rozmyslowicz et al., 2003) and platelet concentrates also exhibit vesiculation without any decrease in the number of platelets (Bode and Miller, 1986). The generation of microparticles from stored platelets is only partially blocked by inhibitors of platelet activation (Bode et al., 1991). The exact contribution that these processes make towards the number of platelet microparticles *in vivo* remains to be elucidated.
4.1.3 The Function of Microparticles

The fact that the baseline levels of microparticles are maintained in a healthy individual and that the majority of microparticles in circulation are derived from platelets (or megakaryocytes) would imply a role for microparticles in the maintenance of haemostasis. Evidence that this is indeed the case is provided by individuals with Scott syndrome, a rare autosomal recessive disorder. Individuals with this condition have mutations which impact upon the function of the scramblase protein and as a direct result of this the numbers of microparticles in these patients are reduced, as is the expression of phosphatidylserine on the surface of the microparticles which are present. This leads to a severe bleeding phenotype in these patients despite apparently normal platelet function (Zwaal et al., 2004).

The presence of phosphatidylserine on the surface of microparticles provides binding sites for a number of activated clotting factors including those associated with both the tenase and prothrombinase complexes (Berckmans et al., 2001)(Sims et al., 1989). Indeed the expression of phosphatidylserine by microparticles allows the concentration of clotting factors to reach the levels necessary to achieve optimum kinetics for the generation of thrombin (Morel et al., 2006). The shielding of phosphatidylserine results in a decrease in the enzymatic activity of the tenase and prothrombinase complexes by a factor of 200 and 1000 respectively (del Conde et al., 2005a). This then is the likely physiological cause of the bleeding seen in patients with Scott syndrome. A similar yet distinct disorder characterised by a deficiency in microparticle generation termed Castamans defect has also been described (Castaman et al., 1996). Patients with this condition present with a bleeding tendency and have a prolonged bleeding time. These conditions provide compelling evidence that microparticles play a crucial role in coagulation.

Their role in haemostasis is not limited to providing a surface for the assembly of the tenase and prothrombinase complexes however. There is evidence to show that endothelial derived microparticles can bind high molecular weight multimers of von Willebrand factor (vWF) and enhance their stability. It has also been shown that vWF bound in this manner has a higher affinity for platelet binding than soluble vWF (Jy et al., 2005). There is also a large body of evidence that microparticles express tissue
factor, a potent activator of the coagulation cascade. The exact cellular origin of tissue factor expressing microparticles is the subject of some debate, whilst it is clear that monocytes can produce microparticles enriched with tissue factor (del Conde et al., 2005b), it was shown that 90% of tissue factor expressing microparticles in the circulation of healthy volunteers originated in equal shares from microparticles derived from platelets and monocytes. Interestingly in relative terms this equates to 91% of all monocyte derived microparticles expressing tissue factor in contrast to only 6% of platelet derived microparticles (Hron et al., 2007).

Initially the tissue factor expressed by platelet derived microparticles was thought to be produced by other cells since tissue factor could not be isolated from megakaryocytes (Muller et al., 2003). It was thought that tissue factor was transferred to platelets following an interaction with tissue factor expressing microparticles derived from leukocytes mediated via the binding of CD15 to P-selectin on the surface of platelets (Rauch et al., 2000). Although it was demonstrated subsequently that platelets can synthesise and translocate biologically active tissue factor to their cell membranes (Panes et al., 2007). The role that these tissue factor expressing microparticles play in vivo is unclear, in vitro experimentation using human blood and in vivo experiments using murine models have postulated a role in the recruitment of these microparticles to the sites of vascular injury where presumably they act as a trigger for fibrin production (Giesen et al., 1999)(Falati et al., 2002). The recruitment of these microparticles was shown to be mediated by the interaction of PSGL-1 on the microparticle surface and P-selectin on the surface of platelets at the site of injury (Falati et al., 2003). Other studies have suggested that the concentration of tissue factor on the surface of microparticles in the circulation of a healthy patient whose blood remains unstimulated with cytokines is below the threshold needed to initiate in vivo thrombin generation (Butenas et al., 2005). Intuitively this result would be anticipated as if the levels of tissue factor were sufficient to induce thrombin generation prior to activation the individuals in this cohort would demonstrate a predisposition towards thrombus formation. Subsequent activation of platelets in these patients did not result in a significant increase in the level of tissue factor expression however. It is worth noting that the binding of P-selectin expressed by platelets or microparticles can induce tissue factor expression in monocytes via binding of PSGL-1 (Barnard et al., 2005) and that this induction of tissue factor may cause the local concentration of tissue factor to rise to a level capable of triggering the formation
of fibrin. It may be the case that tissue factor expressing microparticles are recruited to the sites of vascular injury not to initiate clot formation but to stabilise the thrombus by inducing further fibrin production (Wagner, 2005). The recruitment of endothelial cell derived microparticles bound to vWF would also help maintain clot stability by enhancing platelet aggregate formation.

The involvement of microparticles in haemostasis may also not be limited to thrombus formation, as microparticles have also been shown to express negative regulators of thrombosis such as thrombomodulin, TFPI (Steppich et al., 2005) and Protein C (Satta et al., 1997). It is therefore likely that the development of a thrombus is dependent on the relative signal strengths of the pro-coagulant and anti-coagulant factors.

Although there is significant evidence regarding the role which microparticles play in haemostasis, this is by no means the only function of microparticles in vivo. There is evidence to suggest that microparticles effectively act as long range signalling molecules which can act on cells other than those from which they are derived and that the function of these microparticles is dependent on the molecules they express on their cell membranes (Martínez et al., 2005). Microparticles can interact with endothelial cells to affect both vasoreactivity and angiogenesis. Platelet microparticles can both activate the endothelium and cause vasoconstriction via the modification of arachidonic acid metabolism in the target cells, leading to the generation of thromboxane A2. It has been shown that platelet microparticles can enhance arachidonic acid mediated contractions of the aorta in a rabbit model (Pfister, 2004). Endothelial cell derived microparticles can also impact on the regulation of vascular tone by inhibiting vasodilation. This effect is mediated by the inhibition of NO and was demonstrated in vitro in a rat model (Brodsky et al., 2004).

Microparticles of platelet and endothelial origin can also induce angiogenesis but the mechanisms by which this effect is mediated differs between them. Platelet microparticles isolated from normal individuals have been shown to promote proliferation, migration and tube formation in cultured endothelial cells by the activation of the mitogen-activated protein kinase (MAPK) pathway (stimulation of EGFR)(Kim et al., 2004). In contrast endothelial cell microparticles have been shown to induce angiogenesis facilitated by the binding of metalloproteinases which are
necessary in order to induce proteolytic breakdown essential for vascular invasion (Taraboletti et al., 2002).

The microparticles produced via cellular activation are known to differ from those produced via apoptosis in terms of both size and lipid/protein composition (Martínez et al., 2005) the surface markers have also been shown to be distinct when endothelial microparticles are produced by either activation or apoptosis (Jimenez et al., 2003). Whether the function of these microparticles differs distinctly in line with these observations is unclear, but it has been postulated that microparticle generation may form part of a cellular response to sub-lethal complement attack, since cells can shed complement components from their surface in this manner (Piccin et al., 2007). It has also been suggested that the expression of phosphatidylserine on the surface of apoptotic cells and microparticles might act as a signal for the removal of these cells/microparticles from the circulation via macrophages, since activated macrophages express specific phosphatidylserine receptors (Comfurius et al., 1995).

4.1.4 Microparticles and Disease

Whilst it is known that microparticles play important roles in normal physiology, such as in the maintenance of haemostasis as well as being involved in both vasoconstriction/dilation and angiogenesis, a large number of investigations have studied how microparticle function is subverted in disease.

Given that one of the primary roles of microparticles is the regulation of haemostasis it is perhaps no surprise that elevated levels of microparticles have been reported in a number of thrombotic conditions such as heparin induced thrombocytopenia (HIT), where platelets are activated in an autoimmune reaction, Sepsis, in which bacterial lipopolysaccharide can activate monocytes resulting in tissue factor expression (Satta et al., 1994), Thrombotic Thrombocytopenic Purpura (TTP) (Galli et al., 1996), Paroxysmal Nocturnal Haemoglobinuria (PNH)(Hugel et al., 1999) and Sickle cell disease (Tomer et al., 2001). Interestingly the endothelial microparticles expressed in PNH and sickle cell disease express CD144 and CD105 both of which have relatively short half-lives in the circulation, implying that both disorders are characterised by chronic endothelial damage/activation (Piccin et al., 2007). Elevated numbers of
endothelial and platelet derived microparticles have also been demonstrated in patients with active venous thromboembolism (Heresi et al., 2003). It was also shown that the numbers of platelet microparticles can be combined with the measurement of both P-selectin expression and D-dimer to predict DVT diagnosis with a sensitivity and specificity of 73% and 81% respectively (Rectenwald et al., 2005). An increase in circulating microparticles has been linked with other thrombotic disorders such as ischemic cerebrovascular accidents, transient ischemic attacks (Doeuvre et al., 2009) and multi-infarct dementia (Lee et al., 1993). Interestingly microparticles have also been linked to the pathology of cerebral malaria. The circulating levels of microparticles were only shown to be elevated in patients with severe malaria complicated by coma. It was also shown using a knockout mouse model, in which normal microparticle production was impaired, that the presence of microparticles may be necessary for disease progression to cerebral malaria. The knockout mice showed a complete resistance to cerebral malaria following infection with the parasite (Combes et al., 2006).

There is also evidence of elevated microparticle expression in a number of systemic and chronic conditions such as diabetes mellitus (Diamant et al., 2002), renal failure (Faure et al., 2006) and coronary artery disease, where the levels of circulating endothelial derived microparticles were shown to correlate with the degree of stenosis in a cohort of 84 patients (Bernal-Mizrachi et al., 2003). It was also demonstrated that the number of tissue factor expressing microparticles was two-fold higher in a cohort of 20 patients with colorectal cancer in comparison with a cohort of healthy normal individuals (Hron et al., 2007), thus suggesting a possible mechanism as to the cause of thrombosis in oncology patients. The authors postulated that the circulating levels of microparticles may explain why the location of vascular thrombosis in oncology patients if often distal to the site of the tumour. Another study showed enhanced microparticle tissue factor activity in 50 patients with either pancreatic or breast adenocarcinoma and that increasing activity correlated with disease severity. Interestingly the patients in the cohort with a venous thromboembolic event all had microparticle tissue factor activity in excess of the 99th percentile of the activity demonstrated by a cohort of normal individuals (Tesselar et al., 2007).
It is worthy of note that an elevated level of microparticles is not always associated with a detrimental effect, indeed the elevation in the numbers of platelet microparticles reported in autoimmune thrombocytopenias may well be protective against bleeding and are postulated to be the reason why such patients can show relatively mild symptoms despite profound thrombocytopenia (Jy et al., 1992).

There is also evidence to suggest that the primary cellular origin from which the circulating microparticles are derived may differ between pathological conditions. Platelet microparticles appear to be the most prevalent in Hypertension, Myocardial infarction, cancer and PNH, whereas microparticles of endothelial origin are associated with acute coronary syndromes, type I diabetes mellitus and Lupus anticoagulant. Microparticles derived from leukocytes have been associated with HIV, severe trauma, sepsis and type II diabetes mellitus (Martínez et al., 2005). It is worthy of consideration that an elevation in the numbers of circulating microparticles is by no means causative of disease in all cases and that the disease state itself may give rise to the increased production of microparticles. Elevated levels of microparticles may well be implicated in the exacerbation of disease severity however.

### 4.1.5 Microparticles in Pregnancy

Since increased microparticle expression is associated with systemic inflammatory responses it is perhaps of no great surprise that that the numbers of both platelet and endothelial derived microparticles have been shown to be elevated in normal pregnancy in comparison to non-pregnant healthy women (Bretelle et al., 2003). There is a degree of conflict in the literature however with some groups reporting an initial decrease in the numbers of microparticles during pregnancy followed by a return to normal levels (Lok et al., 2008). As uteroplacental thrombosis is a frequently proposed mechanism for pregnancy loss and since microparticles have been shown to be elevated in a number of pro-thrombotic conditions, there has been some study into the role in which elevated numbers of microparticles may play in pregnancy loss. A study comparing the levels of pro-coagulant microparticles found in a cohort of women with a history of unexplained pregnancy loss with a cohort of women with no history of pregnancy loss found that the circulating levels of procoagulant microparticles were significantly elevated in those women with a history of pregnancy loss (Laude et al., 2001). This result provides...
evidence to explain why many women with recurrent pregnancy loss are found to be ‘normal’ when screened for thrombophilia using traditional assays yet still manifest a clinical history of thrombosis. An interesting finding of this study is the fact that the blood samples were analysed a minimum of 2 months after the last reported obstetric event, thus implying that the circulating levels of pro-coagulant microparticles could be a chronic phenomenon in these patients and that this is only clinically ‘unmasked’ in pregnancy (Greer, 2001). Proof that microparticles can precipitate placental thrombosis was provided by studies in a murine model, where phosphatidylserine expressing phospholipid vesicles were injected into pregnant mice and subsequently induced thrombosis in the placental bed and ultimately resulted in reduced birth weight (Sugimura et al., 1999). The addition of Annexin V which binds with high affinity to phosphatidylserine ameliorated this effect, implying that the presence of phosphatidylserine is necessary to induce thrombosis. Indeed it was postulated by Greer (2001) that the binding of phosphatidylserine expressing microparticles to the trophoblast layer on which annexin V is exposed inhibits the anticoagulant effect of annexin V thereby predisposing the placenta to thrombosis.

4.1.5.1 Microparticles in Pre-eclampsia

The numbers and types of microparticles have been the source of a number of investigations in the field of pre-eclampsia. It is generally shown in the literature that the circulating levels of microparticles in patients with pre-eclampsia are elevated over and above what is seen in association with normotensive pregnancy (Redman and Sargent, 2008). Indeed patients with pre-eclampsia have been shown to have elevated levels of microparticles derived from multiple cellular origins including endothelial cells (Gonzalez-Quintero et al., 2004), leukocytes, platelets (Meziani et al., 2006), T-cells and granulocytes (Van Wijk et al., 2002a) in comparison to normotensive pregnant women. Although as is the case in normal pregnancy, a number of studies have shown discrepant findings, with some reporting decreased levels of platelet derived microparticles in pre-eclamptic patients (Lok et al., 2008). It was postulated by the authors that this may be due to the decreased platelet counts in patients with pre-eclampsia. It is also a possibility that the differing results between studies may be indicative of the different methodologies employed by the various laboratories or even
in the definitions used to define the pre-eclamptic patients themselves (Aharon and Brenner, 2009).

The study of microparticles in pre-eclampsia is an interesting field since placenta derived microparticles potentially provide a neat link between the two stages of disease onset. Indeed this theory is discussed in chapter 3, where Redman and Sargent (2000) proposed a mechanism whereby reduced placental perfusion leading to oxidative stress caused increased levels of syncytiotrophoblast apoptosis. This in turn would lead to increased microparticle production and the long range signalling capacity of the microparticles could result in the systemic maternal syndrome. Increased levels of microparticles would obviously also contribute to the pro-coagulant nature of the condition. Placental microparticles are present in normal pregnancy and are derived from the syncytiotrophoblast layer. These syncytiotrophoblast microparticles have been detected in normal pregnancies by the second trimester and are shown to increase in number significantly into the third trimester. In pre-eclamptic patients the number of these syncytiotrophoblast derived microparticles has been shown to be significantly elevated when compared to normotensive pregnancies (Knight et al., 1998). Further to this, syncytiotrophoblast derived microparticles have been shown to be able to bind to monocytes and stimulate the production of inflammatory cytokines such as TNFα, IL-12, IL-18 and Interferon γ (Germain et al., 2007).

Although these experiments do not offer conclusive proof of a placental derived population of microparticles causing the maternal pre-eclamptic syndrome they are certainly suggestive of a role for these microparticles in systemic endothelial inflammation and perhaps further microparticle production as a result. It is worthy of note that other species of microparticles known to be elevated in pre-eclampsia are also likely to contribute to disease severity regardless of whether their production is initially triggered via placental microparticles or not. Since platelet microparticles can initiate vasoconstriction via the modification of arachidonic acid metabolism (Pfister, 2004), an overexpression of platelet microparticles could in theory exacerbate the hypertension seen in patients with pre-eclampsia. When taken into account with an elevated level of endothelial derived microparticles, also described in pre-eclampsia, which can inhibit vasodilation via inhibition of NO (Brodsky et al., 2004) the evidence for microparticle involvement in the pathology of pre-eclampsia is quite compelling. Indeed
microparticles produced following chronic endothelial damage in disorders such as sickle cell anaemia have been shown to express CD105 (Endoglin) and could sequester TGF-β in a similar manner to sEng, thus providing a secondary mechanism by which vasorelaxation could be inhibited in pre-eclampsia. Evidence in support of these theories was shown using an in vitro model where microparticles isolated from patients with pre-eclampsia significantly inhibited bradykinin-mediated vasorelaxation in myometrial arteries taken from healthy pregnant women in comparison to microparticles isolated from normotensive pregnant controls (Van Wijk et al., 2002b).

Vascular dysfunction mediated by microparticles is not limited to vasoconstriction however, Meziani et al (2006) described that microparticles isolated from pre-eclamptic patients were able to induce vascular hyporeactivity in an ex vivo model using human omental arteries and mouse aortas. This effect was not observed using microparticles isolated from normotensive pregnant controls and could be reversed using an inhibitor of NO. This observation suggests the hyporeactivity was due to increased production of NO. Interestingly the authors also described that the platelet derived microparticles found in the circulation were primarily associated with NO production, whereas those microparticles of leukocyte origin could induce both vasorelaxation via induction of NO production and a vasoconstriction response due to the induction of cellular COX-2 expression. The authors postulated that the platelet derived microparticles may therefore be protective in pre-eclampsia since enhanced expression of NO could counteract the vasoconstrictive signal mediated by the leukocyte derived microparticles. It may be that the impact which microparticles have on hypertension in pre-eclamptic patients depends on the relative balance of signal strength between vasoconstriction and vasodilation. It is of note therefore that leukocytes are highlighted as a primary cellular origin of microparticles in patients with pre-eclampsia (Martínez et al., 2005). It has also been shown that leukocyte derived microparticles can further inhibit vasodilation via the induction of increased levels of caveolin-1 which acts to decrease cellular levels of endothelial NO synthase.

Although the physiological role which microparticles play in pre-eclampsia is not yet fully understood it is clear from the literature that their role is far from benign and that there is a myriad of ways in which they can subvert normal endothelial function and therefore contribute to pathogenesis.
4.1.6 Microparticles as a Therapeutic Target

Since elevated numbers of microparticles are found in a myriad of different pathological conditions and there is a strong evidence base to suggest that microparticles themselves may be implicated in the pathology of some if not all of these conditions it is no surprise that microparticles are coming under scrutiny as potential targets for disease monitoring/treatments. In conditions where there is systemic chronic endothelial damage, such as sickle cell disease and PNH, the monitoring of microparticle levels may provide insights to the efficacy of available treatments (Piccin et al., 2007). Following on from this the identification of microparticles from specific cellular origins such as neurovascular microparticles in CSF or even tears, may provide a valuable tool as biomarkers for disease onset, diagnosis or prognosis (Doeuvre et al., 2009).

Although there are currently no therapies which intentionally target microparticles as a mode of action, a number of therapies already available may mediate some of their beneficiary effects by decreasing the numbers of microparticles or by inhibiting their function. The use of anti-platelet drugs such as clopidogrel and aspirin can lead to decreased levels of both platelet and leukocyte derived microparticles (Serebruany et al., 2003) and therefore reduce the risk of thrombotic events in patients with cardiac disorders. Similarly the use of the statin fluvastatin was shown to reduce the production of endothelial microparticles from human coronary artery endothelial cells in an in vitro model (Tramontano et al., 2004), thereby reducing the potential for microparticle mediated vascular dysfunction in hypertension. It has also been suggested that one of the reasons why plasmapheresis is a superior treatment when compared to plasma infusion in the treatment of TTP is that it results in the removal of circulating microparticles (Piccin et al., 2007). Despite these observations, there is little evidence to suggest any clinical trials have been performed using these agents to ameliorate the pathological effects of microparticles in any other conditions aside from those which the drugs were initially formulated to treat.

There is evidence in the literature however concerning the rationale of using microparticles in the treatment of bleeding disorders. Pro-coagulant leukocyte derived microparticles stimulated by the infusion of soluble P-selectin were shown to be able to correct haemostatic balance in a murine model of haemophilia A (Hrachovinova et al.,
2003). There is also a suggestion that the transfusion of older stored platelets may offer a haemostatic advantage to the patients in which they are administered due to the formation of microparticles during storage (Piccin et al., 2007).

The physiological roles of microparticles are varied, but since they are known to be implicated in thrombosis, this intuitively suggests that they probably have an effect on a patient’s capacity to generate thrombin. The aim of this chapter therefore is to determine the impact that microparticles have on thrombin generation as measured by the CAT thrombin generation assay.

4.2 Materials and Methods

4.2.1 PT/INR
Please refer to section 2.2.1 for details

4.2.2 APTT
Please refer to section 2.2.2 for details

4.2.3 Thrombin Time
Please refer to section 2.2.3 for details

4.2.4 Preparation of Samples: Single Spun Plasma
Blood was collected into 0.109M tri-sodium citrate vacutainers. Samples were centrifuged at 1500 g for 10 minutes. Following this time the plasma was then removed, placed in a corning tube and frozen.

4.2.5 Preparation of Samples: Double Spun Plasma
Blood was collected into 0.109M tri-sodium citrate vacutainers. Samples were centrifuged at 1500 g for 10 minutes. Following this time the top two thirds of the patient’s plasma were removed and aliquoted into LP4 tubes. These aliquots were then centrifuged for a further 10 minutes at 1500 g. The top two thirds of the double spun plasma was then removed, placed in a corning tube and frozen.
4.2.6 Preparation of Samples: Filtration of Patient Plasma
Plasma was filtered using a Ceveron microparticle filtration unit (MFU) 500. Plasma for filtration was first prepared by centrifugation as described in either section 6.2.1 or 6.2.2 above. Centrifuged plasma was then loaded into the 96 well 0.2μm filtration plates, no more than 200μl of sample was placed into each well. The number of wells used was dictated by the size of the plasma sample being filtered. The filter plate was then covered using plate sealer foil. The instrument was then activated, producing a vacuum of ~70kPa. The vacuum sucks the plasma through the filter plate into the pre-loaded 96 well receiver plate. Plasma samples were then aspirated from the receiver plate using a Pasteur pipette and pooled.

4.2.7 Reconstitution of Control Plasma P
Please refer to section 2.2.6 for details

4.2.8 Filtration of Control Plasma P
Following reconstitution as described above the plasma was aspirated into a 5ml plastic syringe driver. A 0.2μm filter was fitted to the end of the syringe and the plasma forced through the filter into a plastic 25ml universal container.

4.2.9 Thrombin Generation
Please refer to section 2.2.5 for details

4.2.10 Protein C Activity
The measurement of the Protein C activity was an automated assay performed on the CS2100i analyser manufactured by Sysmex. Patient’s plasma (12μl) was added to an equal volume of CA System Buffer in a plastic cuvette. To this 120μl of Berichrom Protein C Activator reagent was added after a 60 second incubation at 37°C. The Berichrom Protein C Activator reagent contains a snake venom extract which activates the protein C in the patient’s sample. Following a further 290 second incubation at 37°C 24μl of Berichrom Protein C Substrate reagent was added. The substrate reagent contains a chromogenic substrate consisting of a peptide bound to methoxy-nitroanilide. The action of activated Protein C cleaves the peptide and releases the methoxy-nitroanilide. This can be measured at 405nm. The reaction was allowed to proceed for 100 seconds, after this time the absorbance of the cuvette at 405nm was measured. The
absorbance at 405nm is proportional to the level of Protein C activity in the sample. The absorbance of the cuvette is converted to an activity via interpolation from a stored standard curve produced by measuring in duplicate 5 dilutions of SHP (which has a known protein C activity) and plotting the mean OD result against activity.

The assay was controlled using Coagulation Control N and Coagulation Control A which have target values that are within the normal and abnormal ranges respectively. Berichrom Protein C Activator reagent, Berichrom Protein C Substrate reagent, Coagulation Control N and Coagulation Control A are lyophilised reagents and require reconstitution before use. 10ml of Berichrom Protein C Activator Diluent was added to the Berichrom Protein C Activator reagent. 3ml of distilled water was added to the Berichrom Protein C Substrate reagent and 1ml of distilled water was added to each of the control plasmas. The reagents were then held at room temperature for 15 minutes before use.

4.2.11 Antithrombin Activity

The measurement of the Antithrombin activity was an automated assay performed on the CS2100i analyser manufactured by Sysmex. Patient’s plasma (21μl) was added to 147μl of CA System Buffer in a plastic cuvette, 24μl of this dilution was subsequently transferred to a different cuvette. To this 175μl of Berichrom ATIII reagent THR was added after a 40 second incubation at 37ºC. The Berichrom ATIII reagent THR contains an excess of thrombin to which the antithrombin in the patients plasma binds. Thrombin bound to antithrombin is inactive; the amount of unbound active thrombin remaining following this reaction is therefore inversely proportional to the amount of antithrombin in the patient’s plasma. Following a further 190 second incubation at 37ºC 33μl of Berichrom ATIII Substrate reagent was added. The substrate reagent contains a chromogenic substrate consisting of a peptide bound to acid isopropylamide. The action of the unbound thrombin cleaves the peptide and releases the acid isopropylamide. This can be measured at 405nm. The reaction was allowed to proceed for 60 seconds, after this time the absorbance of the cuvette at 405nm was measured. The absorbance at 405nm is inversely proportional to the level of Antithrombin activity in the sample. The absorbance of the cuvette is converted to an activity via interpolation from a stored standard curve produced by measuring in duplicate 5 dilutions of SHP (which has a known antithrombin activity) and plotting the mean OD result against activity.
The assay was controlled using Coagulation Control N and Coagulation Control A which have target values that are within the normal and abnormal ranges respectively. Berichrom ATIII reagent THR, Berichrom ATIII Substrate reagent, Coagulation Control N and Coagulation Control A are lyophilised reagents and require reconstitution before use. 15ml of Berichrom ATIII reagent THR Diluent was added to the Berichrom ATIII reagent THR. 3ml of distilled water was added to the Berichrom ATIII Substrate reagent and 1ml of distilled water was added to each of the control plasmas. The reagents were then held at room temperature for 15 minutes before use.

4.2.12 Clotting Factor Activity
The measurement of clotting factor activities (FII, FV, FVII, FVIII, FIX, FX, FXI and FXII) were automated assays performed on the CS2100i analyser manufactured by Sysmex. Patient’s plasma (5μl) was added to 45μl of CA System Buffer in a plastic cuvette. To this an equal volume of Coagulation Factor Deficient plasma was added, the Coagulation Factor Deficient plasma used was dependent on the assay being performed; a factor II assay utilises coagulation factor II deficient plasma. Then either following a 190 second incubation at 37ºC 100μl of Innovin was added to the reaction mixture (FII, FV, FVII and FX) or following a 60 second incubation 50μl of Actin FS followed by 50μl of Calcium Chloride after a further 190 second incubation (FVIII, FIX, FXI and FXII) was added to the reaction mixture. This was then incubated at 37ºC for a further 180 seconds. During the incubation the transmission of light through the sample was measured at wavelength of 660nm. The conversion of fibrinogen to fibrin increases the turbidity of the sample and hence decreases the light transmission. Clot detection is defined as the time taken to reach the midway point in terms of light transmittance between the baseline and final transmission values. The clotting time is converted to a clotting factor activity via interpolation from a stored standard curve produced by measuring in duplicate 5 dilutions of SHP (which has a known factor activity) and plotting the mean clotting time in seconds against clotting factor activity. The analysis outlined above is repeated twice more with further dilutions of the patients plasma in CA System Buffer. The analysis described above is classed as a 1:1 dilution; the subsequent dilutions are 1:2 and 1:4. The three dilutions should yield the same level of clotting factor activity (when corrected for dilution) and should yield a line that runs parallel to the standard curve when plotted. The mean of the three dilutions is the reported value.
The assay was controlled using Coagulation Control N and Coagulation Control A which have target values that are within the normal and abnormal ranges respectively. The Coagulation Deficient plasmas, Coagulation Control N and Coagulation Control A are lyophilised reagents and require reconstitution before use. 1ml of distilled water was added to each of the reagents. The reagents were then held at room temperature for 15 minutes before use.

4.2.13 Free Protein S Antigen
The measurement of the Free Protein S antigen was an automated assay performed on the CS2100i analyser manufactured by Sysmex. Patient’s plasma (10μl) was added to 20μl of CA System Buffer in a plastic cuvette. To this 60μl of LIATEST® Free Protein S Buffer was added after a 40 second incubation at 37°C. Following a further 230 second incubation at 37°C 90μl of LIATEST® Free Protein S Latex was added. The latex reagent contains latex beads coated in antibodies against Free Protein S. The binding of the beads to Free Protein S in the patient’s plasma causes agglutination of the beads and subsequently leads to an increased absorbance of light by the cuvette which can be measured at 575nm. The reaction was allowed to proceed for 200 seconds, after this time the absorbance of the cuvette at 575nm was measured. The absorbance at 575nm is proportional to the level of Free Protein S Antigen in the sample. The absorbance of the cuvette is converted to a concentration via interpolation from a stored standard curve produced by measuring in duplicate 5 dilutions of Coagulation Reference Plasma (which has a known Free Protein S Antigen) and plotting the mean OD result against concentration.

The assay was controlled using Coagulation Control N and Coagulation Control A which have target values that are within the normal and abnormal ranges respectively. Coagulation Control N and Coagulation Control A are lyophilised reagents and require reconstitution before use. 1ml of distilled water was added to each of the control plasmas. The reagents were then held at room temperature for 15 minutes before use.

4.2.14 von Willebrand Factor Antigen
The measurement of the von Willebrand factor antigen was an automated assay performed on the CS2100i analyser manufactured by Sysmex. Patient’s plasma (15μl) was added to an equal volume of CA System Buffer in a plastic cuvette. To this 60μl of vWAgl Reagent was added after a 20 second incubation at 37°C. Following a further 250
second incubation at 37°C 90μl of vWAg Buffer was added. The vWAg Reagent contains polystyrene beads coated in antibodies against von Willebrand factor. The binding of the beads to von Willebrand factor in the patient’s plasma causes agglutination of the beads and subsequently leads to an increased absorbance of light by the cuvette which can be measured at 575nm. The reaction was allowed to proceed for 170 seconds, after this time the absorbance of the cuvette at 575nm was measured. The absorbance at 575nm is proportional to the level of von Willebrand factor antigen in the sample. The absorbance of the cuvette is converted to a concentration via interpolation from a stored standard curve produced by measuring in duplicate 5 dilutions of SHP (which has a known von Willebrand factor Antigen) and plotting the mean OD result against concentration.

The assay was controlled using Coagulation Control N and Coagulation Control A which have target values that are within the normal and abnormal ranges respectively. Coagulation Control N and Coagulation Control A are lyophilised reagents and require reconstitution before use. 1ml of distilled water was added to each of the control plasmas. vWAg Reagent requires the addition of 4ml of vWAg Reagent Buffer. The reagents were then held at room temperature for 15 minutes before use.

4.2.15 Flow Cytometry

Analysis was performed on the BD FACS Canto II with a standard set up using FACSDiva software version 6.1.2. 5μl of the appropriate antibodies were added to 50μl of patient’s plasma. This mixture was placed in the dark for 15 minutes at room temperature. Following this time the sample was diluted with the addition of 450μl of Tyrodes salt solution, which was previously filtered using a 0.2μm filter attached to a syringe. 10μl of Sphero AccuCount fluorescent particles were then added to the mixture followed by 1μl of 1.1μm beads. The sample was then gently agitated to ensure sufficient mixing prior to analysis. The sample was aspirated at a low flow rate. The cellular material was gated using forward and side scatter. Separate gates were established for both the 1.1μm beads and the Sphero AccuCount fluorescent particles. The sample was analysed until a total of 400 Sphero AccuCount fluorescent particles were acquired.

4.2.16 Statistical Analysis

Please refer to section 2.2.9 for details
## 4.2.17 Reagents

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<td>Beckman Coulter</td>
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<tr>
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<td>Berichrom Protein C Activator reagent</td>
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<td>vWAg Reagent Buffer</td>
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4.3 Results

4.3.1 Filtration of Control Plasma P

The first stage of analysis in this chapter is concerned with the filtration of control plasma P. This control plasma was used in the inter and intra assay variability experiments described in chapter 2 and yielded unacceptably poor results, in terms of both intra assay trends and high levels of inter assay variability. It was hypothesised that these results were possibly due to the presence of particulate matter in the reconstituted plasma. It is possible that the particulate matter implicated in this analysis includes the presence of microparticles which may have been unevenly reconstituted or distributed throughout the plasma and may have ultimately led to the high variation in the results.

Since microparticles cannot be removed via centrifugation at the forces generated by any of the centrifuges available in the haemostasis laboratory at the Royal London Hospital, it was decided to pass the plasma through a filter with apertures small enough to remove most of the microparticles in the plasma. Since microparticles typically range from 0.2-2µm in diameter (Piccin et al., 2007), a 0.2µm filter was chosen. In order to ascertain the effects of plasma filtration on the thrombin generation of Control plasma P, 48ml of Control plasma P was reconstituted and pooled. Half of this plasma was aliquoted into three 8ml aliquots and frozen at -70°C. The remainder was filtered as described in section 4.2.8 above with a view to freezing in three further 8ml aliquots. Due to the nature of the filters employed, which retained some of the sample volume and the magnitude of the particulate matter, which required the use of multiple filters, a large volume of sample was lost. Indeed the entire filtration process only yielded approximately 13ml of plasma. This was frozen at -70°C in two 6.5ml aliquots. It was decided to freeze the plasma in order to mimic the conditions of the earlier experimentation and to minimise the degeneration of the plasma between the runs. The aliquots of Control plasma P were defrosted one at a time by submersion in a 37°C water bath before analysis via the CAT thrombin generation assay as described in section 4.2.9 above. The analysis time for these experiments was 45 minutes. The non filtered plasma was analysed on three separate plates with 15 replicates on each plate, thus providing 45 data points. The filtered plasma was analysed on two separate plates with 13 replicates on each plate, providing a total of 26 data points. A comparison of the
thrombin generation parameters for the filtered and non filtered Control plasma P samples is shown in figure 4.1 below.

Figure 4.1

A. B.

C. D.

Figure 4.1. Boxplots showing a comparison of the results of the four thrombin generation parameters for both non filtered and filtered Control plasma P. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: Lag time, B: ETP, C: Peak height, D: Time to peak. P values derived using the student’s t-test or Mann Whitney U test as appropriate.

It is clear from figure 4.1 that filtration of Control plasma P had a significant impact on the results of the thrombin generation assay. There is a statistically significant reduction (p= <0.001) in all 4 of the thrombin generation parameters following filtration.
Filtration also reduced the CV of all four of the assayed parameters implying a reduction of variability following filtration. See table 4.1 below. It is of interest however that the CVs for the non filtered plasma are elevated when compared to the same experiment in chapter 2 (table 2.5). This effect may be due to the reduced number of data points in this experiment (45 verses 150).

Table 4.1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non Filtered CV (%)</th>
<th>Filtered CV (%)</th>
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<tr>
<td>Lag Time</td>
<td>21.1</td>
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<td>ETP</td>
<td>10.2</td>
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<td>Peak Height</td>
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<tr>
<td>Time to Peak</td>
<td>9.3</td>
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</table>

Table 4.1. CV values for each of the four thrombin generation parameters for both non filtered and filtered Control plasma P.

The reduction of variability seen post filtration would suggest that there is a decreased level of assay ‘noise’ when the filtered plasma is analysed, although this is a subjective parameter to quantify, it would appear to be the case. Figure 4.2 shows a comparison of typical thrombin generation curves taken from each population. The curves demonstrate the observed findings of plasma filtration for Control plasma P, with the filtered plasma showing a decrease in peak height, ETP, lag time and time to peak when compared to the non filtered plasma. The thrombin generation curve of the filtered plasma also appears smoother than that of the non filtered plasma implying a reduction of assay ‘noise’.
The results from the non filtered Control Plasma P were then analysed to see if there were any significant intra assay trends which were evident in the initial experiments carried out in chapter 2. Whilst there was no evidence of any trends for both the lag time and the time to peak parameters (data not shown) both the ETP and peak height parameters showed a significant trend of decreasing value with the order in which they were pipetted on to the plate (p= <0.001). The results of these analyses are shown in figure 4.3 below. Following these findings the filtered plasma was subsequently analysed in the same manner. The results of this analysis are shown in figure 4.4 below. Interestingly the filtered plasma despite an apparent decrease in variability still showed evidence of identical intra assay trends for both peak height and ETP (p= <0.01 and <0.001 respectively), implying that the decrease in assay noise does not ameliorate the intra assay trends as hypothesised in chapter 2 and that this observed phenomenon is an artefact of Control plasma P itself.
Figure 4.3

A.  

![Graph of Non Filtered Control Plasma P: Peak Height](image1)

B.  

![Graph of Non Filtered Control Plasma P: ETP](image2)

Figure 4.3. Graphs showing the values obtained from the analysis of 45 replicates of non filtered Control plasma P relative to the order in which they were added to the plate, complete with trend lines and $R^2$ as an indication of correlation. A: Peak Height, B: ETP

Figure 4.4

A.  

![Graph of Filtered Control Plasma P: Peak Height](image3)

B.  

![Graph of Filtered Control Plasma P: ETP](image4)

Figure 4.4. Graphs showing the values obtained from the analysis of 26 replicates of filtered Control plasma P relative to the order in which they were added to the plate, complete with trend lines and $R^2$ as an indication of correlation. A: Peak Height, B: ETP

An interesting finding regarding the above filtration experiments is the apparent changes which are observed in the thrombin generation analysis post filtration. The filtration step was introduced as an attempt to remove potentially pro-coagulant microparticles from the plasma, which may be influencing the results generated. Since it is possible any microparticles present have phosphatidylserine rich phospholipid membranes which may increase the rate and magnitude of thrombin generation as well as possibly expressing tissue factor which may also act to enhance thrombin generation, the removal of these microparticles would in theory be accompanied by a decrease in the thrombin generation potential of the sample. This hypothesis fits well with both the
reduction in peak height and ETP seen following the filtration of Control plasma P but not with the reduction of the lag time and time to peak parameters. The removal of pro-coagulant material from the sample would intuitively be accompanied by a prolongation of these times, not the observed reduction. A potential explanation for this is the possibility of mechanical contact activation of the plasma during the filtration process. Sample activation is typically accompanied by a reduction of clotting time (APTT) when measured in the laboratory and can be achieved via the taking of a blood sample with a syringe and the subsequent transferral of the blood sample into a vacutainer.

### 4.3.2 Filtration of Pooled Fresh Plasma

Following the filtration of the pool of Control plasma P it was decided to determine whether or not the effects observed post filtration were artefacts of the filtration of Control plasma P itself or whether a similar pattern is observed following the filtration of a pool of fresh patient plasma.

The plasma pool was obtained via the mixing of plasma from the samples of 80 patients in the Royal London hospital. All patients' samples selected had normal clotting screens as determined by the INR, APTT and Thrombin time, therefore excluding any patients known to be undergoing anticoagulant therapy. The patients' samples had initially been centrifuged at 1500g for 10 minutes. The top two thirds of the plasma from each of the samples was then removed and pooled. The plasma pool was subsequently aliquoted into LP4 tubes and centrifuged at 1500g for 10 minutes. Following this time the top two thirds of the plasma was removed from the LP4 tubes and pooled. This process yielded approximately 80ml of plasma. Half of this plasma pool was then separated into five 8ml aliquots and frozen at -70°C. The remaining half of the plasma was filtered as described in section 4.2.6. This filtration process resulted in a reduction in the volume of sample lost during filtration, when compared with the method described in section 4.2.8, and yielded four 8ml plasma aliquots which were subsequently frozen at -70°C. The pooled plasma aliquots were then thawed in a 37°C water bath and analysed separately using the CAT thrombin generation assay as described in section 4.2.9. A comparison of the thrombin generation parameters for the filtered and non filtered pooled plasma is shown in figure 4.5 below.
Figure 4.5

A.

**Plasma Pool Filtration: Lag Time**

![Boxplot of Lag Time](image)

B.

**Plasma Pool Filtration: ETP**

![Boxplot of ETP](image)

C.

**Plasma Pool Filtration: Peak Height**

![Boxplot of Peak Height](image)

D.

**Plasma Pool Filtration: Time to Peak**

![Boxplot of Time to Peak](image)

Figure 4.5. Boxplots showing a comparison of the results of the four thrombin generation parameters for both non-filtered and filtered pooled fresh plasma. Boxes show 25th to the 75th percentile of the data with the median shown as a horizontal black line. Whiskers show minimum/maximum values. Population outliers defined as values deviating by greater than 1.5 times box height are shown by *. A: Lag time, B: ETP, C: Peak height, D: Time to peak. P values derived using the student’s t-test or Mann Whitney U test as appropriate.

It is clear from the graphs that the results for the filtration of the fresh plasma pool are consistent with those found following the filtration of Control plasma P, i.e. there is a statistically significant decrease of all four thrombin generation parameters following filtration. Examples of the thrombin generation curves produced can be seen in figure 4.6. The curves for both the non-filtered and filtered plasma are typically smoother than those produced via the analysis of Control plasma P, again highlighting the increased assay ‘noise’ evident in Control plasma P analysis.
Interestingly the filtration of the pooled fresh plasma did not impact on the CVs of the thrombin generation parameters in the same manner as the filtration of Control plasma P, which all showed a reduction post filtration. The results of this analysis are shown in table 4.2. Whilst the CVs for both the ETP and peak height parameters are reduced following filtration of the fresh plasma pool, the CVs for the lag time and the time to peak parameters both show an increase post filtration. Neither the results from the filtered or non filtered plasma show much variation from the analysis of the fresh plasma pool discussed in chapter 2 (table 2.15). This implies that the differences in the variation of results following filtration are probably due to chance rather than any effect caused by the filtration process itself. When taken in comparison with the effect of filtration on Control plasma P, which appears to show a decrease in result variation post filtration (although this is by no means proof positive) these results imply that something further is removed from the Control plasma P during the filtration process, which is both different from that responsible for the observed changes in the thrombin generation parameters and absent from the fresh pooled plasma. It is most likely filtration removes particular matter in the Control plasma P reagent, possibly present via inefficient reconstitution of the reagent, which probably disrupts the fluorescent signal in thrombin generation analysis resulting in a reduction in variation post filtration.
Table 4.2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non Filtered CV (%)</th>
<th>Filtered CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time</td>
<td>5.6</td>
<td>8.2</td>
</tr>
<tr>
<td>ETP</td>
<td>6.4</td>
<td>5.0</td>
</tr>
<tr>
<td>Peak Height</td>
<td>5.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Time to Peak</td>
<td>3.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 4.2. CV values for each of the four thrombin generation parameters for both non filtered and filtered pooled fresh plasma.

4.3.3 Filtration of Non-Pooled Samples

Following the results obtained using both pools of commercially produced plasma and fresh patient plasma; it was decided to examine the effects of plasma filtration on individual patients in order to ascertain whether the patterns observed in the experiments described above still held. Although the findings of the first two experiments were both significant and consistent with one another, the fact remained that only two different plasma samples were tested (albeit a number of times each). In order to investigate whether the changes in thrombin generation parameters observed above were conserved in all individuals 19 samples from patients at the Royal London Hospital were analysed. The patients’ samples were prepared as described in section 4.2.5 above, although prior to freezing the plasma was divided into two equal sized aliquots one of which was filtered as described in section 4.2.6. This resulted in one filtered and one non filtered aliquot for each of the 19 patients. The samples were then frozen at -70°C. The patients’ samples were thawed in a 37°C water bath and the thrombin generation of the filtered/non filtered aliquots was subsequently measured as described in section 4.2.9. In order to reduce the impact of inter assay variation on the results both of a patients aliquots were analysed on the same run. The results of this analysis are shown in figure 4.7 below. These graphs show the correlation of the results for the 19 patients analysed for each of the four thrombin generation parameters. Each graph also shows a dashed line corresponding to X=Y, showing what the correlation would resemble if plasma filtration had no impact on the results. The position of the patients trendline relative to the X=Y line is indicative of the average change of the results post filtration. Since the filtered plasma results are shown on the Y axis the presence of the trendline above X=Y is suggestive of an increase of the results post
filtration, if the trendline is below \(X=Y\) it suggests the results are lower post filtration. From the graphs it appears that post filtration the majority of the 19 patients processed show a decreased ETP and peak height and an increase in both the lag time and the time to peak parameters. The average change in each of the parameters can be calculated by calculating the equation of the trendline; the intercept of the y axis is indicative of the magnitude of the change. A negative value is indicative of a decrease in the parameter post filtration where as a positive value indicates an increase post filtration. The y intercept of the trend line, the median change of the cohort of 19 individuals and range of the observed changes are shown for each of the four thrombin generation parameters in table 4.3 below. To calculate the median change, first the magnitude of the difference between a patient’s results pre and post filtration was derived by subtracting the result obtained pre filtration from the value obtained post filtration. The median was then calculated.

**Figure 4.7**

A.  

B.  

C.  

D.  

Figure 4.7 A series of graphs showing the correlation of the results of the CAT thrombin generation assay pre and post plasma filtration in a cohort of 19 individuals. The solid line is the trendline for the correlation of the results of the 19 patients, with \(R^2\) shown as an indication of correlation. The dashed line shows how the patients trend line would look if plasma filtration had no effect on the results. I.e. \(X=Y\). A: Lag Time, B: ETP, C: Peak Height and D: Time to Peak
Table 4.3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range of Change</th>
<th>Median Change</th>
<th>Y Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (Minutes)</td>
<td>-0.33 – 0.83</td>
<td>0.17</td>
<td>0.145</td>
</tr>
<tr>
<td>ETP (nM Thrombin)</td>
<td>-341 – 148</td>
<td>-81</td>
<td>-48</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>-42.8 – 28.0</td>
<td>-12.4</td>
<td>17.4</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>-0.33 – 1.16</td>
<td>0.17</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Table 4.3 A table detailing the range of change obtained in a cohort of 19 individuals for each of the four thrombin generation parameters pre and post plasma filtration. The table also shows the median change of the cohort and the y intercept of the trendlines drawn in figure 4.7 as a further indication of average change.

The average changes observed for the lag time, ETP and time to peak parameters, as defined by the median change and the y intercept, appear consistent in both trend and magnitude, this is not the case for the peak height where the average observed change as defined by the two methods appears contradictory. The median change appears to show a decrease in peak height post filtration and the y intercept implies an increase in peak height post filtration. The reason for this conflict is probably a result of the extrapolation of the trendline in order to calculate the intercept, since the trend line is not parallel to the X=Y line. This result implies that filtration has a greater impact on the reduction of the peak height parameter with increasing peak height. The patients’ trend line is indicative of the fact that any patients with peak height parameters below~140nM Thrombin are likely to demonstrate an increase post filtration. It is worthy of note however that a peak height of <140 nM Thrombin is a highly abnormal result (normal range 258-454 nM thrombin) and corresponds to the peak height generated by spiking heat inactivated plasma with 30% SHP (figure 2.15 B). The majority of patients would therefore demonstrate a decrease in peak height post filtration.

This experiment is suggestive of an average decrease in both ETP and peak height parameters as well as an average increase in both lag time and time to peak parameters post filtration. Using a paired t-test to compare the patients results pre and post filtration revealed that all of these trends were statistically significant (p= <0.01 for all comparisons). What is striking about this result is that whilst the observed post filtration
trends for both ETP and peak height are consistent with those observed in the pooled plasma experiments above, the trends for the lag time and time to peak parameters are not. The pooled plasma experiments above revealed decreasing lag time and time to peak parameters whilst the paired comparison of 19 patients reveals the average change as an increase in these parameters post filtration. The reason for this conflict is probably due to the fact that the paired comparison is based on an average change and although the majority of the patients analysed did follow the observed pattern, this was by no means true of all of the patients analysed. It is worthy of note that this is not just the case for the time to peak and lag time parameters but also the ETP and peak height measurements as well. That is some of the patients analysed in the paired comparison had elevated ETP and peak height parameters post filtration. Figure 4.8 shows Bland Altman plots (mean result of the pre and post filtration result for each patient plotted against magnitude of change) of the results for each of the thrombin generation parameters for the 19 patients analysed.

Figure 4.8

![Bland Altman plots](image)

Figure 4.8 A series of Bland Altman plots showing the mean result for each of the four thrombin generation parameters obtained pre and post filtration against the change post filtration for a cohort of 19 patients. A: Lag time, B: ETP, C: Peak Height, D: Time to Peak.
From figure 4.8 it is clear that although the majority of the patients analysed demonstrate the same pattern of change post filtration a handful of the patients analysed follow the opposite pattern. It is interesting that the pooled plasmas analysed in the above experiments demonstrate the post filtration pattern exhibited by the minority of individuals. The magnitude of any observed changes are also not consistent, this result is perhaps seen more clearly in figure 4.8 rather than in the trendlines of figure 4.7, which seem to show a high level of correlation consistent with a constant change in the results. It is important to note that the average post filtration changes observed in the paired comparison are consistent with the removal of pro-coagulant material (probably microparticles) following the filtration process, since the patients total capacity and maximum rate of thrombin production are reduced in conjunction with an increase in the time taken to initiate thrombin generation and the time to reach the maximum rate of thrombin generation. The fact that neither the magnitude of these changes or indeed the pattern of these changes is conserved implies that the material removed in the filtration process is not conserved from patient to patient. For example it may be the case that those patients who show the largest decreases in ETP following filtration are those who have the highest levels of tissue factor expressing microparticles, those patients who demonstrate decreased lag time and time to peak parameters may well have higher circulating levels of microparticles expressing negative regulators of haemostasis such as TFPI and Protein C.

One hypothesis which seems to be disproved by the paired comparison is the fact that the decreased lag time and time to peak parameters seen following plasma filtration in the pooled plasma experiments are due to plasma activation during the filtration process. Since this trend is not conserved in the 19 patients in the paired comparison this hypothesis does not seem likely. Indeed an analysis performed using a separate cohort of 20 individuals where the PT and APTT of the 20 plasma samples was measured pre and post filtration revealed no significant differences when compared using a paired t-test (data not shown).

4.3.4 Analysis of Plasma Pre and Post Filtration by Flow Cytometry

The next stage of the analysis was to examine the plasma of a cohort of patients pre and post filtration, with a view to discovering the changes in the number and type of
microparticles present following the filtration process. The ultimate aim was to correlate the microparticle changes in the patients’ plasma with the changes observed in their thrombin generation profiles following filtration. In order to ascertain the effectiveness of flow cytometry as a tool for measuring changes in microparticle numbers plasma samples from two patients were analysed pre and post filtration. The patients’ samples were prepared as described in section 4.2.5 above, although prior to freezing the plasma was divided into two equal sized aliquots one of which was filtered as described in section 4.2.6. The analysis was performed as described in section 4.2.15. Four different antibodies were used in this analysis:

- Anti-CD15 FITC, used to detect microparticles of monocyte origin
- Anti-CD42b (Glycoprotein 1b) PE, used to detect microparticles of platelet origin
- Anti-CD45 Pacific Blue, used to detect microparticles of Leukocyte origin
- Anti-Glycophorin A FITC, used to detect microparticles of erythroid origin

Since two of the antibodies utilise the fluorochrome FITC the plasma samples were measured in two separate tubes each containing a pair of antibodies. Anti CD42b and Glycophorin A were used in one tube and Anti-CD15 and CD45 in the other.

The use of the 1.1µm beads allowed the size of the particles present in the patients’ plasma to be determined. The initial gates were placed around the particulate material in the patients’ plasma, the 1.1µm beads and the spherocyt AccuCount Fluorescent particles. Since the 1.1µm beads form a discreet cloud in the analysis, the position of the beads could be use to position two further gates around the particular matter present. These gates were used to divide the cellular material present into two distinct groups with the gates bisecting the 1.1µm beads. These gates are P4 and P5 shown in figure 4.9 below. The material in gate P4 is smaller than 1.1µm in diameter whereas the material gated by P5 is greater that 1.1µm in diameter. The material gated by P4 is therefore more likely to contain exclusively microparticles; the material gated in P5 will contain some microparticles (up to 2µm in diameter) but may also contain some larger cell fragments or intact platelets.
Figure 4.9. Gating strategy for the flow cytometry analysis. P1: All microparticle events. P2: 1.1µm beads. P4: Microparticle events <1.1µm. P5: Microparticle events >1.1µm. P7: Sphero AccuCount Fluorescent particles

It can be seen from figure 4.9 that a distinct population of particulate matter was excluded from the P1 gate and therefore from further analysis. It was felt that this population was likely to be contamination as a result of incomplete removal of particulate matter from the tyrodes salt solution. The material present in gates P4 and P5 was then analysed to in order to ascertain how many of the events were positive for the different antibodies. Example plots are shown in figures 4.10 and 4.11 below.
Figure 4.10. Comparison of flow cytometry results for a patient’s plasma pre and post filtration. Blue plots represent material in gate P4. Orange plots represent material in gate P5. Material in gates Q1-2 and Q1-3 is positive for CD42b expression. Material in gates Q4-2 and Q4-3 is positive for glycophorin A expression. Material in Q3-2 and Q3-3 is negative for both CD42b and Glycophorin A. Material in gates Q2-2 and Q2-3 express both CD42b and Glycophorin A. A: Plasma pre filtration. B: plasma post filtration.
It can be clearly seen from figure 4.10 that filtration of a patient’s plasma results in a dramatic decrease in the number of both CD42b and Glycophorin A positive events. This implies a reduction in the number of both platelet and erythroid derived microparticles. The results in figure 4.11 are much less dramatic with the number of CD15 positive events showing little change post filtration and the number of CD45 positive events increasing slightly. This result is unexpected as it implies the filtration process has little or no effect on the number of monocyte derived microparticles and
may act to enhance the numbers of leukocyte derived microparticles. It is important to note that figures 4.10 and 4.11 are a visual means of assessing the results of filtration on microparticle numbers and are therefore subjective. The actual number of microparticles were calculated using Sphero™ AccuCount Fluorescent particles produced by Spherotech. 10µl of Sphero™ AccuCount Fluorescent particles were added to each of the samples to be analysed by flow cytometry. Since the beads were of a known size (10µm) and therefore appear as a discreet cloud when analysed, they can be gated and counted accurately (gate P7 in figure 4.9). The numbers of microparticles can be calculated using the following equation:

Number of cells per µl of test sample = (A/B)x(C/D)

Where:
A= number of events in the test sample
B= number of events for AccuCount Fluorescent particles
C= number of AccuCount Fluorescent particles per 10µl (10^15)
D= volume of test sample initially used

Tables 4.4 and 4.5 show the numbers of microparticles of each origin pre and post filtration for the sample analysed above for the material gated in P4 and P5 respectively.

Table 4.4

<table>
<thead>
<tr>
<th>Microparticle Origin</th>
<th>Number per µl Pre Filtration</th>
<th>Number per µl Post Filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet</td>
<td>2640</td>
<td>15</td>
</tr>
<tr>
<td>Erythroid</td>
<td>745</td>
<td>28</td>
</tr>
<tr>
<td>Leukocyte (All types)</td>
<td>37</td>
<td>92</td>
</tr>
<tr>
<td>Monocyte</td>
<td>27</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.4. Number of microparticles per µl from gate P4 (<1.1µm in diameter) of each cellular origin in the plasma of a patient pre and post filtration.
Table 4.5

<table>
<thead>
<tr>
<th>Microparticle Origin</th>
<th>Number per µl Pre Filtration</th>
<th>Number per µl Post Filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet</td>
<td>493</td>
<td>4</td>
</tr>
<tr>
<td>Erythroid</td>
<td>157</td>
<td>7</td>
</tr>
<tr>
<td>Leukocyte (All types)</td>
<td>21</td>
<td>41</td>
</tr>
<tr>
<td>Monocyte</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 4.5. Number of microparticles per µl from gate P5 (>1.1µm in diameter) of each cellular origin in the plasma of a patient pre and post filtration.

The data analysis presented in tables 4.4 and 4.5 is consistent with the flow cytometry plots in figures 4.10 and 4.11 above, with the number of platelet and erythroid derived microparticles showing a large reduction post filtration whereas microparticles of monocyte origin seem to be unaffected following the filtration process. The most surprising result is the fact that the number of leukocyte derived microparticles seems to show an increase post filtration. The pattern of change is also conserved between the material gated in P4 and P5. It was a concern that the P5 gate may contain a large number of cellular fragments which were not ‘true’ microparticles and that these could not be excluded in this analysis and would impact upon the results. The conservation of results between the groups would imply this is not the case, although it is by no means proof positive.

Another concern was that the filtration process itself would result in the shearing of the cellular material in the sample and lead to a large increase in the level of cellular material in gate P4 post filtration. This however seems not to be the case in this pilot study as the percentage of events in gate P4 appears conserved post filtration in both tubes analysed (data not shown). The results observed in this study also confirm those published in the literature. Flaumenhaft (2006) reported that platelet derived microparticles are the most abundant in circulation and constitute 70-90% of the total number. In the example shown above platelet derived microparticles constitute 77% and 73% of the total number in gates P4 and P5 respectively.

A number of interesting and unanticipated results were found in this pilot study however. The first of these was the presence of microparticles greater than 0.2µm in
diameter post filtration. The filters used in this experiment were 0.2µm in diameter and therefore should have removed all material greater in diameter than this. This is clearly not the case as there are number of events in gate P5 post filtration which are positive for the cellular antigens used in this assay in both of the tubes analysed. The fact that they are positive for these antigens implies that they are not simply a result of particular contamination from the buffer used in this experiment. This is suggestive of the fact that microparticles demonstrate an ability to alter their shape and pass through the filter apertures without an apparent loss of surface antigen expression. This goes some way to explaining one of the other aberrant findings of this experiment which is the increase in the number of leukocyte microparticles and the conservation of the number of microparticles of monocyte origin post filtration. These results are consistent with the hypothesis that these microparticles can alter their shape to pass through the filter unhindered. What is interesting regarding this study is that there are very few events which are dual positives for CD15 and CD45 implying that the monocyte derived microparticles are a distinct population compared to those of other leukocyte origins.

A further point of note is the change in the number of erythroid microparticles post filtration. Piccin et al. (2007) reported the average size of erythroid microparticles as 0.15µm in diameter. The results in this study do not support these findings. If this were the case, the red cell derived microparticles would pass through the filter unhindered leading to a conservation of the numbers of erythroid microparticles pre and post filtration. The results presented above show a marked decrease in erythroid microparticle numbers post filtration eluding to the fact that they must be large enough to be removed by the filtration process (>0.2µm in diameter).The samples used in this experimentation showed no evidence of haemolysis therefore suggesting that the result obtained in this study was not simply an artefact of filtering red cell fragments from the patients plasma.

It is of importance to note that the discussion presented above is based on a very small sample size (n=2) and may therefore not be representative of the results of a larger cohort. What this study has revealed is that the analysis of microparticles by flow cytometry can accurately determine the numbers of microparticles pre and post filtration and therefore can be used to examine a larger cohort of patients.
4.3.5 Correlation of Thrombin Generation and Microparticle Numbers

The next stage of experimentation was to compare the changes observed in the thrombin generation profile of a cohort of individuals’ pre and post filtration with the change in the numbers of microparticles present in the same samples pre and post filtration. This would allow us to determine whether or not it is the removal of microparticles from the patients' plasma during the filtration process which causes the changes observed in the thrombin generation profile of patients. This experiment would also determine whether microparticles of different cellular origins have a larger impact on thrombin generation than others.

A cohort of 35 patients was used in this experiment. The patients’ samples were prepared as described in section 4.2.5 above, although prior to freezing the plasma was divided into two equal sized aliquots one of which was filtered as described in section 4.2.6. Samples were defrosted in a 37°C water bath prior to analysis. Thrombin generation was performed as described in section 4.2.9. The pre and post samples of each individual were analysed on the same plate in order to ameliorate any effects of inter assay variation on the results generated. Microparticle analysis was performed by flow cytometry as described in section 4.2.15, with the exceptions that the volume of patients' plasma used in the analysis was decreased to 10µl and the volume of SpheriAccuCount fluorescent particles was increased to 25µl. The antibodies used included those described above and Annexin V FITC. Annexin V has high affinity for binding to phosphatidylserine and therefore the degree of Annexin V binding should correlate with total microparticle numbers. The data from this study is shown in Macey et al., 2010b

The median change in the thrombin generation parameters of this cohort post filtration were analogous to those described in table 4.3 above with the vast majority of the cohort manifesting a decrease in both the ETP and peak height parameters of similar magnitudes to those described above. The majority of the cohort also demonstrated either an increase or no change in the lag time and time to peak parameters. Although as detailed above not all patients followed these patterns closely, with some displaying decreasing lag time and time to peak parameters and others increasing ETP and peak heights post filtration.
The variation in the change of microparticle numbers following filtration was marked. All patients showed a decrease in the numbers of both platelet derived (CD42b positive) microparticles and total number of microparticles (Annexin V binding) although the magnitude of these decreases varied dramatically between individuals. One patient demonstrated a modest decrease of 188 CD42b positive events per µl of plasma post filtration whereas the highest change was a decrease of 32320 per µl of plasma. Almost all of the patients analysed (33/35) showed a decrease in the number of microparticles of erythroid origin following filtration, interestingly this was also the case for the CD15 positive microparticles with 30 of the 35 individuals analysed showing a decrease in numbers following filtration. This is in contrast to the pilot study above where the numbers of CD15 positive microparticles were conserved following filtration. The change in the number of microparticles of leukocyte origin (CD45 positive) showed the most variation between individuals in terms of the pattern of change (although not the magnitude of change) post filtration, with 40% of individuals showing an increase in numbers following the filtration process and the remainder showing a decrease in number. The largest increase was 255 CD45 positive microparticles per µl of plasma whereas the biggest decrease was 1122 per µl of plasma. The median change was a decrease of 40 CD45 positive events per µl of plasma.

The most important findings of this analysis were the correlations between the changes in the thrombin generation parameters and the changes in the number of microparticles of different cellular origins. The change in ETP following filtration showed no statistically significant correlations with the changes in the number of microparticles of any origin with the exception of monocyte derived (CD15 positive) microparticles, with which there was a highly significant correlation (p= <0.001) of decreasing ETP with decreasing numbers of CD15 positive events.

The change in peak height post filtration showed a number of statistically significant correlations with changes in microparticle numbers following filtration, with decreasing peak height showing significant correlations with decreasing CD42b positive events (p=≤0.05), Annexin V positive events (p=≤0.01) and CD15 positive events (p=≤0.01). The strongest correlation was however that between decreasing peak height and decreasing numbers of CD15 positive events, which had an R² value of 0.28.
Interestingly both the change in the lag time and time to peak parameters post filtration showed statistically significant correlations with the same microparticle measurements, namely the numbers of CD42b positive and Annexin V positive events (p= <0.05 for both lag time correlations and p= <0.01 for both correlations with time to peak). The pattern of these changes was also conserved with decreasing thrombin generation parameters correlating with decreasing numbers of microparticle events.

The results of this experimentation are promising since they are suggestive of the fact that the measurement of thrombin generation using the CAT assay is sensitive to the presence of microparticles in a patients plasma and that microparticles of different cellular origins have a greater impact on some thrombin generation parameters than they do on others. It is evident from the analysis that the number of CD15 positive (monocyte derived) microparticles has the biggest impact on both the ETP and peak height parameters, whereas the lag time and time to peak parameters are primarily affected by the number of CD42b positive and Annexin V positive events.

4.3.6 The Effects of Plasma Filtration

Although the previous experiment suggests a good correlation between the changes in thrombin generation and the change in the numbers of microparticles post filtration it is conceivable that the correlation is incidental and not causative i.e. something other than the change in microparticle numbers is responsible for the change in an individual’s capacity to generate thrombin following plasma filtration. This was hypothesised since it was unknown whether it was realistically possible to expect the removal of microparticles to have such a large impact in terms of the change in thrombin generation given the relatively high concentrations of tissue factor and phospholipids employed in the reaction. One possibility is that the filtration process may change some of the plasma levels of clotting factors or negative regulators of haemostasis either by mechanical filtration or by adsorbing these clotting factors onto the filter itself.

In order to test this hypothesis 10 plasma pools were prepared. Each pool consisted of four samples taken from patients at The Royal London Hospital. All patients samples selected had normal clotting screens as determined by the INR, APTT and Thrombin time, therefore excluding any patients known to be undergoing anticoagulant therapy.
The samples were spun at 1500g for 10 minutes and the top two thirds of the plasma from each of the four samples were subsequently pooled. Following this the pooled plasma was spun for a further 10 minutes at 1500g. The top two thirds of this double spun plasma were divided into two aliquots one of which was filtered as described in section 4.2.6. The two aliquots were then frozen at -70°C. This process yielded 20 different plasma samples: one filtered and one non-filtered aliquot from each of the ten pools. The samples were subsequently thawed by immersion in a 37°C water bath and analysed for Antithrombin activity, Protein C activity, Free Protein S antigen, von Willebrand Factor antigen and clotting factor activities for factors II, V, VII, VIII, IX, X, XI and XII as described in sections 4.2.10 - 4.2.14. All 20 plasma samples were analysed using the same reagents in order to reduce the effects of inter assay variation on the results. The results obtained pre and post filtration from each of these analyses were compared using a paired t-test. Boxplots showing the results generated from these analyses as well as the p values obtained from the paired t-test comparisons are shown in figure 4.12 below.

Figure 4.12
C. **Plasma Filtration: Free Protein S Antigen**

![Box plot for Free Protein S Antigen](image)

Non Filtered: 70.00 - 100.00
Filtered: 70.00 - 100.00

**P = 0.209**

D. **Plasma Filtration: von Willebrand Factor Antigen**

![Box plot for von Willebrand Factor Antigen](image)

Non Filtered: 200.00 - 300.00
Filtered: 200.00 - 300.00

**P = 0.103**

E. **Plasma Filtration: Factor II Activity**

![Box plot for Factor II Activity](image)

Non Filtered: 70.00 - 90.00
Filtered: 70.00 - 90.00

**P = 0.607**

F. **Plasma Filtration: Factor V Activity**

![Box plot for Factor V Activity](image)

Non Filtered: 120.00 - 140.00
Filtered: 120.00 - 140.00

**P = 0.463**

G. **Plasma Filtration: Factor VII Activity**

![Box plot for Factor VII Activity](image)

Non Filtered: 50.00 - 70.00
Filtered: 50.00 - 70.00

**P = 0.444**

H. **Plasma Filtration: Factor VIII Activity**

![Box plot for Factor VIII Activity](image)

Non Filtered: 100.00 - 200.00
Filtered: 100.00 - 200.00

**P = 0.008**
It is clear from Figure 4.12 that the filtration of plasma as described in section 4.2.6 has no statistically significant effect on the Antithrombin Activity, Protein C activity, Free Protein S antigen, von Willebrand factor antigen, factor II activity, factor V activity, factor VII activity, factor IX activity and factor X activity in this cohort of plasma samples. There was however statistically significant trends in the factor VIII activity, factor XI activity and the factor XII activity post filtration. The changes in the levels of
factors XI and XII post filtration, despite being statistically significant, are unlikely to be responsible for the apparent change in the thrombin generation following filtration, due to the small magnitude of difference in the results pre and post filtration (a mean increase of 1.36 iu/dL for factor XI and an increase of 1.74 iu/dL for factor XII). It is also worthy of note that an increase in the levels of clotting factors post filtration would not be associated with decreasing ETP and peak height parameters or indeed increasing lag time and time to peak parameters following filtration. Intuitively these changes would be caused due to decreasing levels of clotting factors following the filtration process or indeed an increase of the negative regulators of haemostasis (Antithrombin activity, Protein C activity or Free Protein S antigen). The change in the Factor VIII activity following filtration (a mean decrease of 9.02 iu/dL) is consistent with the changes in the thrombin generation parameters most commonly seen following the filtration process. The most likely cause for this decrease is the removal of vWF (a plasma carrier of factor VIII) from the samples following filtration. Although there was no statistically significant difference in the plasma levels of vWF following filtration there is a mean decrease post filtration and the difference is approaching significance. vWF is a large multimeric protein and could be of sufficient size to be affected by the filtration process. What is uncertain in this analysis is whether or not the change in the level of factor VIII activity post filtration is of sufficient magnitude to cause the change in the levels of thrombin generation seen in the prior experimentation. It is interesting to note that the plasma pools prepared in this experiment all have very high levels of factor VIII activity (possibly due to acute phase reactions), indeed the mean factor VIII activity in the cohort prior to filtration was 225.9 iu/dL, with normal levels typically between 50-150 iu/dL. The mean change is therefore a decrease of ~4% which is actually consistent with the intra assay variation of the assay. The fact that the majority of the samples show a decrease of factor VIII activity post filtration is probably indicative that this is more than just an artefact of intra assay variation. Evidence provided in the literature (Al Dieri et al., 2002) shows that there is a hyperbolic relationship between the levels of clotting factor activity and the ETP, therefore suggesting that a difference of 4% in the level of factor VIII activity should have little impact on the level of thrombin generation, especially when the plasma level of factor VIII activity is so great. Indeed further evidence for this hypothesis is provided in chapter 2 where there is little difference in the plasma generating capacity of SHP when the clotting factor levels reached >90% of normal. In order to ascertain whether or not
the change in the levels of factor VIII activity seen post filtration in this experiment could be responsible for the changes in the thrombin generation parameters seen following filtration described previously in this chapter. It was decided to measure the thrombin generation of the 10 plasma pools pre and post filtration. The changes in thrombin generation would then be correlated with the changes in the factor VIII activity and examined to see if there was any evidence of statistically significant trends. The measurement of thrombin generation was performed as described in section 4.2.9. The change in each parameter following filtration was calculated by subtracting the result obtained prior to filtration from that obtained post filtration. The results of the correlation analysis are shown in figure 4.13 below.

Figure 4.13

A. Correlation of Lag Time and FVIII Activity

B. Correlation of ETP and FVIII Activity

C. Correlation of Peak Height and FVIII Activity

D. Correlation of Time to Peak and FVIII Activity

Figure 4.13. A series of graphs showing the correlation between the change in each of the four thrombin generation parameters post filtration with the change in factor VIII activity post filtration in a cohort of 10 plasma pools. Trend lines and $R^2$ are shown as an indication of correlation. A: Lag Time, B: ETP, C: Peak Height, D: Time to Peak

Although it is clear from figure 4.13 that there appears to be a degree of correlation between the change in factor VIII activity post filtration with the change in the ETP, peak height and time to peak parameters post filtration, none of these trends is
statistically significant when the p values of the correlations are determined as described in section 2.3.1.4. It is worthy of note that the number of samples in these correlations is quite small and therefore there may not be sufficient power to establish whether these trends are indeed significant. Even if a larger number of samples could prove a statistically significant correlation it is apparent that the ‘direction’ of the correlations as shown by the trend lines are not supportive of the change in factor VIII activity being causative of a decrease in either the ETP or peak height or indeed an increase in lag time and time to peak post filtration, since the correlations imply the opposite trend i.e. the samples with the biggest decrease in factor VIII are those with the smallest change in ETP/ peak height and the largest decrease in lag time and time to peak parameters. These correlations therefore provide good evidence that the apparent change in factor VIII activity post filtration is not causative of the changes seen in an individual’s thrombin generation following filtration.

4.3.7 The Thrombogenicity of Microparticles

Since the experiments performed in the previous section are suggestive of the fact that the filtration process does not cause changes in the plasma levels of clotting factors which are responsible for the changes apparent in the thrombin generation parameters post filtration, it is increasingly likely that the original hypothesis that these changes are caused via the removal of microparticles is correct. To provide further evidence for this hypothesis it was decided to try and determine whether or not microparticles could initiate thrombin generation and therefore provide proof that the presence or absence of microparticles could impact upon the thrombin generation capacity of a patient’s plasma.

In order to establish the impact which microparticles could have on thrombin generation in the CAT assay it was decided to see if concentrated microparticles could be used to initiate thrombin generation in a patients plasma in the absence of PPP reagent (without the addition of either exogenous tissue factor or phospholipids). This analysis would also therefore establish beyond doubt whether or not microparticles express tissue factor. For this experiment two 4.5ml 0.109M tri-sodium citrate samples spiked with 77µl of 1.3mg/ml CTI were taken from a cohort of 6 healthy volunteers. The use of CTI is essential in these experiments given that the concentration of tissue factor and
phospholipids is very low, the assay would be exceedingly sensitive to contact activation. The samples were then double spun as described in section 4.2.5 although the samples were not subsequently frozen. The plasma collected was then ultra centrifuged at 14000rpm in order to pellet the microparticles and therefore produce microparticle free plasma. The microparticle free plasma was removed from the pellet using a Pasteur pipette, leaving a residual volume of approximately ~100µl of plasma. The microparticle pellet was then re-suspended via the addition of a further 400µl of microparticle free plasma. This process yielded one aliquot of microparticle free plasma and one aliquot of microparticle rich plasma from each of the 6 volunteers. The final concentration of microparticles in the microparticle rich plasma was approximately 10 times that usually found in the patients plasma. The concentration of microparticles was necessary since it was reported by Butenas et al. (2005) that the concentration of tissue factor on the surface of microparticles in the circulation of a healthy patient whose blood remains unstimulated with cytokines is below the threshold needed to initiate in vivo thrombin generation. Following the preparation of the plasma samples the thrombin generation was measured as described in section 4.2.9 with the exception that 20µl of CA system buffer was added to the thrombin generation measurement wells instead of PPP reagent. The analysis time for this experiment was 120 minutes. All 12 plasma samples were analysed on a single plate in order to minimise the effects of inter assay variation. As the analysis proceeded it was clear that all 6 of the microparticle rich plasmas demonstrated thrombin generation curves whereas all 6 of the microparticle poor plasma samples failed to generate recognisable thrombin generation curves. An example of the curves produced following the analysis of one of the patients is shown in figure 4.14 below.

What is apparent from figure 4.14 is that the thrombin generation curve produced following the analysis of the microparticle rich plasma is distinctly different from the curve produced following the analysis of the microparticle poor plasma. This provides compelling evidence for the fact that microparticles can initiate thrombin generation in the CAT thrombin generation assay and therefore must express tissue factor. Unfortunately none of the curves produced by the measurement of any of the 6 microparticle rich plasma samples was complete (the level of thrombin generation did not return to zero over the time course of the reaction) and therefore it is impossible to calculate the ETP and ultimately quantify the impact that the presence of microparticles
has on thrombin generation. The cause of this apparent failure is probably an artefact of the low levels of thrombin generation in these samples rather than a truncated measurement time (personal communication). The median thrombin generation parameters as well as the range of results obtained following this analysis are shown in table 4.6. It is immediately clear from the results that the microparticle rich plasma samples had a higher maximum rate of thrombin generation, a shorter time to the initiation of thrombin generation and a shorter time until the maximum rate of thrombin generation was achieved when compared to the microparticle poor plasma. Indeed these differences are all statistically significant when analysed using the Mann Whitney U test (data not shown). The actual differences between the results of the two cohorts may be even greater than table 4.6 actually suggests since some of the measurements in the microparticle poor plasma cohort are very spurious and are likely caused by mathematical quirks in the analysis software rather than any thrombin generation per se.

Figure 4.14

![Microparticles and Thrombin Generation](image)

Figure 4.14. Example thrombin generation curves produced when measuring microparticle poor plasma and microparticle rich plasma using the CAT thrombin generation assay without the addition of exogenous tissue factor or phospholipids.
An example of this is demonstrated by the lag time of 6.67 minutes in the microparticle poor plasma cohort, this result relates to the thrombin generation curve shown in figure 4.14, which shows little or no thrombin generation at all. Indeed four of the 6 samples in the microparticle poor cohort demonstrated flat curves and therefore no thrombin generation data due to the ‘flat curve error’ discussed in chapter 2. It is worthy of note that some of the values attained in this phase of experimentation do fall below the lower limit of quantification as discussed in chapter two. These limits were established using different concentrations of tissue factor and phospholipids as reaction triggers and without the addition of CTI and are therefore not strictly applicable in this case.

Table 4.6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Microparticle Poor</th>
<th>Microparticle Rich</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Lag Time (Minutes)</td>
<td>&gt;120</td>
<td>6.67 - &gt;120</td>
</tr>
<tr>
<td>Peak Height (nM Thrombin)</td>
<td>0</td>
<td>0 – 3.85</td>
</tr>
<tr>
<td>Time to Peak (Minutes)</td>
<td>&gt;120</td>
<td>74.67 - &gt;120</td>
</tr>
</tbody>
</table>

Table 4.6. The median and range of results produced for each of the thrombin generation parameters following the analysis of microparticle poor plasma and microparticle rich plasma using the CAT thrombin generation assay without the addition of exogenous tissue factor or phospholipids.

What is apparent following this phase of experimentation is that microparticles not only express tissue factor as evidenced by the initiation of thrombin generation in the above experiment but the presence of microparticles in a patient’s plasma is likely to have an impact on a patient’s capacity to generate thrombin. Taken with the results from the previous section the results presented here provide solid evidence that the changes seen in thrombin generation following the filtration process are likely to be caused by the removal of microparticles during filtration.

4.4 Discussion
The initial aim of this chapter was to ascertain the effects of microparticles on thrombin generation as measured by the CAT assay. The starting place for this investigation was the analysis of Control plasma P, a commercially produced lyophilised plasma product initially used to assess both the inter and intra assay variability of the CAT assay (see chapter 2). These experiments were unsuccessful due to unacceptably high levels of inter assay variability and the presence of intra assay trends (decreasing peak height and increasing lag time with plate position). It was thought that the reason for this apparent variability was the presence of particular matter in the Control plasma P itself, which could include microparticles. Due to their procoagulant nature, inefficient or incomplete reconstitution of the plasma leading to uneven distribution of microparticles could be responsible for some of the variation seen in the results. The filtration of Control plasma P did result in a significant decrease in the variability of the results produced by the CAT assay, reducing the CV of the ETP by almost half from 10.2% to 5.4%, with similarly profound decreases in the variation of the peak height and time to peak parameters (45% and 40% decreases respectively). The decrease in CV for the lag time post filtration was the most modest at 23%. These decreases in variability were accompanied by a decrease in assay ‘noise’, with the production of smoother thrombin generation curves as shown in figure 4.2. The filtration of the Control plasma P also produced a visible effect on the plasma in that it became less opaque, although this was not formally quantified. These changes are all consistent with the removal of particulate matter from the plasma via the filtration process. Whether the particulate matter removed contained microparticles which interfered with the analysis is still unclear. The effects of plasma filtration on the results of the thrombin generation assay (statistically significant reductions of all parameters) were conserved between the filtration of Control plasma P and pooled fresh plasma but the fresh plasma pool showed no apparent decrease in variation post filtration. This finding is suggestive of the fact that the cause of the changes in the thrombin generation parameters is distinct from the cause of the decreased variability seen in the Control plasma P. It is most likely therefore that the high degree of variability witnessed in the analysis of Control plasma P is caused by disruption of the fluorescent signal generated by the CAT assay by particulate matter present in the plasma following incomplete reconstitution. The removal of this matter via filtration reduces the assay ‘noise’ and therefore decreases the variability seen in the results. What is interesting regarding this analysis is the fact that
plasma filtration does not ameliorate the intra assay trends prevalent in the analysis of Control plasma P. There was evidence of both decreasing ETP and peak height with increasing plate position in both the filtered and non-filtered Control plasma P in the experiments described in this chapter. These effects are most likely due to some other inherent artefact of the Control plasma P. One potential cause is a predisposition of Control plasma P to calcium independent contact activation with increasing incubation time. Plasma activation is known to lead to an over estimation of ETP (van Veen et al., 2009a), although this is most prevalent in the use of a 1 pM concentration of tissue factor as a reaction trigger in the absence of CTI. This hypothesis can also potentially explain the increase in the lag time with increasing plate position seen in chapter 2. What is evident is that this effect cannot be ameliorated by decreasing the incubation time by the magnitude achieved using an electronic pipette.

The initial filtration experiments performed on both Control plasma P and pooled fresh plasma demonstrated that the filtration of plasma resulted in statistically significant changes in the measured thrombin generation parameters, whilst it was assumed that the removal of pro-coagulant microparticles was responsible for this change, the observed pattern in results did not fit neatly with this hypothesis. Since microparticles have membranes rich in phosphatidylserine which has been shown to allow optimisation of the reaction kinetics for the generation of thrombin (Morel et al., 2006) and can also express tissue factor (del Conde et al., 2005b) (Hron et al., 2007) it was thought that microparticle removal following filtration would ultimately reduce an individuals capacity to produce thrombin and decrease their maximum rate of thrombin production as well as potentially increasing the time taken for the initiation of thrombin generation and the time taken to attain the maximum rate of thrombin production. Whilst the first part of this hypothesis was consistent with the observed results (decreased ETP and peak height following filtration), the observed reduction of both the lag time and time to peak parameters post filtration were unexpected. Whilst it was initially hypothesised that the reduction of these times following filtration was due to sample activation during the filtration process, this theory was discredited by the fact that there were no significant differences in either the PT or APTT measured on plasma taken from a cohort of individuals pre and post filtration. The most likely cause for the decrease in lag time and time to peak parameters post filtration was therefore the removal of negative regulators of haemostasis such as TFPI, thrombomodulin and Protein C all of
which have been reported to be expressed by microparticles (Steppich et al., 2005) (Satta et al., 1997). Intuitively the removal of a negative regulator of haemostasis would also lead to an increase in the ETP and peak height parameters rather than the observed reduction. This however need not necessarily be the case as TFPI is primarily involved in the inhibition of the initiation phase of coagulation, when less than 5% of the total thrombin is formed (Hemker and Béguin, 1995). The removal of microparticles expressing TFPI may therefore have a much greater impact on the initiation of thrombin generation rather than the total level of thrombin produced.

The analysis of non-pooled plasma samples however revealed that whilst the majority of individuals did manifest a reduction in both ETP and peak height parameters following filtration, consistent with the removal of pro-coagulant material, the changes in the lag time and time to peak parameters demonstrated by the two pooled plasma experiments displayed a pattern of change (decrease post filtration) which is evident in a minority of individuals. The majority of subjects in the non-pooled analysis showed an increase in both lag time and time to peak post filtration, which is again consistent with the removal of procoagulant material from their plasma during the filtration process. This analysis was profound as it revealed that the magnitude of change and even the pattern of change in thrombin generation parameters post filtration varied markedly between individuals. This variation is suggestive but certainly not proof positive of the fact that different individuals not only have varying numbers of circulating microparticles but also varying numbers of microparticles of a pro-coagulant nature (expressing tissue factor) as well as those expressing negative regulators of haemostasis.

The measurement of microparticle numbers by flow cytometry in the pilot study revealed that microparticles could be accurately quantified and that they were removed by plasma filtration. This experiment therefore provided circumstantial evidence that the changes in the number of microparticles could be responsible for the changes seen in the measurement of thrombin generation post filtration. The pilot study also highlighted a number of interesting results which were confirmed in the analysis of a larger cohort of individuals. The first of these was the apparent increase in the number of CD45 positive events post filtration. Whilst this finding was by no means the case in all individuals it was a pattern observed in 40% of those patients analysed. The reason for this phenomenon is unknown, a potential explanation is the shearing of CD45 positive
microparticles during the filtration process as this would cause an increase in the numbers of CD45 positive events in gate P4 following filtration. Although if this was the case it would be likely to be accompanied by a decrease in the number of CD45 positive microparticles in gate P5 and this was not the case in these individuals. The increase in CD45 positive microparticles following filtration is potentially indicative of the fact that microparticles are capable of undergoing changes in conformation to facilitate passage through the filter membrane without manifesting an apparent loss of antigen expression, this theory is given weight by the fact that microparticles greater than 0.2μm in diameter can be detected in plasma following the filtration process. One of the most likely reasons for the apparent changes in CD45 expression may be the fact that the mechanical process of passing through the filter can cause a change in the expression levels of CD45. Although a mechanism for this change has yet to be elucidated.

One of the other unanticipated results highlighted by the flow cytometry analysis was the decrease in the number of erythroid microparticles following filtration. It was anticipated that since microparticles of erythroid origin are described in the literature as typically smaller than 0.15μm in diameter (Piccin et al., 2007) they would not be removed via filtration as the filter apertures were 0.2μm in diameter. A total of 33 of the 35 patients analysed by flow cytometry showed evidence of decreasing numbers of erythroid microparticles following plasma filtration, implying that they are removed during the filtration process and that by extension erythroid microparticles must be larger than 0.2μm. The results of these analyses do not therefore support the findings of Piccin et al. (2007).

Other than the results presented above the filtration of plasma produced the anticipated effects on the numbers of microparticles, with decreasing levels of CD42b positive, CD15 positive and Annexin V positive events post filtration, consistent with the removal of platelet derived microparticles, microparticles of monocyte origin and a decrease in the total number of microparticles post filtration.

The investigation into the correlation between the change in thrombin generation parameters and microparticle numbers following filtration resulted in the most interesting findings of this phase of experimentation. The results demonstrated a
statistically significant correlation between the changes in ETP and peak height observed following filtration and the change in the numbers of CD15 positive microparticles post filtration. There was also a statistically significant correlation between peak height and both the number of CD42b positive and Annexin V positive events post filtration. These correlations are consistent with the removal of procoagulant material as hypothesised above. Since 91% of microparticles of monocyte origin (CD15 positive) are known to express tissue factor (Hron et al., 2007) the above correlation is suggestive of the fact that increased levels of microparticles of monocyte origin and hence increased levels of tissue factor can act to enhance both the total thrombin generation capacity and the maximum rate of thrombin generation of an individuals plasma. The mechanism for this is likely to be an increase in tissue factor trigger available to initiate the thrombin generation reaction. The CAT assay used in this experimentation uses a fixed concentration of tissue factor in order to initiate thrombin generation (5pM), individuals with higher circulating levels of tissue factor expressing monocyte microparticles would effectively have a higher concentration of tissue factor available to initiate the thrombin generation reaction than those patients who demonstrate lower levels of circulating monocyte microparticles, the higher levels of tissue factor may lead to a sustained reaction and ultimately higher levels of thrombin generation. Although this hypothesis is theoretically plausible it is worthy of note that the data presented here does not show that CD15 microparticles (of monocyte origin) actually do express tissue factor. It may be that the correlation between the changes in ETP/peak height and the number of CD15 positive microparticles is due to another cause, such as the composition of phospholipids in the microparticle membrane for example.

If the above hypothesis is correct it potentially implies that while most of an individuals thrombin generation capacity is due to the fixed level of tissue factor added in the reaction mixture a proportion of the total thrombin generation is due to the levels of tissue factor expressing microparticles in that patients plasma. This is true to an even greater extent when considering the peak height parameter as this is sensitive to the number of monocyte microparticles and the number of CD42b positive/ Annexin V positive events. It is important to note that the measurement of CD42b positive and Annexin V positive events should yield similar results as the number of Annexin V positive events is roughly analogous to the total number of microparticles present in the
plasma of an individual (since Annexin V binds to the phosphatidylserine expressed in microparticle membranes) and platelet derived microparticles (CD42b positive) constitute between 70-90% of the total number of microparticles in circulation (Flaumenhaft, 2006). It is therefore anticipated that any correlations with thrombin generation parameters should be present for both of these measurements or in neither. The sensitivity of the peak height parameter to the number of microparticles present in a patient’s plasma is probably explained by the increased availability of binding sites for both the tenase and prothrombinase complexes provided by the phosphatidylserine rich microparticle membranes. The peak height is effectively a measurement of the maximum rate of thrombin generation of a sample and it is therefore perhaps unsurprising that an increase in the available material on which the generation of thrombin occurs above and beyond that which is routinely added to the analysis would result in an increased maximum rate of production. Nevertheless it is evident from this research that the maximum rate of thrombin generation in a sample is enhanced by both the presence of microparticles and the number of monocyte microparticles (which may or may not express tissue factor.)

The correlations between the lag time and time to peak parameters were less profound (although still statistically significant) than those between the change in the number of microparticles and the ETP and peak height parameters. The pattern of these correlations were also different to that which was expected. Although as hypothesised above the removal of procoagulant microparticles would intuitively result in the increase of the lag time and time to peak post filtration due to a decrease in the available phospholipids on which the thrombin generation reaction occurs, the correlations appear to show decreasing lag time and time to peak parameters with decreasing microparticle numbers. This picture is consistent with the removal of microparticles expressing negative regulators of haemostasis which primarily impact upon the initiation phase of coagulation such as TFPI (discussed above). Although the observed correlation neatly dovetails with this hypothesis the picture is further confused by the fact that the majority of patients show either no change or an increase in both lag time and time to peak parameters post filtration. These patients also typically show the smallest decrease in the numbers of microparticles following filtration. It is worthy of note that these patients do still show a decrease in microparticle numbers however and therefore it is possible that these effects are due to the removal of procoagulant microparticles which
do not express negative regulators of haemostasis. It appears therefore that the exact
effect that the number of microparticles in a patient’s plasma has on both the lag time
and the time to peak parameters is dependent on whether or not the microparticles
present express negative regulators of haemostasis or not. It is anticipated that an
individual with increased numbers of microparticles in their circulation which do not
express negative regulators of haemostasis will demonstrate a decreased lag time and
time to peak when compared to an individual with a lower level of circulating
microparticles. An individual with a high level of circulating microparticles expressing
negative regulators of haemostasis would demonstrate the opposite effect. What is
apparent in this analysis is that whilst the measurement of the lag time and time to peak
parameters are affected by the number of microparticles present in an individual’s
circulation the exact impact that this is likely to have on the observed results is difficult
to predict without further investigation as to the proteins expressed by those
microparticles.

As discussed in section 4.3.6 it was initially felt that although the correlation between
the changes in the thrombin generation parameters and the changes in microparticle
numbers post filtration were supportive of the hypotheses presented above, it was
uncertain whether changes in the numbers of microparticles could plausibly be
responsible for the magnitude of change seen in the thrombin generation capacity of a
patient’s plasma following filtration. This was because the concentration of tissue factor
and phospholipid used in the reaction trigger was felt to be much higher than the levels
of tissue factor and phospholipid provided by the microparticles themselves. It is clear
from the results presented in section 4.3.6 that the changes in thrombin generation seen
post filtration are not likely to be caused by changes in the plasma concentration of
clotting factors or negative regulators of haemostasis following filtration. Good
evidence is provided by the experiments detailed in section 4.3.7 that microparticles are
capable of initiating thrombin generation without the addition of exogenous tissue factor
and phospholipids and that microparticles therefore can and probably do impact on the
thrombin generation capacity of a patient’s plasma when measured by the CAT
thrombin generation assay. It is worthy of note that the median change in peak height
demonstrated by a cohort of 19 individuals post filtration shown in table 4.3 (-12.4 nM
thrombin) is roughly analogous to the median peak height generated by the
microparticle rich plasma shown in table 4.6 (14.15 nM thrombin). Although these
experiments dovetail nicely it is apparent that the actual concentration of microparticles in the plasma varies dramatically. It is clear from the literature however that the resting level of tissue factor present on the surface of circulating microparticles is insufficient to initiate thrombin generation in vivo (Butenas et al., 2005) but it may be the case that tissue factor expressing microparticles are recruited to the sites of vascular injury not to initiate clot formation but to stabilise the thrombus by inducing further fibrin (and therefore previously thrombin) production (Wagner, 2005). It is evident that although microparticles may not be responsible for the initiation of thrombin generation they obviously play an important role in thrombin generation in vivo, nowhere is this more apparent than in individuals who have deficiencies in microparticle numbers or a loss of microparticle functionality i.e. patients with Scott syndrome or Castamans defect who have marked bleeding tendencies. The fact that microparticles have such an impact on in vivo thrombin generation makes it almost counter intuitive to suggest that they cannot mediate a similar effect in an in vitro assay.

The results of this experimentation suggest that the measurement of thrombin generation by the CAT assay is sensitive to and even partially dependent on both the number of microparticles and also potentially the number of microparticles expressing tissue factor in a patients plasma. Whilst the effects of these parameters can be predicted on both the ETP and peak height, the effects on both the lag time and time to peak are not clear cut.

The results of this investigation do not support those reported by Lawrie et al. (2008) however who demonstrated no measurable effect on any parameter as measured by the CAT assay using the reagents described in section 4.2.9 following the filtration of 20 fresh frozen plasma samples. Interestingly the authors did report similar findings to this investigation when lower concentrations of tissue factor trigger were employed. This effect became more profound when reduced concentrations of tissue factor trigger were used in combination with a reduced concentration of phospholipid. Sinauridze et al. showed an increase in ETP in line with increasing numbers of platelet derived microparticles; which is again contradictory to the findings of this investigation, where we showed no correlation between the ETP and the number of platelet derived microparticles in a patient’s plasma. It is worthy of note however that the experiments performed by Sinauridze et al. employed a different thrombin generation assay to the
CAT assay used in this investigation and that the platelet derived microparticles added to the reaction were the only source of phospholipid available. It is therefore unsurprising that there should be a direct correlation with the numbers of platelet derived microparticles and the resulting endogenous thrombin potential.

The results of this investigation are consistent with the hypothesis discussed in chapter 3 that the increased levels of both ETP and peak height described in patients with pre-eclampsia are due to an increase in the number of microparticles present in the circulation of these women (Macey et al., 2010a). Martinez et al. (2005) suggested that microparticles of leukocyte origin are of particular importance in pre-eclampsia, this result provides further weight to our hypothesis if this also includes microparticles of monocyte origin. The most important aspect of this research is the fact that the CAT assay is sensitive to the presence of microparticles in the circulation of a patient's plasma and that pathological levels of microparticles can cause abnormally high levels of ETP and peak height as seen in the cohort of women with pre-eclampsia in chapter 3. This provides evidence for the potential use of the CAT thrombin generation assay in the monitoring and even the diagnosis of any conditions which display enhanced numbers of microparticles in the circulation, not just pre-eclampsia.

It is worthy of note that it is possible to measure the activity of microparticles in plasma using a microparticle sensitive reagent designed for the CAT assay (essentially a reagent containing no phospholipids), although this was not commercially available at the onset of this study. It is an important distinction to make that the use of this reagent would only reveal the procoagulant activity of the microparticles present, whereas the method employed above gives a global indication of a patient's capacity to generate thrombin following a procoagulant stimulus including both the effects of the plasma concentration of clotting factors and the patients endogenous concentration of phospholipids. The exogenous phospholipid added to the patients’ plasma in the reaction as described in section 4.2.9 is analogous to the phospholipid material provided by the patients’ platelets in a ‘normal’ clotting response; the exclusion of this material would therefore lead to an underestimate of a patient’s true thrombin generation capacity. It is certainly plausible to suggest that the CAT thrombin generation assay could be further optimised from the method described in section 4.2.9 by changing the concentration of tissue factor or phospholipids (or both) in order to make it more...
sensitive to the presence of increased concentrations of microparticles in pathological conditions.
Chapter 5

Discussion
5.1 Assay Validation

The initial aim of this project was to assess the Calibrated Automated Thrombogram thrombin generation assay in order to determine whether or not it was suitable for routine diagnostic use in the haemostasis laboratory. The criteria chosen for assessment in this project included measuring the inter and intra assay variation, investigating the effects of sample handling and storage, ascertaining the limits of detection for the assay and determining the variation of an individuals results over time. Once these experiments had been completed a normal range for the assay was determined and the impacts of both age and sex were ascertained. The assay validation process also examined the effects that the addition of CTI had on the results generated by the CAT assay.

The above parameters were chosen for investigation as an understanding of these effects would enable the accurate interpretation of the results generated by the assay. Without knowing the levels of intra and inter assay variability (or the variation of results seen in an individual over time) for example it is difficult to establish the significance of borderline results generated by the assay. If this was the case, the use of the assay for either the monitoring of therapy or the diagnosis of bleeding/thrombotic tendencies would be called into question.

The initial experimentation yielded undesirable results, although the levels of intra assay variation appeared good, the inter assay variability was high with statistically significant differences between runs when compared using the students t-test for three of the four thrombin generation parameters. There was also evidence of an intra assay trend of decreasing peak height with the order in which the samples were added to the plate. These effects were not ameliorated by decreasing the time taken to set up the assay by using an electronic pipette. The reason for the high level of variation seen in these experiments is almost certainly due to the use of Control plasma P in the analysis. The intra and inter assay variability dropped to acceptable levels when the experiments were repeated using fresh pooled plasma instead of the lyophophilised Control plasma P. The high level of variation associated with Control plasma P is probably an artefact of particulate matter present in the plasma following an incomplete or inefficient reconstitution which disrupts the fluorescent signal generated by the assay. The
filtration experiments revealed that the level of variation observed when measuring Control plasma P could be reduced if the plasma was filtered prior to analysis. Although the optical density of the plasma was not measured pre and post filtration it was observable to the human eye that the plasma became clearer following the filtration process. This observation lends weight to the above hypothesis. What was interesting regarding this result was the apparent failure of the filtration process and subsequent reduction in variation to ameliorate the intra assay trends apparent in the analysis of Control plasma P. Over the course of the experimentation performed in this study the analysis of Control plasma P was associated with a number of intra assay trends including decreasing ETP and peak height with increasing plate position (i.e. the ETP and peak height parameters were elevated in the sample added to the plate first when compared to the sample added to the plate last) and increasing lag time with increasing plate position. These trends were not observed in the analysis of fresh pooled plasma. This implies that Control plasma P is possibly more sensitive to calcium independent auto activation with increased incubation time than fresh plasma and that this effect cannot be overcome by decreasing the incubation times using an electronic pipette.

The use of fresh pooled plasma to determine the inter and intra assay variation produced results which were acceptable for all four of the measured thrombin generation parameters (CVs <10%) and eliminated all of the observed intra assay trends. These results were not perfect however, since there was still evidence of statistically significant differences between runs when analysed by the students t-test, although the magnitude of these differences were below the 10% cut off of acceptability. The majority of these differences were seen in the lag time and the time to peak parameters implying it is the initiation of thrombin generation which is subject to the highest degree of variation. The measurement of the lag time is reported to be more sensitive to decreasing levels of clotting factors in haemophiliacs (except prothrombin deficiency) than the ETP (Al Dieri et al., 2002) and is characteristically increased in patients with lupus anticoagulant (Regnault et al., 2004). It is therefore the use of the CAT assay for these applications which is most likely to be affected by the increased variation in the lag time, something that was not attempted in this study. It is worthy of note that based on the inter assay CV for the lag time reported in this study (8.64% table 2.15), a lag time of >22 seconds above the normal range is certain to be a true abnormal. Since a plasma with 10% normal levels of clotting factors used in the limit of detection
experiment in chapter 2 had a lag time of >50 seconds outside the normal range the assay should be more than capable of detecting patients with significant bleeding tendencies. The diagnosis of haemophilia is also not entirely dependent on the lag time, indeed van Veen et al. (2009) reported that the main differences in the thrombin generation profile of haemophiliacs when compared to normal individuals is a reduced ETP and peak height and an increased time to peak.

The experiments investigating the effects of time between sample collection and processing and sample storage temperature produced pleasing results. It was shown that samples could be stored for up to 24 hours at room temperature before processing without an observable impact on the results produced; this result has large implications for the utility of the assay. Other types of diagnostic assay in the haematology department have restrictive handling procedures which limit their routine use (samples to be analysed for homocysteine for example must be kept on ice until received by the laboratory). The robust nature of the sample stability for the CAT assay means samples can be effectively taken in the community and subsequently transported to the hospital for analysis, without the need for the patient being tested to attend hospital based clinics, thereby saving both time and money. Separated samples can be stored frozen at temperatures ≤-20°C for up to four weeks without impacting upon the results generated. This result not only means that samples for thrombin generation analysis can be stored with samples from the same patient requiring other investigations in the haemostasis laboratory but more importantly samples can be run in batches, as this increases the efficiency of analysis and ultimately reduces the cost of the assay.

The analysis of the assay limits of detection produced some interesting results, not least of which was the apparent inability of the CAT thrombin generation assay to correctly interpret a flat generation curve as a zero result. The lowest results which could be reliably calculated by the analysis software were produced using a 2% dilution of SHP in heat inactivated plasma. As highlighted in chapter 2 although the inability of the assay to calculate a zero result accurately appears a significant limitation, it is highly unlikely that any patients would manifest a pathological condition significant enough to demonstrate the thrombin generation characteristics defined in this experiment. This is because this would necessitate a patient having a thrombin generation profile analogous to that of 2 iu/dL for all plasma clotting factor levels. These levels would only be
obtained in severe consumptive disorders such as major trauma and subsequent massive transfusion or disseminated intravascular coagulation (DIC) and even then this level of clotting factor activity may not be consistent with the patient’s survival.

A frustrating aspect to this phase of the experimentation was the failure to quantify the upper limit of detection, although one of the attempts to do so did lead to an interesting result. It was felt that substrate depletion may be achieved by producing a plasma with a high level of prothrombin. This theory was based on the fact that the dilution experiment to establish the lower limit of quantification described in chapter 2 produced evidence to corroborate the findings of Al Dieri et al. (2002); that there was a linear association between the ETP and the concentration of prothrombin in a sample. In order to produce a plasma with a high concentration of prothrombin it was decided to reconstitute the SHP used in the lower limit of quantification studies with half of the volume of water usually required (500µl as opposed to 1ml). This would produce a plasma with concentrations of all clotting factors (including prothrombin) of approximately 200 iu/dL. This is roughly analogous to twice the normal concentration. Analysis of this plasma however yielded a flat thrombin generation curve and consequently a result which was unquantifiable. Although this experiment was designed to produce a result which could not be calculated by the analysis software, the mechanism by which this was attained was supposed to be due to substrate depletion rather than a failure to produce a generation curve. Interestingly reconstituting the SHP with increasingly higher volumes of water up to the manufacturers specified 1ml resulted in thrombin generation parameters of increasing magnitude, but lower than those attained following the analysis of SHP reconstituted following the manufacturers guidelines (data not shown). This result has important implications for the analysis of diagnostic samples since the apparent reason for this finding was the increased opacity of the SHP with decreasing volume of water used in the reconstitution. When lower volumes of water were used it appeared that the fluorescent signal generated was largely absorbed by the sample itself and was therefore not quantified by the detector, leading to a flat thrombin generation curve. Whether the absorption of fluorescent signal was a consequence of particular matter present in the sample due to inefficient reconstitution of the reagent is unclear. The most important aspect regarding this finding is that there seems to be a level of sample optical density above which the accurate determination of thrombin generation is impossible. This is therefore a concern for the analysis of
patients with a degree of lipaemia or any other causes for raised plasma turbidity, since it is possible that a low thrombin generation result seen in such a patient may be in part due to the nature of the sample itself rather than any predisposition towards bleeding.

The assessment of variation in the thrombin generation profiles of normal individuals across time produced pleasing results. The level of variation seen across time was consistent with the variance in the inter assay variability experimentation. This suggests that an individual’s capacity to generate thrombin is relatively constant across time (provided they remain healthy). An extension of this finding is that the diagnostic power of the assay should be high with little need for repeat testing as the results produced should be reliable. This means that the CAT assay is suitable for both routine diagnosis and therapeutic monitoring in the haemostasis laboratory.

The normal range established for the CAT assay as described in chapter 2 simplifies the interpretation of assay results when analysing adult patients between 20-60 years of age, since no differences were detected in the thrombin generation parameters analysed with either age or sex. The analysis of patients outside this range however is problematical as an appropriate normal range has yet to be established. If the CAT assay is to be used in the routine diagnosis of haemophilia this will have to be rectified as most patients with congenital mutations will typically be identified from a young age.

The analysis of the effect of the addition of CTI on the results produced by the CAT thrombin generation assay produced unanticipated results. It was found that the use of CTI did have an effect on the results produced by the CAT assay when a concentration of 5pM tissue factor was used as a reaction trigger. Specifically it was found that there was a statistically significant reduction in the peak height of the samples spiked with CTI when compared to those samples in which no CTI was added. There were no significant differences in any of the other thrombin generation parameters. These findings contradicted those published in the literature (van Veen et al., 2008) which reported that the addition of CTI was not required when 5pM tissue factor was used as a reaction trigger. This finding implies that part of the maximum rate of thrombin generation, as defined by the CAT assay, is dependent on contact activation. The magnitude of difference between the results obtained with and without CTI is smaller than 10%, which is the limit of variation deemed acceptable in prior experimentation.
The clinical significance of such a small difference is also uncertain, and it was decided that the potential benefits of using CTI in a routine laboratory setting did not outweigh the cost implications.

The assay validation experiments performed in this study were on the whole successful and highlighted few prohibitive limitations, proving that the CAT thrombin generation assay was suitable for routine use in the clinical laboratory setting.

5.2 The Clinical Utility of the CAT Assay

Following the successful validation of the assay, the next stage of experimentation was to determine whether the CAT thrombin generation assay could produce results which were clinically useful. To this end it was chosen to investigate the thrombin generation profiles of women with pre-eclampsia, this cohort was chosen for a number of reasons, not least of which was the fact that there is very little published data on the measurement of thrombin generation in pre-eclampsia, therefore research into this field would provide a novel aspect to this study. Since pre-eclampsia is a condition associated with pro-thrombotic changes in the clotting cascade it was anticipated that these women would have elevated thrombin generation parameters, and therefore the use of CTI to prevent contact activation would be unnecessary. Women of child bearing age also fit neatly into the demographic for which the normal range was established, meaning abnormal results could be reliably detected.

The analysis of thrombin generation in pre-eclampsia produced a number of promising results, most notability that the ETP and peak height parameters in the cohort of women with pre-eclampsia were significantly elevated when compared to those of a cohort of normotensive pregnant women. These findings were consistent with a prothrombotic shift in patients with pre-eclampsia. Whilst these changes were conserved in women with pre-eclampsia of any severity there was no correlation between disease severity and the magnitude of the elevation observed in either the peak height or the ETP. This finding implies that although an increase in thrombogenicity is associated with pre-eclampsia the severity of the condition is affected by other factors which do not directly impact upon a patient’s capacity to generate thrombin. It may also be the case that
optimising the CAT assay may allow for more efficient stratification of disease severity in pre-eclamptic women.

Although this study highlighted elevated thrombin generation parameters in women with pre-eclampsia, utilising this finding in order to predict which women had pre-eclampsia in a retrospective manner proved problematical. The primary reason for this was the substantial overlap between the thrombin generation results of pregnant women with and without pre-eclampsia (figure 3.11) which made choosing a cut off for defining a patient as high or low risk difficult. The combination of the measurement of thrombin generation with the PRECOG score provided results with the greatest clinical utility. Defining a patient as high risk if they had thrombin generation parameters greater than 1SD below the mean of the case cohort (ETP > 2263nM Thrombin and a peak height of >445nM Thrombin) either alone or in conjunction with a high risk PRECOG score, resulted in a sensitivity and specificity of 86% and 43% respectively. Importantly 94% of those individuals who were classified as low risk by both thrombin generation and PRECOG did not have pre-eclampsia. This combination of screening tests provides a means for the separation of a cohort of women who are at very low risk of developing pre-eclampsia from a cohort of women in whom pre-eclampsia is prevalent, whilst minimising the number of false negative results. Such a screening test is much more effective than the use of the PRECOG score alone and therefore highlights a potential new application for the measurement of thrombin generation.

Other potential new applications for the measurement of thrombin generation via the CAT assay were highlighted by the correlations of thrombin generation parameters with end point measurements of pregnancy. It is clear that the measurement of thrombin generation has little application in either determining the chances of women having a LSCS or the risk of developing a PPH. There were however statistically significant correlations suggestive of the fact that high thrombin generation parameters during pregnancy are indicative of low birth weights and increased length of stay in hospital at the time of delivery. Further optimisation of the assay may be necessary to enhance the clinical utility of these correlations. If this can be achieved it would mean that the measurement of thrombin generation could be used as a reliable indicator as to the level of post natal care required, therefore highlighting a novel and clinically valuable application for the CAT thrombin generation assay.
One of the most interesting findings highlighted by the investigation of pre-eclampsia was that the reported changes in both ETP and peak height were conserved in women of Caucasian and Asian ethnic origin but were absent in Black women. Indeed the normotensive pregnant Black women had both elevated ETP and peak height parameters which were analogous to the levels seen in pre-eclamptic patients of either Caucasian or Asian ethnic origins. This result implies that Black individuals may demonstrate levels of thrombin generation which are elevated when compared to individuals of other ethnic origins. The reasons for this have yet to be determined. As highlighted in chapter 3 there are no ethnic differences in any of the normal ranges employed in the haemostasis laboratory at the Royal London Hospital and although some studies have reported the prevalence of deficiencies of natural anticoagulants such as protein C, protein S and antithrombin to be similar in VTE patients of Black ethnic origin when compared to Caucasian patients (Patel et al., 2003) it is worth noting that others have described that healthy Black patients have a normal range for protein C and protein S which is significantly lower than that derived from a White population (Jerrard-Dunne et al., 2003). It has been suggested as a result of this finding that not employing ethnic specific reference ranges could lead to the misdiagnosis of patients of Black ethnic origin (Roberts et al., 2009). The measurement of thrombin generation is most sensitive to the plasma levels of antithrombin, fibrinogen, prothrombin, factor V and free tissue factor pathway inhibitor (TFPI) when high concentrations of tissue factor are used as a reaction trigger (Dielis et al., 2008). Black individuals have elevated levels of fibrinogen as wells as other clotting factors such as factor VIII (Folsom et al., 1992) when compared to Caucasians which may lead to an increased thrombin generation capacity. A reduced level of free protein S may exacerbate this effect since free protein S has been shown to act as a cofactor for TFPI (Hackeng and Rosing, 2009). A natural deficiency of free protein S may therefore result in a reduced efficacy of TFPI and consequently an increased ETP/peak height. It is plausible that a reduced level of natural anticoagulants as well as increased levels of some clotting factors may be responsible for the apparent differences in the thrombin generation profiles observed in the different ethnic groups in this study, although further experimentation may be necessary to prove this hypothesis.

One of the most compelling findings of this phase of the study was the combination of the measurement of ETP with the numbers of platelets expressing P-selectin, P-selectin
expressing microparticles and platelet-monocyte aggregates analysed by flow cytometry in the same cohort of patients with and without pre-eclampsia (Macey et al., 2010a). This analysis provided the most successful screening test for pre-eclampsia with sensitivity and specificity values of 81% and 86% respectively and positive and negative predictive values of 83% and 84% respectively.

The findings of this paper also highlighted the fact that the elevated thrombin generation parameters apparent in the cohort of women with pre-eclampsia may be due to the elevated numbers of microparticles in the circulation of women with pre-eclampsia rather than prothrombotic changes in the plasma concentration of clotting factors or negative regulators of haemostasis. The filtration experiments performed in chapter 4 suggest this is likely to be the case, since plasma filtration had a predictable effect on both the ETP and peak height parameters as measured by the CAT assay (decrease in results) and that the magnitude of these changes showed a statistically significant correlation with decreasing levels of microparticle numbers post filtration. Further evidence in support of this hypothesis was provided by the fact that filtration did not alter the plasma concentrations of clotting factors in a manner which was likely to cause the reported changes in thrombin generation following the filtration process and that microparticles were able to initiate thrombin generation and are therefore likely to have an impact on a patients thrombin generation capacity.

Decreasing ETP results correlated with decreasing numbers of CD15 positive microparticles whereas decreasing peak height results showed a correlation with decreasing numbers of CD15 positive, CD42b positive and Annexin V positive events. It is plausible therefore that the phosphotidylserine rich membranes provided by microparticles in general as well as elevated levels of tissue factor provided by CD15 positive monocyte derived microparticles (although tissue factor expression by CD15 positive microparticles was not demonstrated in this study) enhance the thrombin generation capacity of an individuals plasma by supplementing the exogenous tissue factor and phospholipid added to a patients plasma in order to trigger thrombin generation in the CAT assay. The effect of the presence of microparticles on both the lag time and time to peak parameters is far less predictable but is probably determined by the presence or absence of microparticles expressing negative regulators of haemostasis.
The results of this study suggest that pathological levels of microparticles can act to enhance the level of ETP and peak height of an individual as determined by the CAT thrombin generation assay. This finding is of importance since it means the CAT assay can potentially be employed in the diagnosis of any condition in which the numbers of microparticles are enhanced. As an extension of this the CAT assay could also be used to monitor a patient’s response to therapy if it is known that a treatment causes a reduction in the numbers of microparticles e.g. the use of Clopidogrel and Asprin in patients with cardiac disorders (Serebruany et al., 2003). It is of note that the variation over time experiment performed in chapter 2 revealed that there is little change in the thrombin generation capacity of an individual over the time course analysed, this implies that the level of microparticles present in an individual’s circulation over time must also remain constant. This is consistent with constant baseline levels of microparticles within individuals reported by Brogan et al. (2004). If this is indeed the case it implies that the measurement of thrombin generation is probably sensitive to acquired conditions characterised by changes in the number of microparticles present in the circulation of an individual.

In summary this study has provided evidence that the CAT assay can not only be used in the diagnosis of conditions characterised by changes in the plasma concentrations of proteins involved in haemostasis but is also of potential benefit in the diagnosis and monitoring of conditions characterised by increased numbers of plasma microparticles. This has been demonstrated in principle via the analysis of a cohort of pregnant women with pre-eclampsia and the development of a screen which can be used to differentiate between women with low risk of developing the condition and those of higher risk.

5.3 Future Work

There are a myriad of further research projects that could be attempted following this study, all of which could produce useful data. The primary starting point would be continuing to investigate the use of thrombin generation in the diagnosis of pre-eclampsia. As highlighted in chapter 3, although the data in this study suggests there is an elevation in both the ETP and peak height of a pregnant woman with pre-eclampsia when compared to a normotensive pregnant woman, the analysis was performed retrospectively. A prospective study is essential in order to determine whether or not
these parameters are elevated early in pregnancy or whether they are only characteristic of late stage disease. This would provide conclusive evidence as to whether the measurement of thrombin generation would constitute an effective screening test for pre-eclampsia or not. The fact that neither the ETP or peak height shows any significant trend with increasing gestational age implies that elevated thrombin generation parameters should be apparent at any time during pregnancy. It would be desirable to collect samples from women early in pregnancy and also after 20 weeks (when the diagnosis of pre-eclampsia is possible) in order to compare changes in results over this time. This type of study would need to rely on prospective collection of samples and retrospective analysis, once it is known whether a patient has developed disease or not, in order to minimise the cost. It would also be beneficial to analyse the numbers and types of microparticles present in this cohort of women as the original microparticle analysis performed in the pre-eclampsia cohort was limited to the number of P-selectin expressing microparticles. It would be of interest to characterise the types of microparticles present in women with pre-eclampsia in order to prove the hypothesis that an increase in the number of CD15 expressing microparticles is associated with the increased thrombin generation manifest in women with pre-eclampsia. It would also be of benefit to establish whether or not CD15 positive microparticles do actually express tissue factor.

Before embarking on the prospective study outlined above it would be of use to attempt to optimise the CAT assay to make it more sensitive to the prothrombotic changes seen in women with pre-eclampsia. This may allow differences in the thrombin generation profiles of women with varying levels of disease severity to be determined. Optimisation of the assay may also enhance the sensitivity/specificity of the CAT assay in terms of the diagnosis of pre-eclampsia by decreasing the degree of overlap between the results of the case and control cohorts. Assay optimisation may also increase the degree of correlation between the ETP/peak height and the birth weight and length of stay in hospital at the time of delivery.

It would also be beneficial to further investigate the impact of microparticles on the CAT assay. A starting point for this would be the analysis of plasma pre and post filtration using a low concentration of tissue factor as a reaction trigger. If the analysis is affected by the number of tissue factor expressing microparticles the use of a lower
concentration of tissue factor should make the assay more sensitive to the presence of these microparticles, although the use of CTI would be essential in order to prevent contact activation impacting on the results of the analysis. It would also be of interest to determine the sensitivity of the CAT assay in the detection of elevated thrombin generation parameters in other conditions characterised by increased levels of microparticles in order to prove that the assay is indeed sensitive to these conditions.

An experiment of interest would be to study the persistence of microparticles following delivery in a cohort of patients with and without pre-eclampsia to see when and if they return to a baseline normal level; this could also be correlated with the measurement of thrombin generation. This investigation is of interest in providing a link between pre-eclampsia and cardiovascular disease in later life, since it is known there is an association between the two and that the presence of microparticles are implicated in both diseases, it may be that a persistence of a pathological level of microparticles is causative of this effect.

It would also be of interest to investigate the thrombin generation in a cohort of individuals of Black ethnic origin to see if it is necessary to establish a separate normal range. If there are differences in the thrombin generation capacities of individuals of different ethnic origins it would be beneficial to try and determine the basis for these changes. In the absence of any differences in the plasma concentration of clotting factors it may be that differences in the number and or type of circulating microparticles may be responsible for any differences observed.

A final consideration for further experimentation would be the investigation of the paediatric normal range, which as highlighted above would be essential if the CAT assay is to be introduced in the diagnosis of haemophilia.
Chapter 6

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Chapter 7

Appendix
7.1 Distribution of t

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7.2 Publications

P.T.O