Studies of acquired and inherited coagulopathy using the thrombin generation assay

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I, Emma Louise Fosbury, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

In order to survive, an individual's haemostatic system has to have the capacity to respond promptly to injury and thrombus formation has to occur in a highly regulated fashion. Over the past two hundred years, our understanding of this complex system has increased dramatically. It is perhaps surprising, therefore, that clinicians rely predominantly on assays to assess coagulation that were developed over 50 years ago and that only assess the time to initial clot formation. This reflects the challenge of truly understanding the complex dynamic contributions of platelets, vascular endothelium and coagulation factors under shear force and replicating this in a test-tube. There has therefore been a drive to develop better methods of detecting disordered haemostasis that can be accurately correlated with bleeding or thrombotic risk and that can be utilised to guide treatment.

The thrombin generation assay is currently a research tool that is able to chart the different phases of thrombin generation, recognising that the majority of this occurs after the end-point of traditional coagulation assays. Thrombin is key to effective haemostasis and the thrombin generation assay has been shown to correlate with a range of bleeding and thrombotic states.

Although it is recognised to produce valuable information, it is hampered by the impact of pre-analytical variables, lack of standardisation and inter-operator and inter-laboratory variability. Optimisation of the assay as well as development of normal reference ranges is key. This is in addition to further phenotyping of dysfunctional haemostasis and providing evidence of clinical relevance. This

thesis sets out to address these different areas with studies including the generation of normal reference ranges in addition to examining the sensitivity of the assay to anticoagulation (and its reversal) as well as exploring potential factors contributing to the variable bleeding phenotype seen in patients with severe haemophilia.

Impact Statement

This thesis is primarily centred on the study of thrombin generation, a key component in haemostasis. The clinical subspecialty of haemostasis and thrombosis is far-reaching and involves liason with all specialties and care of patients of all ages. Unfortunately, the tools available to haematologists to enable understanding of defects in the process and thus guide appropriate treatment are blunt and often do not readily provide an effective overview of the different components involved in the process.

Bleeding symptoms are common in the population. Clinicians are frequently asked to investigate patients for a possible underlying bleeding diathesis. In some patients, a defect can be identified. However, there are many in whom uncertainty remains – despite a significant bleeding history - as no defect can be identified with available assays. In contrast, there is often anxiety amongst doctors relating to abnormalities in baseline clotting tests and how these results might translate into bleeding risk in patients needing to undergo surgery or other invasive procedures. Questions are also frequently raised about an individual's thrombotic risk, often in the context of a positive family history or a finding of an abnormality on a thrombophilia screen. At present, guidance is based on the findings of population studies and is challenging to individualise. The PT and APTT can guide the investigation of clotting factor deficiencies but are not really helpful in assessing hypercoagulability. The level of a clotting factor cannot necessarily be translated into a bleeding risk. Levels of greater than 30 IU/dL are generally considered haemostatic, but this does not apply to all factors (FXI is an example of this with levels that correlate poorly with

bleeding risk). Haemostasis involves a complex interplay between the vascular endothelium, platelets and clotting factors. This is extremely difficult to replicate in a laboratory assay. Dynamic, real-time, whole blood assays such as the TEG or ROTEM - as opposed to the PT or APTT - have been developed into point-of-care tests that can help guide clinicians with replacement of blood products in the context of major haemorrhage or liver transplantation.

The thrombin generation assay at present is a research tool. However, there is a drive to produce more fully automated systems to enable its use in the diagnostic laboratory. This is underpinned by a growing body of evidence demonstrating the sensitivity of the assay to both hypo- and hypercoagulable states. It gauges an individual's potential to generate thrombin – the central protease in haemostasis – in the presence or absence of platelets. It allows for a more nuanced interrogation of an individual's haemostatic network in order to both investigate their baseline activity, but also gauge response to clinical intervention.

The studies in this thesis have sought to expand the evidence base by establishing normal reference ranges under a range of assay conditions as well as assessing the assay's potential clinical utility. This adds to the research evidence supporting the development of global assays and their use in clinical practice.

Commonly used abbreviations

aPCC	Activated Prothrombin Complex Concentrate
ABR	Annual Bleeding Rate
APTT	Activated Partial Thromboplastin Time
AT	Antithrombin
BSA	Bovine Serum Albumin
CTI	Corn Trypsin Inhibitor
CV	Coefficient of Variation
CWB	Citrated Whole Blood
ELISA	Enzyme Linked Immunosorbent Assay
ETP	Endogenous Thrombin Potential
FEIBA	Factor Eight Inhibitor Bypassing Agent
FFP	Fresh Frozen Plasma
IQR	Interquartile Range
KDCRPB	Katharine Dormandy Coagulation Research Plasma Bank
LMWH	Low Molecular Weight Heparin
LT	Lag Time
MW	Molecular Weight
NIBSC	National Institute for Biological Standards and Control
OSCA	One Stage Clotting Assay
PCC	Prothrombin Complex Concentrate

PF4	Platelet Factor 4
pM	picoMolar
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PT	Prothrombin Time
rFVIIa	Recombinant activated Factor VII
SHA	Severe Haemophilia A
SHB	Severe Haemophilia B
$T_{1/2}$	Half Life
TBS	Tris Buffered Saline
TF	Tissue Factor
TGA	Thrombin Generation Assay
ttPeak	Time to Peak
UFH	Unfractionated Heparin

1 Introduction

This thesis focuses primarily on thrombin generation. In the process of spending time in the research laboratory, devising experiments and collecting and analysing data, there is an opportunity to extend and consolidate understanding. Whilst it is possible to take a narrow view of a field of research, the real privilege of undertaking a research degree is to learn about an area of interest and to place it in context. Thus, some understanding of the evolution, comparative biology and historical process of evaluation of the mechanisms underlying haemostasis and the development of assays to interrogate the process is an integral part of this thesis. This chapter aims to place the thesis in context and to highlight the challenges involved in understanding the complexities of haemostasis.

1.1 The evolution of haemostasis

It is intriguing, when investigating abnormalities in haemostasis and further understanding the roles of the distinct components, to briefly consider the evolutionary origins of and selection pressures upon the haemostatic system. Comparative biology is an essential tool, lending insights into this complex physiological process.

Haemostasis is a complex interplay between coagulation factors, platelets and the vascular endothelium necessary to preserve a functioning (pressurised)

circulatory system when the latter is breached, in addition to protecting multi-cellular organisms from invasion from pathogens [Aird, 2003].

When seeking to understand the requirement for a haemostatic process, one first needs to think about the derivation of the vascular system and the associated endothelium required to contain a blood circulatory system. Blood vascular systems (necessary to overcome the constraints of diffusion) are found in both invertebrates and vertebrates, but an endothelium is only found in the latter. The evolution of an endothelialised vasculature optimised flow dynamics and barrier function, in addition to performing a role in the localisation of immune and coagulation function. Since then, the vascular system has undergone selective evolutionary pressures shaped by the unique needs of each species [Monahan-Earley *et al.*, 2013].

As the coagulation system cannot be preserved as part of the fossil record, comparative biology is required to help understand its evolution. Biochemical evidence, molecular cloning data and comparative sequence data support the existence of key haemostatic proteases and cofactors in all jawed vertebrates and suggest that the haemostatic system evolved over 450 million years ago [Davidson *et al.*, 2003]. The complexity of haemostasis, as found in modern vertebrates, developed from a primitive system consisting of two or three components that were primarily involved in innate immunity [Spronk *et al.*, 2003]. In mammalian systems, haemostasis exists as a complex network of positive and negative feedback loops with proteases and cofactors controlling the generation of fibrin. The individual coagulation enzymes are also involved in

inflammation and immunity [Schoenmakers *et al.*, 2005], mirroring the roles of the proteins in primitive networks.

1.2 Identification of the components involved in haemostasis

Given that the haemostatic system evolved over 450 million years ago, our understanding of this complex molecular process has only developed over the past 200 years. Prior to that, some initial observations had been made. Hippocrates observed in 400 BC that blood from a wounded soldier congealed as it cooled [Spronk *et al.*, 2003]. In the late 17th century, Malpighi separated and identified fibres from clotted blood using a single lens microscope [Doyle, 2006]. A century or so later, William Hewson observed that the coagulum of blood was derived from the liquid component of blood and not the red cells. In 1801, the term fibrin was coined by Fourcroy who determined that the precursor of fibrin was present in plasma but not serum. Babington introduced the term fibrinogen in 1830 [Douglas, 1999].

The so-called 'fibrin ferment' (identified by several individuals in the second half of the nineteenth century as the initiator of coagulation) was named thrombin by Virchow. However, it was Alexander Schmidt who realised that there must be a precursor to thrombin to account for the fact that circulating blood remained fluid. This led to the proposal of a factor called prothrombin [Douglas, 1999]. Schmidt also drew on work by others, demonstrating that coagulation occurred immediately on mixing blood and macerated tissue in a container identifying a

component termed zymoplastin. By the late nineteenth century, Arthus, Pagès and Sabbatini recognised the key importance of calcium in the process [Quick, 1959, Douglas, 1999].

All of these findings were then summarised by Paul Morawitz in 1905 in his 'classical theory' of coagulation in which he renamed zymoplastin as thrombokinase [Saito *et al.*, 2011]. Howell later modified this term to tissue factor (TF) in 1914. The classical theory proposed that prothrombin was converted to thrombin under the influence of thrombokinase and calcium and thrombin converted fibrinogen to fibrin [Douglas, 1999, Saito *et al.*, 2011]. The method for converting prothrombin to thrombin using tissue was termed the extrinsic pathway and the pathway functioning in the absence of added tissue (albeit much slower than the extrinsic pathway) the intrinsic pathway [Douglas, 1999]. During the late 1930s through to the 1950s, individual clotting factors were identified (often as a result of studying patients with rare bleeding defects) and a consensus was reached regarding their nomenclature using roman numerals (by the formation of the International Committee on the Nomenclature of Blood Coagulation Factors in 1954 [Saito *et al.*, 2011]).

1.3 Developing models of understanding

A principal driver of developing understanding of the haemostatic process was the puzzle of haemophilia. It was understood that, in order to treat the disorder, it was necessary not only to elucidate the missing factor, but also to have a means of detecting the abnormality in the laboratory. In the 1930s, the clinical picture of severe haemophilia was grim. A severely life-limiting disease, it was

associated with profound musculoskeletal deformity overshadowed by a very real fear of death as a result of bleeding from a trivial injury such as a cut to a finger or a bite to the tongue [Biggs, 1967]. Although the first case report of successful treatment of bleeding in a patient with likely haemophilia was published in 1840 (a young boy transfused with whole blood from a 'stout young woman') [Lane, 1840], it was only in the 1980s that the FIX and FVIII genes were cloned [Franchini *et al.*, 2012].

In 1938, R.G. Macfarlane (one of the key researchers of the time in the area of haemostasis) stated that: "It is probable … that there is in normal blood a factor, at present unidentified, that is essential for the rapid activation of prothrombin and that this factor is at fault in haemophilia" [Biggs, 1967]. He spent his career translating the understanding gleaned from his laboratory research into the clinical treatment of these patients.

1.3.1 Waterfall sequence/cascade model of haemostasis

In the mid-1960s, Davie and Macfarlane each published papers proposing a 'cascade' or 'waterfall' model of haemostasis [Davie *et al.*, 1964, Macfarlane, 1964, Houghie, 2004, Hoffman *et al.*, 2007, Saito *et al.*, 2011]. This model was used to explain the functions of the various coagulation factors in producing a fibrin clot and focused principally on the intrinsic (contact) pathway. It was based on the concept that fibrin was formed as a result of sequential reactions between enzymes and substrates with each clotting factor existing as a pro-enzyme that could be converted to an active form and built on modification of the classical theory of Morawitz in light of more recently identified clotting

factors. The cascade model postulated that when blood came into contact with a foreign surface, Factor XII (a contact factor) was activated leading in turn to the activation of Factor XI and then Factor IX ultimately leading to the generation of thrombin (see Figure 1-1).

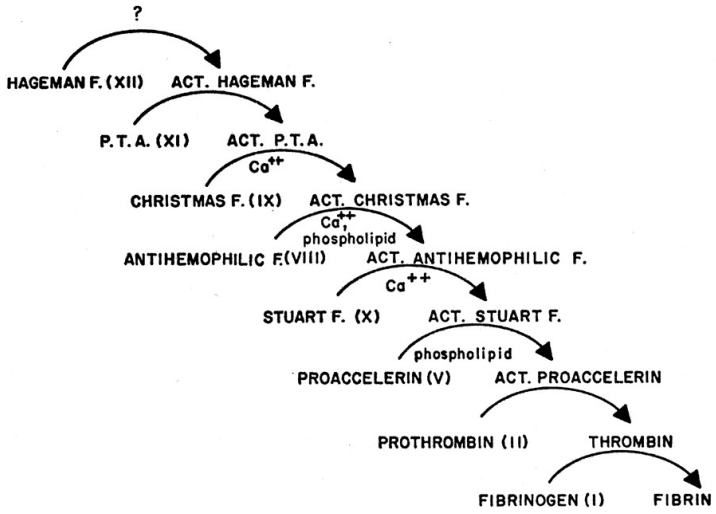


Figure 1-1 Original Davie and Ratnoff waterfall sequence of coagulation [Davie *et al.*, 1964]; this sequence uses the original names for the factors (named after affected patients) prior to the 1954 international consensus agreement that adopted the use of Roman numerals

However, this model could not account for the disparity between patients with FXII (who did not bleed), patients with FXI deficiency (who might or might not bleed) and patients with haemophilia (who did bleed) despite abnormal activated partial thromboplastin test (APTT) results in all. Further studies followed, examining mechanisms independent of FXIa that could activate FIX, and identified the TF.FVIIa complex as a key component, although the exact nature of TF remained unknown [Østerud *et al.*, 1977]. It was understood that

the extrinsic pathway was initiated by the binding of activated FVII (FVIIa) to TF. The pathways then converged on a common pathway at the level of the prothrombinase complex (FXa/FVa) [Hoffman *et al.*, 2007]. This understanding of the activation of FIX by the TF.FVIIa complex clarified the interplay between the intrinsic and extrinsic pathways and led to the classic Y-shaped diagram of the clotting cascade (see Figure 1-2). The model was also updated to clarify that some of the factors were cofactors and did not possess their own intrinsic enzymatic activity.

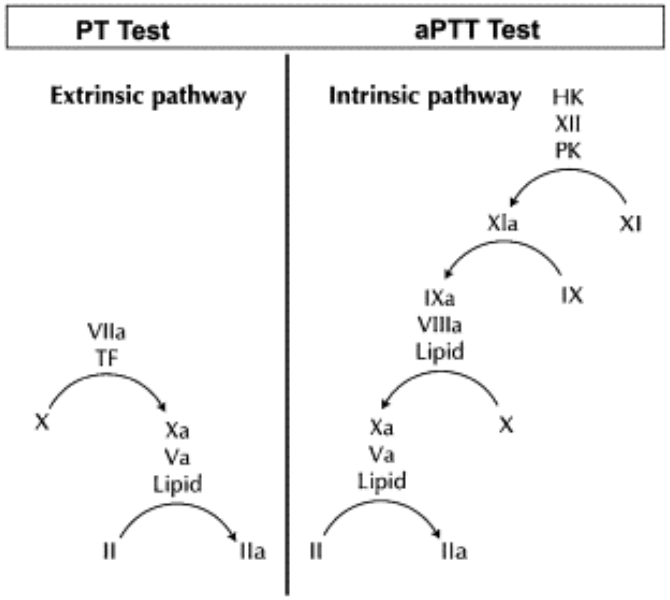


Figure 1-2 Modified cascade model of haemostasis

1.3.2 Cell-based model of haemostasis

Up until this point, the model of haemostasis – whilst acknowledging the necessity of phospholipid for the enzymatic reactions – did not incorporate an

active cellular component beyond the role of providing a phospholipid surface. Hoffman et al. [Hoffman *et al.*, 2001] subsequently proposed a cell-based model, taking into account the differing roles of cells expressing pro- and anti-coagulants (see Figure 1-3), including tissue factor bearing cells, platelets and endothelial cells. This viewed the process in terms of different overlapping phases including initiation, amplification and propagation resulting in a sufficiently large thrombin burst to enable thrombus formation (see Figure 1-3 for illustration).

The cell-based model shifted the emphasis away from a step-wise activation of clotting factors, to a more physiological model recognising a more active role for platelets. It envisaged an initial phase whereby – upon disruption of the vascular endothelium – TF is exposed on fibroblasts. The resultant formation of TF.FVIIa complexes results in activation of FX and FIX and a small amount of thrombin. The thrombin then activates platelets adhering to extravascular matrix components exposed by the breach in the endothelium. The thrombin enhances platelet adhesion as well as activating FV, FVIII and FXI. The activated platelets also release FV from their alpha granules and this is fully activated by thrombin or FXa. FVIII is released from VWF upon activation and the platelet surface now has FVa and FVIIIa bound to it that enables formation of the procoagulant complexes (the prothrombinase and tenase complexes) necessary for the significant burst of thrombin required for successful haemostasis.

Checks and balances play a role at various stages. The processes are localised to the cell surfaces, as activated proteases can be inactivated if they diffuse into the fluid phase e.g. FXa can be inactivated by TFPI. In contrast, FIXa is not

inactivated by TFPI and so can diffuse and attach to the surface of nearby activated platelets. Antithrombin (AT) in plasma inactivates both thrombin and FXa. Platelets do not contain TF (thus TF bearing cells are essential to the process) and, additionally, require activation in order to express the phosphatidylserine phospholipid on the cell surface that is essential for adherence of the procoagulant proteases. Endothelial cells express thrombomodulin on the cell surface that binds thrombin and this complex in turn activates Protein C and leads to inactivation of FVa and FVIIIa.

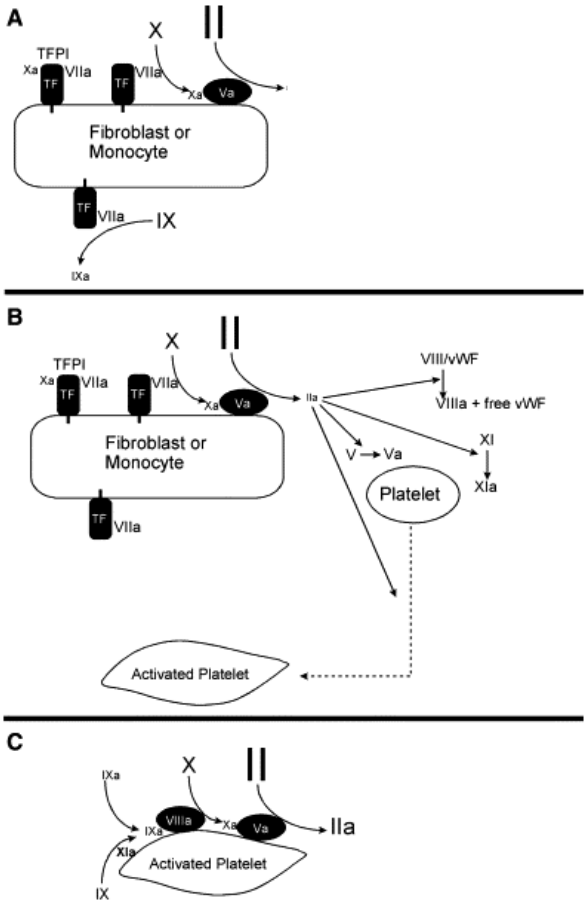


Figure 1-3 Cell-based model of haemostasis (figure reproduced from [Hoffman *et al.*, 2007]). A = Initiation; B = Amplification; C = Propagation.

The authors also tackled the enduring question of why patients with haemophilia bleed and proposed an explanation for the failure of the extrinsic pathway to compensate for a lack of FVIII or FIX. They suggested that, although sufficient FXa is generated, it is on the wrong cell surface i.e. the surface of TF bearing cells. Any FXa (generated by the TF/FVIIa complex) that diffused away from the fibroblast surface would be inactivated by AT or TFPI. In contrast, FX activated by the tenase complex (consisting of FVIIIa.FIXa) on the surface of the activated platelet would not be inactivated and could generate haemostatic amounts of thrombin. Thus, a lack of functioning tenase complexes meant that FXa was only produced in the wrong place and could not, by itself, generate sufficient thrombin.

1.4 The challenge of abnormal coagulation and understanding the underlying pathology

The haemostatic system is a system that is both able to adapt to differing physiological states (as seen in neonates and pregnancy) as well as being profoundly affected by pathological processes such as sepsis, liver and renal disease. In addition, there are patients with inherited defects involving almost all components of haemostasis. Clinicians face the challenge of determining normal function in different physiological settings (i.e. the immature, but effective, system found in the neonate) as well as recognizing abnormal function and determining the potential sequelæ.

1.4.1 Developing assays to assess haemostasis

1.4.1.1 The prothrombin time and activated partial thromboplastin time

The discovery of heparin and the vitamin K antagonists in the first part of the 20th century, in addition to the pursuit of the underlying defect in haemophilia, drove the development of a simple, standardized, reproducible assay that could be used to identify patients with abnormal coagulation. This was initially addressed by the Quick test, also known as the prothrombin time (PT). Quick (basing his work on the theory of Morawitz that in the presence of adequate amounts of tissue, calcium and fibrinogen, prothrombin is the limiting factor) developed a stable thromboplastin reagent from rabbit brain using acetone. He was able to test his one-stage prothrombin test using this reagent on animals fed spoiled sweet clover hay that had been noted to be associated with an acquired haemorrhagic condition in cattle. He fed samples of the sweet clover hay to chicks and rabbits and noted that their PT became prolonged, manifested clinically by bleeding. He was subsequently able to demonstrate correction of the PT when the animals were fed Vitamin K-containing alfalfa [Quick, 1959].

The problem that troubled investigators of the time was that, despite the severity of bleeding, plasma from patients with haemophilia clotted normally with the addition of tissue factor as performed for the PT. There was thus a need to develop a further assay that was sensitive to the underlying defect. This was achieved by the development of a 'partial' thromboplastin that was able to

differentiate between normal plasma and that of a patient with haemophilia (the activated partial thromboplastin time (APTT)). This was initially achieved by Langdell et al., but was modified by Rapaport et al. in the early 1960s with the use of kaolin that shortened and stabilised the assay [White, 2003]. The term partial thromboplastin was a reference to the absence of TF, but the continued presence of phospholipid (thus testing the intrinsic pathway of the classical theory).

1.4.1.2 Global assays

The limitations of the routine coagulation screen (PT/APTT) have long been recognised. Even prior to this, in 1948, Hartert described a different means of assessing haemostasis called thromboelastography [Lancé, 2015]. In contrast to plasma-based assays, thromboelastography (TEG and the closely allied thromboelastometry – ROTEM) is a whole blood assay that continuously measures the viscoelastic changes in blood activated by triggers such as kaolin (a contact activator) thereby tracking clot formation and subsequent lysis. Semi-automated systems have now been developed for these assays. They both operate on similar principles with the addition of whole blood or citrated whole blood (CWB) into a cup into which a probe is inserted. Upon addition of calcium (alone or with a trigger) either the cup or the probe rotates, detecting increasing torsion as a clot forms, reaching maximum stability and then subsequently undergoing lysis [Lancé, 2015, Hans *et al.*, 2016], thus following all stages of haemostasis.

The associated software is able to compute several different parameters giving information about clot formation (see Figure 1-4). The r or CT (clot time) parameter measures the time to reach an amplitude of 2 mm and is influenced by the presence of anticoagulants, clotting factor deficiency or inhibitors. The k or CFT (clot formation time) depicts how fast a visible clot is formed. The angle is a marker of rate and the MA (maximum amplitude) or MCF (maximum clot firmness) reflects the role of platelets and fibrinogen. The first derivative of the tracing (V-curve) (see Figure 1-4) mimics a thrombin generation curve. V_{max} is the maximal velocity of clot formation and time to V_{max} is the time before the V_{max} is reached [Hans *et al.*, 2016]. The addition of different activators and additives (such as platelet inhibitors) allows further refinement of different aspects of the haemostatic process.

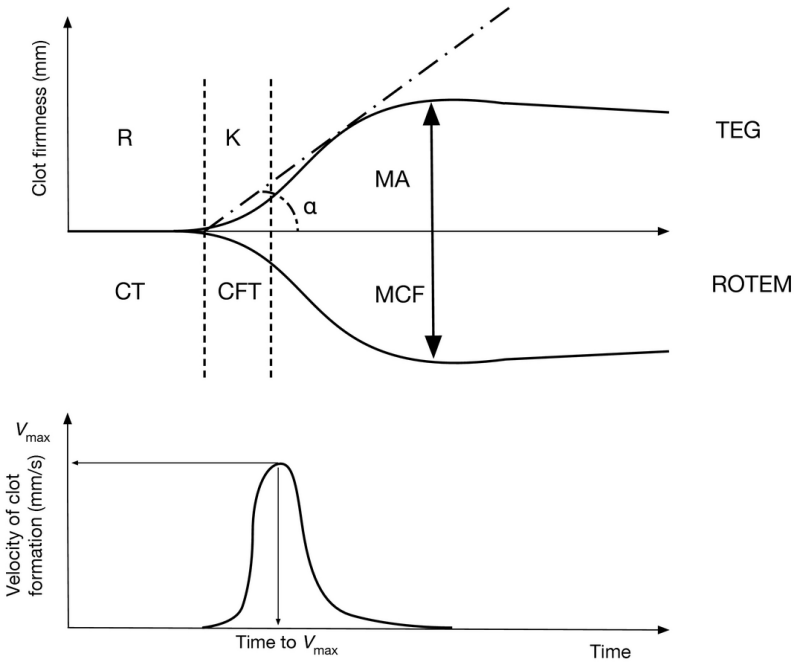


Figure 1-4 TEG/ROTEM traces (reproduced from Hans et al, 2016)

These assays, although initially limited by poor reproducibility and difficulty in interpreting the results, have been developed into point of care tests (POCT) [Tripodi, 2008]. Increasingly, POCT are becoming accessible within the hospital setting (for example in theatres or intensive care units) and are straightforward to use given appropriate training. Algorithms have been developed on a local basis using POCT global assays in certain surgical settings e.g. liver transplantation [Clevenger *et al.*, 2014]. The use of such assays is attractive and can be useful in guiding treatment with blood products and haemostatic agents in bleeding patients.

The thrombin generation assay (TGA) has also been developed and refined since the 1950s and allows the detection of thrombin in platelet poor (PPP) and platelet rich plasma (PRP) using TF as a trigger [Tripodi, 2008]. The assay enables the assessment of the 95% of thrombin generated after the initial formation of a clot, recognizing the fact that it is the capacity to form thrombin (and its subsequent actions) that underpins haemostatic function [Baglin, 2005]. Although still a research tool, technical advances have enabled it to become a more rapid, reproducible assay sensitive to hyper- and hypocoagulable states such as in patients with inherited thrombophilia or bleeding disorders such as haemophilia.

Work started in the 1980s by Hemker et al. [Hemker, 2008] has led to the development of the calibrated automated thrombogram (CAT) – a thrombin generation assay that uses a fluorogenic substrate and continuous individual calibration in order to take into account substrate depletion and the inner filter effect. The associated software determines four parameters. The lag time (LT)

that measures the time to initiation of thrombin generation, the peak that reflects maximum thrombin generation, the time-to-peak (ttPeak) and the endogenous thrombin potential (ETP, otherwise known as the area under the curve (AUC)). The assay is very sensitive to the impact of pre-analytical variables as well as lacking standardisation of the reagents, however, it can produce valuable information about haemostatic dysfunction.

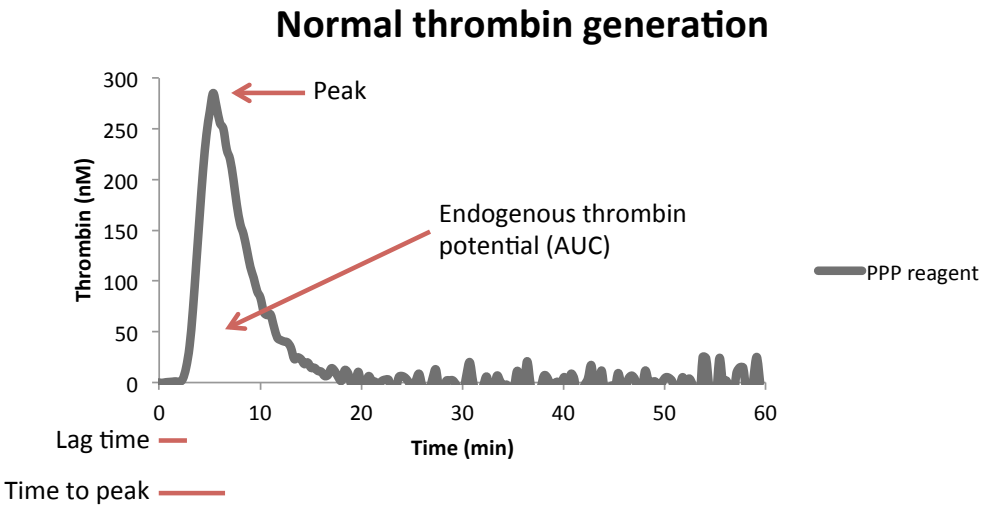


Figure 1-5 A normal thrombin generation curve (platelet poor plasma)

1.4.2 Applications of the thrombin generation assay in clinical practice

The advent of the 21st century has seen a significant increase in work demonstrating the potential applications of thrombin generation [Hemker *et al.*, 2004, Bevan, 2010]

1.4.2.1 Bleeding tendency

Work to further the understanding of inherited bleeding disorders, specifically the impact on thrombin generating potential, has been performed both in reconstituted systems using purified clotting factors [Van't Veer *et al.*, 2000] and samples from patients with haemophilia [Young *et al.*, 2013]. Studies have compared TGA parameters and plasma FVIII and FIX levels and found a significant correlation [Beltran-Miranda *et al.*, 2005, Dargaud *et al.*, 2005]. These studies have also examined the pharmacokinetics of FVIII administration and the impact on the TGA results and demonstrated sensitivity of the assay to modification of the factor levels. In addition, the TGA has also been used to assess responsiveness to bypassing agents (such as FEIBA) in patients with congenital haemophilia and inhibitors [Váradi *et al.*, 2003].

1.4.2.2 Thrombotic tendency

Numerous studies have demonstrated the sensitivity of the TGA to known thrombophilias, with increased thrombin generating potential demonstrated in affected patients (although with significant variability between patients with the same defects) [Van Veen *et al.*, 2008]. Work has also been conducted to show that the assay is sensitive to a variety of anticoagulants as well as being able to detect the impact of potential reversal agents [Gerotziafas, 2007, Gatt *et al.*, 2008, Gatt *et al.*, 2009].

1.4.2.3 Other disorders and novel agents

Thrombin generation assays have aided in greater understanding of the concept of rebalanced haemostasis in patients with liver disease [Gatt *et al.*, 2010], challenging the previously held view that these patients – with abnormal routine tests of coagulation – were predominantly at increased risk of bleeding. In addition, the assay has been used to assess the bleeding tendency of conditions such as ITP (immune thrombocytopaenic purpura) and the impact of novel agents such as thrombopoietin receptor agonists [Alvarez Roman *et al.*, 2014]. There is also growing interest in the use of TGA to assess the pharmacokinetics of novel non-factor replacement agents in haemophilia currently in clinical trials.

1.4.3 Acquired coagulopathy

Acquired coagulopathy is a common scenario encountered in clinical practice. It most often presents with asymptomatic laboratory abnormalities, however active management is required in the context of bleeding and often prior to invasive procedures. There is no consensus definition for this condition. The majority of the literature pertaining to the management of major haemorrhage indicates that prolongation of the PT and APTT to >1.5 the upper limit of normal necessitates intervention [Erber *et al.*, 2006]. There is agreement that baseline coagulation assays are blunt tools in the diagnosis of developing coagulopathy. It is definitely the case that prolongation of these assays does not necessarily indicate an increased risk of bleeding. In contrast, patients can have a normal PT and APTT and still be at an increased bleeding risk.

Common causes of acquired coagulopathy include the use of anticoagulants, vitamin K deficiency, liver and renal disease, dilutional coagulopathy resulting from massive transfusion, disseminated intravascular coagulation, post-cardiac surgery bleeding and the coagulopathy of critically ill patients. Management often relies on the use of blood products such as fresh frozen plasma (FFP) and cryoprecipitate. This is mostly intuitive, based on an assumption that such products provide a physiological replacement of plasma proteins, rather than on robust evidence from randomised controlled trials [Stanworth *et al.*, 2006].

1.4.4 Inherited bleeding disorders

Inherited bleeding disorders range from being common (a conservative estimate of von Willebrand's disease is at least 100 per million [Sadler *et al.*, 2000]) to extremely rare (1: 2 million for prothrombin deficiency [Mumford *et al.*, 2014]). Although on the whole these disorders are rare, they range in severity and can be associated with significant morbidity and mortality. This burden of illness has both evident healthcare and socioeconomic costs as well as 'hidden' costs. As such, a large proportion of the global population of patients with these disorders either do not have access to treatment or only sub-optimal treatment. Haemophilia, the disease that has driven so much research into the mechanisms of haemostasis, is an X–linked inherited bleeding disorder characterised by deficiency or absence of coagulation factor VIII (FVIII) or factor IX (FIX), with an incidence of 1 in 5,000 male births for haemophilia A and 1 in 30,000 male births for haemophilia B [Franchini *et al.*, 2012]. Classically, it is

classified as mild (FVIII or FIX > 5%), moderate (FVIII or FIX 1-5%) or severe (FVIII or FIX < 1%) [Den Uijl *et al.*, 2011].

Phenotypic heterogeneity is well recognised with 10-15% of patients with severe haemophilia A (SHA) exhibiting a moderate bleeding phenotype with rare spontaneous bleeds [Van Den Berg, 2007, Jayandharan *et al.*, 2008, Pavlova *et al.*, 2013]. This is reflected in reduced annualised bleeding rates (ABR), arthropathy and factor concentrate usage.

The mechanisms underlying heterogeneity in bleeding phenotype are multifactorial. The principal determinant is factor level and in severe haemophilia this can range from just below 1% to complete absence [Santagostino *et al.*, 2010]. This will be predicated by the underlying mutation. The lower limit of detection of activity assays is such that levels can only be reported to be < 1 %. Thus, even having only a very low level of expression of functional antigen may lead to a different bleeding pattern that that seen in complete absence of FVIII or FIX.

An understanding of the mechanisms underpinning phenotypic heterogeneity is critically important in a new era of individualised treatment as this knowledge can help optimise prophylaxis in order to improve outcomes in patients. It is the coagulation phenotype rather than just factor VII/IX levels that determine the outcomes in this group of patients.

1.5 MD thesis – rationale and aims

In light of the evidence-base to date, this MD project has focussed on the use of the thrombin generation assay – specifically the CAT. The approach to

measuring the generation of thrombin has undergone many revisions since its inception in the 1950s, but the CAT is the most well described technique. The purpose of the studies described in the thesis has been to address the practicalities of transition of the assay into the diagnostic laboratory, the determination of normal reference ranges and further phenotyping of acquired and inherited coagulopathic states, specifically looking at aspects of initation and regulation of thrombin. The aim has been to utilise the TGA to assess both normal and abnormal coagulation. The thesis has thus been divided into the following sections:

1.5.1 Normal coagulation

In order to understand what is abnormal, it is necessary to understand what is normal. Optimisation and standardisation of the assay protocol in addition to the generation of normal reference ranges under a range of conditions is an integral part of this thesis and the data have then been used to assist in the investigation of abnormal coagulation.

1.5.2 Abnormal coagulation – modification of the initiation and regulation of thrombin generation

1.5.2.1 The clinical use of exogenous heparin – its impact on thrombin generation and exploration of potential means of reversal

This study consists of an *in vitro* proof-of-concept study assessing both the sensitivity of the assay to anticoagulants and the degree of reversibility of

different anticoagulants (unfractionated heparin, low molecular weight heparins, fondaparinux) with the addition of prothrombin complex concentrate, protamine sulphate or both. The second part of the study consists of *ex vivo* spiking of samples from patients receiving treatment with LMWHs with the reversal agents.

1.5.2.2 The possible role of TFPI in the variability of bleeding phenotype in moderate and severe haemophilia

This study consists of 2 parts. The initial part is a cross-sectional study looking for correlation between bleeding phenotype and components of the initiation of the extrinsic pathway in patients with severe haemophilia A and B. In the second part, the thrombin generation assay is used to individually phenotype patients with severe haemophilia A and address the role of tissue factor pathway inhibitor, one of the three key inhibitors of coagulation.

2 The thrombin generation assay

2.1 Initiation and regulation of thrombin generation

2.1.1 Why measure thrombin?

Thrombin has been found in all vertebrate species studied thus far and is the central protease of the haemostatic process [Davie *et al.*, 2006]. Prothrombin (FII), a 70kDa glycoprotein synthesised in the liver and subsequently secreted in blood, is the inactive zymogen precursor of thrombin. The DNA sequence is initially translated into prepro-prothrombin that undergoes a sequence of post-translational modifications resulting in the prothrombin that is secreted into the circulation. Of key importance is the γ–carboxylation of the Gla domain performed by γ-glutamyl carboxylase that confers the ability to adopt the conformation required for binding to anionic phospholipid surfaces at sites of vascular injury and also on activated platelets [Davie *et al.*, 2006]. Prothrombin is converted to thrombin by the prothrombinase complex (FXa/FVa). The resulting thrombin molecule has multiple functions, both pro- and anti-coagulant:

- The facilitation of positive feedback amplification via the activation of FXI, FVIII and FV.
- The proteolytic conversion of fibrinogen to fibrin.
- The conversion of FXIII to FXIIIa. This results in the covalent cross-linking of glutamine and lysine residues on adjacent fibrin molecules.

- The proteolysis of TAFI (thrombin-activatable fibrinolysis inhibitor) resulting in TAFIa (activated TAFI). This carboxypeptidase removes the lysine residues from fibrin thereby impeding clot lysis by fibrinolytic enzymes.
- The activation of platelets via protease activated receptors PAR1 and PAR4 as well as the platelet surface glycoprotein GP1bα [Adam *et al.*, 2003].
- The binding to thrombomodulin – an integral membrane protein found on vascular endothelial cells – that produces an anticoagulant effect by masking a region of the thrombin molecule critical for the binding of procoagulant substrates. The complex of thrombin bound to thrombomodulin activates protein C that, in turn, proteolytically inactivates the cofactors FVIIIa and FVa thereby downregulating haemostasis.
- The stimulation of multiple other cellular responses in cells other than platelets including the expression of adhesion molecules, growth factors and cytokines.

2.1.2 Key components in the initiation of thrombin generation

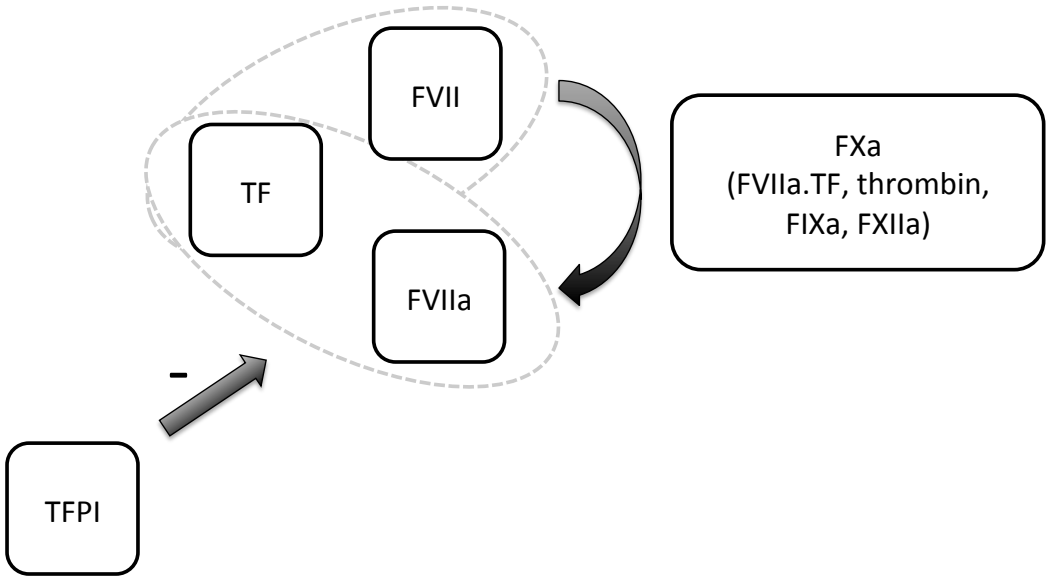


Figure 2-1 Components of interest in the initiation of the extrinsic pathway. Exposure of TF on endovascular cells leads to formation of a complex with FVIIa. FVII is principally activated by FXa, but also by the TF.FVIIa complex itself, thrombin, FIXa and FXIIa [Van't Veer *et al.*, 2000]. Once formed, the complex can be inhibited by TFPI (tissue factor pathway inhibitor) in the presence of its cofactor Protein S, thus forming one of the mechanisms whereby thrombin generation can be suppressed.

2.1.2.1 Tissue factor

From the early days of coagulation research, thrombokinase or tissue thromboplastin (eventually known as tissue factor (TF)) was recognised as a key component in haemostasis [Saito *et al.*, 2011]. However, much of the understanding of the haemostatic process during the first half of the 20th century was gleaned from the study of patients deficient in certain clotting factors and, in the absence of an isolated protein or identifiable deficiency, other proteins

superseded TF in terms of clinical interest. This was compounded by the cascade or waterfall theories published in 1964 that favoured the contact (or intrinsic) pathway of activation via FXII and gave less weight to the role of TF [Davie *et al.*, 1964, Macfarlane, 1964]. The lack of certainty regarding the identity and role of TF was reflected in its confusing nomenclature and it was renamed FIII during work to standardise the terminology in the 1950s and 60s. It was understood that TF formed a complex with FVIIa, phospholipids and calcium that then activated FX, but it was unclear what actually constituted 'tissue factor'. In 1977, Osterud and Rapaport further elucidated that the TF.FVIIa complex also activated FIX as well as FX [Østerud *et al.*, 1977]. TF was eventually purified in 1981 [Bächli, 2000]. Following this, its cDNA was isolated and sequenced and then the 3D crystal structure elucidated. The understanding of the importance of the TF.FVIIa complex in activating FX and FIX, in addition to greater understanding of the role of FIX as well as the identification of TFPI, re-focussed attention on the 'extrinsic' pathway of haemostasis.

It is now known that TF is a 47 kDa cell-bound trans-membrane glycoprotein constitutively expressed by many extravascular cells and with organs and tissues such as the brain and placenta particularly rich in concentration [Key *et al.*, 2007]. In addition, TF is now recognised to play a role in numerous processes including migration and proliferation of vascular smooth muscle cells, development of embryonic blood vessels, tumour neo-vascularisation and metastasis as well as induction of the pro-inflammatory response [Key *et al.*, 2007].

The cascade model of haemostasis was not intended as a literal model of the *in vivo* process, but outlined a step-wise process of proenzyme-enzyme transformation that was supported by the evidence at the time and helped to explain the screening coagulation tests (prothrombin time - PT, activated partial thromboplastin time – APTT). The discovery that the TF.FVIIa complex not only activated FX but also FIX linked the extrinsic and intrinsic pathways. However, despite the growing recognition of the major role played by TF.FVIIa complex, the fact that the extrinsic pathway could not compensate for the pathophysiology of haemophilia A and B could not be explained.

2.1.2.2 Factor VII and Factor VIIa

Factor VII is a Vitamin-K dependent serine protease consisting of 406 amino acids with a molecular weight of approximately 50 kDa. The F7 gene maps to the long arm of chromosome 13, 13q34. The protein is present in plasma in two forms – a single-chain form that is the inactive zymogen and two-chain active form that is present in much smaller amounts (roughly 100 times less) [Perry, 2002]. The conversion of FVII to FVIIa is a result of cleavage of a single peptide bond between Arginine 152 and Isoleucine 153 that results in a light chain of approximately 20 kDa and a heavy chain of ~ 30 kDa. Post-translational modification of the Gla domain at the N terminus of the protein allows binding of calcium that, in turn, causes a conformational change in the molecule that exposes epitopes that facilitate binding both to TF and phospholipids [Perry, 2002].

2.1.2.3 The TF.FVIIa complex

Tissue factor is one of 3 co-factors in the haemostatic process, the others being FV and FVIII. The inactive complex of TF.FVII is unable to activate physiologically significant levels of FX or FIX [Rao *et al.*, 1988]. However, binding of TF to FVIIa increases the catalytic activity of FVIIa by approximately 30,000 fold [Wood *et al.*, 2014]. It is thus important to understand how FVII is activated. It is clear that there are trace amounts of FVIIa circulating under basal conditions. Similarly, there is also evidence that normal individuals produce low concentrations of activated FX in the absence of injury [Rao *et al.*, 1988]. The current hypothesis is that following injury to a vessel wall, the majority of exposed TF will bind FVII. However, there will be trace amounts of FVIIa that also bind TF. The latter complexes will result in small amounts of FXa being generated that, in turn, will activate the inactive TF.FVII complexes and thus greatly increase the amount of FXa produced [Rao *et al.*, 1988]. FXa is thought to be the principle physiological activator of FVII, but the TF.FVIIa complex, thrombin, FIXa and FXIIa have also been reported to activate FVII [Van't Veer *et al.*, 2000].

2.1.3 Regulation of thrombin generation

Given the central role of thrombin in haemostasis, it is critically important that its activity is carefully regulated in order to avoid inappropriate or excessive thrombus formation. Members of the serine protease family (serpins) perform this role – key proteins include antithrombin (AT) in addition to heparin cofactor II (HCII) and protease nexin I (PNI). The activity of these serpins is enhanced by

endogenous (and exogenous) glycosaminoglycans such as heparin, heparan sulphate and dermatan sulphate [Davie *et al.*, 2006]. Importantly, TFPI plays a role in the initiation phase of thrombin generation by inhibiting the TF.FVIIa complex although it does not itself directly interact with thrombin.

2.1.3.1 Antithrombin and heparinoids

Antithrombin (AT) plays a critical role in the regulation of haemostasis by the inhibition of thrombin (FIIa) and FXa (although it also has an inhibitory effect on other components of haemostasis) [Li *et al.*, 2004, Patnaik *et al.*, 2008]. Although AT circulates at high concentrations, it is only once it interacts physiologically with endogenous heparinoids that it becomes an efficient inhibitor. Unless exogenous heparin is being administered, free heparin is not found in the circulation. Thus, the endothelial-bound heparinoids maximise inhibitory activity and play a role in ensuring unimpeded blood flow in the vascular system, in particular the microvascular system [Patnaik *et al.*, 2008]. The key role of AT is underlined by the fact that a mouse knockout model is associated with embryonic lethality, that AT deficiency is associated with an increased risk of venous thrombosis and that heparin – that principally targets AT – is a successful anticoagulant [Huntington, 2003].

2.1.3.2 Tissue Factor Pathway Inhibitor

Not long after the key role of TF was described, Loeb and Dold independently suggested that serum contained a substance that inhibited the procoagulant activity of tissue extracts [Broze, 2003]. Further work on the identity of this

inhibitory substance stalled until 1985, when Sanders et al. reported that the lipoprotein component of plasma contained an inhibitor of the TF.FVIIa complex that required the presence of FX [Broze, 2003]. Research revealed that this substance was released from cultured endothelial cells by heparin. Purification of the substance by competing groups revealed that it was a multivalent, Kunitz-type serine protease inhibitor that, in the presence of FX and calcium, formed a quaternerary complex with TF.FVIIa. It was initially termed tissue factor inhibitor (TFI), lipoprotein-associated coagulation inhibitor (LACI) and extrinsic pathway inhibitor (EPI) but consensus was reached in 1991 when it was officially named tissue factor pathway inhibitor (TFPI) [Broze, 2003].

2.2 The modern thrombin generation assay

The modern thrombin generation assay (TGA) detects the generation of thrombin over time in platelet poor or platelet rich plasma (PPP or PRP) using a fluorogenic substrate and individual calibration. Four key parameters are calculated by specific software. These consist of the lag time (LT, minutes), peak (nM), time to peak (ttPeak, minutes) and endogenous thrombin potential (ETP, nM*min) (see Chapter 1, Figure 1-5). In this following section, the development of the assay is reviewed as well as an assessment of the impact of pre-analytical variables and reagents.

2.2.1 The history of the thrombin generation assay

In 1953, Biggs, Douglas and MacFarlane in Oxford described the thromboplastin generation test [Biggs *et al.*, 1953]. Research was focussed at the time on the activation of prothrombin and they hypothesised that, by plotting thrombin concentration against time, curves could be obtained that demonstrated the speed of thrombin generation and destruction and could, therefore, reflect the rate of production and activity of blood thromboplastin (TF) [Macfarlane *et al.*, 1953]. The assay they described involved taking whole blood into a glass tube in a water bath at 37^0C. Aliquots of blood were added to tubes containing fibrinogen solution (also in the waterbath at 37^0C) and the clotting times were recorded at 1-minute intervals. A thrombin calibration curve was run with each batch of fibrinogen with a standard thrombin solution diluted to 10 different concentrations (~ 0.5 – 20 u/ml). An aliquot of each dilution was added to the fibrinogen solution and clotting times recorded. They describe results from 12 healthy volunteers (6 men and 6 women) and noted several characteristics of the curves obtained:

1) a well defined latent period of 2-4 minutes

2) a sudden, rapid rise in thrombin generation to 7-15 u/ml

3) an initial rapid decline in thrombin generation after approximately 6 minutes that thereafter slowly returned to baseline.

The Oxford group went on to study thrombin generation in blood from patients with thrombocytopaenia and haemophilia. They commented that the effect of a reduction in the number of platelets proportionately reduced the rate and amount of thrombin generated such that they thought that platelets were

involved quantitatively with the amount of intrinsic thromboplastin (TF) produced in the blood. In patients with haemophilia, particularly severe haemophilia, they observed that the latent period was significantly prolonged, in some cases to such an extent that barely any thrombin generation was observed. They thought that this meant that the anti-haemophilic globulin concentration (FVIII had not yet been identified as the underlying deficiency) therefore controlled the time at which intrinsic thromboplastin was formed. The prolonged clotting times seen in such patients was therefore not secondary to reduced potency of thromboplastin, but a prolonged period of time required to generate it. The group saw great potential in the test and could see it being used in diagnosis as well as response to treatment in various bleeding disorders [Biggs *et al.*, 1953, Macfarlane *et al.*, 1953].

Pitney and Dacie described their experience of detecting thrombin generation at around the same time [Pitney *et al.*, 1953]. The difference in their method was the use of recalcified plasma, as opposed to the whole blood used by Biggs et al. They collected whole blood into sodium citrate and then centrifuged it to collect PRP. The rest of the assay was run similarly to the one described above. They also noted the latent time prior to the start of thrombin generation, a peak in the amount of thrombin generated and subsequent decline, such that barely any thrombin was generated after 10 minutes. This confirmed the understanding that the majority of thrombin was generated after the initial formation of a clot i.e. that the clotting time that detected just the initial clot formation gave a view of just a small aspect of the haemostatic mechanism. There were multiple practical and technical challenges with the assay including

substrate exhaustion at high thrombin concentrations and difficulty in detecting a clot at low thrombin concentrations that resulted in imprecise results and significant margin for error [Baglin, 2005]. In the 1980s, a group of researchers including Hemker revisited the assay with the aim of making it more reliable and less imprecise. This work was facilitated by a greater understanding of the structure and function of the thrombin molecule.

2.2.2 The Calibrated Automated Thrombogram (CAT)

In the mid 1980s, Hemker et al. published a modified thrombin generation assay in which the fibrinogen solution was replaced by a chromogenic substrate and the primary plasma sample was defibrinated [Hemker, 2008]. This precluded the use of PRP as turbidity in the plasma (that increases as clot is formed) affects the accurate measurement of optical density [Hemker *et al.*, 2000, Baglin, 2005]. Although the modifications reduced the imprecision of the assay, the chromogenic substrate was recognised to give an erroneous measurement of the thrombin-decay process. It did however mean that a computerised system was developed linked to a time-recording pipette that minimised imprecision relating to sampling [Baglin, 2005].

It was suggested that a fluorogenic substrate was used instead[1]. This circumvented the issues with plasma turbidity, enabling the use of PRP (a more physiological assay) - and even potentially whole blood - but led to challenges

[1] *Substrate – Z-Gly-Gly-Arg bound to 7-amino-4-methylcoumarin*

in determining the thrombin concentration from the fluorescence readings. Unlike the optical density readings with the chromogenic substrate that correlated neatly with the concentration of the cleavage of the substrate and thus the concentration of thrombin, the readings produced with the use of the fluorescent substrate are non-linear i.e. the constant formation of the product produced by cleavage of the substrate results in a steadily decreasing velocity of fluorescence increase. This is compounded by consumption of the substrate – the signal is dependent on the amount of substrate but this decreases over time due to the nature of the reaction. This means that the first derivative of the signal produced does not directly reflect the thrombin generated. This is partly also because the relationship between fluorescence intensity and the concentration of the fluorescent molecules is non-linear [Hemker *et al.*, 2003]. Fluorescent molecules absorb light from other molecules – a phenomenon known as the 'inner filter effect'. Increasing substrate concentrations in order to mitigate consumption increases this 'inner filter effect' [Hemker *et al.*, 2002]. In addition, the magnitude of the fluorescence signal is quenched by the plasma in which it is measured and the degree to which this occurs varies considerably [Baglin, 2005]. The problem was solved by the parallel use of a calibrator – of fixed enzymatic activity – that was added to the test plasma in which no thrombin generation was triggered. The calibration factor could therefore be determined at every time-point i.e. at all fluorescence levels.

2.2.2.1 The importance of the calibrator

Thrombin itself cannot be used as a calibrator as it is rapidly inactivated [Hemker *et al.*, 2013] - predominantly by antithrombin and α-2 macroglobulin (α-2M). Antithrombin is an active site inhibitor therefore, as thrombin is inactivated by antithrombin, cleavage of the substrate would reduce to zero. The calibrator used in the CAT is α-2M as the substrate is still able to bind to the α-2M thrombin complex and be cleaved. α-2M inhibits a wide variety of proteases including thrombin. This molecule contains a 'bait' region and cleavage of this region leads to a conformational change in the α-2M that shields access to the active site of the bound protease (in this case thrombin) and therefore decreases access to protein substrates. Thus the α-2M thrombin complex leads to inactivation of thrombin's haemostatic activity. In the circulation, the α-2M thrombin complex is then cleared. Importantly in the assay, the bound thrombin molecule is still able to cleave the small fluorescence substrate used for the CAT and so, as free thrombin is bound to α-2M, cleavage of the substrate remains constant determined by the concentration of α-2M [Baglin, 2005]. The concentration of the calibrator needs to generate fluorescence intensities that cover the range produced by the sample in which thrombin is generated after TF is added.

The fluorescent substrate binds reversibly to the thrombin active site. There needs to be sufficient substrate to measure the thrombin generated in the assay and avoid substrate depletion. However, by occupying the active site it will also be potentially preventing thrombin's physiological activity and so the assay arguably does not truly reflect the natural process. In addition, the higher the

concentration of substrate, the greater the inner filter effect. Continuous readings are taken from both the test sample and calibration sample throughout the duration of the assay. The calibration factor itself varies in time and between different plasma samples, hence why it should be calculated for each test sample.

Fluorescence intensities are taken at sequential time-points and, from this, the velocity of fluorescence increase can be calculated – the first derivative (dF/dt). However, the relationship between the first derivative and thrombin concentration is not straightforward. It is necessary to take into account the impact of substrate consumption during the assay and the 'inner filter effect' impacting on the fluorescence readings. The substrate declines as the assay progresses and the 'inner filter effect' increases. The data then need to be modified by the calibration factor Cf. This is a value by which each observed dF/dt measured needs to be multiplied in order to obtain the thrombin concentration – Cf itself gradually increases during the experiment. This allows transformation of the raw test data into corrected ideal data. All of this is performed by the accompanying software for the thrombogram.

2.2.2.2 Pre-analytical variables

The TGA is highly sensitive to pre-analytical variables. The problem is that of pre-activation of haemostasis and thus all aspects of sample collection and processing have been scrutinised in order to improve assay precision and reliability [Loeffen *et al.*, 2012]. This includes the type of needle and collection system used for venepuncture, the use of a discard, the requirement for

inactivation of the contact pathway (using corn trypsin inhibitor, CTI), spinning of the samples and the time window for analysis.

Loeffen et al [Loeffen *et al.*, 2012] have produced recommendations for a standardised protocol for TGA (see Table 2-1).

Table 2-1 Recommended procedure to minimise impact of pre-analytical variables (adapted from [Loeffen *et al.*, 2012])

Preanalytical variable	**Recommendation**
Blood collection system	Conventional straight needle recommended as device of first choice; butterfly needles accepted if proper phlebotomy technique used
Blood collection tube	Addition of CTI to the collection tube is recommended to inhibit contact activation. Without CTI addition, the Monovette tube is the one lowest in inducing contact activation
Discard tube necessity	Required
Whole blood incubation	Direct plasma preparation preferred to storage of whole blood for 6 hours
Centrifugation method	Double centrifugation is preferred to single. Recommended protocol: 2000 g for 5 min, 10 000 g for 10 min
Plasma incubation	After plasma thawing, immediate analysis is recommended; when direct measurement is not possible, plasma is most stable when kept at room temperature

2.2.2.3 The importance of inhibition of the contact pathway

Contact factor activation (i.e. the triggering of haemostasis via the intrinsic pathway), through the process of venepuncture and sample collection, is a potential confounding factor when performing coagulation assays in which the trigger is TF. This is therefore a particularly relevant concern in the TGA especially in samples with low levels of thrombin generation where contact factor activation can result in overestimation of the thrombin generating potential.

CTI is a selective FXIIa inhibitor derived from maize. It was originally discovered and purified in the 1970s and used as an inhibitor for trypsin [Hansson *et al.*, 2014]. Since its initial discovery it has also been found to inhibit FXIIa, although not FXa, thrombin or kallikrein. Studies have therefore been performed to assess both the impact of CTI as well as to determine the concentration at which it is most effective. Concerns have been raised that at high concentrations, CTI can start to impact on FXIa and the thrombin feedback loop [Hansson *et al.*, 2014].

TGA tends to employ a TF concentration of 1 or 5 pM in PPP and 0.5 pM in PRP. The concentration used will be chosen dependent on the defect being investigated e.g. samples from patients with haemophilia will be triggered with lower concentrations (1 pM). Another factor to consider in the use of CTI is its expense – an issue that becomes particularly relevant when thinking about employing thrombin generation assays in the diagnostic coagulation laboratory. It has been demonstrated that thrombin generation due to contact activation can be detected in the TGA and that the addition of CTI abolishes this effect.

There is a minimum concentration of CTI required in order to achieve this effect (14 μg/ml final concentration in whole blood) and, in addition, in order to achieve maximum efficacy it is important that the blood is collected into a bottle containing the CTI rather than the CTI being added after the plasma has been separated [Luddington, 2004].

2.2.2.4 Centrifugation - platelets, microparticles and platelet debris

The significance of the protocol for centrifugation of the samples for TGA relates to the impact of platelets, microparticles and platelet debris on subsequent thrombin generation. As discussed, TGA can be run on PRP or PPP. Microvesicles are shed from PRP when stored at room temperature that can impact further on thrombin generation [Vanschoonbeek *et al.*, 2004].

There is notable inter-individual variation in TGA results from PRP and platelet count impacts on thrombin generation [Vanschoonbeek *et al.*, 2004, Gerotziafas *et al.*, 2005]. Many studies therefore adjust the PRP platelet count to 150 x 10^9/L with autologous PPP [Vanschoonbeek *et al.*, 2004] (see Figure 2-2).

The protocol recommended by Loeffen et al [Loeffen *et al.*, 2012] states that double centrifugation should be employed for PPP in order to minimise microparticles and platelet debris that can contribute to variability in results. This is supported by findings from other studies that emphasise the importance of adequate centrifugation to remove the impact of white cells and platelets on results [Dargaud *et al.*, 2005].

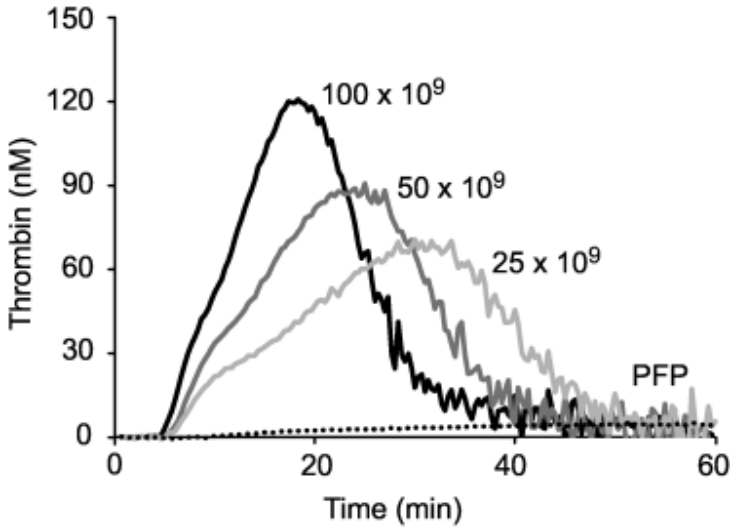


Figure 2-2 Effect of platelet count on thrombin generation (0.5 pM TF used as trigger). Reproduced from [Vanschoonbeek *et al.*, 2004]. This clearly demonstrates the correlation between platelet number and amount of thrombin generated. The higher the platelet count, the greater the degree of thrombin generation.

2.3 Analytical factors - triggers

2.3.1 Tissue factor

There is, as yet, no international standard for the tissue factor trigger for TGA. Reagents are available that have been developed for use with the TGA (Thrombinoscope B.V., Maastricht, The Netherlands). These do not have documented TF concentrations per batch and there is likely batch-to-batch variability. They do contain phospholipid for the PPP reagents. The assumed approximate TF concentrations are:

PRP reagent – 0.5 pM

PPP Low – 1 pM

PPP reagent – 5 pM

PPP High – 20 pM

An alternative source of TF is the PT reagent Innovin® (Dade® Innovin®, Siemens, UK). This is a lyophilised product containing recombinant human TF produced in E.coli that also contains synthetic phospholipids, calcium, a heparin neutralising compound, buffers and bovine serum albumin (as a stabiliser). When used for PPP, exogenous phospholipids are still required.
The National Institute for Biological Standards and Control (NIBSC) have also produced a lyophilised recombinant human TF containing synthetic phospholipids and other components similar to commercial PT reagents that is quantified in units rather than pM (personal correspondence Dr E. Gray, NIBSC).

2.3.2 Thrombomodulin/ Protac™

Thrombomodulin is expressed on endothelial cell surfaces. It binds to thrombin forming a complex that – as a result of allosteric modification of the substrate binding site – activates protein C that, in the presence of protein S, inactivates activated FV and FVIII. Protac™ is an extract of the venom of the snake Akistrodon contortrix – the North American copperhead – that activates protein C and can be used in place of thrombomodulin.
Both thrombomodulin and Protac™ have been used by researchers in addition to TF in TGA in order to try and achieve more physiological results by reflecting

the anticoagulant role of thrombin. An example of this is the use of the TGA in assessing the thrombin generating potential in samples from patients with liver disease where the use of thrombomodulin allows an assessment of the decreased synthesis of physiological anticoagulants [Tripodi *et al.*, 2007, Lisman *et al.*, 2010]. This does add another variable into the assay. Neither thrombomodulin nor Protac™ have been used in the studies in this thesis as the focus was on the procoagulant action of thrombin.

2.4 Analytical factors - quality assurance

One of the challenges of the TGA is reproducibility and intra-assay and inter-assay variability. As a general rule, a coefficient of variation (CV)[2] of 10% or below is considered acceptable for diagnostic assays.

External quality assurance is an important process whereby diagnostic assays can be assessed in order to ensure that they are accurate, precise and specific. It also ensures that different laboratories analysing the same specimen by the same assay achieve similar results (taking into account an accepted degree of variability due to the nature of the assays and handling of biological specimens).

Researchers have identified that the use of different sources and concentrations of tissue factor and phospholipid in the TGA lead to unacceptably high CVs, but that this can be improved by the use of a

[2] for a given data set CV = standard deviation/mean

standardised protocol with defined reagents and concentrations [Dargaud *et al.*, 2007]. In addition, the use of CTI to inhibit FXIIa activation with low TF concentrations also reduces the CV. In one multicentre study, a set of samples were sent for PPP TGA. Interestingly, the laboratory with the highest CV had had the samples processed by a relatively inexperienced member of staff, highlighting the need for operator experience and proficiency. In addition, it has been noted that there are issues with manual dispensing of the substrate – partly due to potential errors or variability in the volume dispensed in addition to concerns about temperature deviation from 37^0C [Dargaud *et al.*, 2010]. Different versions of the software used by the laboratories also appeared to have a role in variability.

An important means of reducing the CV of the assay further is the use of reference plasma in order to be able to normalise results. Normalisation entails the generation of a ratio between the test sample results and a control (reference) sample run under the same conditions at the same time. If a reference range has been generated for this reference plasma, it can additionally also be used as a quality control. This is achieved by running a sample with each run and ascertaining that the results fall within the established range. A subsequent study conducted by the same researchers studied the use of different reference plasmas (both commercial and supplied by NIBSC) [Dargaud *et al.*, 2010]. Although the use of normalised results (regardless of the reference plasma) reduced the inter-centre variability, some reference plasmas produced better results than others (this may reflect methods of collection, numbers of donors and storage method). There is a recommendation by the

authors that a freeze-dried lyophilised reference plasma such as that being trialled by NIBSC should be used as a primary standard.

Increasing automation of the assay in conjunction with a standardised protocol (involving the use of a reference plasma) will aid in minimising the impact of human factors and improving intra- and inter-assay variability. This will make the routine (specialist) use of the assay a more realistic prospect.

2.5 Summary of pit-falls

There are numerous pitfalls of thrombin generation. However, awareness can help address and circumvent these factors. In order for the haemostasis research being conducted using this assay to be consolidated, a universal standardised protocol needs to be agreed upon taking into account the following important factors:

1. Sensitivity to pre-analytical variables
2. The requirement for contact inactivation
3. Calibration of individual samples to take into account the inner filter effect and substrate depletion
4. Use of a reference plasma as a standard

3 The generation of normal reference ranges for the thrombin generation assay

3.1 Rationale

There are, as yet, no published normal reference ranges for the thrombin generation assay and there will, of course, be intra- and inter-laboratory variability. Whilst normal reference ranges have previously been generated within the Royal Free Hospital haemostasis laboratory as part of a research study, there is not a complete set of normal ranges available for a range of different assay conditions used both in the laboratory by other researchers and for the studies in this thesis. Robust normal reference ranges are required in order to be able to recognise abnormal results and to be able to determine whether there is a correlation with clinical phenotype, such that the assay results can be used to aid clinical management.

3.2 Study aim

To establish normal reference ranges for the thrombin generation assay under different assay conditions.

3.2.1 Primary objective

To generate thrombin generation results on samples from healthy volunteers with a mix of age and gender under a range of assay conditions using the calibrated automated thrombogram (CAT) and the technique of Hemker et al [Hemker *et al.*, 2006].

3.2.2 Secondary objective

To assess any statistically significant differences between similar conditions using paired samples t tests (assuming the data are normally distributed) using SPSS 22.0.0.0 (SPSS, Chicago, IL, USA):

- Adjusted v unadjusted platelet count for PRP samples
- Double v triple spinning for CTI PPP samples
- Fresh v frozen PPP samples

3.3 General design

This is an *in vitro* laboratory study using the calibrated automated thrombogram (CAT) and the technique of Hemker et al [Hemker *et al.*, 2006] using a standardised protocol (see Supplementary material 3.7) taking into account previous work on the impact of pre-analytical variables [Loeffen *et al.*, 2012]. Ethical approval and informed consent for the collection of plasma samples from healthy volunteers was obtained via the Katharine Dormandy Coagulation Research Plasma Bank (KDCRPB; REC reference number 14/YH/1272).

3.3.1 Primary end-points

Lagtime LT (min), Peak (nM), time to Peak ttPeak (min) and endogenous thrombin potential ETP (nM*min).

3.3.2 Secondary end-points

Results of paired t tests.

3.4 Protocol for the thrombin generation assay

3.4.1 Normal controls (healthy volunteers)

Volunteers were members of staff working in various departments within the Royal Free hospital. A mix of gender and age was sought. The following inclusion and exclusion criteria were applied.

Inclusion criteria

- Individuals aged 18 or over
- Able to give informed consent
- Reasonable vascular access

Exclusion criteria

- Known inherited or acquired bleeding or thrombotic disorder
- Anti-platelet or anticoagulant medication

3.4.2 Materials and methods

Citrated whole blood samples (CWB) were collected into Sarstedt Monovette® tubes (citrate 3.2% 0.109 M; with and without CTI) as per protocol and processed within two hours. PRP and PPP samples were prepared. CTI PPP was both double spun and triple spun. Aliquots of PPP (± CTI, double spun and triple spun) were also frozen at -80^{0}C for batch analysis at a later point. The platelet count was determined for the PRP samples and then adjusted using autologous PPP to reach a final platelet count of 150 x 10^9/L. The detailed protocol is described in Supplementary material 3.7.

Plates were prepared using a standard format (see Tables 3-1 and 3-2). The range of assay conditions were selected in order to encompass studies examining both hypo- and hypercoagulable states, in addition to allowing comparison of differences in sample preparation, TF concentrations and timing of analysis (i.e. fresh v frozen). Fluorescence was measured using a Fluroskan Ascent fluorimeter (Thermolab Systems, Helsinki, Finland). The endogenous thrombin potential (ETP), lagtime (LT), peak and time to peak (ttPeak) were derived from the raw data by dedicated software (Thrombinoscope™ B.V., Maastricht, The Netherlands).

Table 3-1 TGA assay conditions for normal reference ranges; number in brackets relates to sample identification for assay. Platelet count adjusted to 150 x 10^9/L; CTI = corn trypsin inhibitor

10 ml CTI sample		
PRP	***Unadjusted plt count***	***Adjusted plt count***
	PRP reagent (1)	PRP reagent (2)
	Innovin 0.5 pM (3)	Innovin 0.5 pM (4)
	Innovin 1 pM (5)	Innovin 1 pM (6)
PPP	***Double spun***	***Triple spun***
	PPP LOW (7)	PPP LOW (9)
	Innovin 1 pM (8)	Innovin 1 pM (10)
		Innovin 2 pM (11)
	Quality and normalisation control: normal pool + CTI	
5 ml sample		
PPP	***Double spun***	
	PPP reagent (12)	
	Innovin 5 pM (13)	
	Quality and normalisation control: normal pool	

Table 3-2 Layout of 96 well plate for normal reference range TGA; see Table 3-1 for sample identification 1-13; Cal = calibrator; NP3 = in-house normal plasma pool; all samples run in duplicate

1	2	NP3/CTI PPP Low	-	-	-
3	4	NP3/CTI Innovin 1 pM	-	-	-
5	6	NP3/CTI Innovin 2 pM	-	-	-
7	8	NP3 PPP Reagent	-	-	-
9	10	NP3 Innovin 5 pM	-	-	-
11	-	NP3 Cal	-	-	-
12	13	-	-	-	-
PRP Cal	PPP Cal	-	-	-	-

3.4.3 Statistical analysis

Statistical tests were performed on the raw data using Excel 2011 (Microsoft) and SPSS 22.0.0.0 (SPSS, Chicago, IL, USA). The normal distribution of the data was confirmed by Shapiro-Wilk testing (normality confirmed by $p > 0.05$). Paired t tests were performed to detect any statistically significant difference between results using different assay conditions. Unless otherwise specified, a p value of < 0.05 was considered statistically significant.

3.5 Results

Samples were collected from 41 individuals – 21 male and 20 female. The mean age was 37 years (range 21 – 59 years). All samples were run under all assay conditions including both PRP and PPP. PRP parameters were

expressed as raw results; PPP results were expressed as both raw and normalised results.

Normalised results = Test sample result / Control plasma result

The control plasma was either CTI or non-CTI in-house normal plasma pool (NP3; see Supplementary material 3.9 for further information).

3.5.1 PRP normal ranges

Samples 1-6 (see Table 3-1) encompassed the PRP samples with both unadjusted and adjusted samples run with the same triggers and on the same plate. See Figure 3-1 for an example. As the platelet count was determined on PRP and no volunteer was thrombocytopaenic, all samples were diluted with autologous PPP to achieve an adjusted count of 150 x 10^9/L (mean unadjusted PRP platelet count 373 x 10^9/L).

As expected, the platelet count had an impact on thrombin generation. The results of the PRP samples are displayed in Table 3-3. Within each group, the LT and ttPeak shortened with an increase in the peak and ETP as the concentration of TF in the trigger increased. There were appreciable differences in the results between triggers (ready-made and in-house) theoretically containing a similar concentration of TF. As with all the results of the normal volunteers, there was a wide range seen in all parameters.

Table 3-3 Normal range for PRP with a range of TF reagents and concentrations with unadjusted (U; mean 373 x 10^9/L) and adjusted (A; 150 x 10^9/L) platelet counts; results expressed as mean ± 2SD

Plt count	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
U	PRP reagent	11.41 (3.84 - 18.98)	1826.06 (1104.89 - 2547.23)	137.00 (44.54 - 229.46)	21.97 (12.40 - 31.54)
A	PRP reagent	12.88 (4.05 - 21.71)	1923.94 (1273.71 - 2574.18)	107.83 (50.15 - 165.51)	27.46 (16.55 - 38.36)
U	Innovin 0.5	9.82 (1.09 - 18.55)	1728.41 (1049.39 - 2407.42)	138.23 (24.17 - 252.29)	20.05 (12.10 - 27.99)
A	Innovin 0.5	10.13 (2.25 - 18.01)	1739.02 (1134.48 - 2343.55)	105.49 (40.01 - 170.97)	24.28 (14.27 - 34.29)
U	Innovin 1	6.78 (0.28 - 13.28)	1790.39 (1121.91 - 2458.86)	139.86 (32.98 - 246.75)	17.32 (10.93 - 23.71)
A	Innovin 1	6.58 (2.19 - 10.97)	1836.97 (1190.06 - 2483.88)	111.73 (39.68 - 183.77)	19.86 (12.87 - 26.85)

3.5.1.1 Adjusted and unadjusted platelet count

Review of the data between groups (unadjusted versus adjusted platelet count) demonstrated that there was a difference between all four TGA parameters (see Figure 3-1 for an example). However, the most marked difference was in the peak thrombin generated with reduction in peak values in the samples with an adjusted platelet count. All three groups (i.e. PRP reagent, Innovin 0.5 pM and Innovin 1 pM)) were analysed by paired t-test to ascertain whether there was a statistically significant difference as a result of standardising the platelet count (see Table 3-4).

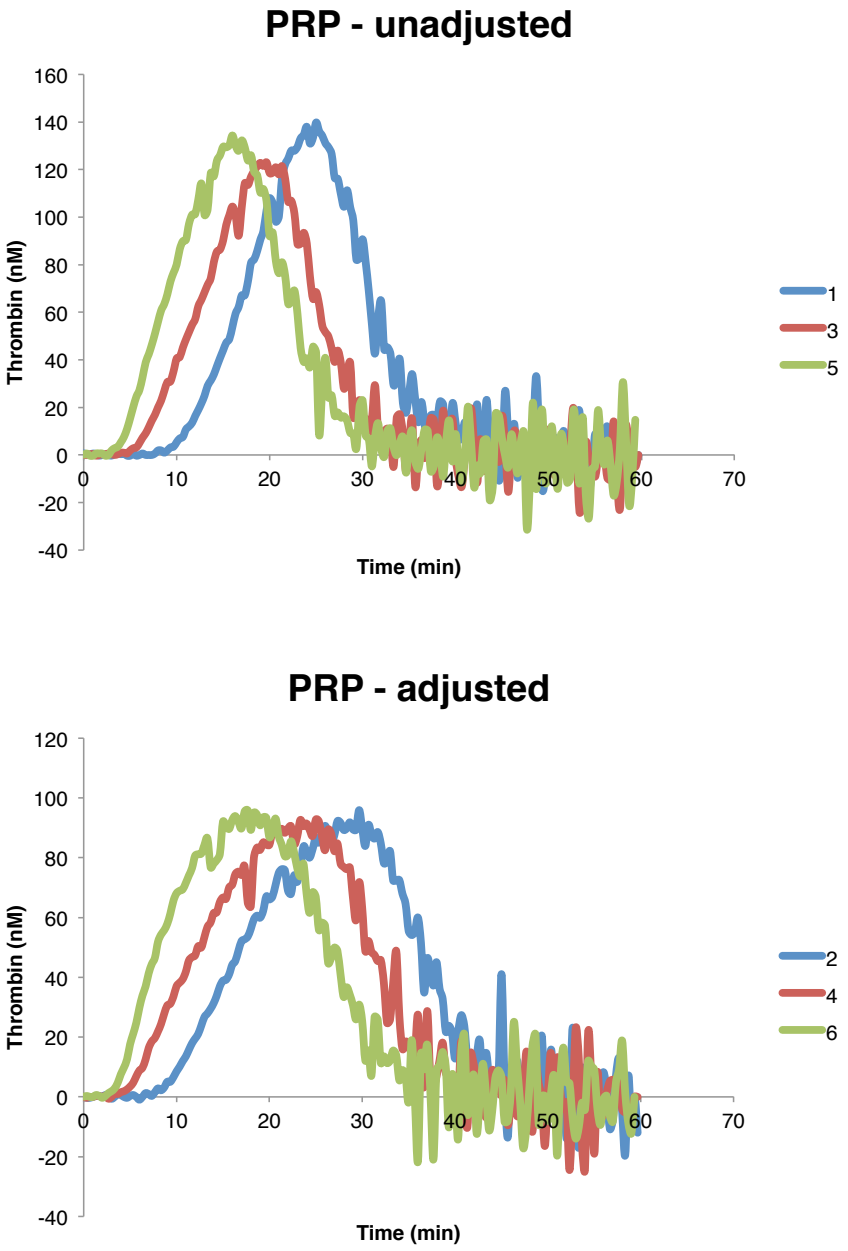


Figure 3-1 Example of normal healthy volunteer PRP TGA curves with unadjusted (395 x 10^9/L) and adjusted (150 x 10^9/L) platelet counts; 1,2 = PRP reagent; 3,4 = Innovin 0.5 pM; 5,6 = Innovin 1 pM. Note the difference in peak thrombin generation.

Table 3-4 Paired t test results for unadjusted (U) versus adjusted (A) platelet counts with different TF trigger reagents

PRP Reagent

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - U - LT - A	-1.54553	4.26930	.69257	-2.94881	-.14224	-2.232	37	.032
Pair 2	ETP - U - ETP - A	311.7105	1248.2817	202.4980	-98.5895	722.0105	1.539	37	.132
Pair 3	Peak - U - Peak - A	28.86421	58.90567	9.55576	9.50240	48.22602	3.021	37	.005
Pair 4	ttPeak - U - ttPeak - A	-5.53342	5.68816	.92274	-7.40307	-3.66377	-5.997	37	.000

Innovin 0.5 pM

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - U - LT - A	-.85694	4.10379	.68396	-2.24547	.53158	-1.253	35	.219
Pair 2	ETP - U - ETP - A	383.5278	890.8873	148.4812	82.0949	684.9607	2.583	35	.014
Pair 3	Peak - U - Peak - A	25.66389	49.74284	8.29047	8.83333	42.49444	3.096	35	.004
Pair 4	ttPeak - U - ttPeak - A	-4.53694	4.62708	.77118	-6.10252	-2.97137	-5.883	35	.000

Innovin 1 pM

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - U - LT - A	.19667	3.70735	.61789	-1.05772	1.45105	.318	35	.752
Pair 2	ETP - U - ETP - A	-152.4722	796.6284	132.7714	-422.0125	117.0680	-1.148	35	.259
Pair 3	Peak - U - Peak - A	28.38361	60.96793	10.16132	7.75503	49.01219	2.793	35	.008
Pair 4	ttPeak - U - ttPeak - A	-2.53639	3.30976	.55163	-3.65625	-1.41653	-4.598	35	.000

There was a statistically significant difference between adjusted and unadjusted platelet counts for the peak thrombin generated and the ttPeak with all three TF reagents tested. This is in accordance with the main role of platelets in propagation of thrombin generation. Also, when the curves were examined (see Figure 3-1), the area under the curve (i.e. the ETP) could be seen to be similar as, with the higher platelet count the curve had a higher, sharper peak whereas with the adjusted platelet count, the curve – although the peak was less pronounced – had a greater spread. This would suggest that although the ETP value might not differ greatly between samples, this may not detect the potentially clinically important difference between a rapid, pronounced peak of thrombin compared to more gradual, less pronounced peak.

3.5.2 PPP normal ranges

The normal ranges for the PPP samples were expressed both as raw and normalised data (see Tables 3-5 – 3-10). Again, there was a wide range of results for all parameters. There appeared to be less variability in the means between the different reagents and TF concentrations between 1-2 pM. The normalised data were expressed as a ratio and review of the parameters showed that the mean of the normal volunteers were hypercoagulable as compared with the normal pool i.e. shortened LT and ttPeak, with higher peak and ETP.

There was a marked difference in increase in thrombin generation at the higher TF concentration of 5 pM (see Tables 3-9 and 3-10). Interestingly, the means of the normalised ratios are all much closer to 1.0.

3.5.2.1 The impact of increasing TF concentrations

Increasing the TF concentration in the trigger impacts on the results and is an important consideration when deciding what question the assay is being used to study. The higher the TF concentration, the greater the peak thrombin generation but also the shorter the LT. There was a fairly subtle difference between 1 and 2 pM Innovin, but a more pronounced difference between 1 and 5 pM Innovin. This effect is more marked in PRP (see Figure 3-1) where a clear effect on the LT can be seen with different reagents but also differing TF concentrations. There is a concurrent increase in the ETP and shortening in the ttPeak.

Table 3-5 CTI PPP – double spun versus triple spun data; raw results expressed as mean ± 2SD; samples analysed same day

CTI PPP – samples analysed same day; raw results

DS/TS	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
DS	PPP low	4.92 (2.55 - 7.30)	1418.20 (849.95 - 1986.45)	159.55 (55.13 - 263.97)	9.27 (5.74 - 12.80)
DS	Innovin 1	4.02 (2.06 - 5.98)	1610.81 (1063.72 - 2157.90)	154.95 (73.78 - 236.13)	9.88 (5.32 - 14.44)
TS	PPP low	4.82 (2.56 - 7.08)	1351.00 (798.03 - 1903.97)	154.68 (53.53 - 255.84)	9.25 (5.77 - 12.72)
TS	Innovin 1	4.03 (2.01 - 6.05)	1522.20 (958.59 - 2085.81)	147.72 (67.22 - 228.21)	9.86 (5.27 - 14.45)
TS	Innovin 2	4.09 (1.10 - 7.07)	1514.51 (826.48 - 2202.55)	148.36 (54.69 - 242.03)	9.71 (3.84 - 15.57)

Table 3-6 CTI PPP – double spun versus triple spun data; normalised data expressed as mean ± 2SD; samples analysed same day

CTI PPP – samples analysed same day; results expressed as a ratio

DS/TS	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
DS	PPP low	0.89 (0.56 - 1.22)	1.38 (0.38 - 2.38)	1.77 (0.22 - 3.31)	0.85 (0.56 - 1.14)
DS	Innovin 1	0.81 (0.47 - 1.16)	1.24 (0.70 - 1.78)	1.50 (0.60 - 2.39)	0.83 (0.44 - 1.23)
TS	PPP low	0.88 (0.56 - 1.19)	1.38 (0.51 - 2.25)	1.7 (0.24 - 3.17)	0.85 (0.57 - 1.14)
TS	Innovin 1	0.82 (0.48 - 1.17)	1.16 (0.65 - 1.67)	1.41 (0.55 - 2.26)	0.84 (0.45 - 1.23)
TS	Innovin 2	0.82 (0.51 - 1.14)	1.15 (0.78 - 1.53)	1.36 (0.55 - 2.17)	0.84 (0.43 - 1.26)

Table 3-7 CTI PPP – double spun versus triple spun data; raw results expressed as mean ± 2SD; frozen samples

CTI PPP – frozen samples; raw results

DS/TS	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
DS	PPP low	4.18 (2.98 - 5.38)	1423.82 (750.20 - 2097.43)	165.80 (75.34 - 256.27)	8.39 (6.54 - 10.24)
DS	Innovin 1	3.31 (2.47 - 4.14)	1714.38 (1164.92 - 2263.83)	184.64 (116.13 - 253.15)	8.24 (6.10 - 10.38)
TS	PPP low	4.46 (2.43 - 6.48)	1372.96 (815.75 - 1930.17)	156.46 (62.91 - 250.00)	8.80 (5.87 - 11.74)
TS	Innovin 1	3.70 (2.21 - 5.20)	1594.14 (1155.09 - 2033.19)	162.73 (87.68 - 237.78)	9.33 (5.57 - 13.09)
TS	Innovin 2	3.52 (2.07 - 4.98)	1676.51 (1140.60 - 2212.43)	180.33 (93.86 - 266.80)	8.79 (5.08 - 12.51)

Table 3-8 CTI PPP – double spun versus triple spun data; normalised data expressed as mean ± 2SD; frozen samples

CTI PPP – frozen samples; results expressed as a ratio

DS/TS	TF conc. pM	LT	ETP	Peak	ttPeak
DS	PPP low	0.79 (0.5 - 1.09)	1.35 (0.53 - 2.17)	1.74 (0.44 - 3.04)	0.83 (0.54 - 1.11)
DS	Innovin 1	0.85 (0.56 - 1.14)	1.13 (0.72 - 1.54)	1.3 (0.65 - 1.95)	0.83 (0.52 - 1.14)
TS	PPP low	0.86 (0.48 - 1.23)	1.43 (0.77 - 2.09)	1.79 (0.46 - 3.12)	0.85 (0.55 - 1.15)
TS	Innovin 1	0.85 (0.46 - 1.24)	1.15 (0.77 - 1.54)	1.33 (0.61 - 2.04)	0.86 (0.50 - 1.23)
TS	Innovin 2	0.89 (0.56 - 1.22)	1.15 (0.63 - 1.66)	1.3 (0.52 - 2.07)	0.87 (0.53 - 1.22)

Table 3-9 PPP – double spun data; results expressed as mean ± 2SD; samples analysed same day

PPP – samples analysed same day; raw results

DS/TS	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
DS	PPP reagent	2.88 (1.78 - 3.98)	1811.75 (1203.49 - 2420.01)	288.50 (164.12 - 412.89)	6.03 (3.44 - 8.62)
DS	Innovin 5	1.92 (0.39 - 3.45)	1797.91 (1248.09 - 2347.73)	224.66 (175.46 - 273.86)	5.39 (2.77 - 8.01)

PPP – samples analysed same day; results expressed as a ratio

DS/TS	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
DS	PPP reagent	0.97 (0.66 - 1.28)	1.00 (0.58 - 1.42)	1.18 (0.63 - 1.72)	0.87 (0.57 - 1.18)
DS	Innovin 5	1.07 (0.58 - 1.57)	1.00 (0.67 - 1.33)	0.86 (0.63 - 1.09)	1.04 (0.71 - 1.38)

Table 3-10 PPP – double spun data; results expressed as mean ± 2SD; frozen samples

PPP – frozen samples; raw results

DS/TS	TF conc. pM	LT (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
DS	PPP reagent	2.81 (1.74 - 3.88)	1952.41 (1391.40 - 2513.41)	295.96 (203.42 - 388.51)	5.88 (3.58 - 8.18)
DS	Innovin 5	1.58 (1.00 - 2.16)	1831.12 (1279.20 - 2383.03)	243.36 (198.85 - 287.87)	4.75 (3.43 - 6.06)

PPP – frozen samples; results expressed as a ratio

DS/TS	TF conc. pM	LT	ETP	Peak	ttPeak
DS	PPP reagent	0.90 (0.60 - 1.21)	1.05 (0.72 - 1.38)	1.19 (0.70 - 1.67)	0.85 (0.53 - 1.18)
DS	Innovin 5	1.03 (0.67 - 1.39)	1.00 (0.70 - 1.30)	0.91 (0.68 - 1.14)	1.05 (0.71 - 1.39)

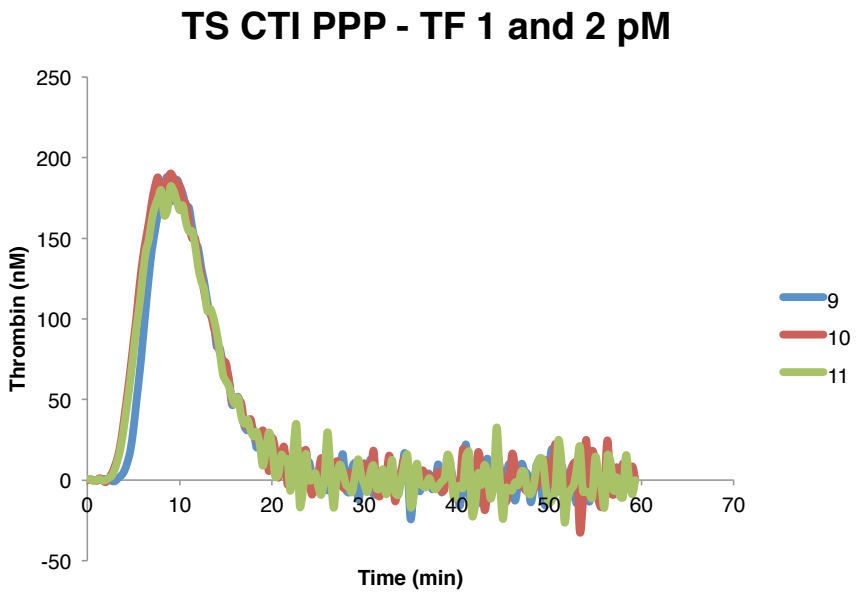


Figure 3-2 Example of normal healthy volunteer TS (triple spun) CTI PPP samples run with different TF reagents and concentrations demonstrating negligible difference in results; 9 = PPP low; 10 = Innovin 1 pM; 11 = Innovin 2 pM

3.5.2.2 Double spinning versus triple spinning CTI samples

The aim of triple spinning is to maximise the removal of platelet debris and microparticles that may contribute to thrombin generation in samples being triggered by low concentrations of TF. The samples usually run with TF concentrations less than 2 pM tend to be hypocoagulable samples, e.g. samples from patients with haemophilia. When studying thrombin generation triggered by the extrinsic pathway in these PPP samples with reduced thrombin generation potential, it is all the more important not only to inhibit contact activation (with good blood sampling technique and the addition of CTI) but also to remove extraneous phospholipid. The amount of phospholipid present is then controlled by the amount included in the trigger.

For the normal reference ranges, there was no statistically significant difference between double spinning and triple spinning CTI PPP samples (see Table 3-11). However, if individual results are examined, it would appear that a difference starts to become apparent in those with lower peak thrombin generation with a smaller peak seen in the triple spun samples (see Figures 3-3 and 3-4). It may be surmised therefore that CTI PPP samples from patients with reduced thrombin potential (such as in inherited bleeding disorders) should be triple spun. A further study comparing double spun and triple spun samples from such patients would need to be conducted in order to prove this.

Table 3-11 Paired t test results for double spun versus triple spun samples (samples analysed same day)

CTI PPP – PPP Low reagent; raw results

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - DS - LT - TS	.09846	.58094	.09302	-.08986	.28678	1.058	38	.297
Pair 2	ETP - DS - ETP - TS	73.3750	248.5441	41.4240	-10.7202	157.4702	1.771	35	.085
Pair 3	Peak - DS - Peak - TS	4.86846	26.73007	4.28024	-3.79643	13.53335	1.137	38	.262
Pair 4	ttPeak - DS - ttPeak - TS	.02051	.72834	.11663	-.21559	.25661	.176	38	.861

CTI PPP – Innovin 1 pM; raw results

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - DS - LT - TS	.05889	1.51137	.25189	-.45248	.57026	.234	35	.817
Pair 2	ETP - DS - ETP - TS	97.0278	411.0242	68.5040	-42.0428	236.0984	1.416	35	.166
Pair 3	Peak - DS - Peak - TS	7.47639	55.17448	9.19575	-11.19197	26.14475	.813	35	.422
Pair 4	ttPeak - DS - ttPeak - TS	.06833	3.38091	.56349	-1.07560	1.21227	.121	35	.904

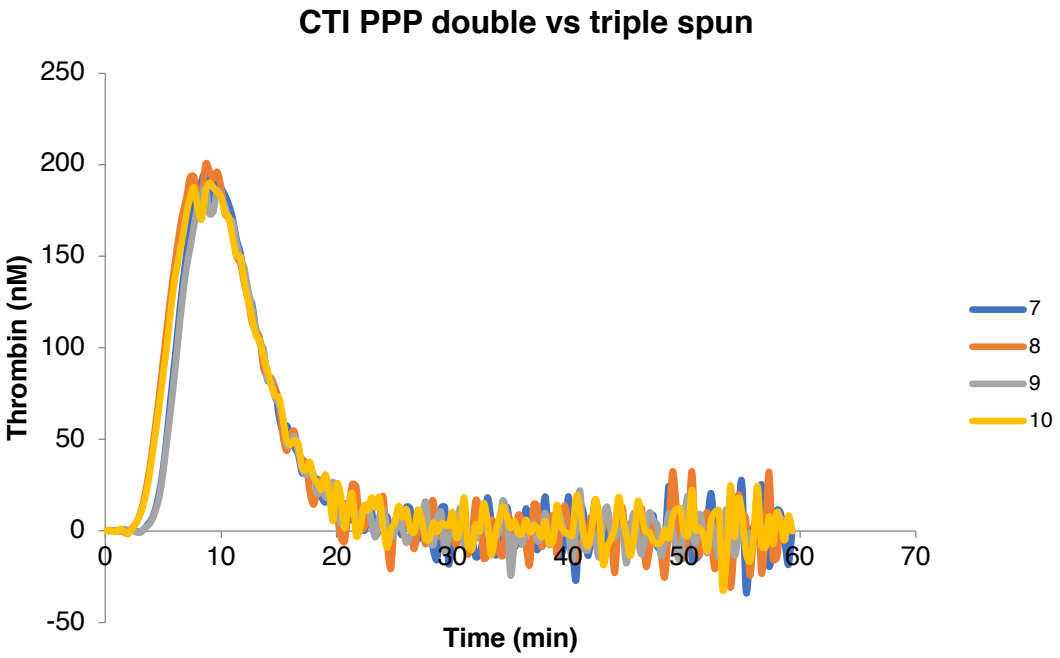


Figure 3-3 CTI PPP double spinning versus triple spinning; 7,9 = PPP Low; 8,10 = Innovin 1 pM; 7,8 = double spun samples; 9,10 = triple spun samples

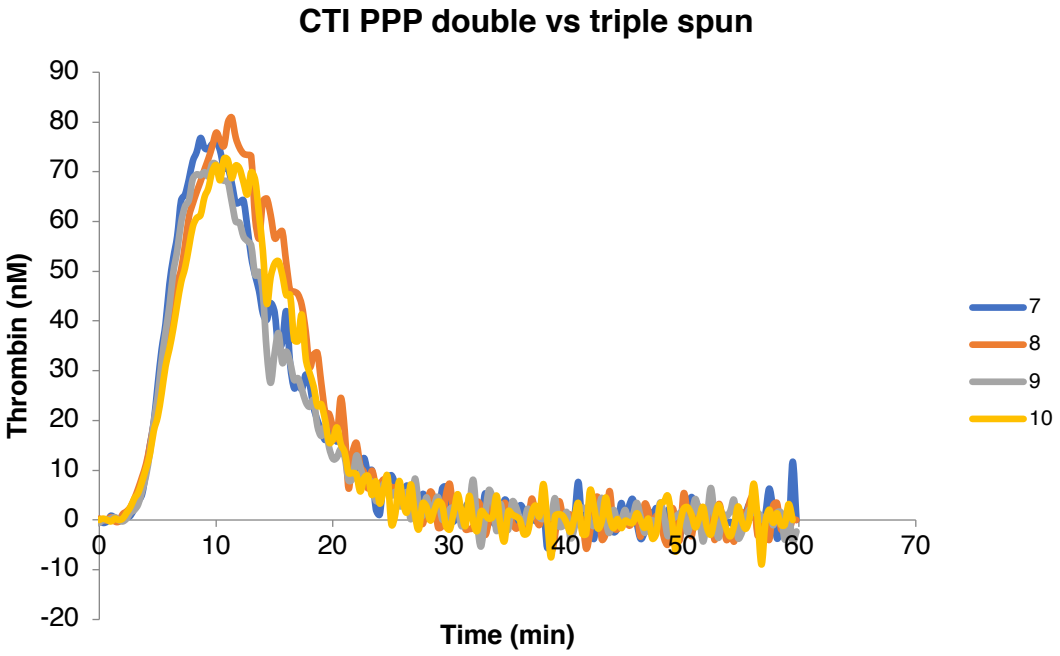


Figure 3-4 As for previous figure; samples from a different healthy volunteer – note difference in peak thrombin with markedly lower results than the volunteer depicted in Figure 3-3; 7,9 = PPP Low; 8,10 = Innovin 1 pM; 7,8 = double spun samples; 9,10 = triple spun samples

3.5.2.3 Fresh v frozen PPP samples

PPP samples were analysed both within 2 hours of collection and also after a period of storage at -80^{0}C. Results were expressed again both as raw and normalised data (see Tables 3-5 – 3-10). Samples were compared for any significant difference in results after a period of storage (maximum 6 months). Figure 3-5 is an example of one of the volunteers' samples run with different TF triggers both within 2 hours of venepuncture and on thawed frozen samples. It can be seen that in this example, there is little difference between fresh and frozen.

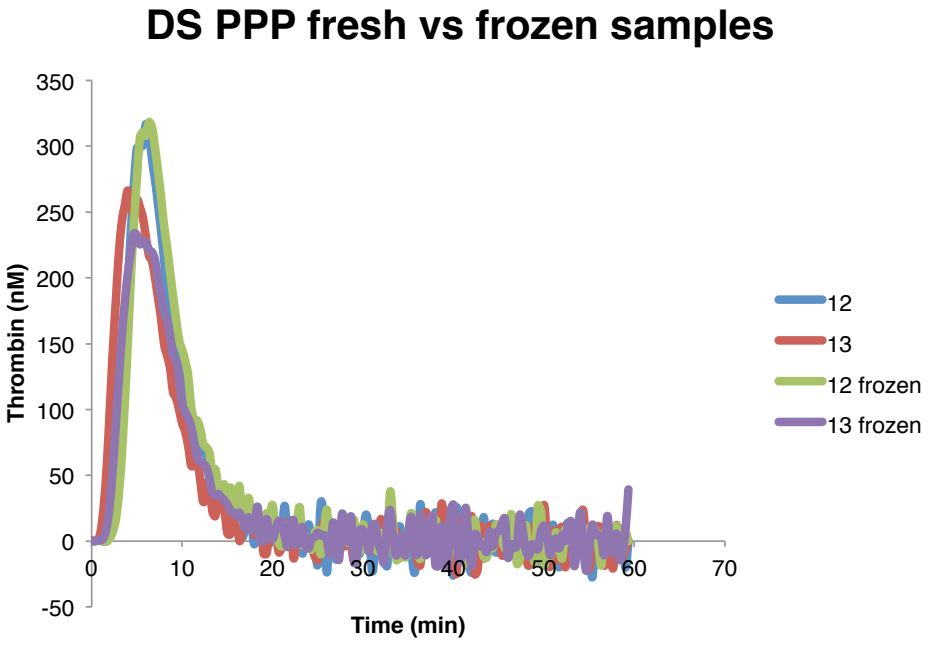


Figure 3-5 Example of normal healthy volunteer samples (analysed same day and frozen samples); 12 = PPP reagent; 13 = Innovin 5 pM

Statistical analysis was performed on the raw data to assess for any significant difference between results obtained from fresh versus frozen samples (see tables 3-12, 3-13). This was done for the range of TS PPP samples (PPP Low, Innovin 1 pM and Innovin 2 pM) as well as DS PPP (PPP Reagent and Innovin 5 pM). No significant differences were found at the lower TF concentrations (PPP Low and Innovin 1 pM). However, there were significant differences found for the LT, Peak and ttPeak using Innovin 2 pM and Innovin 5 pM but not for the PPP reagent (apart from the ETP). This would perhaps suggest a potential impact of the reagent used.

Table 3-12 Paired t test results for fresh (SD) versus frozen samples; PPP Low, Innovin 1 pM, Innovin 2 pM

Same day analysis vs frozen samples; TS CTI PPP; PPP Low reagent

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - SD - LT - frozen	.38179	1.60597	.25716	-.13880	.90239	1.485	38	.146
Pair 2	ETP - SD - ETP - frozen	-7.3472	297.1892	49.5315	-107.9016	93.2071	-.148	35	.883
Pair 3	Peak - SD - Peak - frozen	-.34128	70.48618	11.28682	-23.19026	22.50769	-.030	38	.976
Pair 4	ttPeak - SD - ttPeak - frozen	.43795	2.43031	.38916	-.34987	1.22576	1.125	38	.267

Same day analysis vs frozen samples; TS CTI PPP; Innovin 1 pM

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - SD - LT - frozen	.33789	1.28239	.20803	-.08362	.75940	1.624	37	.113
Pair 2	ETP - SD - ETP - frozen	-68.3289	364.3472	59.1049	-188.0869	51.4290	-1.156	37	.255
Pair 3	Peak - SD - Peak - frozen	-14.13816	55.09767	8.93802	-32.24831	3.97200	-1.582	37	.122
Pair 4	ttPeak - SD - ttPeak - frozen	.52474	2.81084	.45598	-.39916	1.44864	1.151	37	.257

Same day analysis vs frozen samples; TS CTI PPP; Innovin 2 pM

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - SD - LT - frozen	.56250	1.71846	.27171	.01291	1.11209	2.070	39	.045
Pair 2	ETP - SD - ETP - frozen	-170.0270	517.1300	85.0156	-342.4467	2.3927	-2.000	36	.053
Pair 3	Peak - SD - Peak - frozen	-31.97500	53.84414	8.51351	-49.19519	-14.75481	-3.756	39	.001
Pair 4	ttPeak - SD - ttPeak - frozen	.91425	3.03130	.47929	-.05521	1.88371	1.908	39	.064

Table 3-13 Paired t test results for fresh (SD) versus frozen samples; PPP Reagent, Innovin 5 pM

Same day analysis vs frozen samples; PPP reagent

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - SD - LT - frozen	.06750	.30589	.04837	-.03033	.16533	1.396	39	.171
Pair 2	ETP - SD - ETP - frozen	-140.6579	299.1745	48.5325	-238.9941	-42.3217	-2.898	37	.006
Pair 3	Peak - SD - Peak - frozen	-7.45875	43.98955	6.95536	-21.52729	6.60979	-1.072	39	.290
Pair 4	ttPeak - SD - ttPeak - frozen	.15175	.64594	.10213	-.05483	.35833	1.486	39	.145

Same day analysis vs frozen samples; Innovin 5 pM

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LT - SD - LT - frozen	.34256	.84273	.13495	.06938	.61575	2.539	38	.015
Pair 2	ETP - SD - ETP - frozen	-32.4211	391.2885	63.4754	-161.0344	96.1923	-.511	37	.613
Pair 3	Peak - SD - Peak - frozen	-18.33154	35.56643	5.69519	-29.86084	-6.80224	-3.219	38	.003
Pair 4	ttPeak - SD - ttPeak - frozen	.64205	1.40310	.22468	.18722	1.09688	2.858	38	.007

It is not clear why significant differences should emerge between the fresh and frozen samples with higher TF concentrations, as intuitively one might expect the opposite to be the case with more variability seen at lower TF concentrations that might contribute to this difference. It may be that it is a reflection of the greater variability seen when Innovin is used to make up the trigger for the assays, rather than using a 'ready-made' trigger (see Supplementary material 3.9 Table 3-18 for coefficients of variability with different reagents). Assessment of both the raw and normalised data does not demonstrate marked differences and there is co-linearity in the trends with increasing TF concentrations. These data may serve to emphasise the fact that samples in a study should all be assessed under the same conditions i.e. not mixing results from samples analysed on the day of collection and those that have been frozen and being mindful of the TF reagent being used.

3.6 Discussion

Normal reference ranges have been generated for the TGA under a range of conditions and the principal finding is that there is wide variability amongst healthy volunteers. In addition, consistency and standardisation have been found to be key to achieving robust results, with the application of a protocol that takes into account the impact of pre-analytical variables including venepuncture technique and sample processing. Moreover, a reference sample needs to be used for internal quality control as well as normalisation of the data. The wide range of results in these 41 volunteers raises a number of questions and potential criticisms. The first issue is that of the number of volunteers

tested, as 41 is a relatively small number particularly when compared to other assays in the diagnostic laboratory where over 100-150 samples might be tested to generate a normal reference range. This in reality relates to practicality – both in terms of time required in order to set up and run the assay, but also in availability of appropriate individuals who could come to the centre and be bled according to the protocol. Additionally, individuals were excluded if they were taking anticoagulant medication or anti-platelet agents or had a clear bleeding history. However, they were not subjected to more stringent questioning that may have revealed other factors that could influence results (for example, the use of oral contraceptives, hormone replacement therapy, or other medication (certain anti-depressants have an impact on platelet function)).

The second issue is that of normality – what is normality and how is it best defined? Without embarking on a philosophical detour, 'normal' was taken to mean 'conforming to a standard: usual, typical or expected'. Individuals therefore played a part in self-selection, putting themselves forward as volunteers as they viewed themselves as normal. They were aware of the aim of the study. The exclusion criteria were a secondary means of avoiding obvious haemostatic dysfunction.

If the range is accepted to represent normal results, it suggests a wide spectrum of thrombin generating capacity amongst individuals that, in turn, potentially suggests a degree of redundancy in the system. Alternatively, there may have been individuals included who had a degree of haemostatic dysfunction that had not yet become apparent in the absence of a haemostatic challenge such as a surgical procedure. The volunteers were also not tested for

thrombophilic defects, merely excluded if they themselves had experienced a thrombotic event.

The results also emphasise the need for clarity in designing a TGA protocol as it can be modified to study different states of haemostatic dysfunction. As demonstrated, different means of sample processing have an appreciable, statistically significant, impact on results. Platelet number has an obvious role in thrombin generation. Platelets accelerate the propagation phase of coagulation and increase the maximum concentration of thrombin generated due to the presence of procoagulant phospholipids and release of clotting factors upon activation [Gerotziafas, 2007]. Adjusting the platelet count with careful processing to minimise activation allows comparison of the role of the platelet component between individuals. When testing low levels of thrombin generation, the use of CTI and triple spinning reduces the interference from contact activation and platelet microparticles and debris. There may also be a difference between results from fresh and frozen PPP samples and so only using one approach during a study will also minimise variability in results.

This study therefore accomplished the aim of generating a range of normal references ranges for the CAT using the technique of Hemker et al. [Hemker *et al.*, 2006]. It also highlighted the variables that needed to be considered going forward to the studies examining inherited and acquired coagulopathy.

3.7 Supplementary material – further details of TGA protocol

3.7.1 Citrated whole blood samples

A TGA protocol was devised taking into account local practice and the recommendations of Loeffen et al [Loeffen *et al.*, 2012] (see also Chapter 2 Table 2-1). As part of this, a standard protocol for venepuncture was adopted as per local practice (following the Royal Free London NHS Foundation Trust guidelines) in order to minimise inter-operator variability. Atraumatic venepucture (usually of the ante-cubital veins) was performed with the use of gentle tourniquet, 21-gauge butterfly needles (BD Vacutainer® Safety-Lok™ Blood collection set with pre-attached holder – 21 gauge needle with 0.8 x 19mm x 305mm) and BD Vacutainer® tubes (Becton Dickinson, 0.109M tri-sodium citrate) or Sarstedt Monovette® tubes (citrate 3.2% 0.109 M, 1:10; aspiration technique). A discard sample was always taken (removing the first 2-3 mls blood) in order to minimise the impact of contact activation. Corn trypsin inhibitor (CTI; Haematologic Technologies, Cambridge Bioscience, UK) was added as required to Monovette tubes prior to use at a final concentration of 20 μg/ml.

3.7.2 Sample processing

Samples were processed within 2 hours of collection. Overt haemolysis was very rarely detected and did not appear to be an issue with samples taken via butterfly needle as found in other studies [Loeffen *et al.*, 2012]. Platelet rich plasma (PRP) was obtained by centrifugation at 220g (1000 rpm) for 15 minutes (with no brake) and careful aspiration avoiding contamination with the buffy coat or red cells. The platelet count of the PRP was determined on a Sysmex XS-1000i analyser (Sysmex, UK) and then adjusted using autologous PPP as required [Gerotziafas *et al.*, 2005]. Platelet poor plasma (PPP) was obtained by double spinning (2000g for 15 minutes each time with brake). CTI samples were either double spun or triple spun with an additional centrifugation step at 6000g for 3 minutes.

In order to generate in-house normal pools, individual PPP samples were pooled with gentle mixing before being divided into aliquots and frozen at -80^{0}C. Spun samples were kept on ice until all samples could be added to the pool. The number of individuals contributing to the pool varied depending on availability of volunteers, phlebotomists, centrifuges and the practicality of completing processing within 2 hours of collection (n = 35 for NP2 and n = 15 for NP3 – abbreviations allocated by author for ease of identification). Individual samples and pooled normal plasma were frozen in aliquots and stored at -80^{0}C. Prior to analysis, these were thawed for 10 minutes in a water-bath at 37^{0}C and then analysed in batches (individual samples) or used as a relevant quality or normalisation control.

3.7.3 Reagents

'PRP reagent', 'PPP Low', 'PPP reagent' and 'Thrombin calibrator' in addition to a 'FluCa buffer' and fluorogenic substrate were all obtained from Thrombinoscope BV (Diagnostica Stago, France). The former contain different concentrations of TF with the addition of phospholipid as required – approximately 0.5 pM TF for PRP reagent, 1 pM for PPP Low reagent and 5 pM TF for the PPP reagent (the exact concentrations are not given). Alternatively, Innovin PT reagent containing relipidated recombinant human TF (Dade® Innovin®, Siemens, UK) was obtained for use in the creation of in-house trigger reagents with the addition of phospholipid (PL) for PPP. PL was obtained from Rossix (Quadratech Diagnostics Ltd, UK) – a stabilised 0.5 mM phospholipid emulsion containing a mixture of highly purified phosphatidyl choline (PC, 42%), phosphatidyl serine (PS, 28%) and sphingomyelin (SM, 30%). This was used at a final concentration of 4 μM. The latter were diluted in OBS buffer (Owrens barbiturate buffer pH 7.35 and sodium chloride (0.9%)). The same batches for all reagents were used where possible.

In addition to collection of samples for a local plasma pool, a lyophilised reference plasma specifically for use in the TGA was obtained from NIBSC (Potters Bar, Hertfordshire, UK; kindly provided by Dr Elaine Gray).

3.7.3.1 Calculations for Innovin dilutions

Preparation of 5 pM, 2 pM, 1 pM and 0.5 pM final concentrations of TF using Innovin as the TF source

TF concentration for batch used for all assays calculated to be ~8000 pM using ACTICHROME® TF assay (see Supplementary material 3.8)

1:10 dilution, followed by a 1:4 dilution

Reagents diluted in OBS pH 7.4 buffer

Final concentration in well:

5pM (37.5 μl 1: 1:4 dilution + 10 μl phospholipid + 187.5 μl OBS = 250 μl in total, 20 μl then added to well)

2 pM (15 μl 1:4 dilution + 10 μl phospholipid + 225 μl OBS = 250 μl in total, 20 μl then added to well)

2 doubling dilutions of 1:4 dilution

1 pM (15 μl first 1:2 dilution + 10 μl phospholipid + 225 μl OBS = 250 μl in total)

0.5 pM (15 μl subsequent 1:2 dilution + 10 μl phospholipid + 225 μl OBS = 250 μl in total)

3.7.4 Calibrated Automated Thrombogram equipment

The assays were performed using Immunlon 2HB, round-bottom 96-well plates (Dynex technologies, UK). Fluorescence was measured using a Fluroskan Ascent fluorimeter (Thermolab Systems, Helsinki, Finland) with an excitation filter at 390 nm and an emission filter at 460 nm.

All samples were tested in duplicate with a calibrator well for each individual (also run in duplicate). A normal control (consisting of the in-house normal pooled plasma) was run with the same triggers used for the assay – this doubled as a quality control and a normalisation control. There was no practical

normalisation control for the PRP samples. Ready-made triggers were used or modified trigger reagents were prepared (see above). 20 μl of trigger or calibrator was added to each well followed by 80 μl of test plasma or control. The plate was then warmed in the fluorimeter at 37^0C for 5 minutes before manual addition of FluCa buffer (Hepes (pH 7.35) and Calcium Chloride) to which flurogenic substrate (*Z-Gly-Gly-Arg bound to 7-amino-4-methylcoumarin)* was added immediately before use using a repeat pipette for speed. The final step of adding calcium and the fluorogenic substrate was performed manually in preference to the automatic dispenser due to large volumes of reagent utilised by the latter and the fact that experiments were set up usually using roughly half of a 96-well plate. The data collection was then commenced with readings taken at 20-second intervals over an hour.

The endogenous thrombin potential (ETP), lagtime (LT), peak and time to peak (ttPeak) were derived from the raw data by dedicated software (3.0 Commercial Release, Version 3.0.0.29 (2004) Thrombinoscope™ B.V., Maastricht, The Netherlands).

3.8 Supplementary material – assessing the TF concentrations of different reagents

3.8.1 TF assay

TF levels were measured using a chromogenic ACTICHROME® TF activity assay (Sekisui Diagnostics, Invitech, UK). All samples were run in duplicate. A TF standard was used for plasma samples with relipidated human TF added to 5% TFPI depleted human plasma. TF standards (further diluted in 5% TFPI depleted plasma) and test samples (neat) were added to the wells of a 96-well clear flat-bottom microtitre plate (Corning Costar, Sigma-Aldrich) along with buffer, human FVIIa and human FX. After a 15 minute incubation at 37^0C, SPECTROZYME® FXa substrate was added followed by a further 30 minute incubation at 37^0C. The reaction was stopped with the addition of glacial acetic acid and the plate read at 405 nm and 490 nm on a SpectraMax i3 multi-mode microplate platform (Molecular Devices, UK). The $\Delta A_{405\text{-}490}$ of the sample was used to interpolate the TF concentration. The TF standard dilutions were used to create a standard curve on Excel 2011 (Microsoft) plotted using a 2^{nd} order polynomial regression. The test sample concentrations were calculated using the equation generated. Local normal ranges were generated with 20 healthy volunteer samples (individuals who had consented to participate in the normal reference range study; see Section 3.4.1).

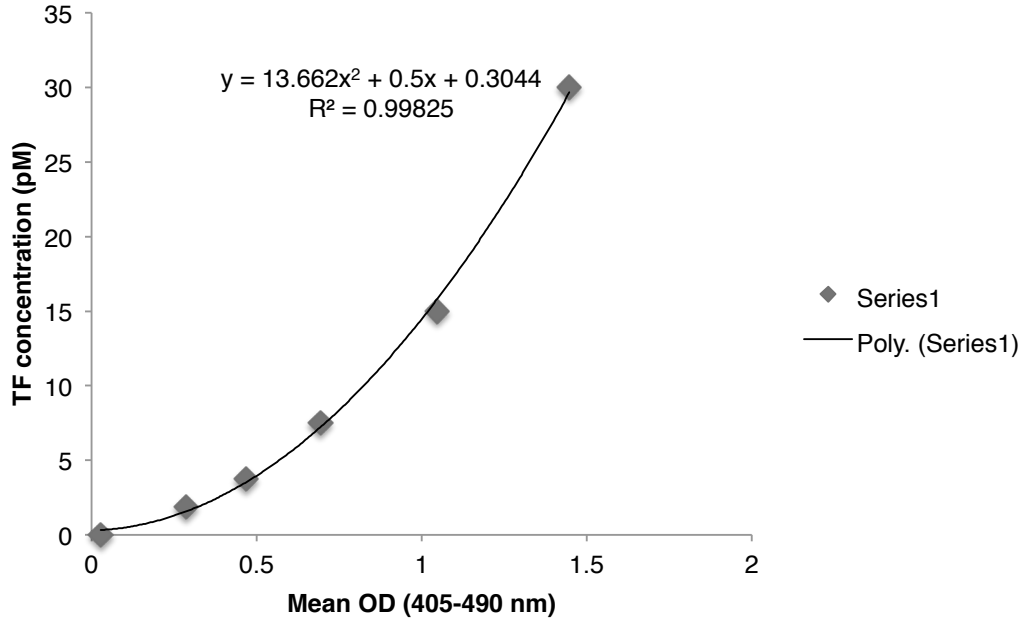


Figure 3-6 TF assay standard curve

3.8.2 Results

The concentration of TF in the neat Innovin reagent was calculated from an average of results of 1:1000 and 1:2000 dilutions. The lower concentrations were calculated in order to achieve a final target concentration in the TGA well (including the test plasma, trigger and substrate solutions).

Table 3-14 ACTICHROME chromogenic TF assay results

TF Reagent	TF concentration (pM)
Innovin (neat)	8289 (mean)
Innovin 2 pM	3.06
Innovin 1 pM	1.25
Innovin 0.5 pM	0.66
PPP Low (approx. 1 pM)	1.86

A previous batch of Innovin had been found to contain 6000 pM of TF. The batch tested and used for subsequent experiments was found to have a higher concentration and so calculations were adjusted accordingly. The lower concentrations tested gave results roughly equivalent to that expected.

3.9 Supplementary material – results and coefficients of variation for reference plasmas

One of the particular challenges of the TGA, as discussed previously, is obtaining an adequate degree of reproducibility. This can be aided by the use of a reference plasma that can act both as a quality and normalisation control. Three different plasmas were trialled as reference plasmas. Two of these were in-house normal plasma pools consisting of both CTI and non-CTI samples. The third was a lyophilised plasma pool manufactured by NIBSC that has been used by other research groups [Dargaud *et al.*, 2010, Bagot *et al.*, 2015]. All 3 plasmas were run together 12 times under the same conditions. The in-house normal plasma pools NP2 and NP3 had been run separately a number of times during development of the TGA protocol.

These reference plasmas were run concurrently using the range of conditions used to generate the normal reference ranges for PPP (see Section 3.4). The mean ± 2SD were generated in addition to the coefficient of variation (CV) (Tables 3-15 – 3-18).

Table 3-15 Results for the NIBSC reference plasma; n = number of runs

NIBSC reference plasma, raw data (mean ± 2SD) [n = 12]

NIBSC RP	LT	ETP	Peak	ttPeak
PPP Low	3.34 (2.38 - 4.29)	1488.69 (1138.25 - 1839.13)	192.41 (133.29 - 251.53)	7.47 (6.19 - 8.75)
Innovin 1 pM	3.47 (1.78 - 5.16)	1748.64 (1360.68 - 2136.56)	206.41 (142.37 - 270.44)	8.14 (5.88 - 10.40)
Innovin 2 pM	2.79 (1.80 - 3.78)	1617.50 (1162.00 - 2073.00)	200.16 (158.76 - 241.56)	6.97 (5.58 - 8.35)
PPP reagent	2.00 (1.33 - 2.67)	1978.91 (1572.76 - 2385.06)	311.40 (257.38 - 365.42)	4.98 (4.08 - 5.88)
Innovin 5 pM	1.52 (0.47 - 1.99)	1844.00 (1298.36 - 3142.36)	263.88 (147.63 - 411.51)	4.73 (2.25 - 6.98)

Table 3-16 Results for the NP2 plasma; n = number of runs

NP2 – in-house normal pooled plasma, raw data (mean ± 2SD) [n = 22]

NP2	LT	ETP	Peak	ttPeak
CTI – PPP Low	4.04 (2.76 - 5.31)	1453.56 (1210.84 - 1696.29)	153.71 (116.80 - 190.62)	8.52 (6.93 - 10.11)
CTI – Innovin 1 pM	3.71 (1.39 - 6.02)	1565.73 (1061.98 - 2069.49)	171.12 (72.15 - 270.08)	9.20 (4.60 - 13.80)
CTI – Innovin 2 pM	2.92 (2.14 - 3.70)	1653.00 (1221.86 - 2084.14)	201.97 (167.75 - 236.19)	6.88 (4.87 - 8.89)
PPP Low	3.57 (2.97 - 4.17)	1411.70 (1107.11 - 1716.29)	186.77 (158.29 - 215.26)	7.62 (6.24 - 9.00)
Innovin 1 pM	2.84 (2.21 - 3.46)	1637.10 (1406.58 - 1867.62)	221.66 (200.91 - 242.40)	6.78 (4.88 - 8.67)
Innovin 2 pM	2.33 (1.94 - 3.31)	1753.63 (1110.03 - 2397.22)	245.96 (198.94 - 292.98)	6.05 (4.53 - 7.57)
PPP reagent	2.49 (1.94 - 3.04)	1821.38 (1458.93 - 2183.82)	303.20 (255.45 - 350.95)	5.41 (4.5 - 6.32)
Innovin 5 pM	1.35 (0.46 - 2.23)	1880.50 (1532.67 - 2228.33)	306.71 (237.58 - 375.84)	4.07 (2.40 - 5.74)

Table 3-17 Results for the NP3 plasma; n = number of runs

NP3 – in-house normal pooled plasma, raw data (mean ± 2SD) [n = 55]

NP3	Mean LT	Mean ETP	Mean Peak	Mean ttPeak
CTI – PPP Low	5.45 (4.11 - 6.78)	943.96 (700.22 - 1187.70)	86.61 (59.49 - 113.73)	10.80 (9.10 - 12.50)
CTI – Innovin 1 pM	4.72 (2.60 - 6.84)	1341.81 (938.74 - 1744.87)	114.58 (65.95 - 163.20)	11.45 (7.97 - 14.93)
CTI – Innovin 2 pM	4.44 (2.61 - 6.27)	1404.38 (1034.62 - 1774.14)	124.47 (62.98 - 185.97)	10.85 (7.41 - 14.29)
PPP Low	4.67 (3.88 - 5.46)	1382.00 (1266.30 - 1497.70)	145.14 (119.35 - 170.93)	9.18 (8.13 - 10.23)
Innovin 1 pM	3.43 (2.60 - 4.26)	1638.93 (1476.53 - 1801.33)	196.68 (172.26 - 221.11)	8.46 (6.94 - 9.98)
Innovin 2 pM	2.96 (2.39 - 3.52)	1652.25 (1324.19 - 1980.31)	208.62 (158.09 - 259.14)	7.17 (6.32 - 8.03)
PPP reagent	3.00 (2.34 - 3.66)	1778.65 (1365.79 - 2191.52)	249.77 (173.76 - 325.78)	6.75 (4.96 - 8.54)
Innovin 5 pM	1.58 (1.01 - 2.14)	1796.43 (1461.93 - 2130.93)	265.70 (220.05 - 311.34)	4.72 (3.35 - 6.09)

Table 3-18 Coefficients of variation for reference plasmas (NIBSC, NP2, NP3) with different TF reagents

NIBSC RP	TF conc.	LT	ETP	Peak	ttPeak
No CTI	PPP low	14.3%	11.8%	15.4%	8.6%
	Innovin 1	24.3%	11.1%	15.5%	13.9%
	Innovin 2	17.7%	14.1%	10.3%	9.9%
	PPP reagent	16.7%	10.3%	8.7%	9.0%
	Innovin 5	34.5%	14.8%	22.0%	26.2%

NP2	TF conc.	LT	ETP	Peak	ttPeak
CTI	PPP low	15.8%	8.3%	12.0%	9.3%
	Innovin 1	31.2%	16.1%	28.9%	25.0%
	Innovin 2	13.4%	13.0%	8.5%	14.6%
No CTI	PPP low	8.4%	10.8%	7.6%	9.1%
	Innovin 1	11.0%	7.0%	4.7%	14.0%
	Innovin 2	13.0%	18.4%	9.6%	12.6%
	PPP reagent	11.0%	9.9%	7.9%	8.4%
	Innovin 5	32.9%	9.2%	11.3%	20.5%

NP3	TF conc.	LT	ETP	Peak	ttPeak
CTI	PPP low	12.3%	12.9%	15.7%	7.9%
	Innovin 1	22.5%	15.0%	21.2%	15.2%
	Innovin 2	20.6%	13.2%	24.7%	15.9%
No CTI	PPP low	8.5%	4.2%	8.9%	5.7%
	Innovin 1	12.1%	5.0%	6.2%	9.0%
	Innovin 2	9.6%	9.9%	12.1%	6.0%
	PPP reagent	11.0%	11.6%	15.2%	13.2%
	Innovin 5	17.8%	9.3%	8.6%	14.5%

The NIBSC lyophilised plasma does not contain CTI. The results generally showed shorter LT and ttPeak as well as higher peaks. The CVs of the results were mostly greater than 10%. A CV of 10% is generally considered acceptable for a diagnostic assay.

The results for NP2 – the first in-house plasma pool generated within the time frame of this thesis – were lower than for the NIBSC plasma, however there was still a great deal of variability with high CVs.

NP3 – the second plasma pool generated - appeared to be the most reliable. This was likely partly a reflection of a more streamlined collection with fewer volunteers (15 versus 35) allowing for more precise sample processing. The trends of the results were as expected. In addition, the CVs were better particularly for the Thrombinoscope reagents with results much closer to 10%. NP3 (with and without CTI) was therefore used for the majority of the studies in this thesis both as a quality and normalisation control. The results of the NIBSC reference plasma could be used as a primary standard against which future in-house (non CTI) normal pools could be compared.

4 The inhibition of thrombin and FXa by low molecular weight heparin – a study of the anticoagulant effect and its potential reversal using the thrombin generation assay

4.1 Introduction and study rationale

Low molecular weight heparins (LMWHs) and, to a lesser extent, unfractionated heparin (UFH) are common anticoagulants used for the prophylaxis and treatment of venous thromboembolic disease (VTE) [Levi *et al.*, 2011]. Their most significant side effects are bleeding and heparin induced thrombocytopaenia (HIT).

Although protamine sulphate may be used to neutralise the effects of UFH, it is only partially effective in neutralising LMWHs [Schroeder *et al.*, 2011]. Studies of complications of LMWHs cite a risk of major bleeding of between 0.5 – 2% [Weitz, 1997] and a risk of minor bleeding of approximately 10% [Firozvi *et al.*, 2006]. Given that LMWHs have superseded UFH in their use in both the inpatient and outpatient setting, clinicians can face a dilemma when patients on these medications present with bleeding or requiring urgent invasive intervention with an associated risk of bleeding.

The current options for reversing the anticoagulant effect of LMWHs are sub-optimal. The problems are two-fold. Firstly, a reversal agent is required that can neutralise the varying anti-Xa and anti-IIa activities of these agents and

secondly, there also needs to be a mechanism by which the effect as well as the reversal thereof can be monitored (as the anti-IIa assay is not part of routine practice and the anti-Xa assay only detects part of the anticoagulant effect). The thrombin generation assay (TGA) has been found in previous studies to be sensitive to both the anticoagulant effect of the LWMHs and also able to detect reversal of this effect. The rationale for this study was to develop an appropriate protocol for the TGA to detect both the anticoagulant effect of UFH and LMWH and also the reversal thereof (using a combination of agents) and then use this tool to study spiked normal plasma samples in addition to patient samples. The aim was to demonstrate the potential for reversibility, but also data to support when potential reversal agents might be clinically useful.

4.2 Background

4.2.1 The antithrombin-thrombin-heparin complex

Heparin is one of the oldest biological compounds used therapeutically as an anticoagulant. Fat-soluble compounds that were found to cause excessive bleeding when injected into experimental animals were extracted from canine liver in the early 20th century. A distinct water-soluble compound was identified shortly afterwards and named heparin (due to the site of its derivation) and was produced commercially, but its use was complicated by numerous side effects. Improvements in the purification process ensued and heparin was introduced into clinical practice in the 1940s [Wardrop *et al.*, 2008]. LMWHs were discovered by chance in the 1970s and introduced into practice in the 1980s

with the advantage of more predictable pharmacokinetics and convenience of administration [Gray *et al.*, 2008].

In order to fully appreciate the mechanism of action of heparin (both UFH and LMWH), it is important to understand the structures of both heparin and its principal target and substrates – AT, thrombin and FXa. Elucidation of the crystal structure of the AT-heparin-thrombin complex using a heparin mimetic has demonstrated the bridging role of heparin [Li *et al.*, 2004] (see Figure 4-1).

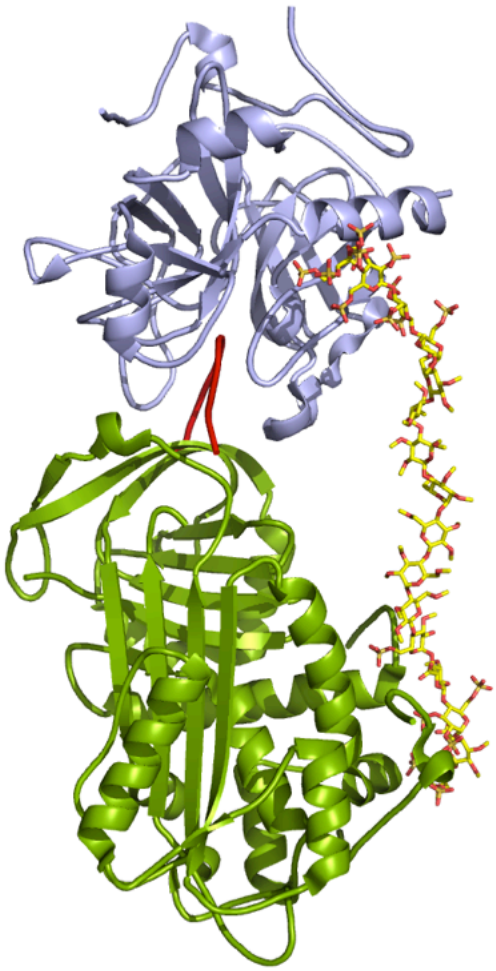


Figure 4-1 Ribbons depiction of the crystal structure of the Antithrombin (green with red RCL) – Thrombin (purple) – Heparin (yellow/orange) complex drawn using PyMol; Protein Data Bank ID: 1TB6. Courtesy of Dr K. Gomez.

AT contains a reactive centre loop (RCL) that incorporates a hinge region (see Figure 4-1; depicted by red loop). This is a highly conserved region in serpins that interacts with the relevant protease, in this case thrombin. The interaction between serpin and protease not only produces a conformational change in the former but also in the target protease [Huntington, 2003]. AT has two main targets. Firstly thrombin, that itself has two anion-binding exosites (I and II) sitting either side of its catalytic site (exosite II has been identified as the heparin-binding site and is critical for the heparin-accelerated inhibition of thrombin by AT) [Huntington, 2003, Crawley *et al.*, 2007]; secondly FXa, that has an open active site and interacts with heparin in the presence of calcium. Thrombin becomes bound irreversibly to AT and cleared from the circulation.

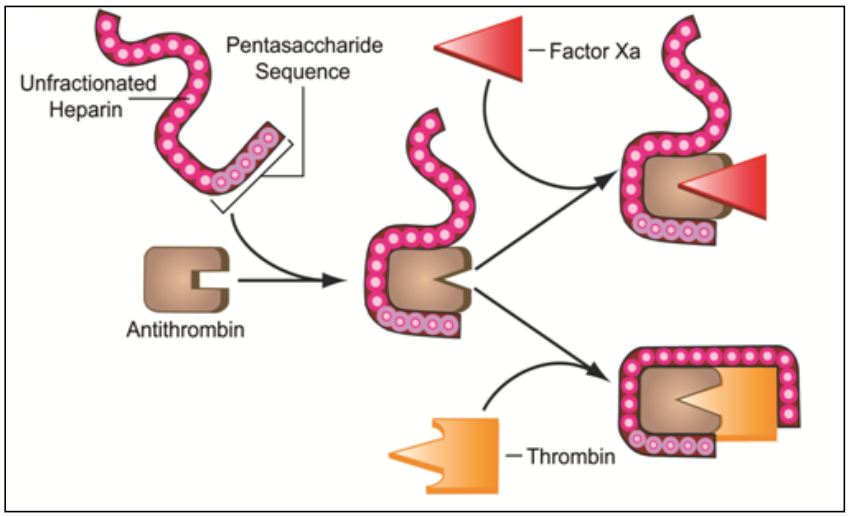


Figure 4-2 Mechanism of action of UFH; figure reproduced from [Weitz, 1997]

The process is accelerated by heparin (see Figure 4-2). AT binds to heparin specifically via a unique pentasaccharide sequence found in approximately one third of unfractionated heparin chains. The pentasaccharide sequence is

necessary to induce a conformational change in AT thereby exposing it's reactive centre and potentiating by 1000-fold the inhibition of FXa [Levi *et al.*, 2011]. In addition, a thrombin-binding domain is provided by the associated linear polysaccharide component of heparin that, providing it is of sufficient length (at least 18 disaccharide repeats), enables formation of a ternary bridging complex between AT, heparin and thrombin [Patnaik *et al.*, 2008, Liu *et al.*, 2014]. A secondary anticoagulant effect of heparin is mediated by its binding to the C terminal domain of tissue factor pathway inhibitor (TFPI) resulting in release of TFPI from the endothelium and further inhibition of FXa as well as the TF.FVIIa complex [Sandset, 1996, Weitz, 1997].

4.2.2 UFH, LMWH and synthetic pentasaccharides

Unfractionated pharmaceutical heparin (UFH) by definition has not been fractionated or subjected to depolymerisation techniques and is a heterogeneous product comprising chains of different molecular weights with both anti-thrombin (IIa) and anti-Xa effects. The LMWHs are produced by chemical or enzymatic depolymerisation of UFH and differing methods result in different pharmacokinetics and anticoagulant profile – most significantly a reduced anti-IIa effect due to loss of high molecular weight chains [Laux *et al.*, 2009].

UFH comprises polysaccharide chains that contain a spread of molecular weights (MWs) and their physicochemical properties vary little between different preparations. LMWH preparations all contain mean MWs less than half that of UFH [Gray *et al.*, 2008] with a narrow range and vary in the ratio of anti-Xa/anti-

IIa effect. However the *in vitro* derived anti-Xa/anti-IIa ratios do not correlate well with *in vivo* pharmacokinetics of anti-Xa and anti-IIa activity [Samama, 2000]. Fondaparinux is a synthetic pentasaccharide with sole anti-Xa effect (as it lacks the polysaccharide chain that can bind thrombin) (Levi et al., 2011). The advantages of synthetic compounds such as Fondaparinux include simplified pharmacodynamics and reduction of side-effects that relate to other structures in heparin or to particularly large molecular size [Mulloy *et al.*, 2016]. Differences in bioavailability, plasma clearance and half-life ($t_{1/2}$) mean that there can be a wide range of peak anti-Xa and anti-IIa effect depending on the anticoagulant [Samama, 2000] (see Table 4-1).

Table 4-1 Molecular mass and anti-Xa/anti-IIa activities of UFH, LMWH and Fondaparinux (reproduced with data from [Gerotziafas, 2007])

Anticoagulant	Mean molecular mass (Da)	Anti-Xa/anti-IIa ratio	Anti-IIa concentration in plasma (IU/ml) when anti-Xa activity is 1 IU/ml
UFH	15 000	1	1
LMWH – Tinzaparin	6750	2	0.5
LMWH – Enoxaparin	4200	3.9	0.2
Fondaparinux	1725	specific anti-Xa activity	0

UFH is mainly administered intravenously and, due to its variable pharmacokinetics, pharmacodynamics and short half-life ($t_{1/2}$), requires frequent laboratory monitoring. LMWHs have more predictable pharmacokinetics, a longer $t_{1/2}$ and can be administered subcutaneously, as can Fondaparinux. All of

these anticoagulants have an immediate onset of action but varying half-lives, with a more rapid loss of anti-IIa effect than anti-Xa [Samama, 2000]. The half-life can vary from 60-90 minutes in the case of UFH to 15-20 hours for Fondaparinux.

4.2.3 Monitoring of LMWHs

Due to the different profiles in anti-IIa and anti-Xa effect, routine laboratory assays can only partially reflect the anticoagulant effect of LMWHs (unlike UFH that has approximately a 1:1 ratio). The most common assay used for UFH is the activated partial thromboplastin time (APTT). This is not the case for the LMWHs and synthetic pentasaccharides such as Fondaparinux where monitoring is done by the anti-Xa assay. International standards are available for the former, but not the latter [Gatt *et al.*, 2008]. Dosing is based on weight, but there are situations such as when patients are bleeding or progressively thrombosing despite 'adequate' anticoagulation where monitoring is necessary. Peaks and troughs can be ascertained and treatment adjusted accordingly. In some cases, for example at extremes of weight or in pregnancy, anti-Xa levels can correlate poorly with the administered dose of LMWH [Chowdary *et al.*, 2015]. This may reflect the fact that the assay is performed on platelet poor plasma (PPP) that does not reflect the degree of neutralisation by platelet factor 4 (PF4) released by platelets *in vivo* [Gerotziafas, 2007]. More recently, global haemostatic assays have been used in the assessment of anticoagulant efficacy and reversal and have been demonstrated to be sensitive and superior to traditional tests in the latter [Gatt *et al.*, 2008, Chowdary *et al.*, 2015].

The TGA has been used to study the impact of LMWHs on both PRP and PPP. LMWHs vary markedly in activity, pharmacokinetics and pharmacodynamics depending on the manner by which they are derived from UFH. They also vary in their ability to release TFPI from the vascular endothelium and so the measurement of anti-Xa and anti-IIa activity does not truly reflect their anticoagulant effect [Gerotziafas, 2007]. A previous study compared different LMWH preparations with UFH and Fondaparinux and found that at a given anti-IIa activity, LMWHs are more potent at suppressing thrombin generation than UFH. This is in PRP, and the authors suggest that this is due to less efficient inhibition of LMWHs by PF4 released by platelets than UFH. When compared at equivalent anti-Xa activities, it was found that Tinzaparin had inhibitory effects very similar to UFH, but that Enoxaparin was less potent at suppressing thrombin generation. The authors concluded that the major inhibitory effect of LMWHs on thrombin generation in PRP was a result of their anti-IIa activity [Gerotziafas, 2007].

A study by Gatt and colleagues [Gatt *et al.*, 2008] assessed the effect of LMWHs on thrombin generation using PPP. They found a dose-dependent effect on all TGA parameters, with increasing concentrations of the anticoagulants leading to prolongation of the LT and ttPeak as well as reduction in peak and ETP. They found variation between the different preparations. Furthermore, they assessed potential reversal and confirmed the effective neutralisation of UFH by protamine as assessed by the TGA. They also demonstrated that higher doses of protamine (10-15 μg/ml) could almost completely reverse the effects of Tinzaparin. It had a notable effect on

Enoxaparin, but was unable to demonstrate complete reversal. They postulated that the more marked effect on Tinzaparin related to the increased sulphation of this preparation.

4.2.4 Current reversal strategies

Current guidance, given the lack of a specific antidote, recommends the use of protamine sulphate and possible consideration of recombinant activated FVII (FVIIa) [Gatt *et al.*, 2008, Makris *et al.*, 2013]. Protamine sulphate is a highly charged cation derived from salmon sperm that binds to negatively charged UFH and produces a complete reversal of both it's anti-IIa and anti-Xa effects by forming an inactive complex [Levi *et al.*, 2011]. However, although it can be used to reverse the anti-IIa action of the LMWH, it is not effective at completely negating the anti-Xa effect [Makris *et al.*, 2000]. Even at very high protamine/heparin ratios, protamine is still not effective and the reason for this has not been clear, although it must be borne in mind that the preparations contain a mix of chains of differing size. It is possibly due to charge differences in the ultra-LMWH molecules contained in LMWHs or even that the molecules are too small to interact with protamine [Schroeder *et al.*, 2011, Van Veen *et al.*, 2011]. Some work has suggested that the degree of reversibility of the various LMWH preparations correlates with their respective sulphate charge density and that reduced levels in LMWHs may account for the inability of protamine to neutralize the anti-Xa effect [Crowther *et al.*, 2002]. A study by Crowther et al. compared the neutralisation of UFH and LMWH with protamine and demonstrated that only approximately 60% anti-Xa activity was neutralised for

the latter but more than 90% anti-IIa activity was abolished for both. It found that the incomplete anti-Xa neutralisation was not due to reduced binding of protamine to LMWH, but reflected the reduced sulphate charge density. Others also suggest that it is likely that structural factors relating to the production of different preparations of LMWH have an influence [Schroeder *et al.*, 2011]. Protamine also has no effect on Fondaparinux that has pure anti-Xa activity. Importantly, it has also been noted that at higher doses protamine can have an anticoagulant effect itself by down-regulating thrombin generation by inhibition of FV activation [Ni Ainle *et al.*, 2009].

The study by Gatt et al formed the basis for this study [Gatt *et al.*, 2008]. To summarise, the group used the TGA to examine the efficacy of different reversal agents (protamine, rFVIIa, activated prothrombin complex concentrate (aPCC, FEIBA) and fresh frozen plasma (FFP)) in neutralisation of the anticoagulant effect of five heparinoids (UFH, Tinzaparin, Enoxaparin, Danaparoid and Fondaparinux).

They first demonstrated sensitivity of the TGA to therapeutic levels of the anticoagulants using a trigger of 5 pM TF. As discussed above, protamine was shown to fully neutralise the effect of UFH, but not the LMWHs. The authors demonstrated that rFVIIa (as suggested in the reversal guidelines) had some impact but did not achieve complete reversal. FFP was not shown to have any beneficial effect, but some improvement in normalisation of TGA parameters (specifically the endogenous thrombin potential, ETP) was demonstrated with FEIBA (aPCC): from 20% improvement in parameters when added to samples spiked with tinzaparin to 55% improvement in samples spiked with

Fondaparinux. This intuitively would appear to make sense as aPCC contains FVIIa, FXa and some FIIa as well as FII, FVII, FIX and FX. Although no evidence to support the use of prothrombin complex concentrate (PCC) was found in the Gatt study, the author of this study hypothesised that the addition of 4-factor PCC containing FII, FVII, FIX and FX, particularly when combined with protamine, could improve the coagulation profile in samples spiked with LWMH.

PCC are plasma-derived products containing significant amounts of the Vitamin K-dependent factors II, IX and X with some also containing FVII (3-factor versus 4-factor). In addition, due to concerns regarding thrombotic side effects, they contain varying levels of Protein C, Protein S, heparin (in the form of UFH) and antithrombin. They are licensed for use in inherited or acquired deficiency of Vitamin-K dependent clotting factors and are mostly prescribed for the immediate reversal of warfarin, thus making them readily available in the clinical setting. There is some evidence for their efficacy in the reversal of the effect of the synthetic pentasaccharides [Desmurs-Clavel *et al.*, 2009], direct thrombin inhibitors and FXa inhibitors such as rivaroxaban [Dickneite *et al.*, 2014].

4.3 Study Aim

The aim of this study was to confirm sensitivity of the thrombin generation assay to UFH, LMWH and Fondaparinux and subsequently provide proof-of-concept for the reversibility of the anticoagulant effect of LMWH preparations using 4-factor PCC alone and in combination with protamine sulphate.

4.3.1 Primary objective

Evaluation of the dose-dependent reversal of UFH, Tinzaparin, Enoxaparin and Fondaparinux by PCC alone or in combination with protamine sulphate in spiked pooled normal plasma samples.

4.3.2 Secondary objective

Evaluation of the reversibility of LMWHs as above with *ex vivo* spiking of samples from patients treated with LMWHs.

4.4 Study design

This was primarily a laboratory study involving spiking of normal pooled plasma samples or patient samples and assessing the reversal of anticoagulant effect using the calibrated automated thrombogram (CAT) and the technique of Hemker et al. [Hemker *et al.*, 2006].

4.4.1 Primary endpoint

The generation of TGA parameters (LT (min), Peak (nM), ttPeak (min), ETP (nM*min)) from spiked normal plasma samples and patient samples with the subsequent addition of a range of concentrations of PCC with and without protamine sulphate.

4.4.2 Secondary endpoints

The use of statistical analysis including:

- Descriptive statistics (% change from baseline for TGA parameters i.e. the absence of anticoagulant or reversal agent or both) for the range of different conditions
- Analysis to determine any correlation between parameters, timing and dose of anticoagulant
- Pairwise comparison to determine any statistically significant impact of reversal agents

4.5 Materials and methods

4.5.1 Pooled plasma samples

In vitro spiking experiments were performed using pooled plasma prepared from healthy volunteers (NP3). Ethical approval and informed consent was obtained via the Katharine Dormandy Coagulation Research Plasma Bank ((KDCRPB; REC reference number 14/YH/1272). Citrated whole blood (CWB) samples were obtained from ante-cubital veins using gentle tourniquet and 21-gauge butterfly needle and collected into Sarstedt Monovette™ tubes (citrate 3.2% 0.109 M, 1:10; aspiration technique). Samples were immediately put on ice. Plasma was double spun (2000g for 15 minutes) in order to obtain platelet poor plasma (PPP), pooled, divided into aliquots and frozen at -80^{0}C within 2 hours of collection.

4.5.2 Patients

Suitable patients being treated with LMWH were identified by Katharine Dormandy Haemophilia and Thrombosis Centre (KDHC) staff or by the author. Patients were approached to participate if they were receiving treatment with the above, were not on any other anti-coagulants or anti-platelet agents and were able to provide informed consent (obtained through the KDCRPB). Patient samples were collected as for the healthy volunteers. A sample was also collected into a citrated tube (Becton Dickinson Vacutainer, 0.109M tri-sodium citrate) for the anti-Xa assay. The anti-IIa assay was performed later on frozen PPP aliquots. Platelet rich plasma (PRP) was obtained from the CWB sample containing CTI by centrifugation at 220g (1000rpm) for 15 minutes. The platelet count was then adjusted to 150 x 10^9/L using autologous PPP. PPP was obtained via double centrifugation (2000g for 15 minutes).

4.5.3 Reagents

All medications were licensed products for use in patients. Unfractionated heparin (UFH, non-proprietary heparin sodium) 1000 IU/ml, final concentrations 0 – 0.5 anti-Xa units/ml; Tinzaparin (Innohep®, Leopharma) 4500 IU/0.5ml, final concentrations 0 – 0.8 anti-Xa units/ml; Enoxaparin (Clexane®, Sanofi-Aventis) 100 mg/ml, final concentrations 0 – 0.8 anti-Xa units/ml; Fondaparinux (Arixtra®, GlaxoSmithKline) 2.5 mg/0.5ml, final concentrations 0 – 0.8 anti-Xa units/ml; 4 factor prothrombin complex concentrate (Beriplex®, CSL Behring) 500 IU (FIX used for calculations, measured in-house for batch = 22.4 IU/ml; FII 22 IU/ml;

heparin 0.7 anti-Xa units/ml [3]); protamine sulphate (non-proprietary) 10 mg/ml used at standard final concentrations of 2.5-5 μg/ml (as used in previous studies: see [Gatt *et al.*, 2008]). Samples of NP3 pooled plasma were spiked initially with the anticoagulant and subsequently the procoagulant. The concentrations used for the anticoagulants aimed for a range of anti-Xa activity to reflect results from patients with final concentrations confirmed by anti-Xa assay. Whole blood versus PRP versus PPP was spiked initially and anti-Xa assays were performed to demonstrate equivalent results such that all spiking was done on PRP or PPP (data not shown). The spiking dose of PCC (reconstituted as directed by the manufacturer and added neat) was added at a concentration at 0.6 IU/ml and calculated to be equivalent to a therapeutic dose of 25 IU/kg. 0.3 and 0.9 IU/ml were used as low and high dose equivalents (12 IU/kg and 36 IU/kg). All other medications were diluted in Tris buffered saline (TBS, pH 7.4) with 1% bovine serum albumin (BSA). See Supplementary material 4.8 for calculations.

Thus, there were multiple different groups. Each anticoagulant was added at a range of calculations (see Table 4.2). Then, for each concentration, PCC at a concentration of 0.3 IU/ml, 0.6 IU/ml and 0.9 IU/ml was added. In addition, protamine sulphate was added at 2.5 μg/ml and 5 μg/ml. Finally, PCC 0.6 IU/ml and protamine 2.5 μg/ml were added in combination.

[3] *final heparin concentration per 80 μl aliquot of plasma added to thrombin generation assay well calculated to be ≤ 0.056 anti-Xa units; see also Figure 4-7 to demonstrate that addition of 4 factor PCC to normal (unspiked) plasma produces an increase in thrombin generation*

Table 4-2 Range of anticoagulant concentrations added to normal pooled plasma

UFH	0.1, 0.2, 0.3, 0.5 anti-Xa units/ml *
Tinzaparin	0.2, 0.4, 0.6, 0.8 anti-Xa units/ml *
Enoxaparin	0.2, 0.4, 0.6, 0.8 anti-Xa units/ml *
Fondaparinux	0.2, 0.4, 0.6, 0.8 anti-Xa units/ml *

4.5.4 Thrombin generation assay

Thrombin generation (TGA) was measured as per the method of Hemker et al. [Hemker *et al.*, 2006]. In brief, 80 μl PRP or PPP was added to 20 μl PRP or PPP Reagent (Thrombinoscope B.V., Maastricht, The Netherlands) in an Immunlon 2HB, round-bottom 96-well plate (Dynex technologies). 20 μl Hepes/BSA buffer containing 100 mM calcium chloride ($CaCl_2$) and 5 mM fluorogenic substrate was added to initiate the assay. Each sample was tested in duplicate with an internal calibrator. Fluorescence was measured with an excitation filter at 390 nm and an emission filter at 460 nm using a Fluroskan Ascent fluorimeter (Thermolab Systems, Helsinki, Finland). The endogenous thrombin potential (ETP), lagtime (LT), peak and time to peak (ttPeak) were derived from the raw data by dedicated software (Thrombinoscope™ B.V., Maastricht, The Netherlands)[4].

[4] For further details of the TGA protocol see Chapter 3

4.5.4.1 TF source and concentration

Different TF concentrations were used in initial spiking experiments, but 5 pM was found to give the best results for PPP samples (data not shown). Lower concentrations were too sensitive to the anticoagulants. In order to minimise variability and, given the better CV results found using the Thrombinoscope reagents whilst generating the normal reference ranges (see Chapter 3: Supplementary material 3.9), the PPP reagent (that contained approximately 5 pM TF) was selected. The Thrombinoscope PRP reagent (containing approximately 0.5 pM TF) was used for the PRP samples.

4.5.4.2 Reference plasma

In-house generated normal pooled plasma (NP3) was used both as quality control and to normalise PPP data (see Chapter 3: Supplementary material 3.9).

4.5.4.3 TGA parameters and reporting of data

The principal parameters of interest in this study were the LT and peak as, in a clinical setting of bleeding or necessity for urgent intervention, a clinician would want to know that an adequate and timely thrombin burst was being produced. In addition, the reporting of an ETP becomes problematic at very low levels of thrombin generation. For the *in vitro* spiking study, all the results were normalised against the internal control (a sample of normal pooled plasma run without the addition of PCC or protamine). In the case of the *ex vivo* spiking where both PRP and PPP were analysed, the raw data are reported for the

PRP experiments (in the absence of a satisfactory normalisation control), whereas the data for the PPP experiments were normalised against an internal control in order to minimise inter-assay variability [Dargaud *et al.*, 2007].

4.5.5 Anti-Xa and anti-IIa assay

Anti-Xa activities were measured using a HemosIL Liquid Anti Xa kit (Instrumentation Laboratory (IL), USA) on an ACL TOP 700 coagulometer (IL, USA). Anti-IIa activities were measured using a 2-stage chromogenic assay (Hyphen BioMed, France). Calibration curves were obtained using international standards for unfractionated heparin and LMWH (National Institute for Biological Standards and Control (NIBSC), UK). Double spun PPP samples were frozen, stored at -80^{0}C and then processed in batches using a SpectraMax i3 multi-mode microplate platform (Molecular Devices, UK). The anti-Xa assays were performed by the routine diagnostic laboratory at the Royal Free Hospital. Assistance with the anti-IIa assay was provided by Dr C. Hamid.

4.5.6 Statistical analysis

Statistical tests were performed using Excel 2011 (Microsoft) and SPSS 22.0.0.0 (SPSS, Chicago, IL, USA). Descriptive statistics were employed including percentage change from baseline with reference made to in-house derived normal ranges for spiked normal plasma in addition to median and inter-quartile ranges (IQR) for patient data. Correlation testing was used to assess the strength and direction of the relationship between the TGA dependent

variables and the between-patient variables (anti-Xa levels and time since dosing). Following this, analysis was conducted in order to detect any statistically significant difference between the different spiking groups for the patient samples, followed by pairwise comparisons with a Bonferroni correction (SPSS, 2013). A p value of < 0.05 was considered statistically significant unless stated otherwise.

4.6 Results

4.6.1 Sensitivity of the assay to pro- and anticoagulants

The TGA demonstrated sensitivity to all four anticoagulants showing a dose-dependent reduction in thrombin generation with increasing concentrations of each agent. Each anticoagulant had a distinct pattern of response in the thrombin generation curve (see Figures 4-3 – 4-6) reflecting the different anti-Xa:anti-IIa ratios with Fondaparinux having the least profound impact on the TGA results. Tinzaparin showed the greatest similarity to UFH. Enoxaparin at equivalent anti-Xa activities had less impact on thrombin generation. Of interest, addition of increasing concentrations of Fondaparinux resulted in prolongation of the LT.

Secondly, the assay was also sensitive to increasing concentrations of PCC (see Figure 4-7). Of note, an increase in thrombin generation was also seen with the addition of protamine, in the absence of any anticoagulant (approximately 30% increase in peak) (Figure 4-8). The mechanism underlying this phenomenon is unclear.

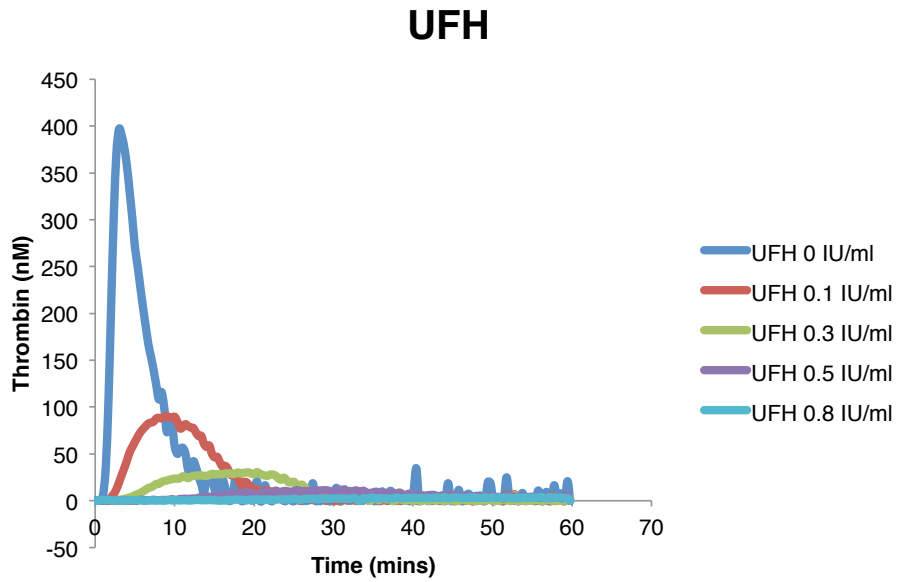


Figure 4-3 TGA: normal plasma spiked with increasing concentrations of UFH 0 – 0.8 IU/ml

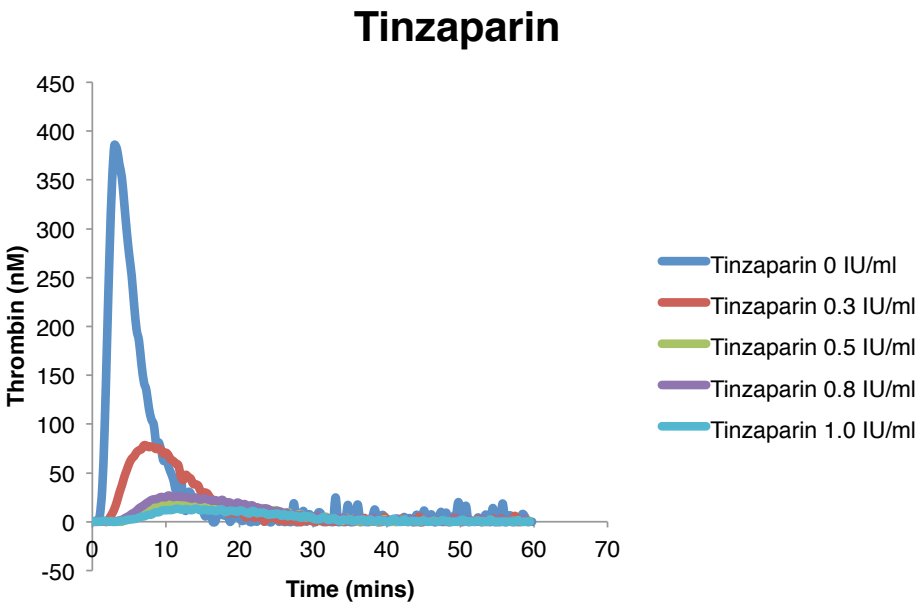


Figure 4-4 TGA: normal plasma spiked with increasing concentrations of Tinzaparin 0 – 0.8 IU/ml; note similarity of curves to those with addition of UFH (Figure 4-3)

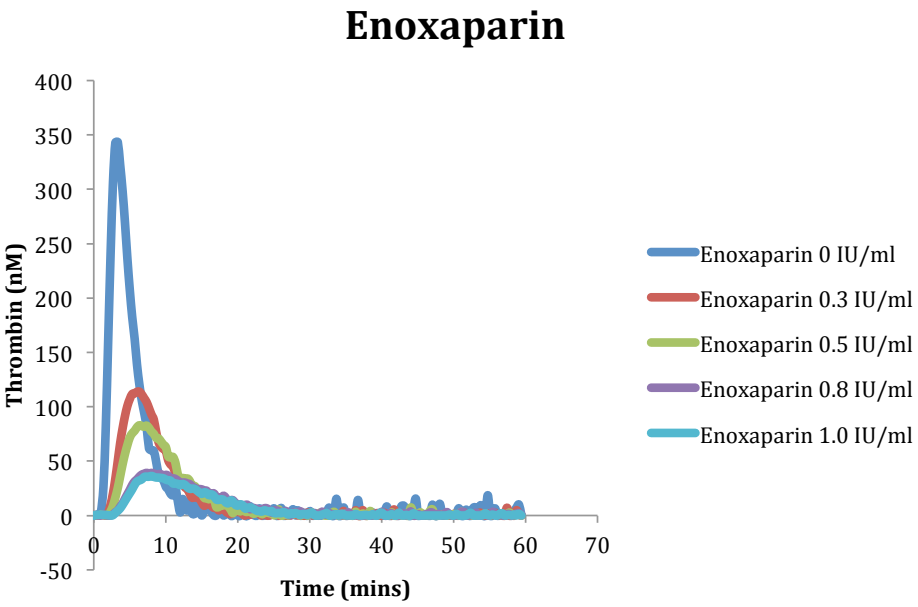


Figure 4-5 TGA: normal plasma spiked with increasing concentrations of Enoxaparin 0 – 1.0 IU/ml; note less suppression of thrombin generation at similar concentrations to UFH and Tinzaparin

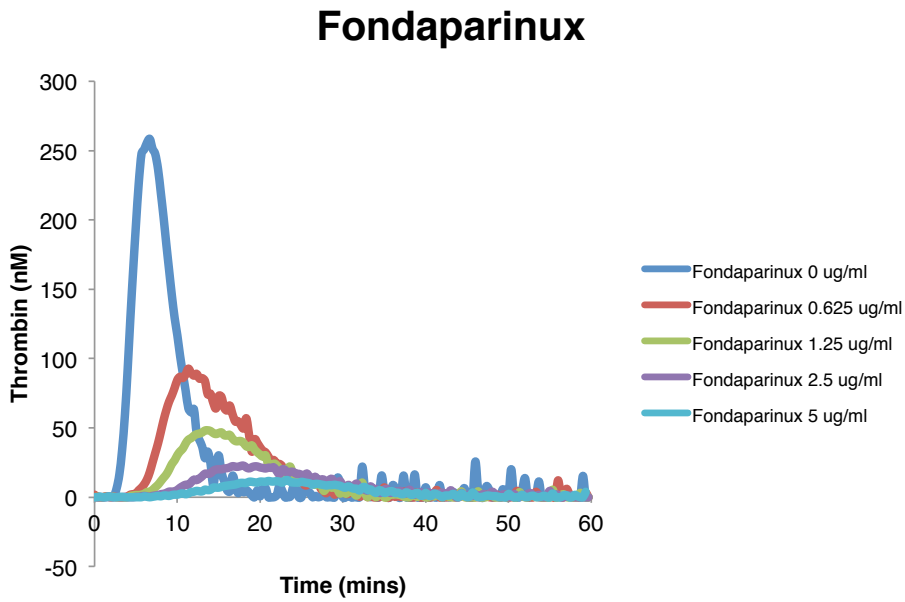


Figure 4-6 TGA: normal plasma spiked with increasing concentrations of Fondaparinux 0 – 5μg/ml; note prolongation of LT with increasing concentrations as compared to the other anticoagulants (see Figures 4-3, 4-4 and 4-5).

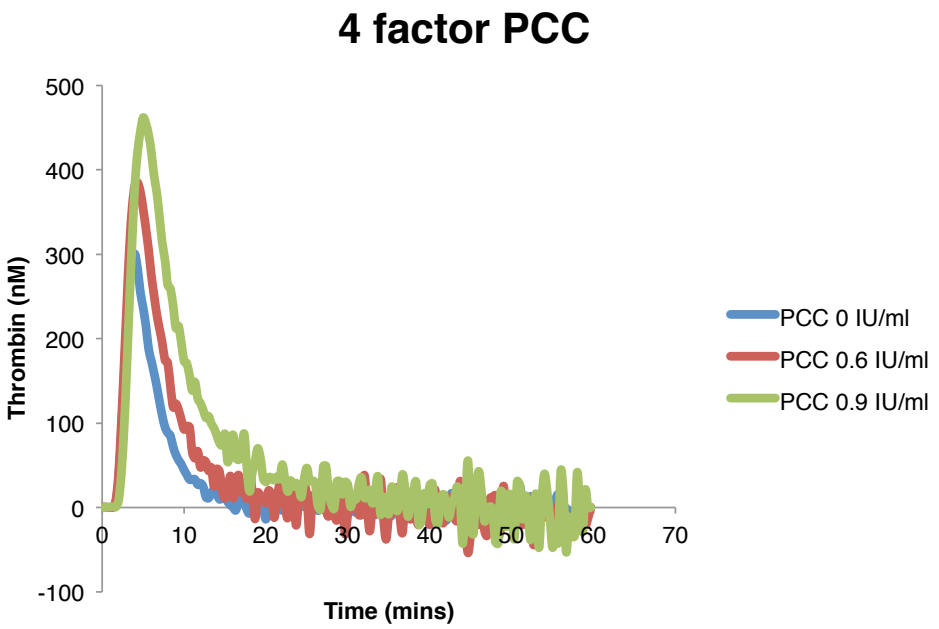


Figure 4-7 TGA: normal plasma spiked with 2 different concentrations of 4 factor PCC 0.6 and 0.9 IU/ml (equivalent to 25 IU/kg and 36 IU/kg).

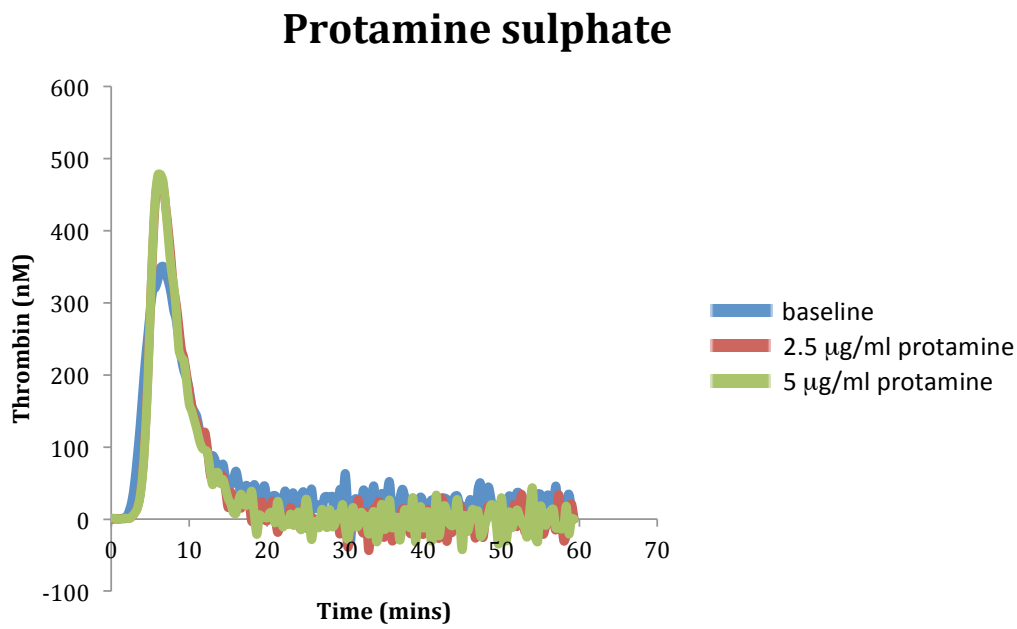


Figure 4-8 TGA: normal plasma spiked with 2 different concentrations of protamine 2.5 and 5 μg/ml; note increase in peak thrombin generation as compared to baseline.

4.6.2 *In vitro* spiking – impact of reversal agents on thrombin generation in normal pooled plasma

Given the number of combinations of different concentrations of anticoagulant combined with PCC and protamine, there was a large amount of data created. In order to find the most informative way of representing these results, it was decided to calculate the percentage change from baseline in the TGA parameter of interest. This meant that the result of the control sample (without the addition of either the anticoagulant and/or the reversal agent) was allocated the value 0.

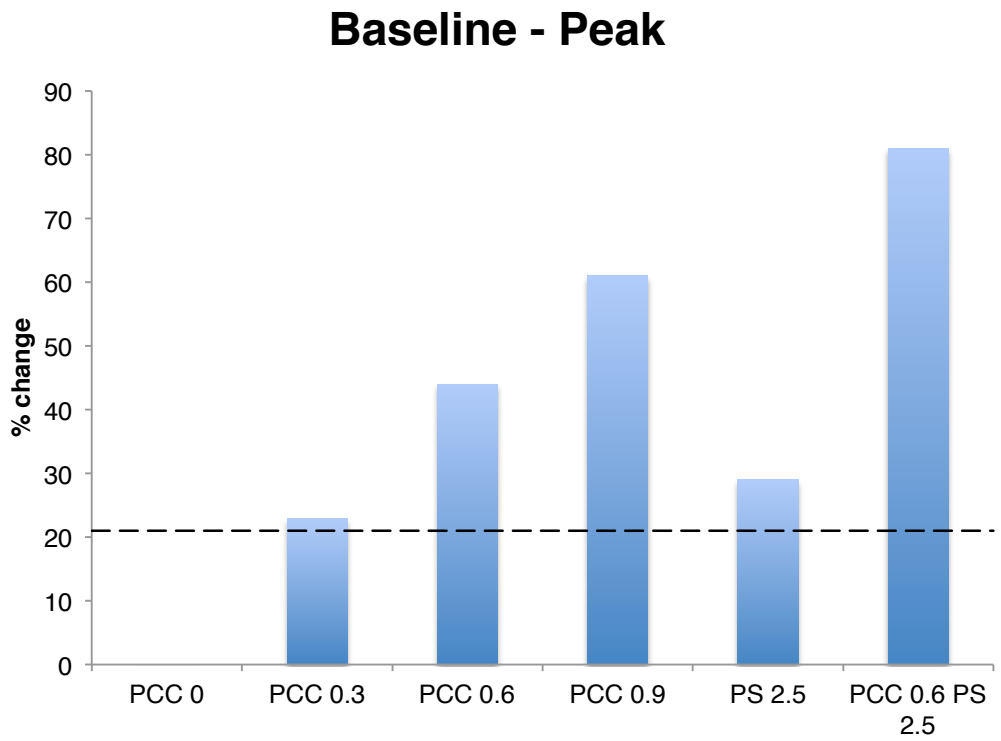


Figure 4-9 *In vitro* spiking of normal pooled plasma with PCC ± protamine sulphate. Unspiked sample allocated a value of 0. Other categories depict the % increase in peak value (Y axis). Dashed line = upper limit of normal range (+ 2SD). PCC units IU/ml; protamine μg/ml.

Peak

Figure 4-9 clearly demonstrates the effect of adding sequential concentrations of PCC to a non-anticoagulated sample. In addition, it highlights the increase in peak seen with protamine alone. There is a further increase in the peak value with the addition of both PCC and protamine (0.6 IU/ml and 2.5 μg/ml respectively).

The following Figures (4-10 – 4-13) again demonstrate percentage change from baseline. This time the different concentrations of anticoagulant are grouped together and the impact of the addition of the reversal agents demonstrated as per Figure 4-9. The upper and lower limits of the peak for the normal pool (± 2SD) are highlighted.

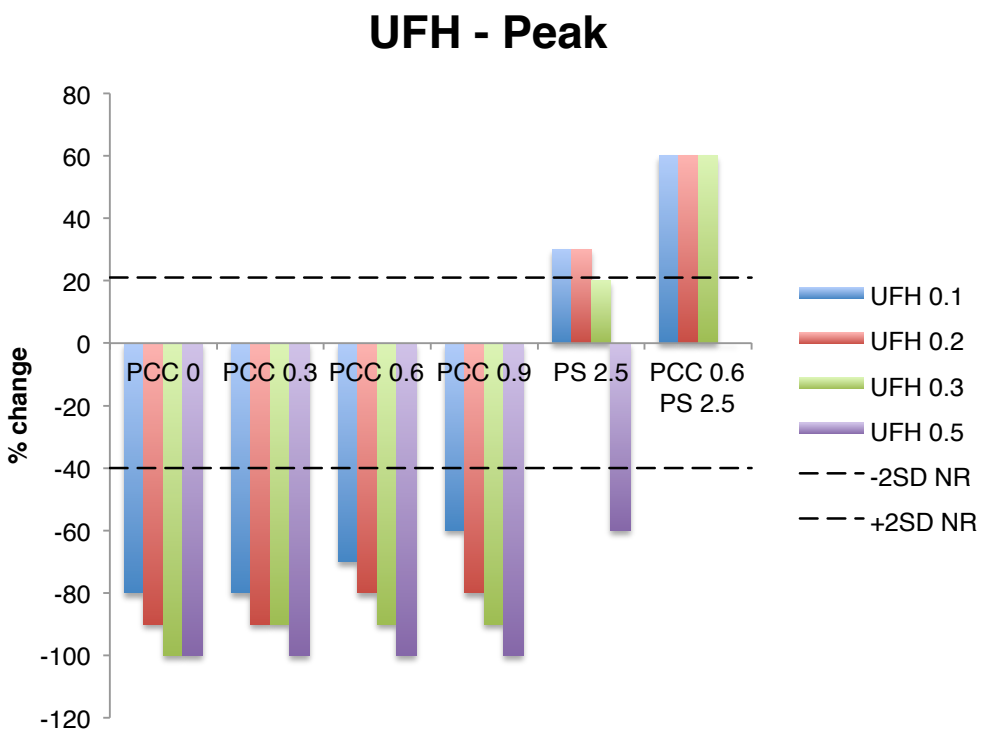


Figure 4-10 UFH: percentage change from baseline peak (no anticoagulant or procoagulant) upon addition of UFH, PCC and/or protamine (baseline given value = 0); upper and lower limit of normal range highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

The assay, as expected, demonstrated the reversibility of UFH suppression of the peak by protamine (Figure 4-10). This was true for all but the highest concentration of UFH as the protamine dose was insufficient for complete neutralisation. The combination of PCC and protamine brought the peak of all doses either to within the normal range or above. There were similar findings for the peak results in the Tinzaparin experiment (see Figure 4-11).

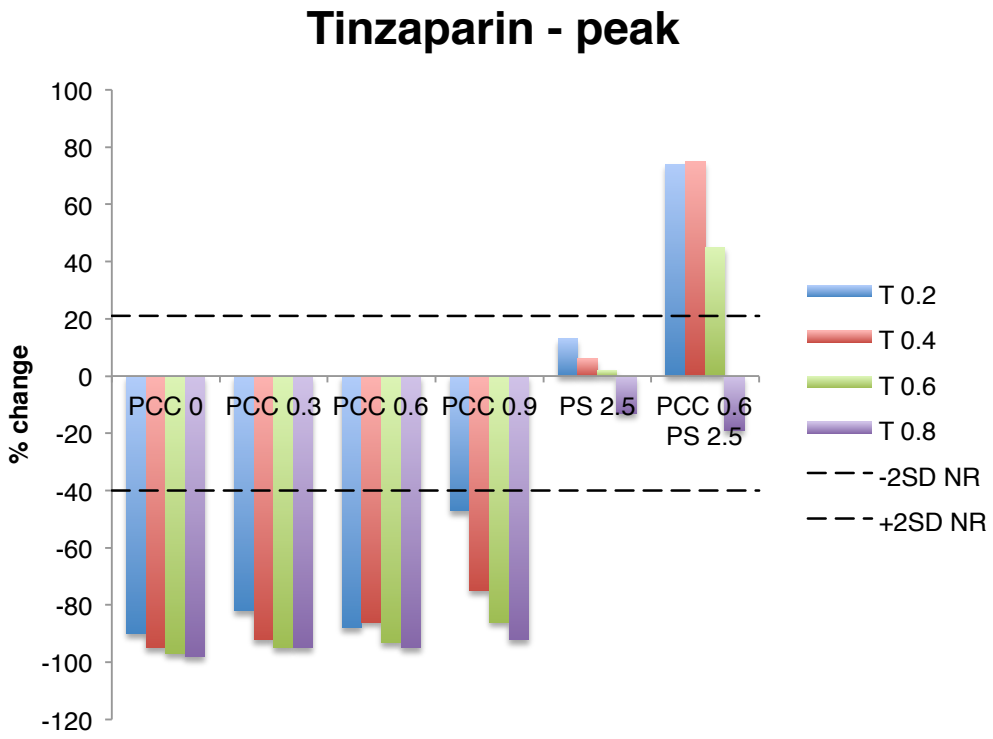


Figure 4-11 Tinzaparin: percentage change from baseline peak (no anticoagulant or procoagulant) upon addition of Tinzaparin, PCC and/or protamine (baseline given value = 0); upper and lower limit of normal range highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

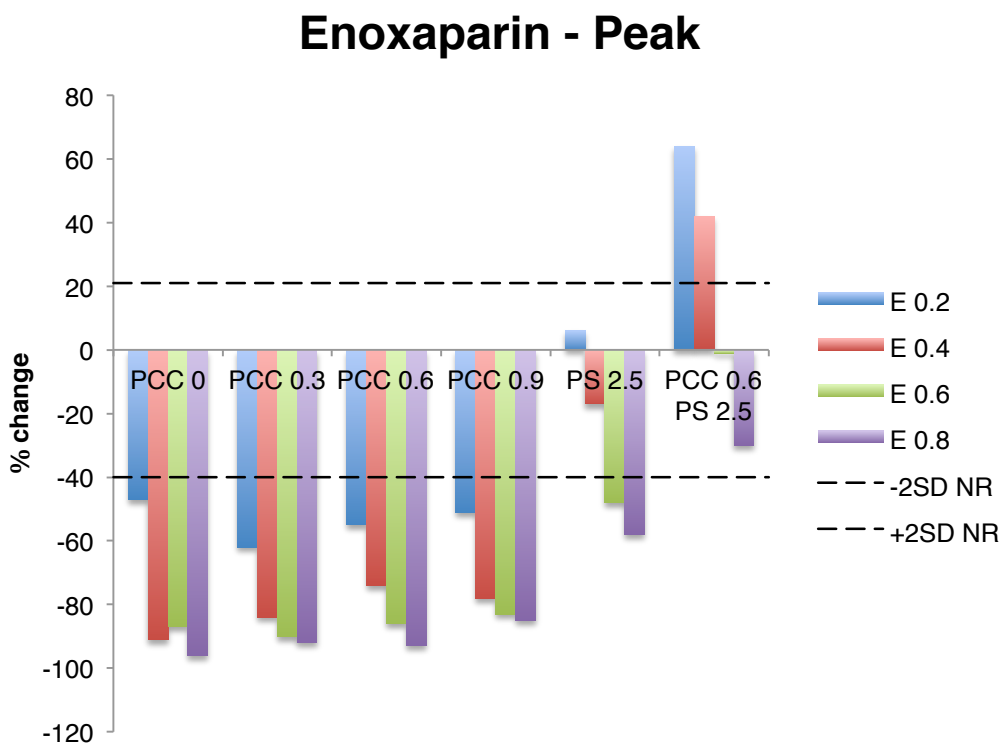


Figure 4-12 Enoxaparin: percentage change from baseline peak (no anticoagulant or procoagulant) upon addition of Enoxaparin, PCC and/or protamine (baseline given value = 0); upper and lower limit of normal range highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

Protamine was less effective at reversing Enoxaparin and only the combination of PCC and protamine bought all results into the normal range (Figure 4-12). In contrast, PCC was more effective than protamine or the combination in returning the Fondaparinux peak results to within the normal range (Figure 4-13).

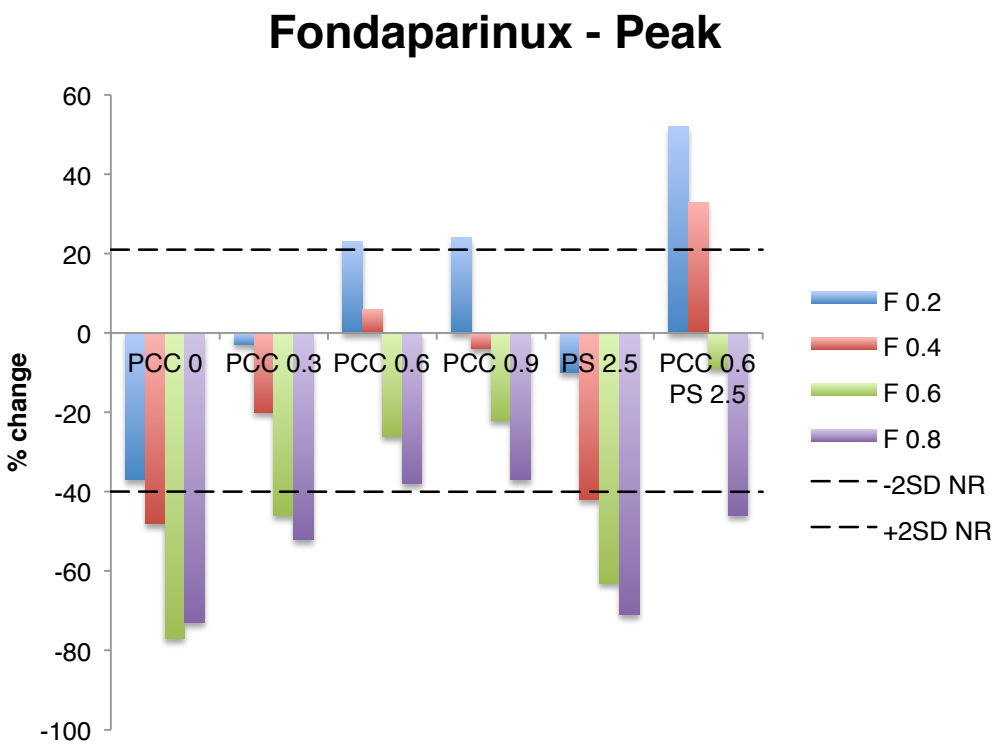


Figure 4-13 Fondaparinux: percentage change from baseline peak (no anticoagulant or procoagulant) upon addition of combinations of Fondaparinux, PCC and/or protamine (baseline given value = 0); upper and lower limit of normal range highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

LT

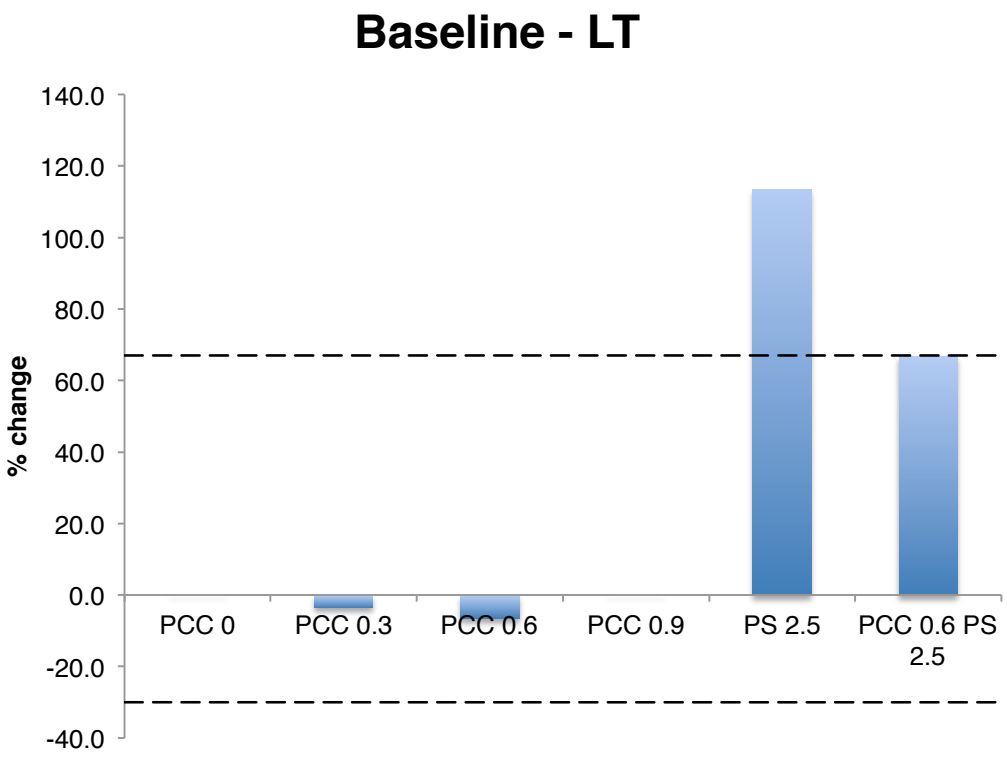


Figure 4-14 *In vitro* spiking of normal pooled plasma with PCC ± protamine sulphate. Unspiked sample allocated a value of 0. Other categories depict the % change in LT value. Dashed lines = upper and lower limits of normal range (± 2SD). PCC units IU/ml; protamine μg/ml.

The impact of the addition of the reversal agents on the LT of the control (non-anticoagulated) sample is shown in Figure 4-14. PCC alone had minimal impact with some shortening of the LT at 0.3 IU/ml and 0.6 IU/ml, but not 0.9 IU/ml. Protamine caused a marked increase (prolongation) in the LT that was somewhat tempered by the addition of 0.6 IU/ml PCC in combination.

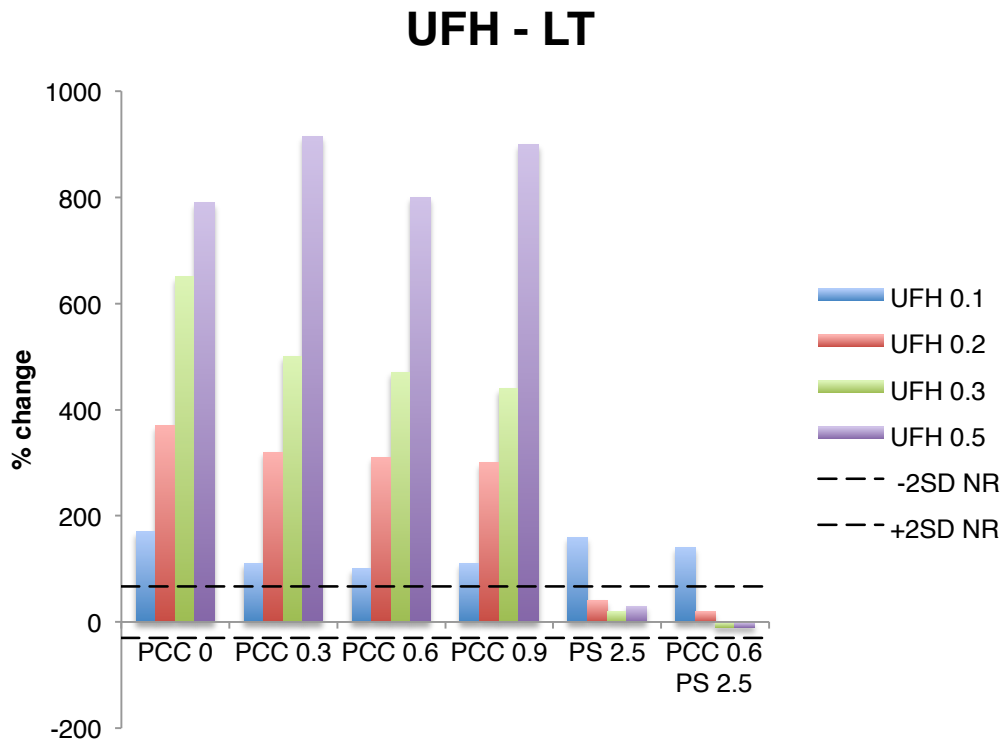


Figure 4-15 UFH: percentage change from baseline LT (no anticoagulant or procoagulant) upon addition of combinations of UFH, PCC and/or protamine; baseline LT given value = 0; upper and lower limits of NR highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

In terms of the LT, in the case of UFH protamine brought the LT back down to within the normal range, although did not achieve this completely for the lowest concentration of UFH (Figure 4-15). A similar pattern was seen for Tinzaparin, but in this case the highest dose of PCC (0.9 IU/ml) also demonstrated effect (Figure 4-16). The combination of PCC and protamine achieved equivalent results to protamine alone. UFH and Tinzaparin showed very marked prolongation of the LT compared to the other agents.

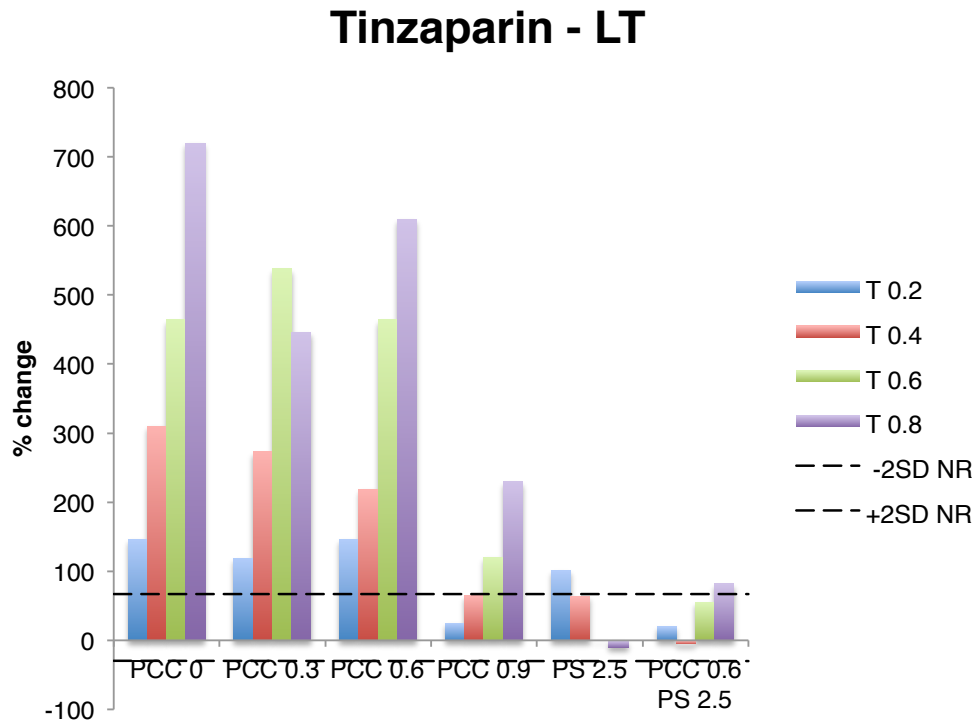


Figure 4-16 Tinzaparin: percentage change from baseline LT (no anticoagulant or procoagulant) upon addition of combinations of Tinzaparin, PCC and/or protamine; baseline LT given value = 0; upper and lower limits of NR highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

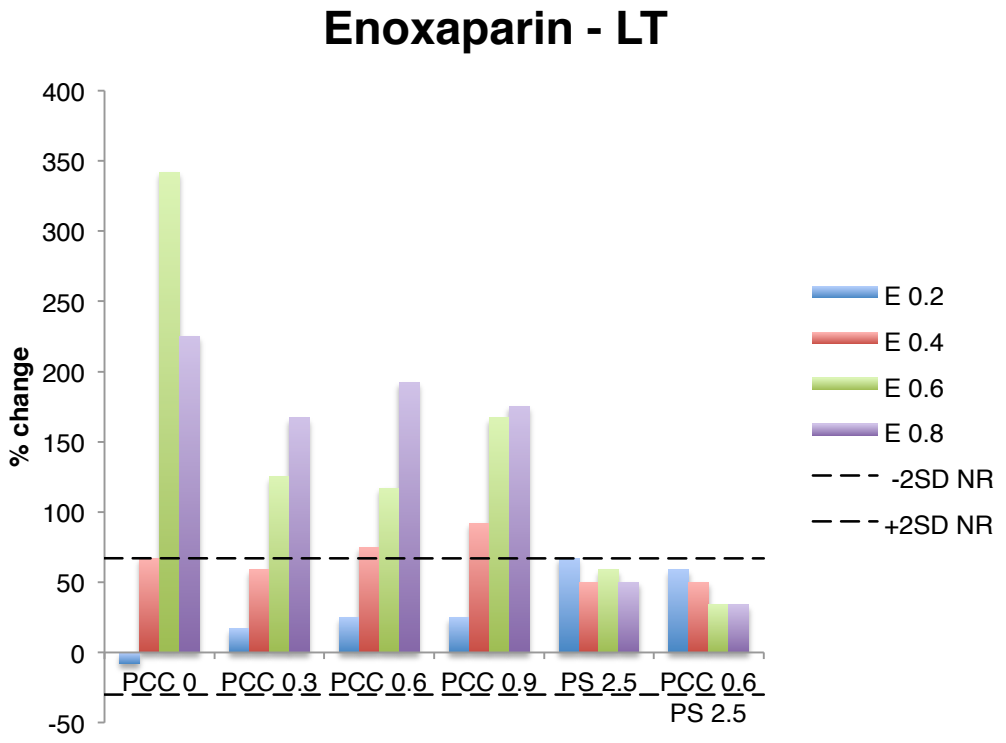


Figure 4-17 Enoxaparin: percentage change from baseline LT (no anticoagulant or procoagulant) upon addition of combinations of Enoxaparin, PCC and/or protamine; baseline LT given value = 0; upper and lower limits of NR highlighted (±2SD NR). Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

For Enoxaparin, there was success at normalising the LT at the higher doses of PCC (although this was not complete for the higher doses of Enoxaparin), protamine and the combination (see Figure 4-17). The Fondaparinux LT was normalised by PCC, but prolonged by the addition of protamine (Figure 4-18).

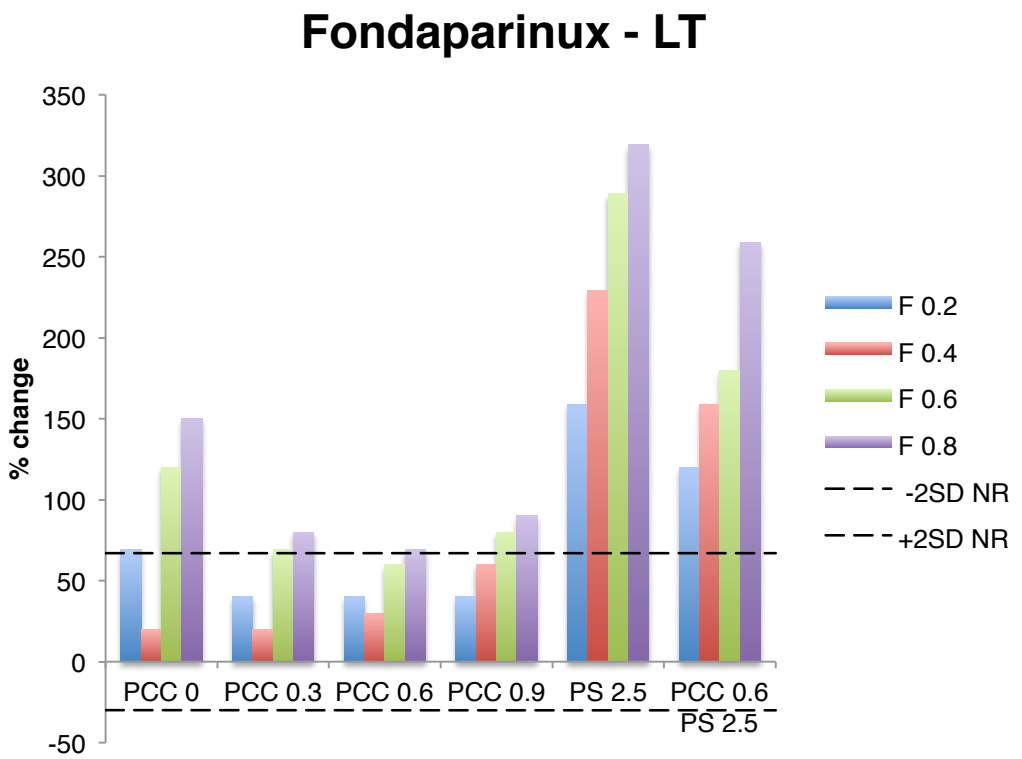


Figure 4-18 Fondaparinux: percentage change from baseline LT (no anticoagulant or procoagulant) upon addition of combinations of Fondaparinux, PCC and/or protamine; baseline LT given value = 0; upper and lower limits of NR highlighted. Concentrations of anti-coagulant expressed as anti-Xa activity, PCC as IU/ml and protamine μg/ml.

In summary, as in previous studies, complete reversal of the effects of UFH by protamine could be demonstrated by TGA. The impact of protamine on normalisation of the peak lessened as the proportion of anti-IIa activity decreased. The most consistent reversal of the impact on peak results for all four anticoagulants was achieved by the combination of PCC and protamine. PCC alone caused marked prolongation of the LT in those agents with significant anti-IIa activity (UFH, Tinzaparin). Protamine alone and in combination with PCC caused the greatest normalisation of the LT in the case

of UFH, Tinzaparin and Enoxaparin. However, in the case of Fondaparinux with sole anti-Xa activity, protamine (alone and in combination with PCC) caused notable prolongation of the LT.

Thus, for both parameters, the greatest success in terms of reversal of anticoagulant effect for all four agents was seen with the combination of PCC 0.6 IU/ml and protamine sulphate 2.5 μg/ml.

4.6.3 *Ex vivo* spiking – impact of reversal agents on thrombin generation in patient samples

14 patients were recruited to the study (see Table 4-3 for demographics and treatment information). In the case of 3 individuals, samples were obtained at more than one time-point. 11 patients were treated with Tinzaparin, 2 with Enoxaparin and 1 with Dalteparin (Tinzaparin is the LMWH of choice used in the Royal Free London NHS Foundation Trust). The principal reason for treatment was acute VTE, however some patients with a history of VTE were being covered during high-risk periods i.e. surgery and pregnancy. 10 patients were female, 4 male and the average age was 53.3 years (range 23 – 82 years). Median weight was 71.5 kg (range 51-102kg). The mean anti-Xa level was 0.4 anti-Xa U/ml (range 0 – 0.7 U/ml) taken at a median 4 hours post dose (see Table 4-3). 8 patients were receiving treatment dose Tinzaparin (approximately 175 IU/kg), 5 patients were receiving split dose i.e. bd at treatment intensity (both Tinzaparin and Enoxaparin) and the one patient on Dalteparin was receiving a treatment dose. No patients were identified who were receiving Fondaparinux within the time frame of the study. The anti-Xa

results of the patient samples encompassed the therapeutic range (Table 4-3; Figure 4-19). The anti-IIa results were mostly undetectable as might be expected a few hours post dose. The TGA did demonstrate increased sensitivity as described in the literature as demonstrated by Figure 4-20 – a short pharmacokinetic study at 3 time points following treatment dose Tinzaparin with corresponding anti-Xa and anti-IIa results.

Table 4-3 Patient demographics and indication for anticoagulation, LMWH product, dose and timing of sample with anti-Xa and anti-IIa results (*TPX = thromboprophylaxis)

Study ID	Product	Age	Gender	Indication	Dose IU/kg	Hrs post dose	Anti-Xa	Anti-IIa
1	Tinzaparin	23	F	DVT	169	19	0.00	0.00
2	Tinzaparin	36	F	PE	86 bd	4	0.16	0.00
2	"	"	"	"	86 bd	8	0.19	0.00
2	"	"	"	"	86 bd	12	0.11	0.09
2	"	"	"	"	100 bd	6.5	0.32	0.00
3	Tinzaparin	37	M	SVC thrombus	181	1.5	0.35	0.02
4	Tinzaparin	82	F	DVT	181	24	0.19	0.00
4	"	"	"	"	181	4	0.68	0.31
5	Tinzaparin	67	F	DVT	170	2	0.59	0.11
6	Tinzaparin	53	M	DVT	175	4	0.15	0.12
7	Tinzaparin	78	M	DVT/PE	124	5	0.26	0.00
8	Tinzaparin	46	M	DVT	176	3	0.70	0.03
9	Enoxaparin	43	F	DVT	1.4	4	0.51	0.00
10	Dalteparin	34	F	Pregnancy TPX*	210	2	0.70	0.00
11	Enoxaparin	52	F	Portal vein thrombosis	0.8	4	0.43	0.00
12	Tinzaparin	57	F	Sup. Thrombophlebitis	100	7	0.34	0.00
13	Tinzaparin	69	F	Previous ileofemoral DVT, Cancer surgery	59 bd	4	0.31	0.00
13	"	"	"	"	169	4	0.53	0.28
13	"	"	"	"	169	6	0.50	0.17
13	"	"	"	"	169	8	0.40	0.00
14	Tinzaparin	69	F	DVT	196	6	0.36	0.00

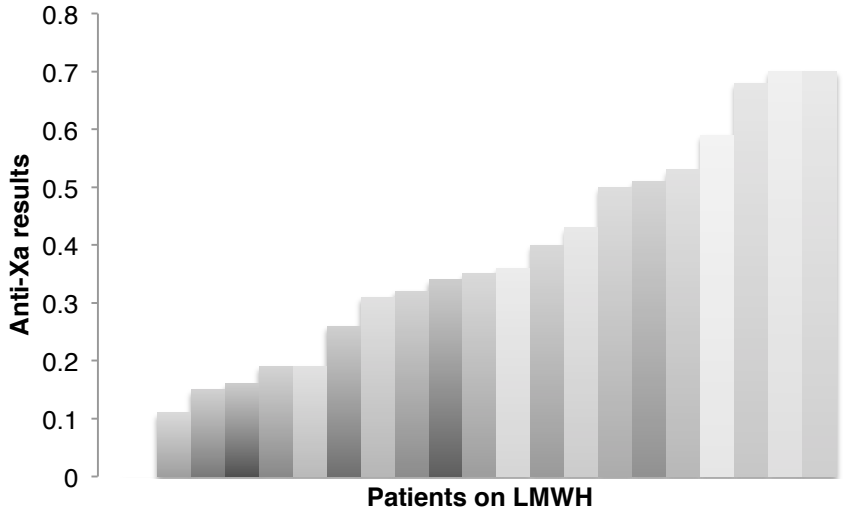
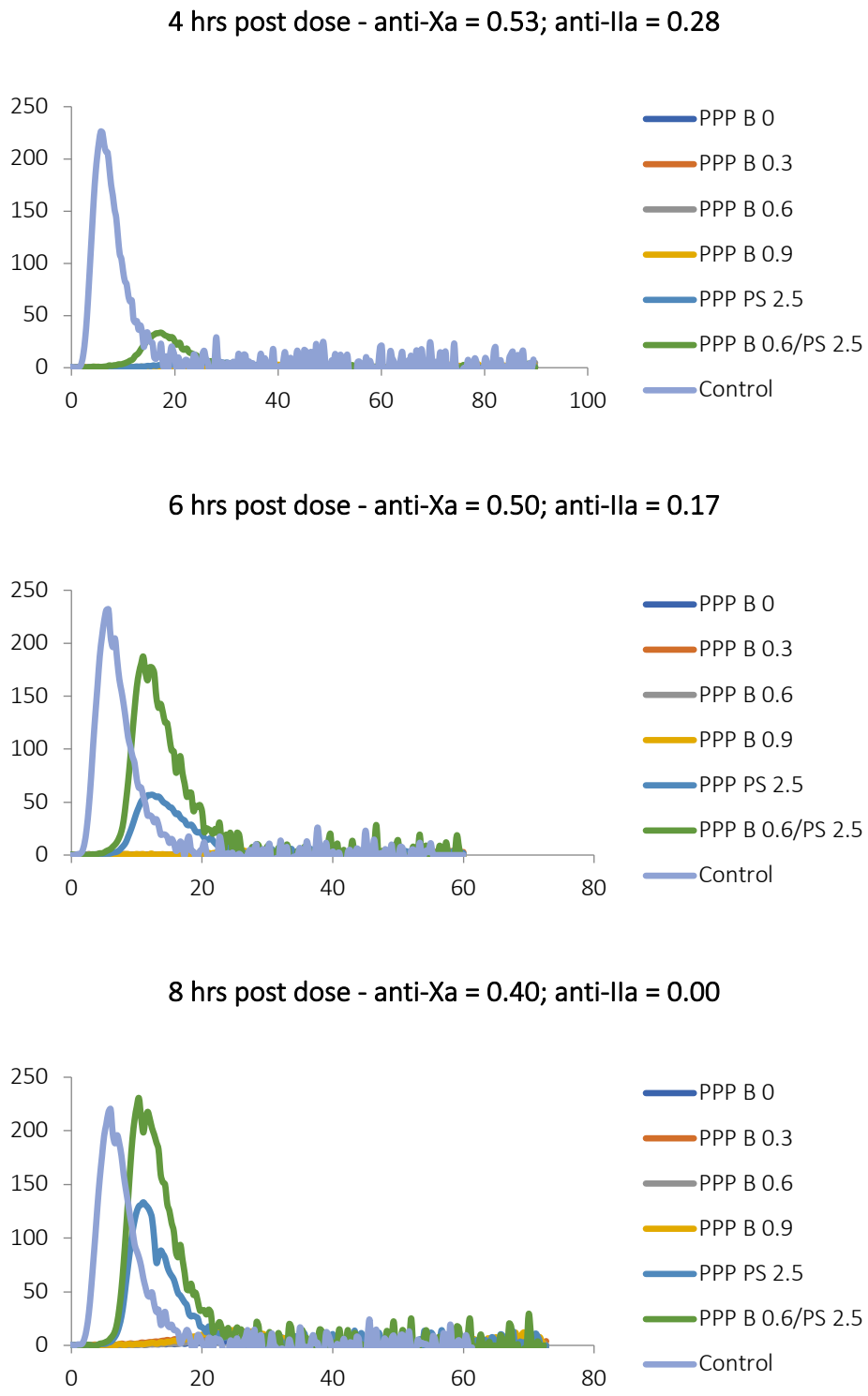


Figure 4-19 Anti-Xa results for samples from patients recruited to study

As this was a small observational study, the aim was to assess the impact of the reversal agents on samples with a range of anti-Xa results taken at differing time-points (post administration of anticoagulant). It was apparent from the *in vitro* spiking work that there was a dose-dependent relationship between the anticoagulant and efficacy of reversal (i.e. the higher the anti-Xa, the less marked the reversal). In patients, the anti-Xa result is a reflection of both the dose and the time post dose.

Figure 4-20 Limited PK study on patient treated with Tinzaparin (169 IU/kg od); B = Beriplex (PCC); PS = protamine sulphate

Samples taken at 4, 6 and 8 hours post dose. Platelet poor plasma; ex-vivo spiking with PCC and/or protamine. X axis = time (min); Y axis = thrombin (nM)



4.6.3.1 Statistical analysis

Due to the presence of outliers and data not being normally distributed (as assessed by Shapiro-Wilk's test $p < 0.05$), a non-parametric Spearman's rank-order correlation was used to assess the strength and direction of the relationship between the TGA dependent variables and the between-patient variables (anti-Xa levels and time since dosing). Following this, a Friedman test was conducted in order to detect any statistically significant difference between the different spiking groups for the patient samples, followed by pairwise comparisons with a Bonferroni correction (SPSS, 2013).

The TGA has 4 different parameters. Data for ETP were not analysed as results were confounded by inability of the software to calculate results in anti-coagulated samples with minimal thrombin generation. However, it was important to ascertain the correlation between the three remaining parameters – LT, peak and ttPeak – in order to determine which parameters to use as dependent variables. Clinically, it was felt by the author that the LT and peak thrombin generation were of interest in the context of arresting bleeding or allowing safe intervention.

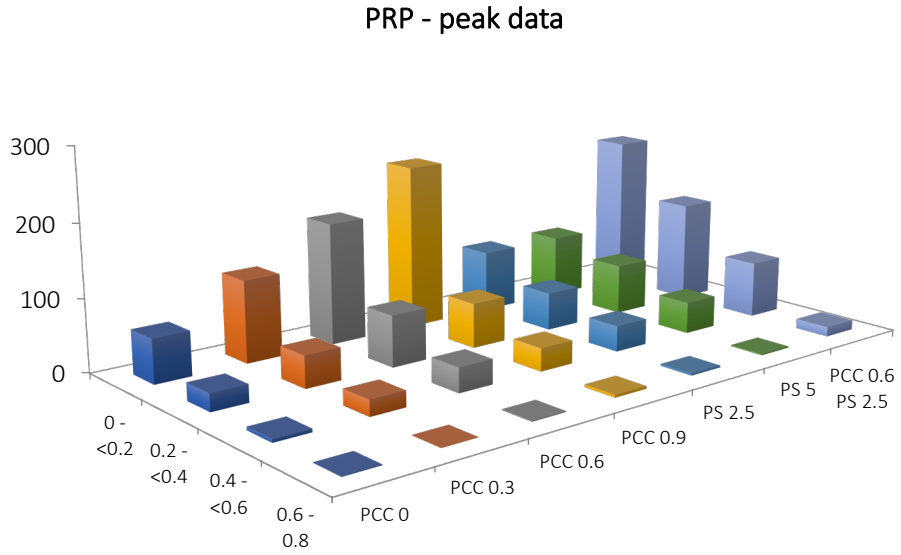
The PPP peak and LT were not associated when assessed by Spearman's correlation (correlation coefficient = -5.25, $p = 0.054$), however the LT and ttPeak were (correlation coefficient = 0.824, $p = 0.0001$). The same was found for PRP (data not shown). The peak and LT were thus the two variables carried forward for further assessment. Each of these 2 variables were then examined

to see assess the correlation between the variable, anti-Xa result and time post dose, both for the results from PRP and PPP.

Review of the data (Figures 4-21 and 4-22) reflected the assumption that the higher the anti-Xa (> 0.6 anti-Xa units/ml) or the closer to the dose (within 4 hours), the less the degree of reversal seen on the TGA peak. The question was whether there was a statistically significant difference between baseline (no reversal) and each of the reversal groups.

Figure 4-21Thrombin generation assay results for PRP – Peak; raw data (mean value for each data set)

i) Patients divided into categories based on anti-Xa result



ii) Patients divided into category based on time post dose of LMWH

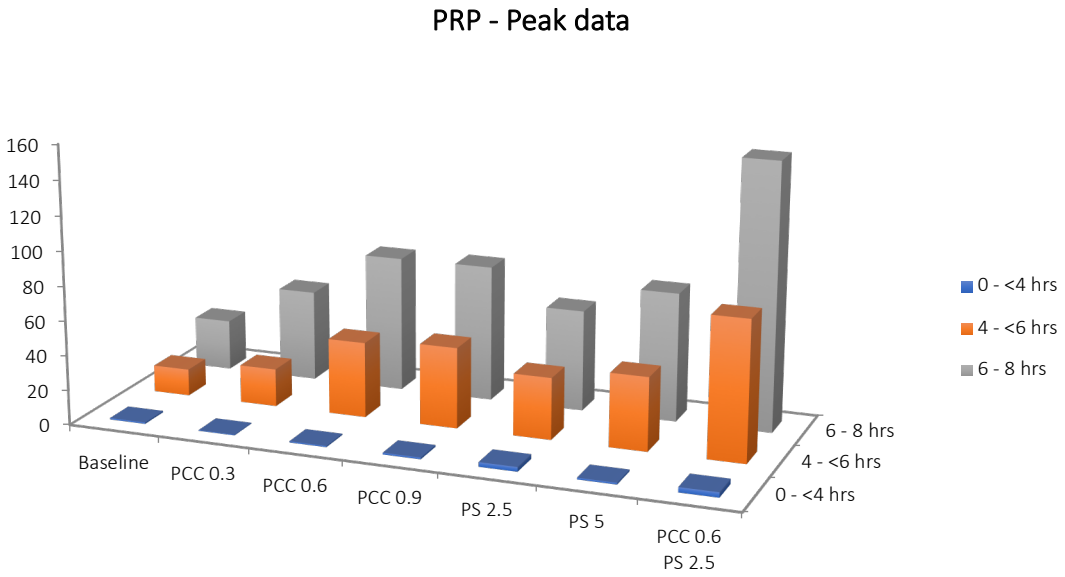
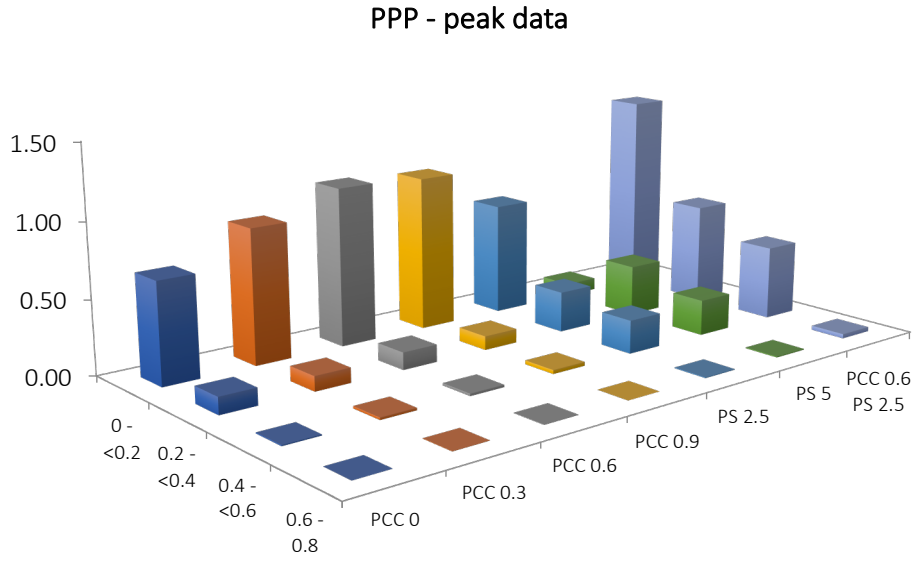
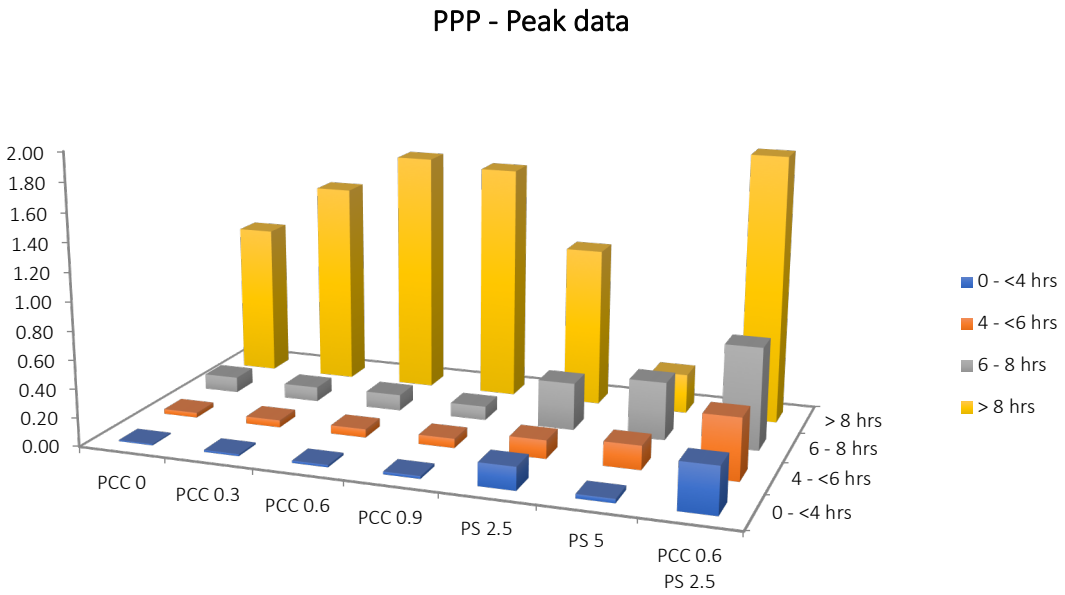


Figure 4-22 Thrombin generation assay results for PPP – Peak; results normalised using an internal control (normal pooled plasma) and expressed as a ratio

i) Patients divided into categories based on anti-Xa result



ii) Patients divided into category based on time post dose of LMWH



Spearman's correlation of the PRP peak, anti-Xa result and time post dose showed a significant relationship (Peak – anti-Xa: correlation coefficient = -0.727, p = 0.003; peak – time post dose: correlation coefficient = 0.659, p = 0.01; anti-Xa – time post dose: correlation coefficient = -0.690, p = 0.006) (see Table 5-3). Thus, a between groups comparison for the TGA peak was conducted using a Friedman test with post hoc analysis. The peak was statistically significantly different between the treatment groups (χ^2 (7) = 43.714, p = 0.0001). Pairwise comparisons with a Bonferroni correction showed that the change was significant for: baseline – protamine 5 μg/ml (p = 0.02); baseline – PCC 0.6 IU/ml (p = 0.014); baseline – PCC 0.9 IU/ml (p = 0.005) and baseline – PCC 0.6 IU/ml/protamine 2.5 μg/ml (p = 0.0001).

The LT did not correlate significantly with the anti-Xa or time post dose (see Table 4-4). When a between groups comparison was performed with a Friedman test (χ^2 (7) = 33.879, p = 0.0001), pairwise comparisons did not prove that this was due to a significant difference between baseline and the individual treatment groups.

Table 4-4 PRP Spearman correlations

Correlations

			Peak - baseline	Hrs post dose	Anti-Xa
Spearman's rho	Peak - baseline	Correlation Coefficient	1.000	.659*	-.727**
		Sig. (2-tailed)	.	.010	.003
		N	14	14	14
	Hrs post dose	Correlation Coefficient	.659*	1.000	-.690**
		Sig. (2-tailed)	.010	.	.006
		N	14	14	14
	Anti-Xa	Correlation Coefficient	-.727**	-.690**	1.000
		Sig. (2-tailed)	.003	.006	.
		N	14	14	14

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

			Hrs post dose	Anti-Xa	LT - baseline
Spearman's rho	Hrs post dose	Correlation Coefficient	1.000	-.690**	-.126
		Sig. (2-tailed)	.	.006	.667
		N	14	14	14
	Anti-Xa	Correlation Coefficient	-.690**	1.000	.420
		Sig. (2-tailed)	.006	.	.135
		N	14	14	14
	LT - baseline	Correlation Coefficient	-.126	.420	1.000
		Sig. (2-tailed)	.667	.135	.
		N	14	14	14

**. Correlation is significant at the 0.01 level (2-tailed).

In terms of the PPP peak, Spearman's correlation was also significant for peak – anti-Xa (correlation coefficient = -0.836, p = 0.0001), peak – hours post dose (correlation coefficient = 0.727, p = 0.002) and anti-Xa – hours post dose (correlation coefficient -0.563, p = 0.023) (see Table 5-4). LT and ttPeak were also associated (0.900, p = 0.0001). LT was also associated with the anti-Xa although not time post dose (see Table 4-5). A Friedman test was significant for PPP peak (χ^2 (7) = 21.928, p = 0.001) with a significant comparison between baseline and the combination of PCC and protamine (p = 0.0001). This was also true for the PPP LT (χ^2 (7) = 28.149, p = 0.001), with the comparison between baseline and protamine 5 μg/ml also being significant (p = 0.011).

Table 4-5 PPP Spearman correlations

Correlations

			Anti-Xa	Hrs post dose	Peak - R
Spearman's rho	Anti-Xa	Correlation Coefficient	1.000	-.563*	-.836**
		Sig. (2-tailed)	.	.023	.000
		N	16	16	15
	Hrs post dose	Correlation Coefficient	-.563*	1.000	.727**
		Sig. (2-tailed)	.023	.	.002
		N	16	16	15
	Peak - R	Correlation Coefficient	-.836**	.727**	1.000
		Sig. (2-tailed)	.000	.002	.
		N	15	15	15

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

			Anti-Xa	Hrs post dose	LT - R
Spearman's rho	Anti-Xa	Correlation Coefficient	1.000	-.563*	.856**
		Sig. (2-tailed)	.	.023	.000
		N	16	16	15
	Hrs post dose	Correlation Coefficient	-.563*	1.000	-.444
		Sig. (2-tailed)	.023	.	.097
		N	16	16	15
	LT - R	Correlation Coefficient	.856**	-.444	1.000
		Sig. (2-tailed)	.000	.097	.
		N	15	15	15

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

In order to assess the clinical significance, the results were interpreted with reference to in-house normal reference ranges (see Table 4-6). Median (IQR) patient results for peak and LT in PRP and PPP were reviewed. In PRP and PPP, only the combination of PCC and protamine brought the median peak value into the normal reference range. The LT showed shortening, but not to within the normal range.

In summary, the data demonstrate that peak thrombin generation correlates with anti-Xa and timing of dose. Spiking of samples with reversal agents produces statistically significant improvements in peak thrombin (and LT) with the combination of PCC and protamine returning peak results to within the normal reference range.

Table 4-6 Patient samples; PRP and PPP TGA median (IQR) values for peak, LT. Green shading = median within normal range

PRP (raw data)	Peak (nM)	LT (min)
Normal ref. range	*107.83 (50.15 – 165.51)*	*12.88 (4.05 – 21.71)*
Baseline	8.57 (0.96 – 33.41)	51.54 (34.61 – 65.59)
PCC 0.3	20.06 (2.93 – 57.90)	49.67 (38.62 – 57.84)
PCC 0.6	25.55 (4.61 – 83.80)	56.74 (44.60 – 60.91)
PCC 0.9	25.06 (5.84 – 91.10)	54.00 (43.84 – 61.14)
PS 2.5	37.07 (6.99 – 64.94)	23.64 (15.87 – 51.67)
PS 5	46.64 (24.84 - 79.96)	22.46 (15.84 – 48.12)
PCC 0.6 PS 2.5	93.02 (35.69 – 135.05)	28.32 (22.13 – 53.59)
PPP (normalised)		
Normal ref. range	*1.18 (0.63 – 1.72)*	*0.97 (0.66 – 1.28)*
Baseline	0.05 (0.00 – 0.37)	2.50 (1.31 – 4.18)
PCC 0.3	0.05 (0.01 – 0.35)	2.70 (1.32 – 3.68)
PCC 0.6	0.06 (0.01 – 0.39)	3.53 (1.74 – 5.81)
PCC 0.9	0.07 (0.02 – 0.24)	2.90 (1.83 – 5.62)
PS 2.5	0.25 (0.08 – 0.62)	2.65 (1.78 – 3.51)
PS 5	0.19 (0.04 – 0.40)	3.45 (2.64 – 4.15)
PCC 0.6 PS 2.5	0.72 (0.15 – 1.12)	2.89 (2.07 – 4.34)

4.7 Discussion

This study was a proof-of-concept study using the TGA as a means of determining the efficacy of reversal of LMWH using 4-factor PCC and protamine sulphate. To our knowledge, this is the first time that this combination of reversal agents has been studied using the TGA.

The study has confirmed the sensitivity of the assay to both anticoagulants and procoagulants as previously reported in the literature [Gatt *et al.*, 2008]. In addition, the study has highlighted the impact differing ratios of anti-Xa:IIa activity seen in LMWH preparations have on the ability of procoagulants to reverse the anticoagulant effect. Whilst protamine is most effective when used in the context of agents with significant anti-IIa activity, PCCs would appear to have an effect against anti-Xa activity as seen in the spiking study with Fondaparinux. The most significant impact is seen when a combination of both PCC and protamine is employed.

The findings are replicated in patient samples, even when no anti-IIa activity can be detected by conventional assay. The data analysis of patient samples indicates that spiking with PCC in combination with protamine is able to achieve peak thrombin generation within the normal range and that this effect is most marked when the anti-Xa is less than 0.6 anti-Xa units/ml or more than 4 hours since dosing has elapsed. Even though the effects are less marked on the LT, there is still a degree of shortening observed. The results in PRP are greater than PPP, indicating an important role of platelets in a more physiological setting. The clinical requirement for a timely burst of thrombin generation in the

acutely bleeding patient or one that needs an urgent procedure may be achieved.

It should be noted that the majority of patient samples were obtained from patients being treated with Tinzaparin with an anti-Xa:IIa ratio of 2:1 (this is the preparation on the Royal Free Hospital Trust formulary). It could be argued that more significant reversal might be seen in patients treated with LMWH preparations with a higher ratio, such Enoxaparin or Dalteparin. There are understandable concerns regarding the use of PCC in patients who are already considered to be pro-thrombotic, however PCC are used routinely and safely in the reversal of vitamin K antagonists in similar patients. By reversing the anticoagulant effect, one is returning a patient to a prothrombotic baseline (hence why they are prescribed anticoagulation) so there needs to be a strong indication for reversal. Current guidance advises the use of protamine that, although effective, has anticoagulant effects at higher doses.

There are definite limitations of spiking studies, the principal one being that they are disproportionately influenced by high molecular weight units with anti-IIa effect and may not reflect the potential *in vivo* reversibility by PCC. The second part of the study using patient samples sought to address this limitation, in addition to enabling the use of PRP that allowed examination of a more physiologically comparable scenario. Platelets are recognised to accelerate the propagation phase of coagulation and increase the maximum concentration of thrombin generated due to the presence of procoagulant phospholipids and release of clotting factors upon activation [Gerotziafas, 2007].

The study is limited by sample number, although it was possible to recruit enough patients to cover a therapeutic range of anti-Xa levels. In addition, samples were spiked *ex vivo* and so, even though performed in PRP, are not entirely physiological. A clinical trial would be the best means of assessing efficacy but may be difficult to conduct, as patients would need to be identified who are in urgent need of reversal. In addition, there are novel non-specific antidotes currently under investigation that are claimed to be effective against multiple different anticoagulants including LMWHs, so it may be that this approach could be used in the interim whilst these compounds are assessed for safety and efficacy.

Protamine is a well-established compound in the reversal of unfractionated heparin but would appear to have interesting pro-coagulant features that do not appear to have been previously investigated. Further studies would be required to elucidate potential mechanisms. In addition, it would be interesting to spike heparinised samples with increasing concentrations of FII and FX individually to further understand the individual roles of the components of PCC. It would also be helpful to look at more samples from patients treated with different LMWH preparations such as Enoxaparin or Dalteparin.

In conclusion, the data demonstrate that the combination of protamine (2.5 μg/ml) with a standard dose of PCC (25 U/kg) when added to plasma samples containing LMWH (both spiked normal plasma and ex vivo patient samples) results in return of TGA variables (peak and LT) to within the normal range. Clinical outcomes were not recorded in this study and therefore further studies would need to be conducted to see whether normalisation of TGA variables

correlates with reduction in bleeding and whether there are any unwanted sequelae such as increased rates of thrombosis.

4.8 Supplementary material - calculations

4-factor PCC (Beriplex®)

Average 70 kg person – plasma volume 40 ml/kg

Plasma volume = 40 x 70 = 2800 mls

Dose of PCC = 25 IU/kg

Total dose = 25 x 70 = 1750 IU

PCC/ml = 0.6 IU/ml

Calculations based on FIX concentration 22.4 IU/dL

Using the same calculation: 0.3 IU/ml equivalent to 12 IU/kg; 0.9 IU/ml equivalent to 36 IU/kg.

Formula used for calculations: $c_1v_1 = c_2v_2$

c = concentration; v = volume

UFH	0.1, 0.2, 0.3, 0.5 anti-Xa units/ml *
Tinzaparin	0.2, 0.4, 0.6, 0.8 anti-Xa units/ml *
Enoxaparin	0.2, 0.4, 0.6, 0.8 anti-Xa units/ml *
Fondaparinux	0.2, 0.4, 0.6, 0.8 anti-Xa units/ml *

* *Confirmed by anti-Xa activity*

Aliquots of pooled normal plasma spiked with different concentrations of anticoagulant first, then sub-divided into test samples and spiked with reversal agent.

Protamine sulphate 2.5 and 5 μg/ml used as per Gatt et al. [Gatt *et al.*, 2008]

5 Inter-individual variability in the initiation phase of the extrinsic pathway: a potential modifier of phenotypic heterogeneity in haemophilia – Part 1

5.1 Severe haemophilia and variable bleeding phenotype

Variability in the bleeding phenotype of patients with severe haemophilia is well recognised. By convention, the classification of the disorder is determined by the baseline level of the missing clotting factor i.e. a factor level of less than 1 IU/dL means that a patient has severe haemophilia. However, in reality, not all patient's phenotypes can be so clearly predicted and a significant proportion of patients with severe haemophilia do not bleed as expected (10-15% in SHA) [Van Den Berg, 2007, Jayandharan *et al.*, 2008, Pavlova *et al.*, 2013]. The experience within the Katharine Dormandy Haemophilia Centre has been that even patients with the intron 22 inversion (i.e. 50% of patients with SHA resulting in no functional product of the F8 gene) show variability such that some do not require any prophylaxis and can treat themselves on demand or in anticipation of increased physical activity. This has also been observed in patients with non-null mutations [Pavlova *et al.*, 2013]. Of particular interest,

washout samples from patients with null mutations have thrombin generating capacity (i.e. when no exogenous FVIII/FIX is present). Further, the thrombin-generating potential of plasma can vary between individuals with similar antigen levels and genotypes and has been found to predict severity [Dargaud *et al.*, 2005]. Variation in individual pharmacokinetics affecting the FVIII/IX half-life ($t_{1/2}$) will also play a role in disease modification. An important contributor to this – and a surrogate marker for FVIII half-life ($t_{1/2}$) – is von Willebrand factor (VWF). VWF stabilises the FVIII protein in circulation and selectively concentrates FVIII at sites of injury. It is notable that FVIII only has a normal $t_{1/2}$ in conjunction with VWF and that VWF levels correlate with the pharmacokinetic parameters of infused FVIII concentrate in patients with haemophilia [Deitcher *et al.*, 1999, Lalezari *et al.*, 2014].

Thus although the factor level is key, genetic variables impacting on other components of the haemostatic system or polymorphisms of genes regulating inflammation and joint integrity may also play a role (the latter in the manifestation of musculoskeletal pathology). Environmental factors such as access to treatment, employment and physical activity levels in addition to psychosocial factors such as adherence to treatment and resultant presence or absence of joint disease all contribute further.

It has been postulated that there may be disease-modifying genes (particularly those known to confer a thrombophilic risk) that may ameliorate bleeding phenotype [Franchini *et al.*, 2009]. Studies have demonstrated a correlation between thrombophilic risk factors (for example, low antithrombin (AT) levels) and patients with a milder disease severity [Shetty *et al.*, 2007]. Factors

impacting on the fibrinolytic pathway and platelet function may also correlate [Pavlova *et al.*, 2013].

Regulation of the extrinsic pathway has recently started to garner interest in this context. It has previously been assessed in the context of thrombotic disorders. However, the concept of 'rebalancing' haemostasis derived from greater understanding of the changes that occur in liver disease has been pursued with the hope of potential therapeutic benefit in patients with bleeding disorders. The natural anticoagulants – AT, tissue factor pathway inhibitor (TFPI) and protein C (PC) - have thus become of interest in the latter.

5.2 The story of TFPI

TFPI is key in the initial regulation of the TF.FVIIa complex and resultant FX activation. Deficiency or absence of TFPI has not been described as a clinical phenomenon. However, recent discoveries have pointed to the clinical implications of increased or decreased TFPI levels. This is in addition to the knowledge that there is an interaction between heparin and TFPI, resulting in release of the latter from the endothelium.

East Texas bleeding disorder has been described in the last few years in a cohort with moderate bleeding symptoms [Wood *et al.*, 2014]. A mutation in the FV gene has been found to lead to an alternative splicing event and removal of the majority of the FV B domain. This results in a 'FV-short' isoform. However, this still has the acidic region of the B domain and this binds tightly to the basic region of TFPIα. The result is a 10-20 fold increase in circulating TFPIα levels and subsequent bleeding symptoms [Wood *et al.*, 2014].

The TFPI gene is located on chromosome 2 and comprises approximately 90 kb [Broze *et al.*, 2012]. It is an alternatively spliced protein of which there are 4 isoforms – 2 major and 2 minor [Mast, 2016]. The 2 principal isoforms are TFPIα and TFPIβ. TFPIα (also known as full-length or free TFPI) contains an acidic N-terminal region, 3 tandem Kunitz domains and a basic C-terminal region (see Figure 5-1). Each Kunitz domain plays a role in the different functional properties of the inhibitor: K1 binds to the active site of FVIIa, K2 to the active site of FXa and K3 to Protein S. The TFPIβ isoform comprises the K1 and K2 Kunitz domains attached to a glycosyl phosphatidyl inositol-anchored (GPI) C-terminus (see Figure 5-2).

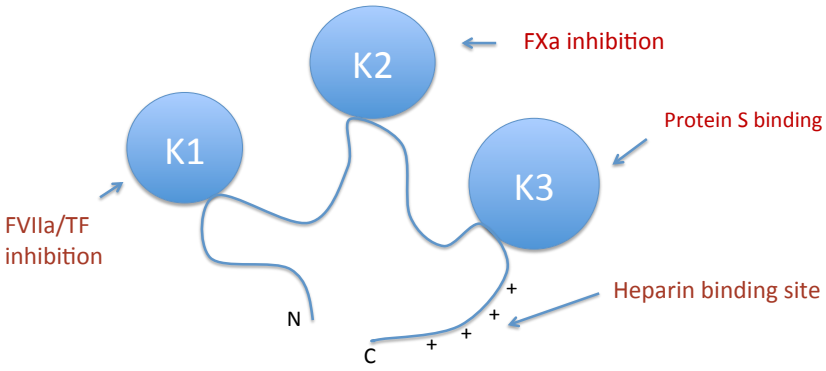


Figure 5-1 TFPIα isoform

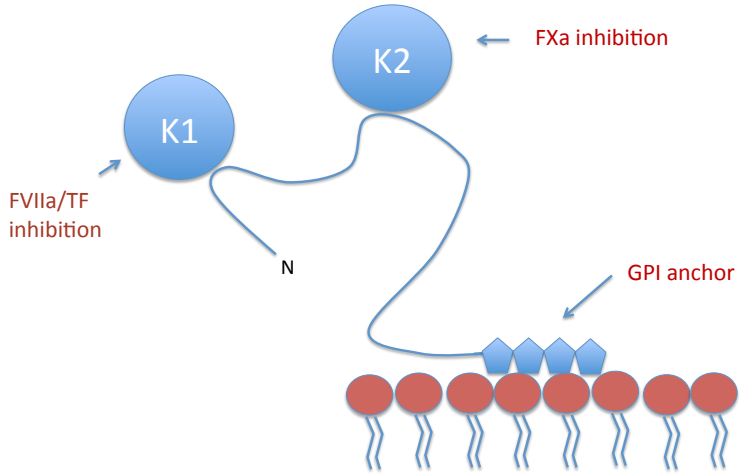


Figure 5-2 TFPIβ isoform

Approximately 80% of the plasma pool of TFPI is bound to lipoproteins (predominantly low density lipoprotein (LDL)) and the physiological significance of this component is unclear. The remaining 20% of the plasma pool is composed of TFPIα and C-terminally truncated versions of TFPI. Platelets contain 8-10% of TFPIα that is released upon activation. TFPIβ is bound to cell surfaces including the vascular endothelium via the GPI anchor [Ellery *et al.*, 2014, Wood *et al.*, 2014].

TFPI is believed to bind to the TF.FVIIa.FXa ternary complex via K1 and K2 with the K2 binding to FXa being the rate-limiting step. The 2 major isoforms α and β are expressed in different cell types and optimised for different functions. TFPIα is dependent on its co-factor Protein S to bind to cell surfaces and inhibit FXa whereas TFPIβ with its GPI anchor is able to inhibit TF.FVIIa and FXa on the endothelium. TFPIα localised in platelets is able to inhibit the prothrombinase complex in the initial stages of thrombus formation (but not the

propagation phase) [Augustsson *et al.*, 2014, Wood *et al.*, 2014]. It does this by the binding of the K2 domain to FXa and the basic C-terminus to the acidic B domain found in FXa-activated FVa and platelet-derived FVa. This acidic region is not found in thrombin-activated FVa [Wood *et al.*, 2014].

These structural and functional characteristics mean that TFPI is the principal inhibitor of the initiation of coagulation via the extrinsic TF.FVIIa pathway. It also plays a role in modulating the severity of bleeding and thrombotic disorders [Wood *et al.*, 2014]. It is constitutively expressed by microvascular endothelial cells and vascular smooth muscle cells but is also expressed in megakaryocytes and platelets, T cells, monocytes and macrophages [Winckers *et al.*, 2013]. TFPI expression in endothelial cells can be upregulated by heparin and thrombin amongst others and the administration of heparin results in the release of glycosaminoglycan-associated TFPIα [Winckers *et al.*, 2013].

More recently, preliminary work has demonstrated a correlation between TFPI levels and disease severity in haemophilia A and B [Shetty *et al.*, 2007, Tardy-Poncet *et al.*, 2011]. This was prompted by an observation of lower bleeding rates in patients with haemophilia B compared to haemophilia A. One study was a small observational study assessing free TFPI levels by ELISA in 30 patients with haemophilia A and 21 patients with haemophilia B (with a mix of mild, moderate and severe in each cohort). A lower level of free TFPI was found in patients with haemophilia B than haemophilia A (regardless of severity) [Tardy-Poncet *et al.*, 2011]. It was also found that patients had fewer bleeding events and lower factor concentrate use with lower levels [Shetty *et al.*, 2007]. Low TFPI levels have been found to be associated with a shorter lag time in

thrombin generation [Sandset, 1996]. Also, inhibition of TFPI has been studied as a potential therapeutic tool in haemophilia [Knappe *et al.*, 2013, Waters *et al.*, 2013] and animal studies have demonstrated a decreased bleeding tendency after infusion of a monoclonal antibody. A recently published phase 1 study shows variation in response to the anti-TFPI antibody in humans [Chowdary *et al.*, 2015]. There are on-going clinical trials investigating the safety, pharmacokinetics and efficacy of monoclonal antibodies targeting TFPI with the final data yet to be published.

5.3 The importance of individualised prophylaxis

The plasma levels of FVIII/FIX are the principal determinants of bleeding in people with haemophilia and, when factor levels are less than 1% (1 IU/dL) bleeding is characteristically spontaneous, recurrent and all tissues and organs are susceptible [Mannucci *et al.*, 2001, White *et al.*, 2001]. Before the introduction of replacement therapy, bleeding was the predominant cause of death and most patients with severe haemophilia did not survive beyond the second decade [Lee, 2014]. Whilst replacement therapy has resulted in decreased mortality from bleeding, recurrent bleeding into joints and muscles continues to be seen and joint arthropathy, muscle damage and disability are the most frequent sequelæ of the disease. The introduction of prophylaxis in the late 1950s, characterised by self-infusion of FVIII or FIX two to four times a week, has brought about a paradigm shift in the management of severe haemophilia. Most patients in developed countries can now hope to have near normal life expectancy with variable disability, although there continues to be

slight excess mortality in patients with severe haemophilia A [Reitter *et al.*, 2009]. A major aim of prophylaxis in the management of haemophilia (aside from the prevention of fatal bleeding) is the prevention of joint damage and the arrest or slowing of progression of existing joint damage.

Multiple studies have unequivocally demonstrated the superiority of prophylaxis as compared to on-demand or episodic therapy [Manco-Johnson *et al.*, 2007, Valentino, 2014] and this is now the gold standard. However, debate continues amongst clinicians over when prophylaxis should start, what dose should be used and how frequently it should be administered. It is increasingly recognised that a uniform approach can lead to undertreatment of some individuals and overtreatment of others. Most current prophylactic regimens are weight based. Efficacy and adequacy are monitored by trough levels in addition to self-reported bleeds. A greater understanding of an individual's propensity to bleed would help clinicians plan a more appropriate prophylactic regimen.

5.4 Rationale of the study

There is a need to be able to gather information about variables impacting on bleeding phenotype in severe haemophilia in order to be able to optimise care. TFPI is an important inhibitor of coagulation and is being pursued as a target for novel therapies in haemophilia (with and without inhibitors). Given the preliminary data showing significantly lower levels of TFPI in patients with haemophilia B versus haemophilia A and the correlation with bleeding phenotype, in addition to the finding of substantially faster and enhanced clotting in blood from healthy individuals with low levels of free and total TFPI as

assessed by TGA and ROTEM [Knappe *et al.*, 2013], the author reasoned that further data relating to the initiation of the extrinsic pathway collected in a cross-sectional study could potentially shed further light on phenotypic heterogeneity.

5.5 Study Aim

This study aims to evaluate the levels of the components contributing to the initiation of the extrinsic pathway - including free and total TFPI, FVII, FVIIa, TF and FVIIa.AT complex - in a cohort of patients with moderate and severe haemophilia A and B in the absence of bleeding.

5.5.1 Objectives

5.5.1.1 Primary objective

The main objective of the study is to ascertain the levels of components of the initiation of the extrinsic pathway both in patients with moderate/severe haemophilia A and B (in the absence of bleeding) and in normal volunteers.

5.5.1.2 Secondary objective

To perform statistical analysis to:

1) Determine normal reference ranges
2) Determine any correlation between results of the different components
3) Determine any significant differences in levels between type of haemophilia or bleeding phenotype compared to the normal population

4) To perform multinomial logistic regression analysis in order to determine whether any of the variables are predictive of bleeding phenotype.

5.5.2 Study design

This is a laboratory-based cross-sectional study using commercially available assays to characterise the levels of constituent components of the initiation phase of the extrinsic pathway.

5.5.2.1 Primary end-point

Levels of the following haemostatic components:

- TF (ACTICHROME® TF activity chromogenic assay)
- FVII (PT-based assay)
- FVIIa (STACLOT® FVIIa-rTF clotting assay)
- TFPI (free and total) (ASSERACHROM® ELISA)
- FVIIa.AT complex (ASSERACHROM® ELISA)

5.5.2.2 Secondary end-points

- Descriptive statistics to determine normal reference ranges
- Tests of association to determine whether there is any correlation between the different independent variables
- Tests to determine any statistically significant differences between the disease types or bleeding phenotypes

- Multinomial linear regression to determine whether any variable is predictive of bleeding phenotype

5.6 Study group

5.6.1 Patient selection

Patients with a diagnosis of congenital haemophilia registered at the Katharine Dormandy Haemophilia and Thrombosis Centre were approached to participate in the study. Ethical approval and informed consent was obtained via the Katharine Dormandy Coagulation Research Plasma Bank ((KDCRPB; REC reference number 14/YH/1272).

Patient inclusion

- Moderate/severe haemophilia A or B
- Aged 18 or over
- Ability to give informed consent

Patient exclusion

- Patients with inhibitors
- Patients experiencing a bleeding episode

5.6.2 Assessment of bleeding phenotype

Bleeding phenotype was independently assessed by 4 clinicians (not including the author), each with greater than 10 years experience treating patients with haemophilia. A consensus was taken as to whether patients bled less than expected, as expected or more than expected whilst on a standard prophylactic regimen (excluding factors such as poor adherence – see Table 5-1).

Table 5-1 Bleeding phenotype scoring system

1 = Less than expected	A less severe bleeding phenotype, i.e. on on-demand treatment with fewer than 10 joint bleeds/year
2 = As expected	As expected for a patient with SHA/B on appropriate prophylaxis
3 = More than expected	A severe bleeding phenotype i.e. a patient known to develop bleeds with missed doses, continuing to have breakthrough bleeds despite adequate prophylaxis in the absence of chronic synovitis and patients with joint damage despite adequate prophylaxis

5.7 Materials and methods

5.7.1 Patient samples

Blood samples were taken as per the departmental protocol (see Chapter 3: Supplementary Material 3.7) into S-Monovette® tubes (Sarstedt; 3.2% 0.109 M trisodium citrate). Samples were processed within 2 hours of collection. PPP was obtained by double-spinning at 2000 g for 15 minutes and then aliquots were stored at -80^{0}C.

5.7.2 Laboratory methodology

5.7.2.1 Tissue factor

TF levels were measured using a chromogenic ACTICHROME® TF activity assay (Sekisui Diagnostics, Invitech, UK). All samples were run in duplicate. A TF standard was used for plasma samples with relipidated human TF added to 5% TFPI depleted human plasma. TF standards (further diluted in 5% TFPI depleted plasma) and test samples (neat) were added to the wells of a 96-well clear-bottom microtitre plate (Corning Costar, Sigma-Aldrich) along with buffer, human FVIIa and human FX. After a 15 minute incubation at 37^{0}C, SPECTROZYME® FXa substrate was added followed by a further 30 minute incubation at 37^{0}C. The reaction was stopped with the addition of glacial acetic acid and the plate read at 405 nm and 490 nm on a SpectraMax i3 multi-mode microplate platform (Molecular Devices, UK). The $\Delta A_{405\text{-}490}$ of the sample was used to interpolate the TF concentration. The TF standard dilutions were used to create a standard curve on Excel 2011 (Microsoft) plotted using a 2nd order

polynomial regression. The test sample concentrations were calculated using the equation generated. Local normal ranges were generated with approximately 20 healthy volunteer samples (collected during the TGA normal reference range study – see Chapter 3).

5.7.2.2 Factor VII

The FVII assay was measured by one-stage PT assay on an ACL TOP coagulometer (IL, USA) using PT Recombiplastin 2G reagent (IL, USA) and FVII deficient plasma (Diagnostic Reagents, UK). The normal range was determined by the hospital's diagnostic laboratory.

5.7.2.3 Factor VIIa

FVIIa was measured using a STACLOT® FVIIa-rTF clotting assay run on a KC4 Delta™ semi-automated coagulation analyser (Amelung, UK). The kits contained both a calibrator and quality control. These and the test samples were diluted in buffer (either 1:10 or a range of dilutions for the calibrator). They were then added to cuvettes with FVII depleted human plasma (obtained by selective immuno-adsorption) and recombinant soluble TF and phospholipids and then mixed. After a 180 second incubation at 37^0C, 0.025 M $CaCl_2$ was added and the clotting time measured by the analyser. The calibrator dilutions were used to create a standard curve on Excel 2011 (Microsoft) using log scales and a linear trendline. Providing the control results fell within the accepted range supplied for the kit, the test sample concentrations were calculated using the equation generated. Local normal ranges were generated

with 20 healthy volunteer samples (collected during the TGA normal reference range study - see Chapter 3).

5.7.2.4 Factor VIIa.Antithrombin complex

FVIIa.AT complexes were measured by ELISA (ASSERACHROM®, Diagnostica Stago, UK). All samples were run in duplicate. A calibrator and control were supplied (lyophilised preparations containing known quantities of human FVIIa.AT complex). Plastic microwells were ready-coated with mouse monoclonal anti-human FVIIa $F(ab')_2$ fragments. Dilutions of the calibrator and control in addition to test samples (diluted 1:21 in reagent) were added and the plate incubated at room temperature for 2 hours. The detection antibody - a mouse monoclonal anti-human AT antibody coupled with peroxidase - was subsequently added after the wells had been washed and then the plate was incubated for a further 2 hours. After washing of the wells tetramethylbenzidine (TMB) reagent was added, left for 5 minutes and the reaction was stopped with the addition of sulphuric acid (H_2SO_4). After 15 minutes, the absorbance was read at 450 nm on a SpectraMax i3 multi-mode microplate platform (Molecular Devices, UK). The calibrator dilutions were used to create a standard curve on Excel 2011 (Microsoft) using log scales and a linear trendline. Providing the control results fell within the accepted range supplied for the kit, the test sample concentrations were calculated using the equation generated. Local normal ranges were generated with 20 healthy volunteer samples (collected during the TGA normal reference range study - see Chapter 3).

5.7.2.5 Tissue Factor Pathway Inhibitor - free and total

Free and total TFPI levels were measured by ELISA (ASSERACHROM®, Diagnostica Stago, UK). These assays do not differentiate between the two isoforms, instead they detect either the ~20% of TFPI not bound to lipoproteins (free) or the entire TFPI pool (total). All samples were run in duplicate. The methods were identical apart from the detection antibodies and the dilution of the samples (1:10 in buffer for free TFPI; 1:20 for total TFPI). The kits were supplied with appropriate calibrator (human plasma containing TFPI) and control (buffer containing TFPI) with lot-specific antigen levels pre-determined. Plastic microwells were supplied coated with $F(ab')_2$ fragments from mouse anti-TFPI monoclonal antibodies. A mouse monoclonal antibody-peroxidase conjugate (either anti-free TFPI or anti-TFPI) was subsequently added in addition to the diluted calibrator, controls and test samples. This was incubated at room temperature for 2 hours and then washed. An ortho-phenylenediamine (OPD)/urea peroxide substrate was then added and incubated for 8 minutes prior to stopping the reaction with H_2SO_4. The plates were then kept at room temperature for 30 minutes before reading at 492nm on a SpectraMax i3 multi-mode microplate platform (Molecular Devices, UK). The calibrator dilutions were used to create a standard curve on Excel 2011 (Microsoft) using log scales and a linear trendline. Providing the control results fell within the accepted range supplied for the kit, the test sample concentrations were calculated using the equation generated. Local normal ranges were generated with 20 healthy volunteer samples (collected during the TGA normal reference range study - see Chapter 3).

5.7.3 Statistical design

Statistical tests were performed using Excel 2011 (Microsoft) and SPSS 22.0.0.0 (SPSS, Chicago, IL, USA). The normal reference ranges were obtained by calculating the mean ± 2 SD in order to encompass 95% of results. Patient results were expressed as the median (10^{th}, 90^{th} percentiles).

Patient data were assessed for normality and outliers in order to determine whether the use of non-parametric tests was required. Appropriate tests were selected to assess for correlation between variables and then to compare independent groups (disease type or bleeding phenotype) for any statistically significant differences between groups.

In order to determine whether any of the components could be used as a predictor of bleeding phenotype, multinomial logistic regression was selected as this method can be used to predict the nominal dependent variable (i.e. bleeding phenotype: less severe, as expected, more severe) given one or more independent variables (i.e. the components assessed in the study).

5.8 Results

5.8.1 Patients

64 patients were recruited to the study (see Table 5-2). 2 patients had combined deficiencies – 1 with SHA and mild VWD and 1 with moderate haemophilia A and FXI deficiency (31 IU/dL). The median age was 34 years (range 18 – 86 years).

Table 5-2 Patients recruited to study (total 64)

Disease severity	Haemophilia A	Haemophilia B
Severe	45	11
Moderate	6	2

5.8.2 Bleeding phenotype

The bleeding phenotype of each patient was assessed independently (by 3 consultant haematologists and 1 clinical nurse specialist) using the scoring system outlined in Table 5-1. The researcher determined a consensus (majority) score. There was a fairly similar spread of patients within the categories for haemophilia A and B (see Figure 5-3). There was a higher percentage of patients within both groups with a bleeding phenotype less severe than expected (~ 25% = 1; the literature suggest 10-15% of patients with SHA have a more moderate phenotype) and this most likely reflects a degree of selection bias as these were patients of interest.

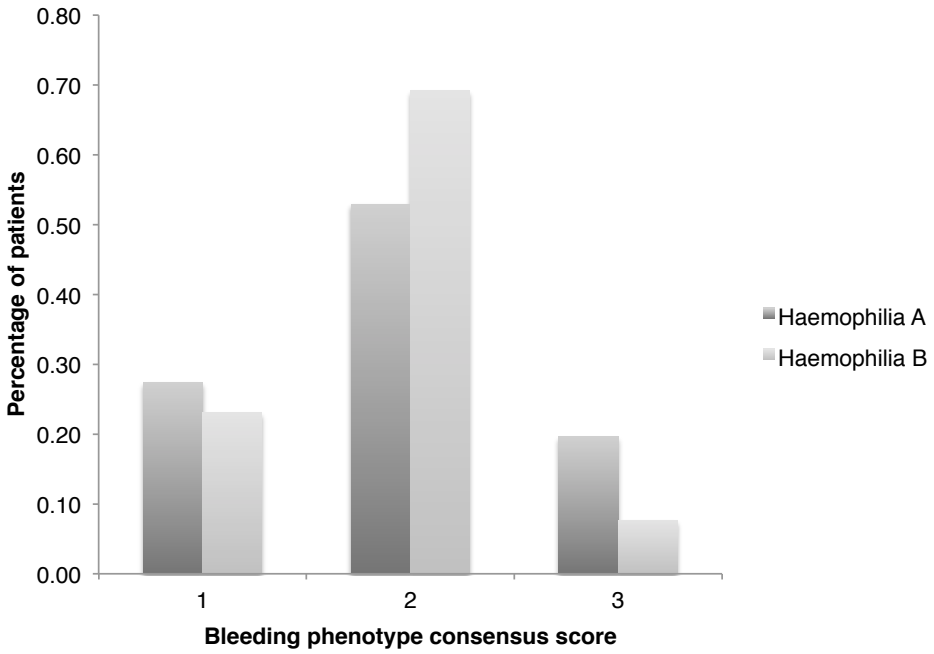


Figure 5-3 Bleeding phenotype consensus score (see Table 5-1 for scoring system) 1 = less than expected, 2 = as expected, 3 = more than expected

5.8.3 Normal ranges

See Table 5-3 for the normal ranges generated for the assays.

Table 5-3 Normal ranges * normal range determined by diagnostic laboratory

Assay	**Normal range (± 2SD, mean)**
TF (pM)	-0.31(i.e. not detected) - 2.39 (1.04)
FVII (IU/dL)	45 - 180*
FVIIa (mIU/dL)	10.60 - 62.12 (36.36)
Free TFPI (ng/ml)	2.8 - 13.8 (8.3)
Total TFPI (ng/ml)	31.7 - 88.8 (60.2)
FVIIa.AT complex (pM)	41.7 - 243.0 (142.3)

5.8.4 Correlation of study assay results

Data were assessed for correlation between the different components (see Table 5-4). The data were not normally distributed as determined by significance in the Shapiro-Wilk test ($p < 0.005$). Thus, Spearman's correlation was performed. There was a weak but significant correlation between TF and FVII (correlation coefficient r_s 0.312). There were strong (significant) correlations between FVII – FVIIa (0.753) and FVII - FVIIa.AT complex (0.689). This was also true for FVIIa – FVIIa.AT complex (0.830). There was moderate (significant) correlation between free and total TFPI (0.528).

Table 5-4 Spearman's correlation to determine the strength and direction of association between variables

Correlations

			TF	Factor VII	Factor VIIa	Free TFPI	Total TFPI	FVIIa.AT complex
Spearman's rho	TF	Correlation Coefficient	1.000	.312*	.145	.037	-.012	.298*
		Sig. (2-tailed)	.	.013	.254	.770	.923	.017
		N	64	63	64	64	64	64
	Factor VII	Correlation Coefficient	.312*	1.000	.753**	-.042	-.025	.689**
		Sig. (2-tailed)	.013	.	.000	.743	.848	.000
		N	63	63	63	63	63	63
	Factor VIIa	Correlation Coefficient	.145	.753**	1.000	-.057	-.110	.830**
		Sig. (2-tailed)	.254	.000	.	.657	.388	.000
		N	64	63	64	64	64	64
	Free TFPI	Correlation Coefficient	.037	-.042	-.057	1.000	.528**	.010
		Sig. (2-tailed)	.770	.743	.657	.	.000	.940
		N	64	63	64	64	64	64
	Total TFPI	Correlation Coefficient	-.012	-.025	-.110	.528**	1.000	-.046
		Sig. (2-tailed)	.923	.848	.388	.000	.	.719
		N	64	63	64	64	64	64
	FVIIa.AT complex	Correlation Coefficient	.298*	.689**	.830**	.010	-.046	1.000
		Sig. (2-tailed)	.017	.000	.000	.940	.719	.
		N	64	63	64	64	64	64

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5-5 Ranges for the study population; 10th, 90th percentiles (median)

Assay	All patients	Haemophilia A	Haemophilia B
TF **(ND – 2.39 pM; mean 1.04)**	0.34 – 0.64 (0.45)	0.34 – 0.64 (0.45)	0.34 – 0.64 (0.45)
FVII **(45 – 180 IU/dL)**	55 – 119 (84.8)	66 – 120 (87.9)	42.4 – 96.5 (56.4)
FVIIa **(10.60 – 62.12 mIU/dL; mean (36.36)**	9.2 – 67.3 (34.1)	23.9 – 67.8 (41.5)	3.2 – 18.6 (7.5)
Free TFPI **(2.8 – 13.8 ng/ml; mean 8.3)**	7.6 – 21.3 (10.2)	7.5 – 18.3 (10.2)	8.1 – 22.2 (9.4)
Total TFPI **(31.7 – 88.8 ng/ml; mean 60.2)**	48.9 – 82.0 (62.7)	49.3 – 79.5 (63.0)	48.3 – 87.7 (61.3)
FVIIa.AT complex **(41.7 – 243.0 pM; mean 142.3)**	50.8 – 202.5 (122.7)	97.4 – 220.8 (132.4)	32.3 – 84.0 (47.9)

5.8.5 Comparison of variables by disease type

For the statistical analysis, given that the assumption of normality was not met and that there were outliers that were felt to represent genuine results, the Mann Whitney U test was used to compare the results between haemophilia A and B for the study assays (see Table 5-6).

Table 5-6 Mann Whitney U tests for significance

Mann Whitney U independent samples test – Haemophilia A v Haemophilia B	
Assay	**p value**
TF	0.448
FVII	**0.001**
FVIIa	**< 0.0005**
Free TFPI	0.809
Total TFPI	0.714
FVIIa.AT complex	**< 0.0005**

5.8.5.1 Tissue factor

Tissue factor levels were measured in healthy volunteers and patients. The levels in the normal volunteers were significantly higher than the patient results ($p < 0.0005$) (see Figure 5-4 and Table 5-5).

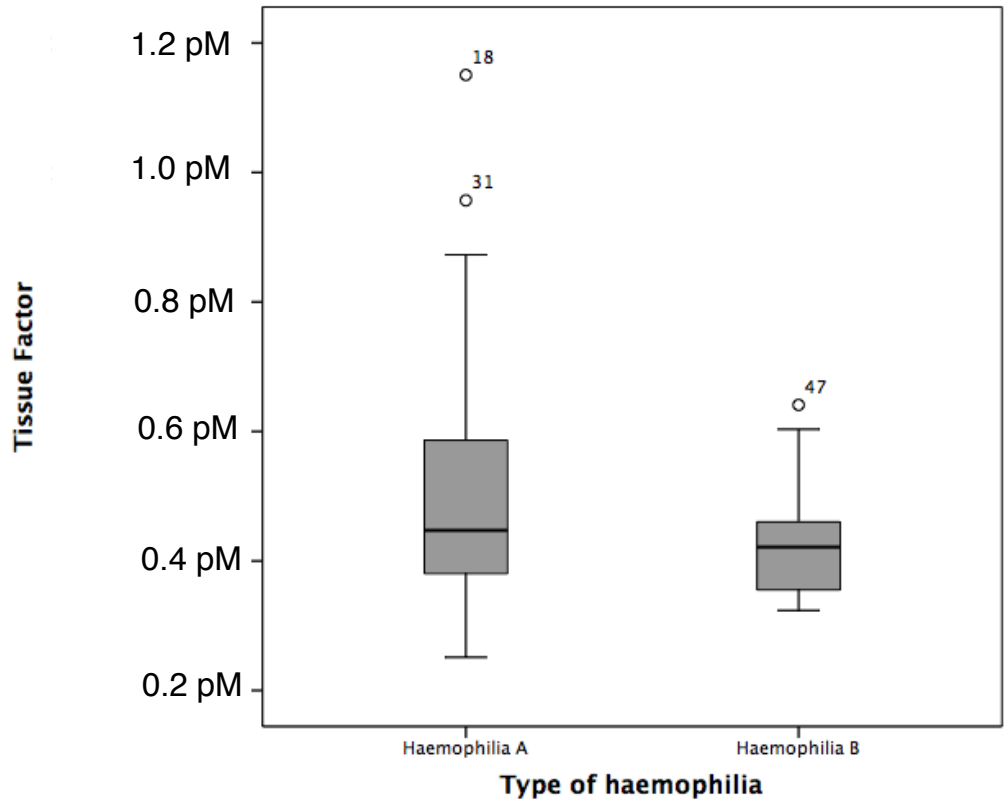


Figure 5-4 Tissue factor boxplot for haemophilia A and B (pM; min, 25th percentile (Q1), median, 75th percentile (Q3), max)

5.8.5.2 Factor VII and VIIa

The FVII levels were significantly lower – although mainly still within the normal range - in the patients with haemophilia B (severe and moderate) than haemophilia A ($p = 0.001$) (see Table 5-5 and Figure 5-5). One patient with severe haemophilia B was diagnosed incidentally with FVII deficiency (level 22 IU/dL, FII and FX levels within the normal range).

The FVIIa levels were also significantly lower in the patients with haemophilia B than A ($p < 0.0005$) with 7/13 (54%) below the lower limit for the normal range

(see Table 5-5 and Figure 5-6). The difference between the patients and the normal range was not of statistical significance.

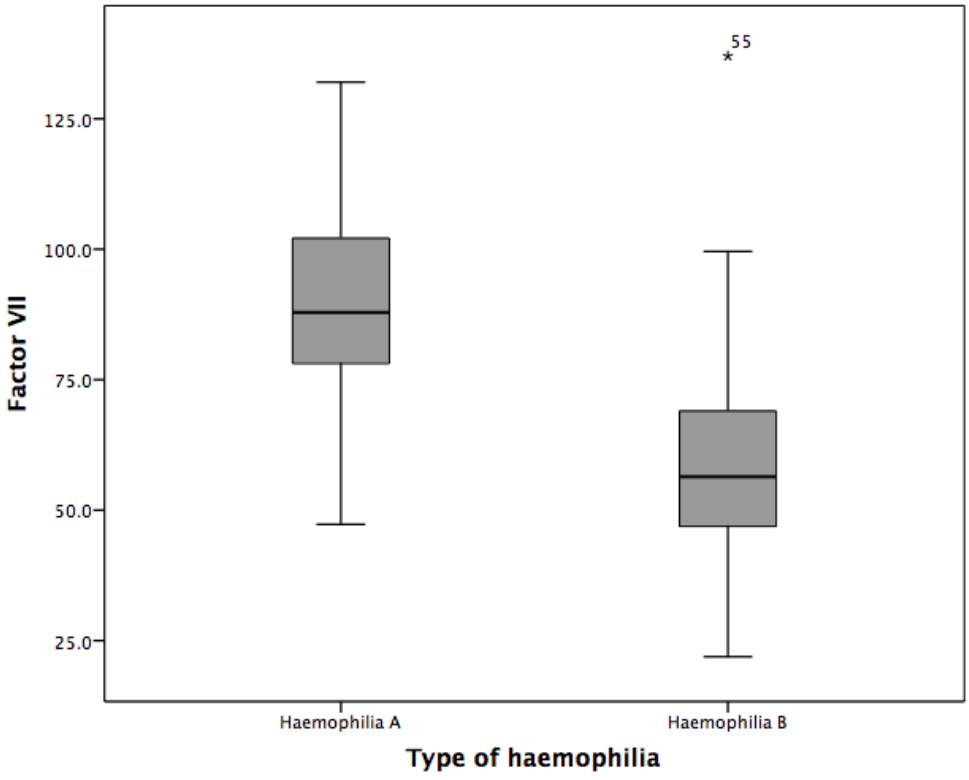


Figure 5-5 Factor VII boxplot for haemophilia A and B (IU/dL; min, 25th percentile (Q1), median, 75th percentile (Q3), max)

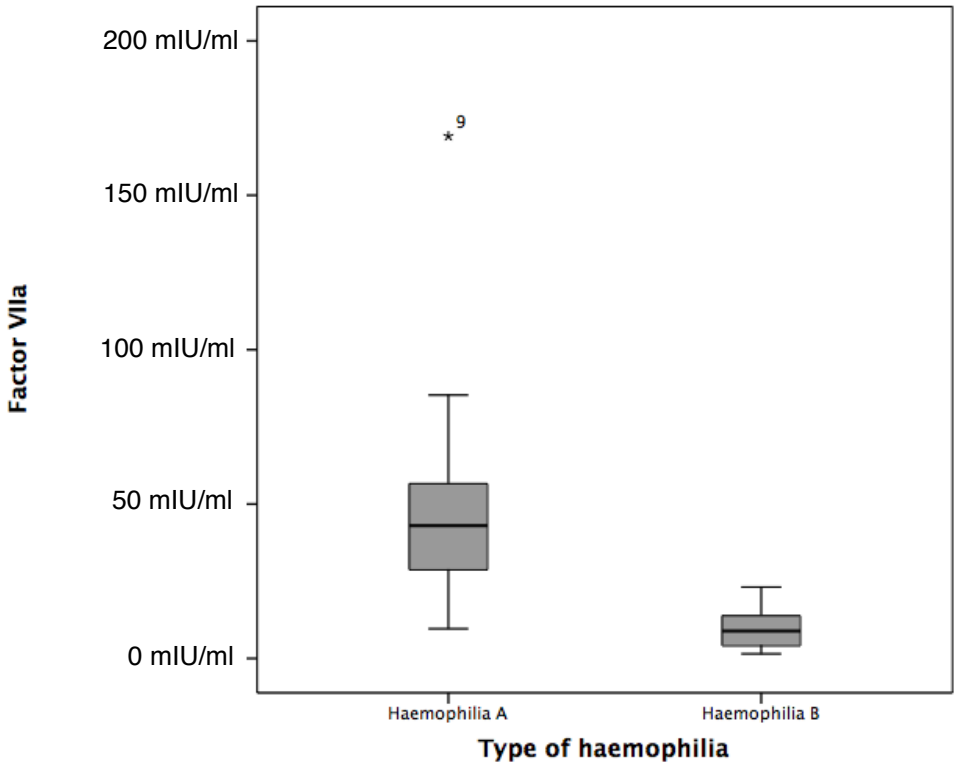


Figure 5-6 Factor VIIa boxplot for haemophilia A and B (mIU/dL; min, 25th percentile (Q1), median, 75th percentile (Q3), max)

5.8.5.3 Free and total TFPI

Free TFPI levels were similar in both haemophilia A and B and, interestingly, many levels were higher than the upper limit of the normal range (this was statistically significant, p = 0.001) (see Table 5-5 and Figures 5-7, 5-8).

Total TFPI levels were again similar in both groups and largely within the normal range, although there were a couple of outliers with higher levels.

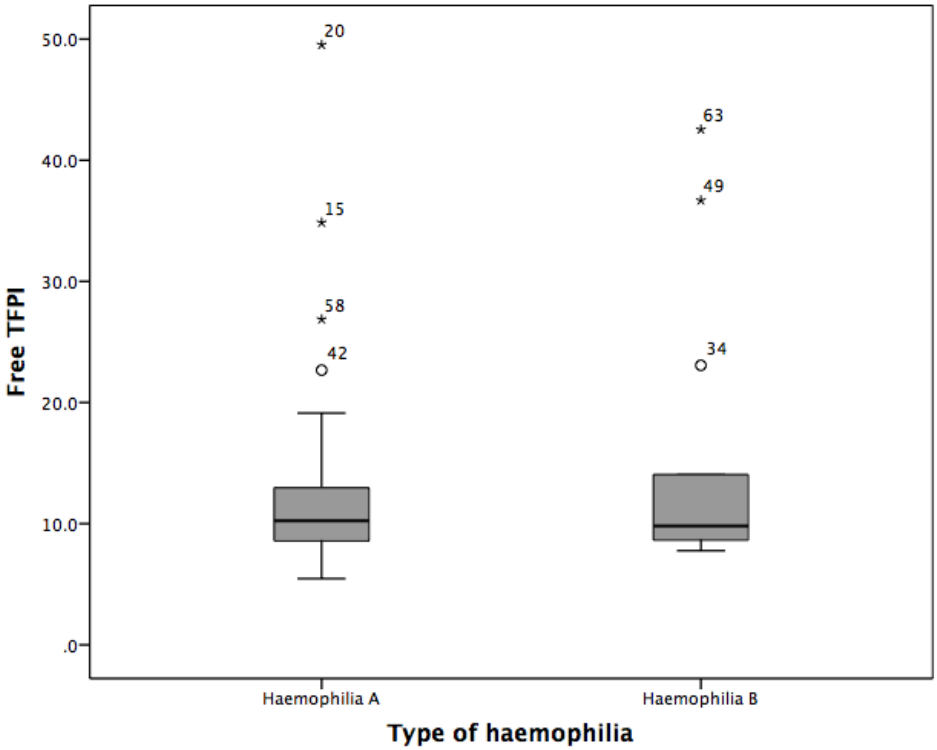


Figure 5-7 Free TFPI boxplot for haemophilia A and B (ng/ml; min, 25th percentile (Q1), median, 75th percentile (Q3), max)

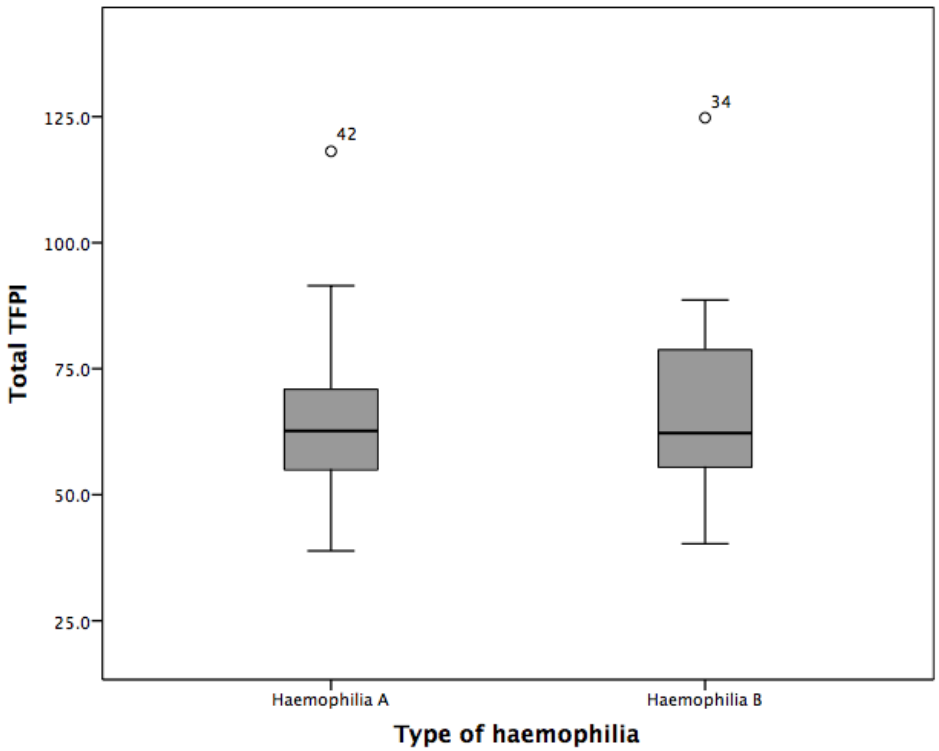


Figure 5-8 Total TFPI boxplot for haemophilia A and B (ng/ml; min, 25th percentile (Q1), median, 75th percentile (Q3), max)

5.8.5.4 FVIIa.AT complex

Overall the results for both diseases combined and the patients with haemophilia A fell within the normal range. However, the results for patients with haemophilia B were significantly lower ($p < 0.0005$) with 5/13 (38%) patients with results below the lower limit of the normal range (see Table 5-5 and Figure 5-9).

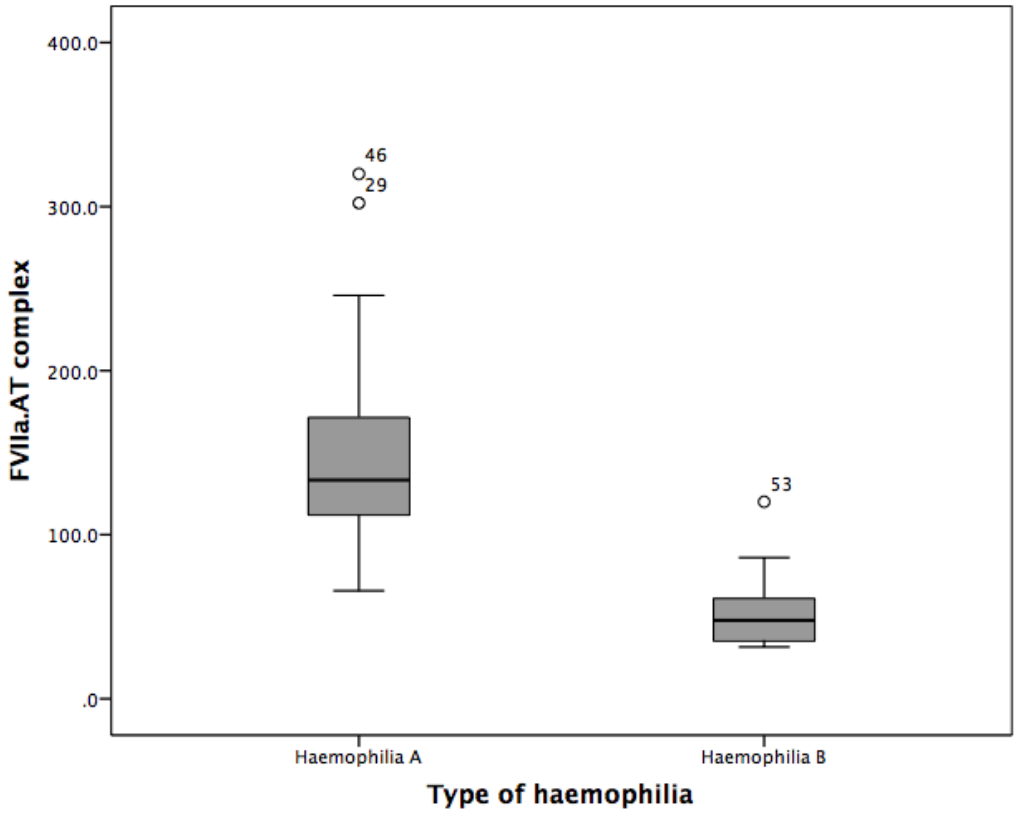


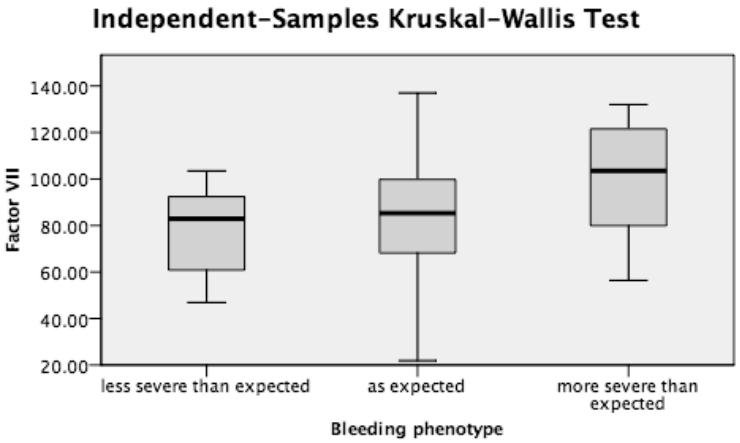
Figure 5-9 FVIIa.AT complex boxplot for haemophilia A and B (pM; min, 25th percentile (Q1), median, 75th percentile (Q3), max)

5.8.6 Comparison of variables by bleeding phenotype

In order to compare the levels of the components between the different bleeding phenotypes, the non-parametric Kruskal Wallis test was performed. This was done for the whole patient cohort (i.e. haemophilia A and B combined). Although none of the parameters reached statistical significance (see Table 5-7), the box plots for FVII and free TFPI are included below (Figures 5-10, 5-11). There is a suggestion that the higher the FVII level, the worse the bleeding phenotype. Free TFPI is the variable that is closest to reaching statistical significance and there does appear to be a higher median level with no outliers in the 'more severe than expected' group (Figure 5-11).

Table 5-7 Kruskal Walis results analysing the levels of components in cohorts of different bleeding phenotypes; p value significant if < 0.05

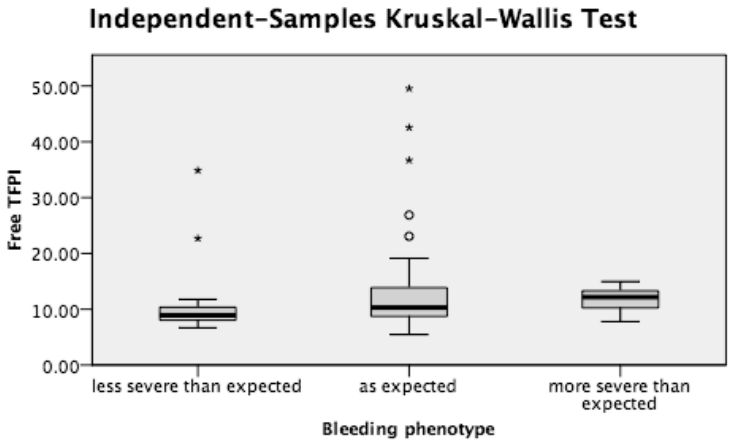
Assay	**Independent samples Kruskal Wallis test p value**
TF	0.284
FVII	0.126
FVIIa	0.685
Free TFPI	0.066
Total TFPI	0.280
FVIIa.AT complex	0.610



Total N	63
Test Statistic	4.143
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.126

1. The test statistic is adjusted for ties.
2. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

Figure 5-10 Kruskal Wallis test assessing differences in FVII levels between different bleeding phenotypes



Total N	64
Test Statistic	5.427
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.066

1. The test statistic is adjusted for ties.
2. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

Figure 5-11 Kruskal Wallis test assessing differences in free TFPI levels between different bleeding phenotypes

5.8.7 Predictors of bleeding phenotype - multinomial logistic regression

Sample size is of significant importance when considering the predictive ability of a multinomial regression model. In this study, numbers were limited by practical issues such as the rarity of the disorders of interest as well as the limitations of time and patient availability. It was important to bear this issue in mind. There are various rules of thumb when it comes to calculating sample size in this setting – none of which are perfect or set in stone. 2 different

approaches suggest either setting the number (N) of participants as a function of the predictor (e.g. 15 cases per predictor (as suggested by Stevens, 1996)) or as a function of the study (i.e. that N should exceed the number of predictors by 50 (as suggested by Harris, 1985)). Another suggestion is that N = participants ≥ 50 + 8*m (where m = independent variables) [Tabachnick *et al.*, 2014]. Other assumptions that needed to be considered include the absence of multicollinearity (when two or more independent variables are highly correlated), the presence of a linear relationship between the continuous independent variables and the logit transformation of the dependent variable and the absence of outliers or high leverage points [Lund *et al.*, 2013].

64 patients were recruited to the study. With a minimum requirement of 15 participants for each independent variable in order to perform a regression analysis, it was decided that, in theory, 4 variables could be included. Various combinations of the 6 independent variables were examined. As the TF, free and total TFPI variables were consistently non-significant ($p > 0.5$) and thus non-predictive, multinomial regression was performed on the covariates FVII, FVIIa and FVIIa.AT complex. However, given the significantly lower levels of all three in patients with haemophilia B, the analysis was performed only on patients with moderate/severe haemophilia A (n = 51).

There was no multicollinearity as assessed by Tolerance values > 0.1. Linearity of the continuous variables with respect to the logit of the dependent variable was assessed via the Box-Tidwell (1962) procedure. A Bonferroni correction was applied using all three terms in the model resulting in statistical significance

being accepted when $p < 0.0167$ [Tabachnick *et al.*, 2014]. Based on this assessment, all continuous independent variables were found to be linearly related to the logit of the dependent variable. Cook's distance was calculated for the three variables and one case was found to be an outlier and appeared to be exerting an undue influence on the regression line (outlier > 4/n i.e. 4/50 – 0.08 (number of cases – 1 with missing data)). This outlier was therefore deleted for the purposes of the analysis.

The final model predicted the bleeding phenotype over and above the intercept-only model with statistical significance, $\chi^2(6) = 21.716$, $p = 0.01$ (see Table 5-8). The Pearson and Deviance results were also non-significant, indicating a good fit of the model (see also Table 5-8). The results of the likelihood ratio tests demonstrated that FVII and FVIIa were statistically significant (see Table 5-9).

Table 5-8 Regression model fit

Model Fitting Information

Model	Model Fitting Criteria	Likelihood Ratio Tests		
	-2 Log Likelihood	Chi-Square	df	Sig.
Intercept Only	99.237			
Final	77.521	21.716	6	.001

Goodness-of-Fit

	Chi-Square	df	Sig.
Pearson	80.051	90	.764
Deviance	77.521	90	.823

Table 5-9 Statistical significance of independent variables

Likelihood Ratio Tests

Effect	Model Fitting Criteria	Likelihood Ratio Tests		
	-2 Log Likelihood of Reduced Model	Chi-Square	df	Sig.
Intercept	93.478	15.957	2	.000
FactorVII	99.052	21.530	2	.000
FactorVIIa	88.491	10.969	2	.004
FVIIa.ATcomplex	78.773	1.252	2	.535

The chi-square statistic is the difference in -2 log-likelihoods between the final model and a reduced model. The reduced model is formed by omitting an effect from the final model. The null hypothesis is that all parameters of that effect are 0.

Parameter estimates were given for the bleeding phenotype categories 1 (less severe than expected) and 2 (as expected) with reference to category 3 (more severe than expected) (see Table 5-10). In category 1, FVII and FVIIa were statistically significant. In category 2, FVII was statistically significant. In the case of FVII, the B value was – 0.240 (p = 0.001) and – 0.121 (p = 0.020) for category 1 and 2 respectively. This meant that lower FVII levels were predictive of an 'as expected' or 'less severe' bleeding phenotype. The B value for FVIIa in category 1 was 0.193 (p = 0.006). This meant that higher FVIIa levels were predictive of an 'as expected' or 'less severe' bleeding phenotype. This analysis however does not suggest any causative relationship.

Table 5-10 Parameter estimates

Parameter Estimates

HA bleeding phenotype[a]		B	Std. Error	Wald	df	Sig.	Exp(B)	95% Confidence Interval for Exp(B)	
								Lower Bound	Upper Bound
less severe than expected	Intercept	11.728	3.808	9.487	1	.002			
	FactorVII	-.240	.072	11.166	1	.001	.787	.683	.905
	FactorVIIa	.193	.070	7.554	1	.006	1.213	1.057	1.393
	FVIIa.ATcomplex	.012	.017	.465	1	.495	1.012	.978	1.047
as expected	Intercept	6.733	2.752	5.986	1	.014			
	FactorVII	-.121	.052	5.454	1	.020	.886	.800	.981
	FactorVIIa	.083	.045	3.451	1	.063	1.087	.995	1.186
	FVIIa.ATcomplex	.015	.013	1.194	1	.274	1.015	.989	1.041

a. The reference category is: more severe than expected.

5.8.8 Potential confounders – thrombophilia results

Thrombophilia screens were performed for the majority of patients (all or part of the screen incomplete for 11/64 patients – mainly relating to insufficient blood sample and lack of opportunity to re-test). 11/53 (21%) of patients had abnormal results.

- 2 patients had a positive lupus anticoagulant
- 3 patients had an isolated low AT, Protein C or Protein S result
- 1 patient had combined low AT and Protein S results
- 2 patients were heterozygous for the FV Leiden mutation; 1 patient was homozygous
- 2 patients were heterozygous for the PGM mutation (G20210A)

When the patients were examined by bleeding phenotype, only the patients with a low Protein S result (54 IU/dL; NR 60-140) and low AT results (67 IU/dL; NR 79-121) had a bleeding phenotype score of 3.

5.8.9 Potential confounder - Von Willebrand Factor

Results were available for 58/64 patients. A one-way ANOVA was performed to see if there was any difference between the bleeding phenotype categories and no statistically significant difference was detected. No results were below the lower limit of the normal range. It would appear that, although VWF antigen has a logical impact on FVIII $t_{1/2}$, from the analysis of this dataset it does not correlate with bleeding phenotype at baseline.

5.9 Discussion

The aim of this study was to assess the levels of the components of the initiation of the extrinsic pathway in patients with moderate and severe haemophilia A and B in the absence of bleeding. A secondary objective was to determine whether there were any differences between patient cohorts or bleeding phenotypes and whether any variable demonstrated predictive value for bleeding phenotype. This was a cross-sectional laboratory study using both clotting and chromogenic assays as well as ELISAs measuring levels in plasma samples. There was a fairly even spread of bleeding phenotype in the patients with haemophilia A and B.

The results revealed the following:

- Tissue factor levels were significantly lower in patients than the healthy volunteers, but were not predictive of bleeding phenotype.
- In contrast to the previous small study by Tardy-Poncet et al. [Tardy-Poncet *et al.*, 2011], there were no significant differences found in free or total TFPI levels in haemophilia A or B patients. Interestingly, many patients had free TFPI levels above the upper limit of the normal range. When the data were assessed by multinomial logistic regression, the TFPI levels were not predictive of bleeding phenotype.
- The key findings of this study were the results relating to FVII, FVIIa and FVIIa.AT complex that both highlighted a difference between haemophilia A and B patients but that also – in regression analysis – identified a predictive role of FVII and FVIIa levels and bleeding

phenotype. All three components were significantly lower in haemophilia B than haemophilia A. The statistical analysis demonstrated that lower levels of FVII but higher levels of FVIIa in patients with haemophilia A (moderate and severe) were predictive of a less severe bleeding phenotype.

How can these data be interpreted? It is important to question whether these findings could relate to assay artefact, particularly the results in the patients with haemophilia B with low or undetectable levels of FIXa and the potential impact of this on feedback loops in functional assays (especially for the FVIIa assay). Functional testing of FVIIa with a one-stage clotting assay has been performed previously in healthy volunteers and patients with SHA/B. The study in question found that FVIIa levels are significantly lower in SHB than SHA (~10% of normal in the former, ~60% normal in the latter) and that both are lower than the levels in healthy volunteers (levels of about 1% of circulating FVII). There were no significant differences found in FVII antigen levels. Repetitive measurement of basal FVIIa levels showed that levels remained fairly constant over time [Wildgoose *et al*., 1992].

There are concerns that the lower levels of FVIIa found using clotting assays in patients with haemophilia B relate to lower levels of FIXa required for the activation of FVII. This has previously been addressed by Miller et al. [Miller *et al.*, 1985]. They added purified human FIX to achieve a level of I U/ml to plasma from patients with haemophilia A and B (albeit only 3 patients in total) and repeated the assay with no increase in the results seen. In the assay used in this study, patient's samples were diluted 1:10 in FVII depleted plasma. Levels

of FVIII and FIX were assayed in the deficient plasma in the kit and found to be present at equivalent levels and within the normal range (data not shown). Another issue with the assays is the limited sensitivity to low levels of the component of interest that may influence the reliability of results.

All samples were obtained from patients in the absence of overt bleeding and mainly at their trough (patients were attending principally for routine clinic review). It might be surmised that these samples were therefore a reflection of the function of coagulation under basal activation levels. The method for categorising bleeding phenotype was specifically chosen to be a clinical assessment rather than an assessment purely based on annual bleeding rate (ABR), annual concentrate usage or other single parameters. The latter would have made it difficult to assess the moderate patients, however the principal reason for choosing this approach was a desire to obtain a holistic view of bleeding phenotype that incorporated multiple factors (such as joint disease, job, physical activity levels, compliance with treatment) as assessed by experienced clinicians with established, long-term relationships with the patients.

The findings of the study were not as might have been anticipated. The regression analysis found that lower FVII levels but higher FVIIa levels in patients with moderate/severe haemophilia A were predictive of a less severe bleeding phenotype. In addition, there were significant differences in the levels of these components between haemophilia A and B. This suggests an

important role for FVII and FVIIa (and, to a degree, FVIIa.AT complex for which FVIIa is proposed to be a strong predictor [Martinelli *et al.*, 2016]). It could be that the results reflect differences in the basal activation of coagulation in these patients and potentially the degree of activation of FVII. It may be that there are compensatory mechanisms within the feedback loops of the coagulation process that vary within individuals and also within disease types i.e. haemophilia A versus B. Of note, the patients with haemophilia B had a similar spread of phenotype to haemophilia A, although there were fewer patients with a more severe phenotype (numbers however were small).

All patients had lower levels of circulating TF. Given this finding, it could be that slightly higher levels of FVIIa could be key in the initiation of coagulation. However, it is then difficult to account for the higher than normal levels of circulating free TFPI levels. One would expect this to have a greater impact on switching off the triggering of haemostasis. There are other variables that may have an impact such as weight and lipid profile on TFPI levels that were not accounted for.

The study is limited by sample size. There are practical reasons for this, but it would be important to recruit further patients and see if the findings can be replicated. The assessment of static levels of clotting components in the plasma compartment obviously is unable to assess the complexity of haemostatic function within the vascular and extravascular compartments.

With this in mind, the second part of the study was designed to investigate the variability in bleeding phenotype with a functional assay.

5.10 Supplementary material - laboratory methodology

The following assays were routinely performed by the diagnostic haemostasis laboratory at the Royal Free Hospital.

5.10.1 FVIII

Measurement of FVIII was by one stage APTT clotting assay using an ACL TOP 700 coagulometer (Instrumentation Laboratory, USA). Clotting times were measured and then compared to a six-point calibration curve of reference plasma (Cryocheck, Precision BioLogic Inc. (PBI), Halifax, Canada). The average of three parallelism points were calculated and reported.

5.10.2 VWF antigen

Von Willebrand antigen was measured in patients and controls by an in-house ELISA assay. In brief, ELISA plates were coated with rabbit polyclonal anti-human VWF (Dako Ltd, UK). After thorough washing, dilutions of the laboratory reference plasma (Cryochek, Precision BioLogic Inc. (PBI), Halifax, Canada), patient and control plasmas were added and after further washing, the plate was tagged with anti-VWF horseradish peroxidase antibody (Dako Ltd, UK). Finally, the reaction was visualised by the addition of substrate containing 10 mg O-phenylenediamine dihydrochloride (Sigma Chemical Co Ltd, Poole UK) and stopped using a 1.5M H_2SO_4 solution. Absorbance was measured at 490 nm.

5.10.3 Thrombophilia tests

Protein C (PC) and Protein S (PS) were measured on an ACL TOP coagulometer using the Prochrom™ protein C chromogenic activity kit (IL, USA) and the Free Protein S kit (IL, USA). Antithrombin (AT) was measured on a CS2000i (Sysmex, Milton Keynes, UK) using the Berichrom AT activity assay (Siemens, Germany). Factor V_{Leiden} and 3′UTR prothrombin gene mutation was measured using the Gene Xpert® FII & FV test (Cepheid, USA) which is a qualitative genotyping test for detection FII (G20210A) and FV Leiden (G1691A).

5.10.4 Lupus anticoagulant

Lupus anticoagulant was detected using dilute Russell's viper venom test (DRVVT; Sekisui Diagnostics LLC, Pfungstadt, Germany) on the ACL TOP 700 coagulometer (IL, USA).

6 Inter-individual variability in regulation of the initiation pathway: a potential modifier of phenotypic heterogeneity in Haemophilia – Part 2

6.1 Sensitivity of the thrombin generation assay to bleeding phenotype and a functional assessment of the role of TFPI in patients with Haemophilia A

The initial cross-sectional part of this study analysed levels of free and total TFPI – in addition to other components of the initiation of coagulation – in order to interrogate any correlation between variables, examine differences in levels between patient cohorts or bleeding phenotypes and, using regression analysis, assess whether any independent variable was predictive of phenotype.

The regulation of the initiation of haemostasis has been an area of increasing interest with greater understanding of the role of TFPI and promising results from phase 1 clinical trials of a monoclonal antibody targeting TFPI used in patients with haemophilia [Chowdary *et al.*, 2015]. The use of ELISAs to measure free and total TFPI is well established, but functional assessment is more challenging. Dahm et al. [Dahm, 2005] have used a modified dilute

prothrombin time (dPT) with and without the addition of an anti-TFPI antibody, expressing results as a ratio with the sample with the anti-TFPI antibody as the denominator. These results were then normalised against the results from a reference plasma. This was then compared to the results of the free and total TFPI ELISAs and a TFPI chromogenic substrate activity assay in over 400 volunteers. Their assay results were found to correlate with the static assays.

The primary hypothesis of this next study was that the lag phase and amount of thrombin generated as assessed by the thrombin generation assay (TGA) is a potential reflection of phenotype in patients with haemophilia. A secondary hypothesis was that TFPI potentially influences TGA parameters and plays an additional role in modifying bleeding phenotype.

The study therefore set out to assess the sensitivity of the TGA to phenotypic heterogeneity with the use of a neutralising anti-FVIII antibody to abolish the influence of varying FVIII levels using the rationale that even modest amounts of FVIII make a significant difference to thrombin generation (thus the study participants were limited to patients with Haemophilia A). In addition, the aim was to develop a protocol with the use of an anti-TFPI antibody to gain information about the functional activity of TFPI and variation between patients and bleeding phenotypes. By using patients who had already been recruited to the cross-sectional study, results were available for the TF, FVII, FVIIa, free and total TFPI and FVIIa.AT complex assays. This would enable correlation of these components with the TGA results.

In addition to the use of the anti-FVIII and anti-TFPI antibodies, it was felt that it would be valuable to assess thrombin generation using a range of tissue factor

(TF) in the trigger reagent in order to look for any evidence of a threshold effect between patients.

6.2 Study aim

To assess the sensitivity of the TGA to bleeding phenotype in patients with haemophilia A, gain understanding of the influence of TFPI on thrombin generation and look for correlation between results of TF, FVII, FVIIa, free and total TFPI and FVIIa.AT complex and TGA results.

6.2.1 Primary objective

To recruit 20 patients with haemophilia A from the cross-sectional study and develop a TGA protocol using a range of TF triggers with the addition of both a neutralising FVIII antibody as well as an anti-TFPI antibody to determine the potential sensitivity of TGA to bleeding phenotype.

6.2.2 Secondary objective

To compare the results from the cross-sectional study with the TGA parameters in these 20 patients and use statistical analysis to determine any correlation.

6.3 Study design

An exploratory laboratory study using a modified TGA protocol incorporating the use of an anti-FVIII and an anti-TFPI antibody.

6.3.1 Primary end-point

TGA parameters using the technique of Hemker et al. ([Hemker *et al.*, 2006] and the calibrated automated thrombogram (CAT).

6.3.2 Secondary end-points

Statistical analysis to determine the sensitivity of the TGA to samples from patients with haemophilia A (with and without the use of neutralising antibodies to FVIII and TFPI) as well as look for differences between bleeding phenotypes and any correlation between TGA parameters and bleeding phenotype or levels of initiation pathway components.

6.4 Study group

6.4.1 Patient selection

The patients approached for this part of the study had already given samples for the cross-sectional study (see Chapter 5) and thus their consent was still valid (providing they were willing to provide further samples). They were generally approached when they attended for routine clinic review and therefore were already having blood tests including trough FVIII levels.

Inclusion criteria

- Patients with Haemophilia A who had already provided consent and samples for the cross-sectional study

Exclusion criteria

- Patients with an inhibitor
- Patients experiencing a bleeding episode

Target recruitment

The target number for recruitment of patients with haemophilia A was 20, with a mix of causative genetic mutations (null as well as non-null) and bleeding phenotypes.

6.4.2 Patient samples

Blood samples were taken as per the departmental protocol (see Chapter 3: Supplementary Material 3.7) into S-Monovette® tubes (Sarstedt; 3.2% 0.109 M trisodium citrate) with CTI added (final concentration 20 μg/ml). Samples were triple spun (i.e. 2000g for 15 minutes x 2 and then 6000g for 3 minutes) before being divided into aliquots and frozen at -80^{0}C. Samples were processed within 2 hours of collection.

6.4.3 Healthy volunteer sample

A male healthy volunteer (consented for samples for the normal reference range study) was bled to obtain triple-spun citrated CTI PPP to run as a normalising and quality control for the study.

6.4.4 The use of neutralising antibodies

6.4.4.1 Neutralising FVIII activity

A sheep polyclonal anti-human FVIII antibody (Haematologic Technologies Inc (HTI), Cambridge Bioscience, UK; concentration 25.9 mg/ml; activity 2278 BU/mg) was used to neutralise any residual FVIII activity (endogenous or exogenous). FVIII levels were available from routine clinic samples taken at the same time (determined by the local diagnostic laboratory).

The aim was to determine a concentration that was effective without a period of incubation (in order to avoid another variable that might impact on the results and allow comparison with the normal reference ranges that had not been incubated). Given that the patient samples were predominantly trough levels and taking into account the need to avoid over-diluting the plasma samples for the TGA, an exploratory assay was set up with the addition of approximately 40, 20 and 10 BU of antibody activity to samples from a male healthy volunteer (triple spun CTI PPP) (see Figure 6-1). Samples were also tested for residual factor levels following addition of the neutralising antibody (see Table 6-1). The antibody was diluted in tris buffered saline stabilised with bovine serum albumin (TBS/1% BSA).

As can be seen from the TGA results in Figure 6-1, the addition of antibody greatly suppressed the thrombin generation. There was a dose effect seen, but no measurable FVIII activity was detected in the samples containing the antibody by one-stage APTT-based assay[5]. 20 BU activity was chosen for

[5] FVIII method, see Chapter 5: Supplementary Material (section 5.10)

subsequent experiments. The calculations are documented in Supplementary Material 6.8.

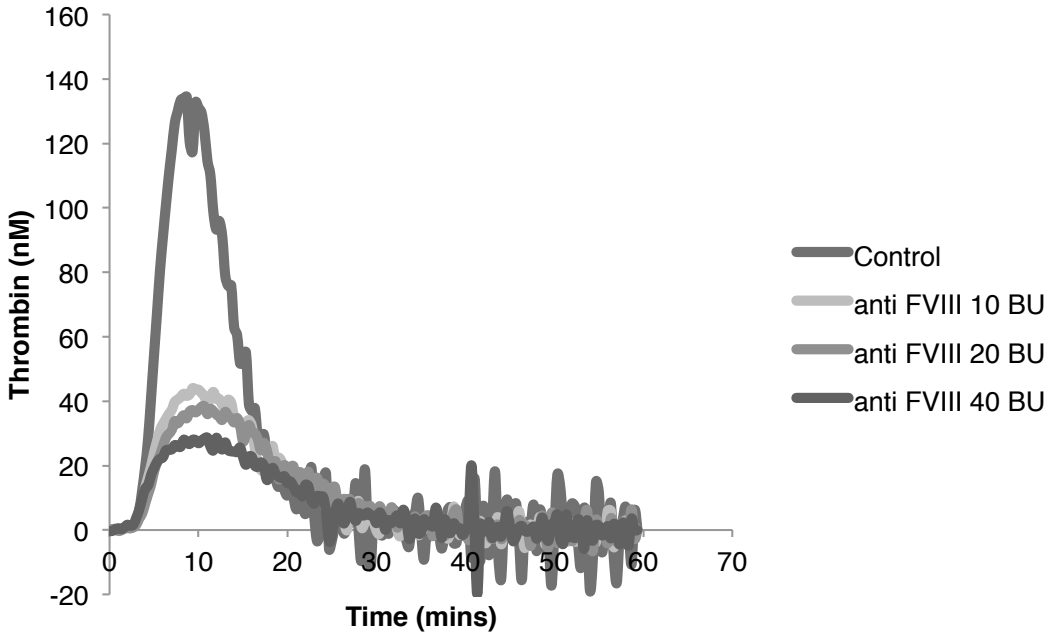


Figure 6-1 Titration of sheep anti-human FVIII antibody; sample from healthy volunteer; trigger = PPP Low (~1 pM TF)

6.4.4.2 Neutralising TFPI activity

A sheep anti-human TFPI antibody (9.3 mg/ml; HTI, Cambridge Bioscience, UK) was selected to neutralise TFPI activity in the TGA. However, no activity data regarding anti-TFPI neutralising efficacy was available.

An ELISA was used to measure free and total TFPI levels in the cross-sectional study. However, there were concerns that the direct addition of the sheep antibody to plasma would potentially interfere with the capture and detection antibodies of the ELISA. It was decided therefore to also use a surrogate functional marker.

One of the patients involved in the study also participated in a phase 1 clinical trial of a monoclonal anti-TFPI antibody and local samples had been taken at several time-points during the pharmacokinetic study. These samples had had both ELISA measurements and TGA performed by the researcher (see Table 6-1 and Figures 6-2, 6-3, 6-4) (unpublished data; consent for use in thesis granted by sponsor of clinical trial).

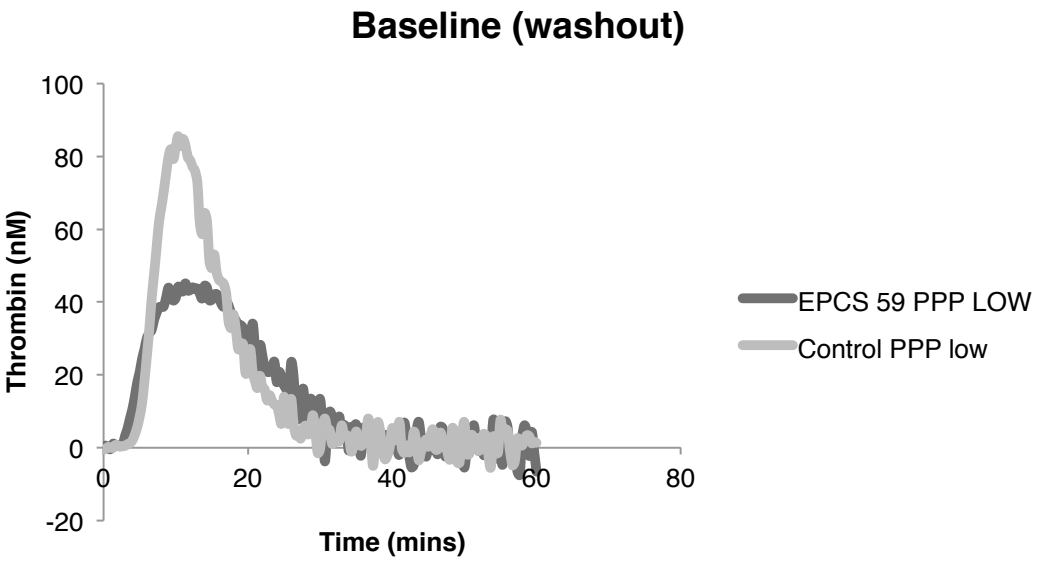


Figure 6-2 SHA patient. TGA curve on baseline sample with normal pooled plasma control (FVIII one-stage 2 IU/dL, chromogenic <1 IU/dL); trigger = PPP Low

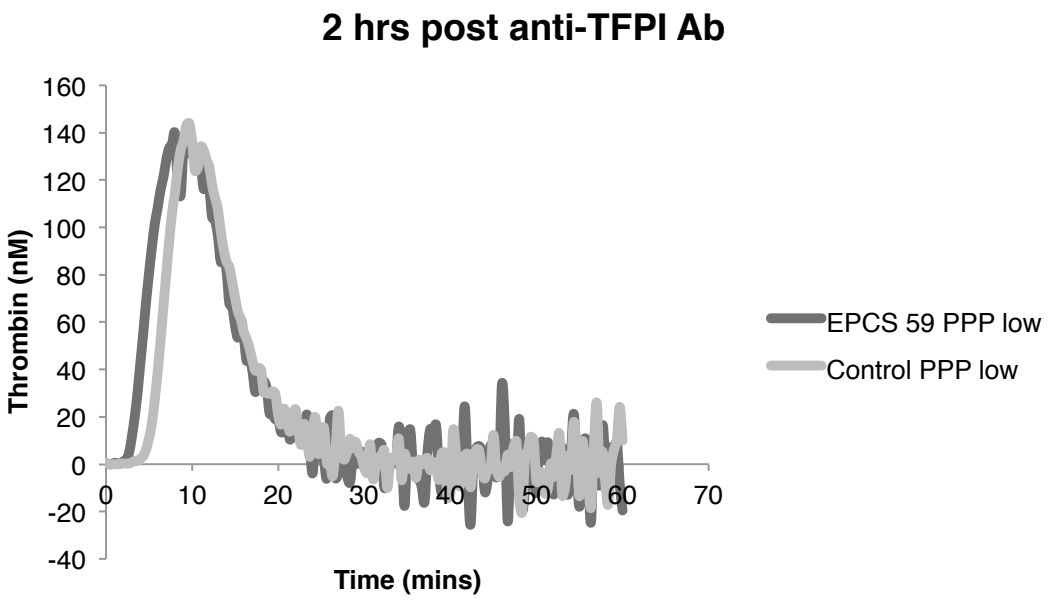


Figure 6-3 SHA patient. TGA curve 2 hours post intravenous administration of anti-TFPI antibody; trigger = PPP Low

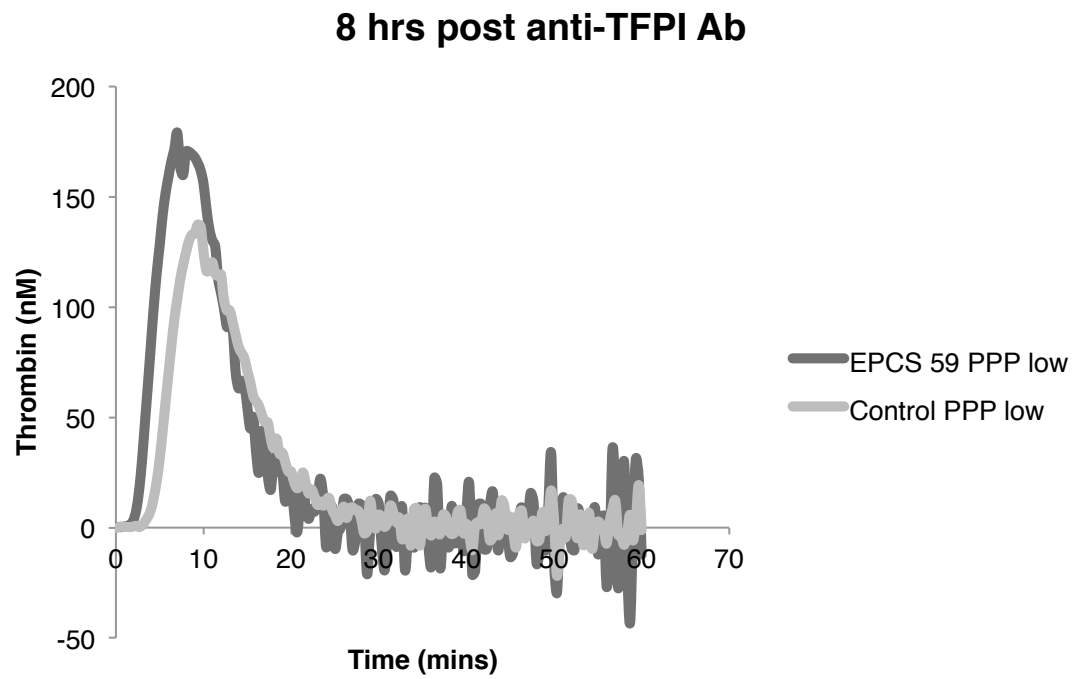


Figure 6-4 SHA patient. TGA curve 4 hours post administration of anti-TFPI antibody; trigger = PPP Low

A decision was made to dilute the stock antibody 1:10 in TBS/1% BSA and then add an equivalent volume to that used with the 20 BU activity anti-FVIII antibody. This was calculated to be 0.2 mg/ml. A TGA assay was run in order to compare to the samples from the patient who had received the anti-TFPI antibody as part of the clinical trial (see Figure 6-5). This gave similar results. A TGA assay was subsequently set up with a range of concentrations: 0.4 mg/ml, 0.2 mg/ml and 0.1 mg/ml added to male healthy volunteer plasma. The same samples were also run on the free and total TFPI ELISAs in order to ascertain whether a reduction could be detected (see Table 6-1 and Figure 6-6). For the calculations see Supplementary Material 6.8.

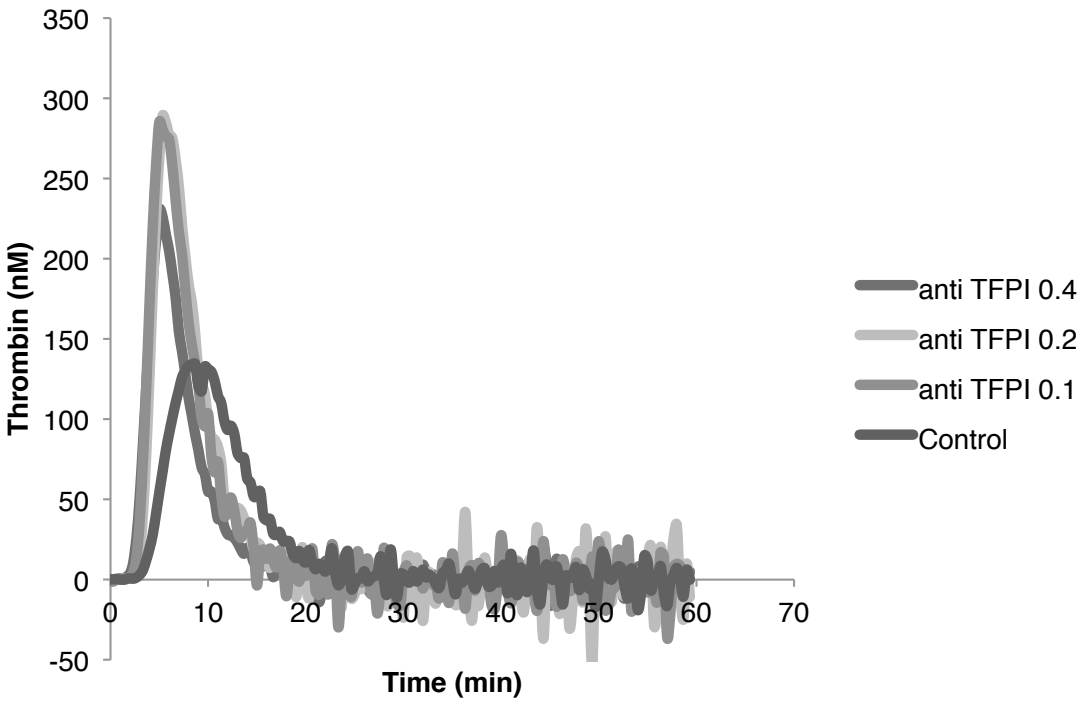


Figure 6-5 Healthy volunteer plasma spiked with range of concentrations of anti-TFPI antibody (mg/ml); trigger = PPP Low

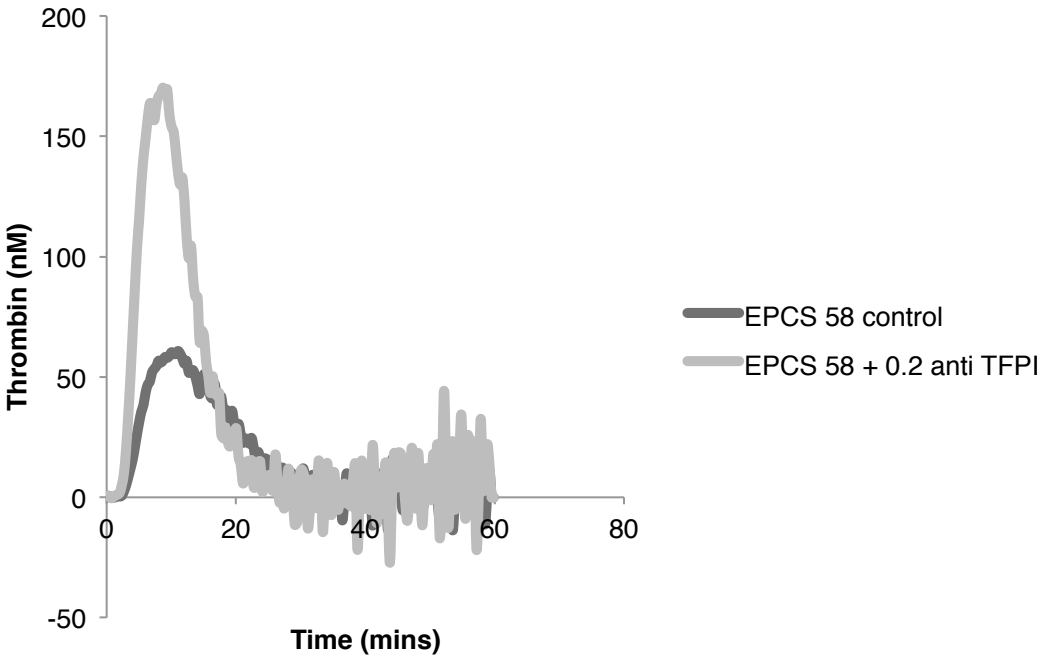


Figure 6-6 Washout sample from patient with SHA with addition of 0.2 mg/ml anti-TFPI antibody; trigger = PPP Low. Compare with Figures 6-2 – 6-4.

Table 6-1 Results of FVIII levels, free and total TFPI levels from trial patient given humanised anti-TFPI antibody and addition of sheep anti-FVIII and anti-TFPI antibodies to healthy volunteer (HV) plasma

Plasma sample	FVIII levels (IU/dL) NR 50-150	Free TFPI levels (ng/ml) NR 2.8-13.8	Total TFPI levels (ng/ml) NR 31.7-88.8
Trial pt baseline	-	9.6	59.9
Trial pt 2 hrs post anti-TFPI Ab	-	1.0	7.4
Trial pt 4 hrs post anti-TFPI Ab	-	1.8	9.7
Trial pt 8 hrs post anti-TFPI Ab	-	3.4	10.2
HV baseline	-	11.2	58.6
HV + 0.4 mg/ml anti-TFPI Ab	-	0.0	7.3
HV + 0.2 mg/ml anti-TFPI Ab	-	0.7	8.1
HV + 0.1 mg/ml anti-TFPI Ab	-	2.1	8.2
HV + 40 BU anti-FVIII Ab	< 1	-	-
HV + 20 BU anti-FVIII Ab	< 1	-	-
HV + 10 BU anti-FVIII Ab	< 1	-	-

6.4.4.3 Controlling for sheep IgG

In order to control for any non-specific effect of the sheep antibodies, purified sheep IgG (Bethyl Laboratories Inc, Cambridge Bioscience, UK) was obtained. This was at a concentration of 5 mg/ml.

The neutralising antibodies had IgG concentrations of 25.9 mg/ml (FVIII) and 9.3 mg/ml (TFPI). Given the higher protein concentration of the anti-FVIII antibody, the equivalent final concentration added to the plasma sample (~ 2.5 mg/ml; 20 BU) was calculated for the sheep IgG. Concentrations higher and lower were prepared (~5mg/ml and ~ 1.25 mg/ml) and all three were added to healthy volunteer plasma and run on the TGA (see Figure 6-7). No significant non-specific effect on thrombin generation was detected.

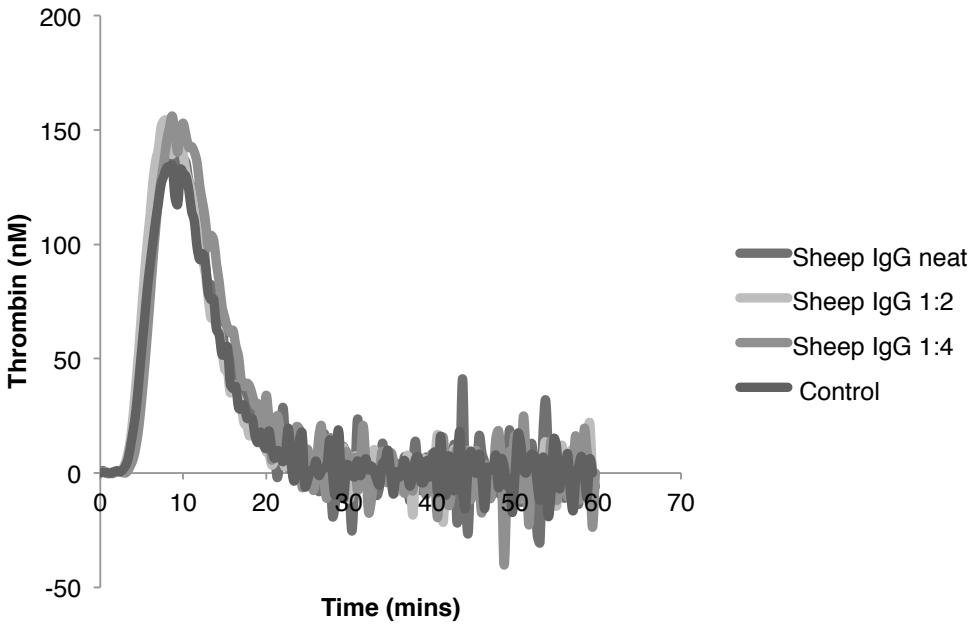


Figure 6-7 TGA curve; healthy volunteer (triple spun CTI) plasma with titration of control sheep IgG; trigger = PPP Low

6.4.5 TF concentrations

The data generated for the normal reference ranges, incorporating the use of a normal plasma pool as a quality and normalisation control, demonstrated that the coefficients of variation (CVs) were closer to 10% when ready-made TF reagents were used (see Chapter 3: Supplementary Material 3.9). However, this study aimed to use 3 different concentrations of TF (that also required the addition of phospholipid (PL)) and it was therefore more straightforward to use a separate source of TF and prepare study-specific triggers.

Initial experiments were conducted in the absence of TF (akin to the NATEM that solely re-calcifies the citrated whole blood sample). However, negligible thrombin generation was produced and there was a very prolonged LT (data not shown). In order to incorporate a standard TF concentration employed with the running of PPP samples from patients with haemophilia as well as a higher concentration to try and maximise potential thrombin generation (in the absence of FVIII) in addition to a lower than normal TF concentration to assess any potential 'threshold' effect, 2 pM, 1 pM and 0.5 pM TF concentrations (incorporating synthetic PL at a final concentration of 4 μM) were used (see Supplementary material 6.8 for the calculations). Innovin® (Dade®, Siemens, UK) was used as the TF source. PL was obtained from Rossix (Quadratech Diagnostics Ltd, UK) – a stabilised 0.5 mM phospholipid emulsion containing a mixture of highly purified phosphatidyl choline (PC, 42%), phosphatidyl serine (PS, 28%) and sphingomyelin (SM, 30%). The ACTICHROME® TF activity assay was used to assess the TF concentration of the batch of Innovin® used

(see Chapter 3: Supplementary material 3.8 for methodology). This was also used to confirm the calculated concentrations.

6.4.6 FVIII levels

FVIII levels were ascertained at the time of the study samples (see Table 6-2; see Chapter 5: Supplementary material 5.10 for methodology). Not all samples were washout samples and some patients were on clinical trials of extended half-life FVIII concentrates and thus had measurable levels at 96 hours when they attended for study visits. One patient did not have a FVIII level taken at the time of the samples.

6.4.7 TGA protocol

Thrombin generation (TGA) was measured as per the method of Hemker et al. [Hemker *et al.*, 2006]. Supplementary Material 6.8 describes the methodology for the calculations of antibody concentrations and addition to plasma samples as well as layout of the 96-well plate. Each plate included the same healthy volunteer PPP with identical addition of antibodies and triggered with 2 pM TF. These results could be used both as a quality and normalization control. 80 μl PPP (including addition of antibodies or buffer) was added to 20 μl TF trigger in an Immunlon 2HB, round-bottom 96-well plate (Dynex technologies). 20 μl Hepes/BSA buffer containing 100 mM calcium chloride ($CaCl_2$) and 5 mM fluorogenic substrate was added to initiate the assay. Each sample was tested in duplicate with an internal calibrator. Fluorescence was measured with an

excitation filter at 390 nm and an emission filter at 460 nm using a Fluroskan Ascent fluorimeter (Thermolab Systems, Helsinki, Finland). The endogenous thrombin potential (ETP), lagtime (LT), peak and time to peak (ttPeak) were derived from the raw data by dedicated software (Thrombinoscope™ B.V., Maastricht, The Netherlands).

6.4.8 Statistical analysis

Descriptive statistics were used to assess results that were then assessed for normality. Based on these results, appropriate tests were selected to determine differences between groups as well as assess for the strength and direction of any correlation.

6.5 Results

6.5.1 Patients

20 patients with haemophilia A were recruited to this study (see Table 6-2). Of these, 2 had moderate haemophilia and 2 patients were brothers. 7 patients had no functional product of the F8 gene based on mutation analysis – predominantly due to the intron 22 inversion. The spread of bleeding phenotype scores are demonstrated in Figure 6-8. The average age was 38 years (range 19 – 64 years).

Table 6-2 Patient information; OSCA = one stage clotting assay; EHL = extended half life product; inv22 = inversion 22; tx = treatment

Patient study ID	Bleeding phenotype	Moderate or severe	? functional product based on mutation	OSCA FVIII (IU/dL)	Chromogenic FVIII (IU/dL)	Timing of sample	Weight (kg)
EPCS 1	1	Severe	Uncertain	3	7	96 hr washout (EHL)	59.1
EPCS 2	3	Severe	No (inv 22)	17	19	3.5 hr post 1500 IU	97
EPCS 6	2	Severe	No (inv 22)	1	2	96 hr washout (EHL)	85.5
EPCS 21	3	Severe	Uncertain	1	<1	75 hr washout	82.6
EPCS 25	2	Moderate	Uncertain	1	2	Baseline	60
EPCS 26	2	Severe	No (inv 22)	<1	-	Washout	63.9
EPCS 31	3	Moderate	Uncertain	9	6	Baseline	95.8
EPCS 34	2	Severe	Uncertain	<1	<1	56 hr post tx	73
EPCS 35	2	Severe	Uncertain	<1	<1	56 hr post tx	67.9
EPCS 38	3	Severe	Uncertain	<1	2	101.75 hr post tx (EHL)	73.5
EPCS 47	1	Severe	Uncertain	<1	3	26 hr post tx	90
EPCS 48	2	Severe	No (inv 22)	112	129	2.5 hr post 3000 IU	79
EPCS 50	2	Severe	Uncertain	<1	<1	42.5 hr post 2000 IU	49
EPCS 57	1	Severe	No (inv 22)	-	-	*Uncertain*	57.9
EPCS 58	2	Severe	No	<1	<1	72.5 hr post 2500 IU	93.3
EPCS 60	1	Severe	Uncertain	<1	1	Washout	87.3
EPCS 62	2	Severe	Uncertain	2	4	48 hr post 2000 IU	78.3
EPCS 63	2	Severe	Uncertain	6	5	51 hr post 2000 IU	81.9
EPCS 64	3	Severe	Uncertain	6	2	44.5 hr post 3000	76.9
EPCS 66	3	Severe	No (inv 22)	<1	1	Washout	70.9

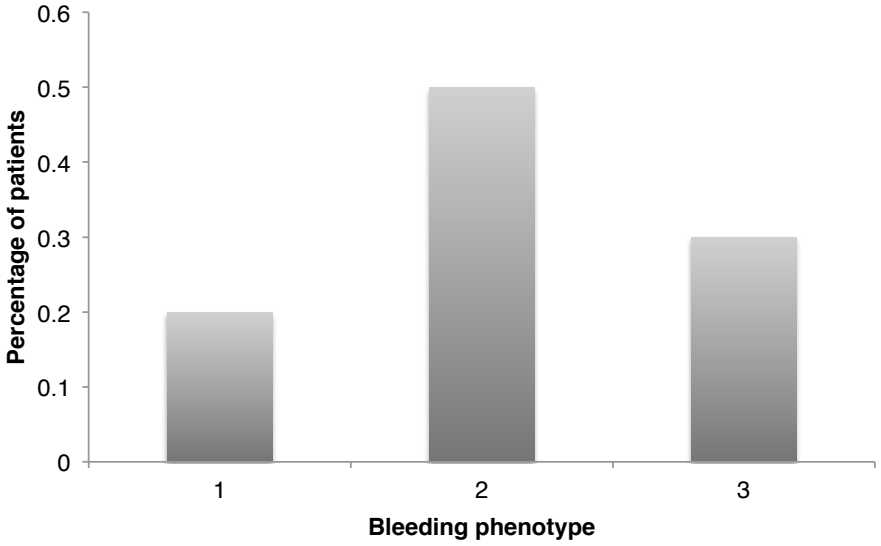


Figure 6-8 Bleeding phenotype score 1 = less than expected, 2 = as expected, 3 = more than expected

6.5.2 Baseline thrombin generation (FVIII neutralised)

TGA Peak values were plotted for samples run with the addition of the neutralising FVIII antibody and triggered with 2 pM Innovin (see Figure 6-9). This enabled comparison with a normal reference range generated with 40 healthy volunteers (see Chapter 3). 6/20 patients (30%; 2 x phenotype 1, 4 x phenotype 2) had baseline peak thrombin values (in the absence of FVIII) that were within the lower end of the normal range. A further 3/20 (15% - all bleeding phenotype 3) had results within 5 nM of the lower end of the normal range.

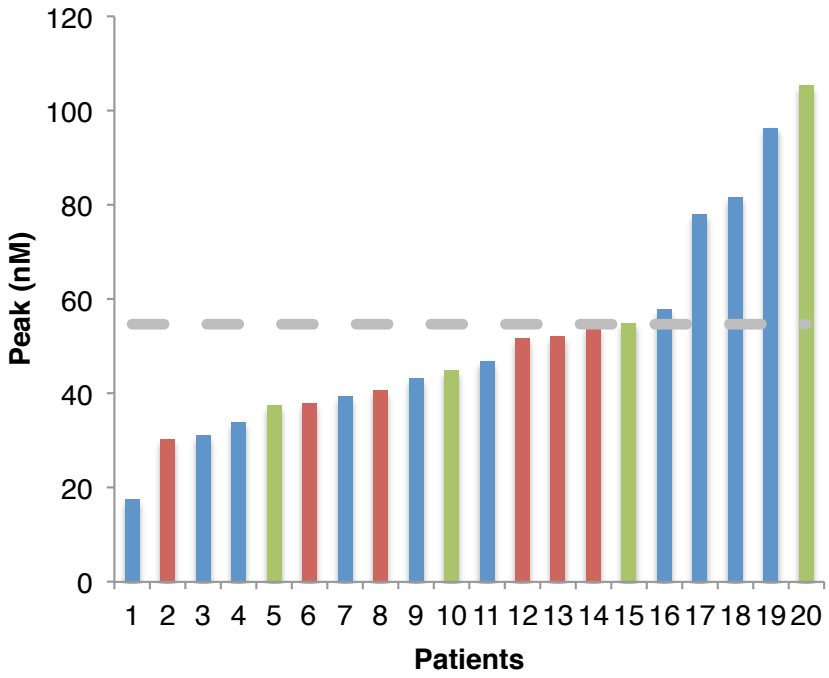


Figure 6-9 TGA Peak results (nM) for patient samples with addition of anti-FVIII antibody – trigger = Innovin 2 pM; bleeding phenotype 1 (less than expected), 2 (as expected), 3 (more than expected); lower limit of normal range generated with triple spun CTI PPP with trigger 2 pM highlighted (dashed line)

6.5.3 Impact of neutralising FVIII activity

Samples were run with and without the addition of a polyclonal neutralising anti-FVIII antibody. Although the majority of samples were trough or washout samples, they had variable levels of FVIII activity as measured by both the one stage clotting (OSCA) and chromogenic assays. The comparison of the curves in those samples with levels < 1IU/dl enabled detection of those patients with no functional product of the F8 gene, with over-lapping or super-imposed curves indicating that the addition of the neutralising antibody did not make any difference to the thrombin generation patterns and hence the supposition that

there was no FVIII present (see Figures 6-10 – 6-14). This assumption would need to be confirmed by measurement of the FVIII antigen (that can more accurately measure levels < 1 IU/dL), as an alternative possibility is that the antibody was unable to completely neutralise all FVIII activity.

6.5.4 Impact of neutralising TFPI

The addition of the neutralising anti-TFPI antibody (in combination with the anti-FVIII antibody) had a striking effect on thrombin generation, normalising peak values to well within the normal range (2 pM Innovin) lending weight to the clinical utility of a therapy reducing levels of TFPI (TGA Peak 141.34 - 226.76 nM (mean 184.05), normal range = 54.69 – 242.03 nM; 0.71 - 1.15 (normalised data), normalised normal range = 0.55 – 2.17). Regardless of the variable amounts of residual FVIII, the addition of the anti-TFPI antibody in the absence of the anti-FVIII antibody gave similar results (TGA Peak 122.16 - 239.75 nM (mean 180.95)).

TGA output for patients with baseline one-stage clotting assay (OSCA) and chromogenic assay (C) results < 1 IU/dL with and without the addition of a FVIII neutralising antibody

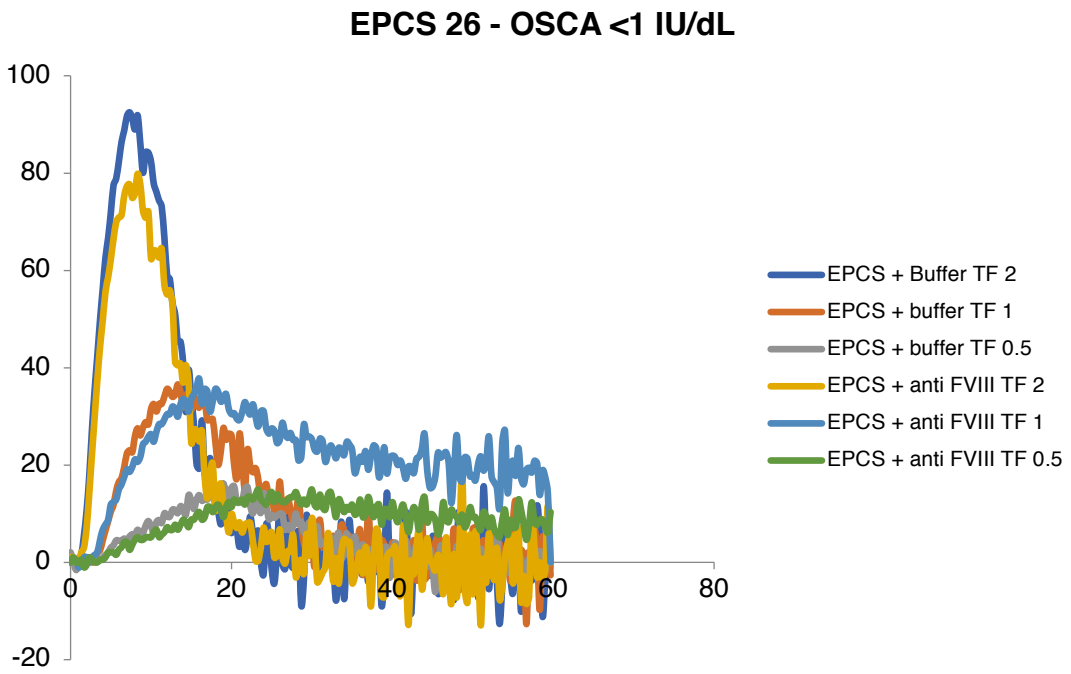


Figure 6-10 Patient with inversion 22; note close over-lapping of curves with and without anti-FVIII antibody added; TS CTI PPP samples run with and without the addition of an anti-FVIII antibody (buffer added to sample without antibody to take into account dilution effect on thrombin generation); range of TF concentrations: 2, 1 and 0.5 pM, x axis = time (min), y axis = Thrombin (nM)

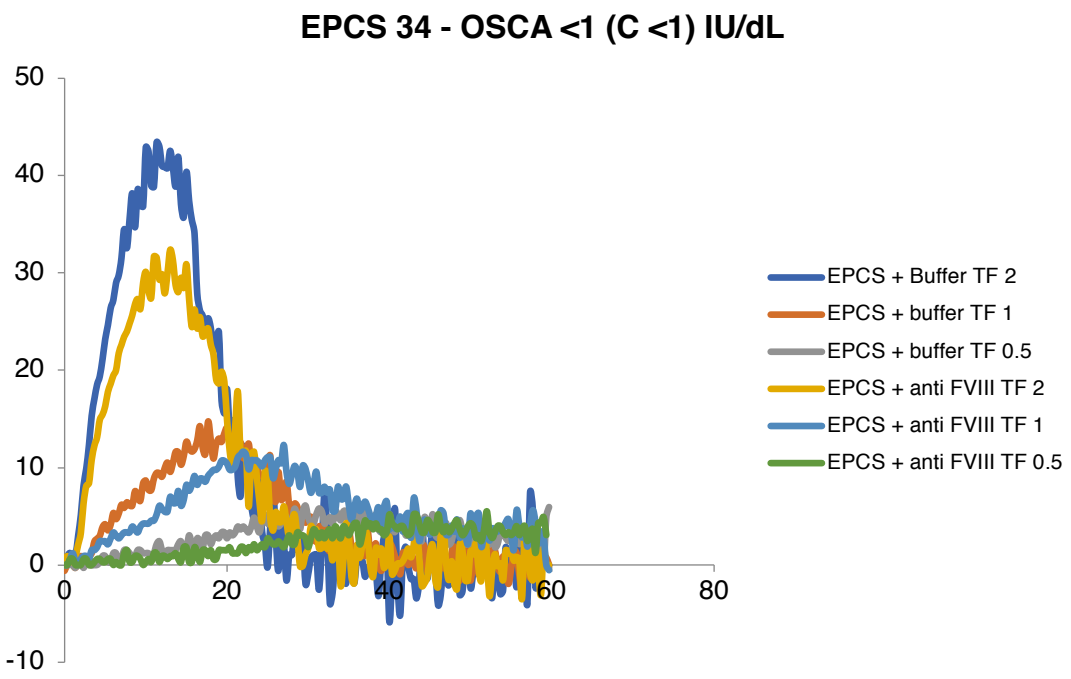


Figure 6-11 Patient with uncertain functional product of F8 gene; brother of patient EPCS 35 below; note difference in magnitude and peak of TGA curves; range of TF concentrations: 2, 1 and 0.5 pM, x axis = time (min), y axis = Thrombin (nM)

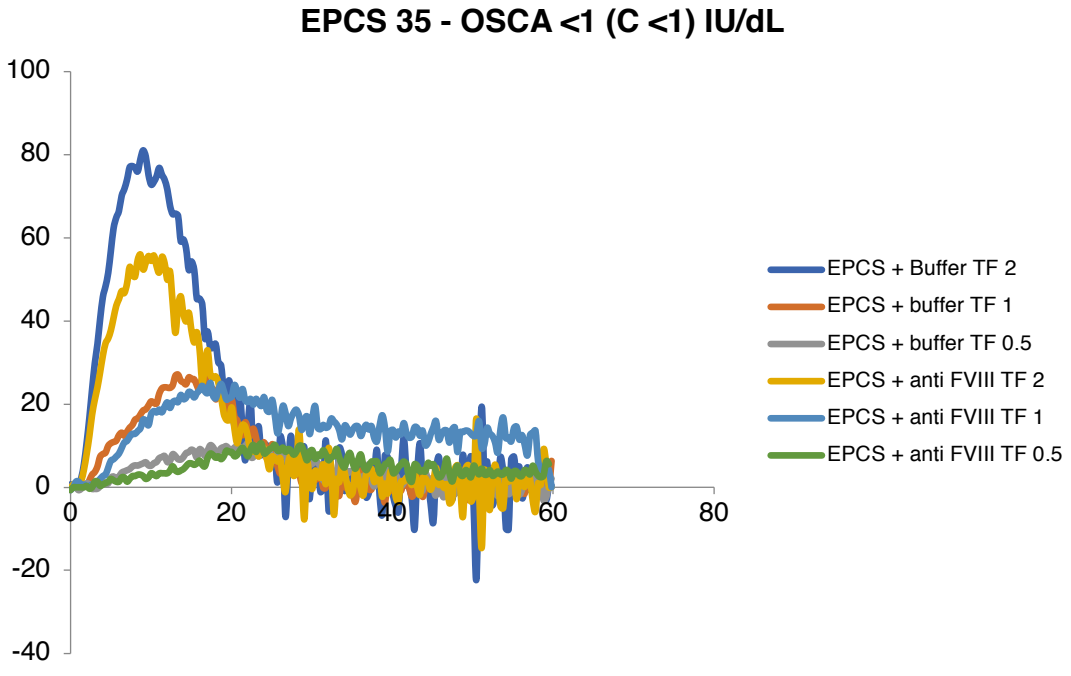


Figure 6-12 Patient with uncertain functional product of F8 gene; brother of patient EPCS 34 above

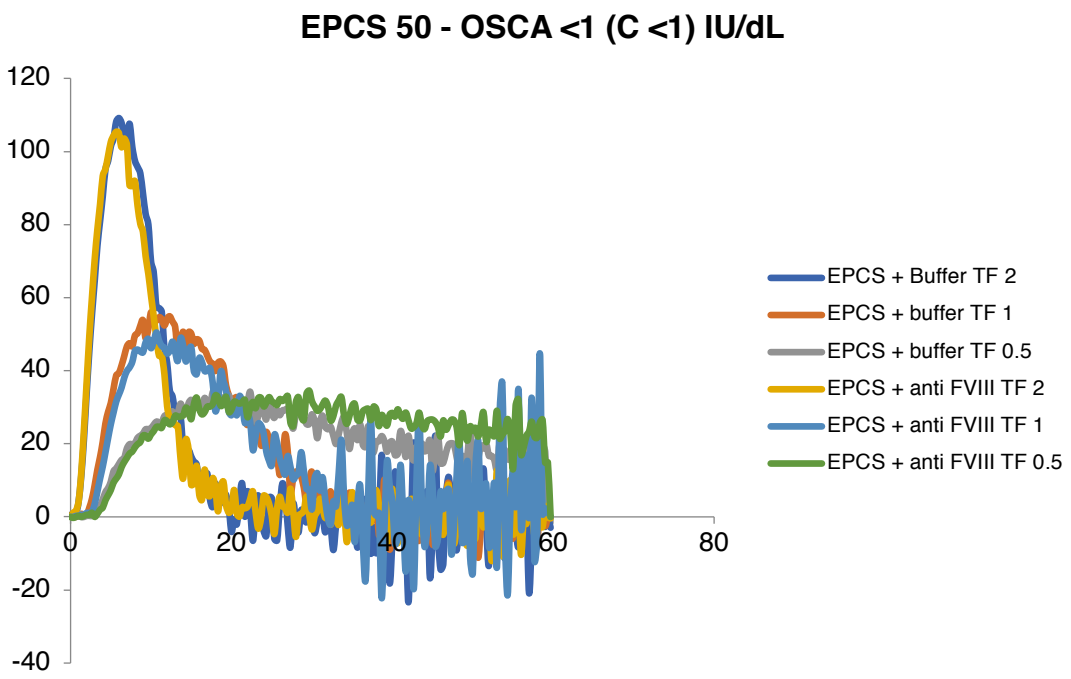


Figure 6-13 Patient with a mutation introducing a splice site into intron 19 – appearances suggest no functional FVIII present; range of TF concentrations: 2, 1 and 0.5 pM, x axis = time (min), y axis = Thrombin (nM)

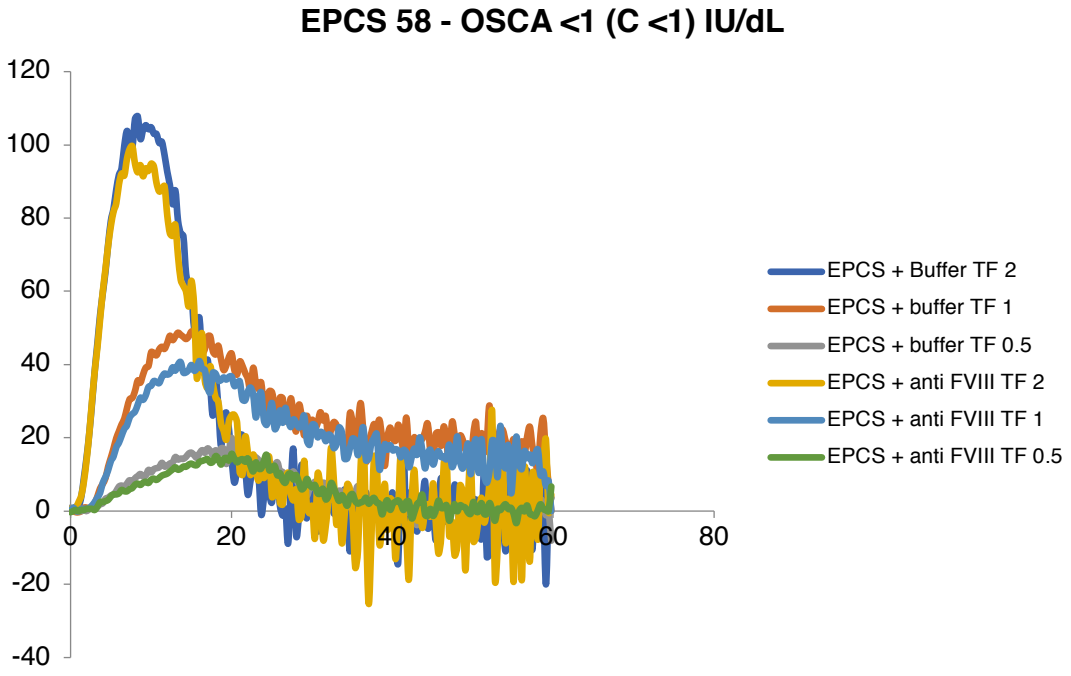


Figure 6-14 Patient with no functional product of F8 gene (mutation resulting in introduction of a STOP codon into exon 2 in the A1 domain)

6.5.5 TGA parameters in the absence of FVIII – comparison between phenotypes and correlation with free and total TFPI

6.5.5.1 Lag Time (LT)

LT results were assessed for the samples treated with the anti-FVIII antibody in order to examine for any significant differences between phenotypes or correlation with free or total TFPI.

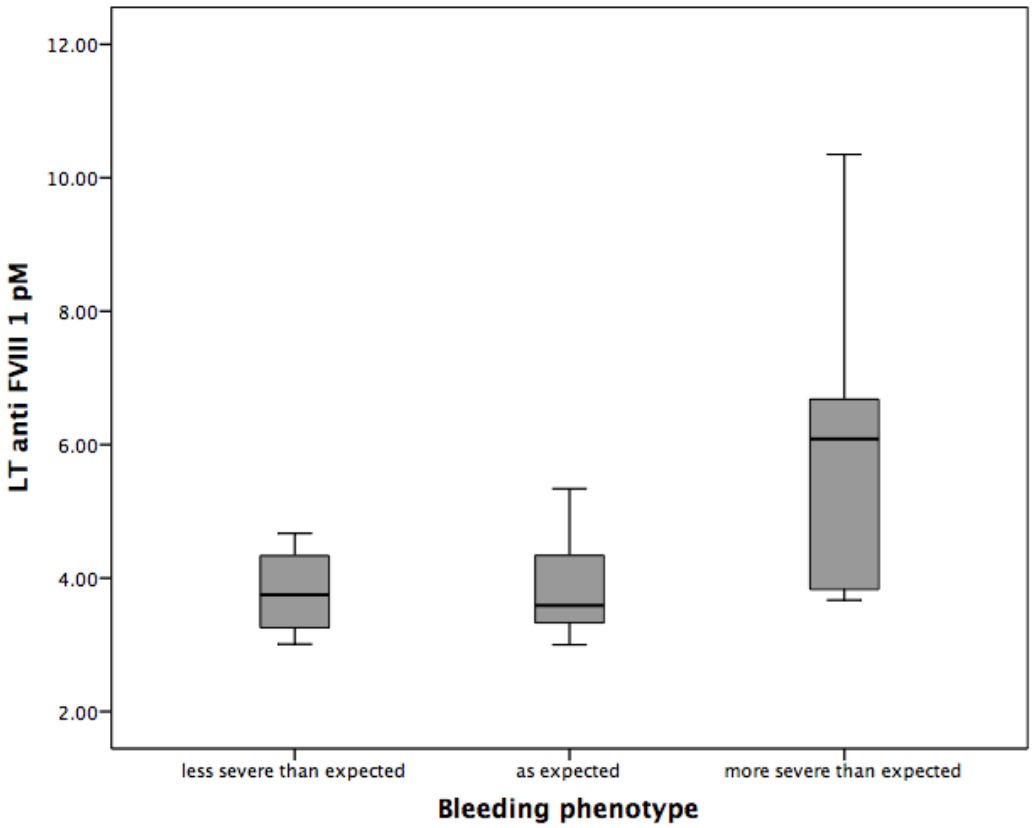


Figure 6-15 Boxplots for LT values of samples with neutralised FVIII separated as per bleeding phenotype (s; min, 25th percentile (Q1), median, 75th percentile (Q3), max); trigger 1 pM TF

When values were compared between bleeding phenotypes, there was a trend towards prolongation of the LT with the ‘more severe than expected’ phenotype. When assessed using the Kruskal Wallis test (for the samples triggered with 1 pM TF), the LT was statistically significantly different between the 3 groups ($p = 0.037$). Post hoc analysis using a Bonferroni correction ($p < 0.017$) demonstrated a significant difference in the LT between the ‘less severe than expected’ and ‘more severe than expected’ categories ($p = 0.014$).

Table 6-3 Spearman correlations between free and total TFPI levels and LT in samples with no FVIII activity; all TF concentrations and including normalised data (NR) for 2 pM TF samples

Correlations

			free TFPI	total TFPI	LT anti FVIII 2 pM
Spearman's rho	free TFPI	Correlation Coefficient	1.000	.549*	.059
		Sig. (2-tailed)	.	.012	.805
		N	20	20	20
	total TFPI	Correlation Coefficient	.549*	1.000	.315
		Sig. (2-tailed)	.012	.	.176
		N	20	20	20
	LT anti FVIII 2 pM	Correlation Coefficient	.059	.315	1.000
		Sig. (2-tailed)	.805	.176	.
		N	20	20	20
	NR - LT anti FVIII 2 pM	Correlation Coefficient	.351	.462*	-.598**
		Sig. (2-tailed)	.130	.040	.005
		N	20	20	20
	LT anti FVIII 1 pM	Correlation Coefficient	.382	.502*	.530*
		Sig. (2-tailed)	.096	.024	.016
		N	20	20	20
	LT anti FVIII 0.5 pM	Correlation Coefficient	.178	.305	.416
		Sig. (2-tailed)	.452	.191	.068
		N	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

The data were subsequently analysed to assess for any correlation between the LT and free and total TFPI levels. There was a strong correlation as expected between free and total TFPI (0.549; p = 0.012). However, although there was no significant correlation between free TFPI and the LT, there were moderate correlations between total TFPI levels and the normalised data (NR) for the LT triggered with 2 pM (0.462; p = 0.040) and the raw data for the LT triggered with

1 pM in the presence of the anti-FVIII antibody (0.502; p = 0.024) (see Table 6-3). The higher the total TFPI level, the longer the LT in those samples with no FVIII activity.

6.5.5.2 Peak

When the peak values were assessed for any significance in the difference between categories, the independent samples Kruskal Wallis test was significant for the normalised results of the samples triggered with 2 pM TF (p = 0.047). Post hoc analysis with a Bonferroni correction applied showed that there was a significant difference between the 'as expected' and the 'more severe than expected' phenotypes (p = 0.014).

Correlation analysis with a Spearman correlation showed that total TFPI correlated strongly with peak values in the absence of FVIII (2 pM) (-0.496; p = 0.026) (see Table 6-4). Thus, the higher the total TFPI level, the lower the peak amount of thrombin generation. This would be consistent with the correlation seen with prolongation of the LT.

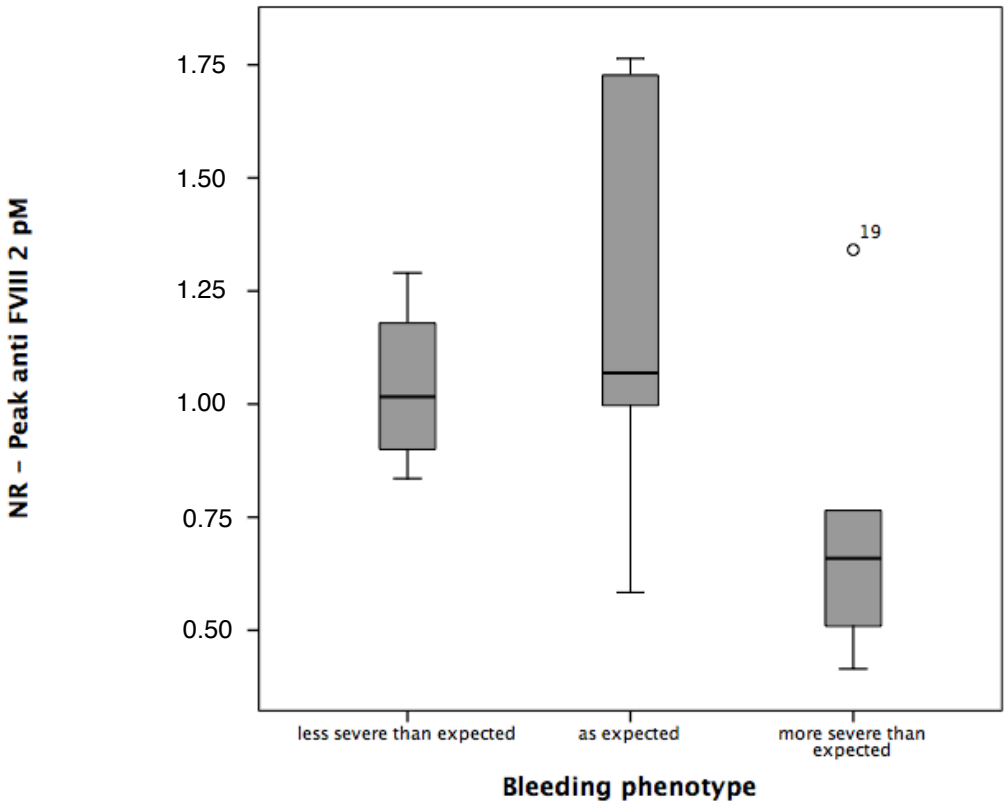


Figure 6-16 Boxplots for Peak values of samples with neutralised FVIII (NR = normalised data) separated as per bleeding phenotype (nM; min, 25th percentile (Q1), median, 75th percentile (Q3), max); trigger = 2 pM TF

Table 6-4 Spearman correlations between free and total TFPI levels and peak in samples with no FVIII activity; all TF concentrations (2 pM, 1 pM, 0,5 pM) and including normalised data (NR) for 2 pM TF samples

Correlations

			free TFPI	total TFPI	Peak anti FVIII 2 pM
Spearman's rho	free TFPI	Correlation Coefficient	1.000	.549*	-.249
		Sig. (2-tailed)	.	.012	.290
		N	20	20	20
	total TFPI	Correlation Coefficient	.549*	1.000	-.496*
		Sig. (2-tailed)	.012	.	.026
		N	20	20	20
	Peak anti FVIII 2 pM	Correlation Coefficient	-.249	-.496*	1.000
		Sig. (2-tailed)	.290	.026	.
		N	20	20	20
	NR - Peak anti FVIII 2 pM	Correlation Coefficient	-.239	-.424	.827**
		Sig. (2-tailed)	.310	.062	.000
		N	20	20	20
	Peak anti FVIII 1 pM	Correlation Coefficient	-.244	-.162	.850**
		Sig. (2-tailed)	.299	.494	.000
		N	20	20	20
	Peak anti FVIII 0.5 pM	Correlation Coefficient	-.061	-.165	.635**
		Sig. (2-tailed)	.799	.486	.003
		N	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

6.5.5.3 ttPeak

The same analysis was undertaken for the ttPeak results for the samples treated with the anti-FVIII antibody.

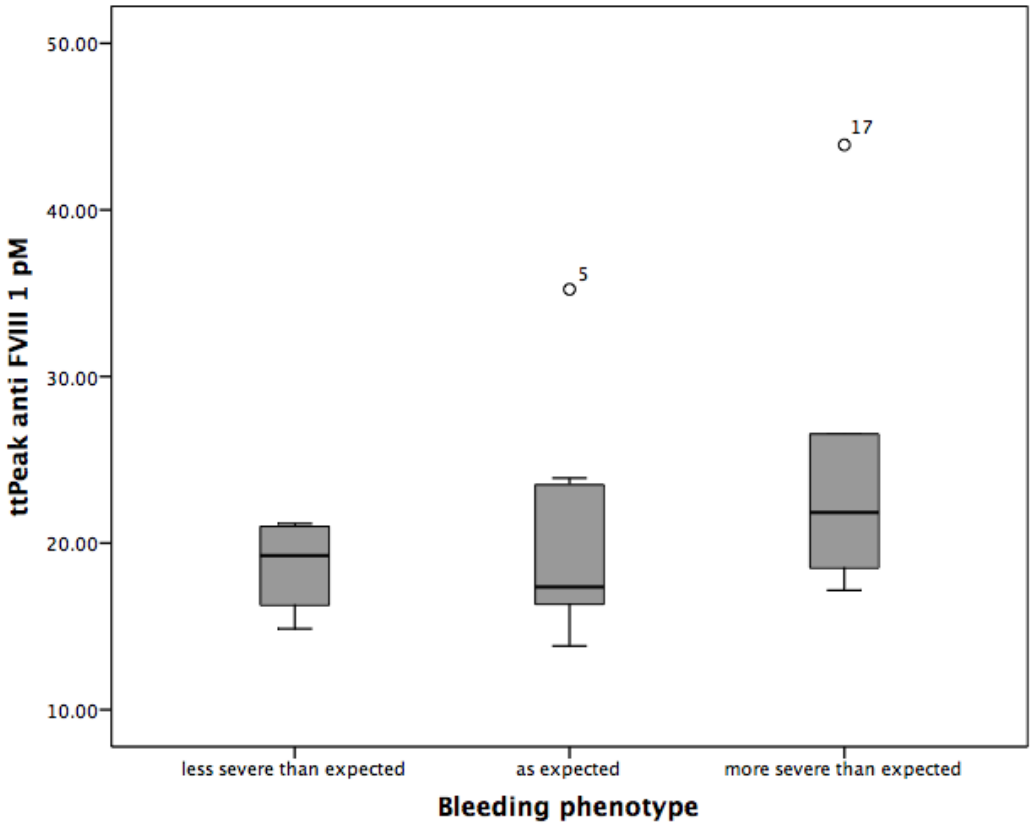


Figure 6-17 Boxplots for ttPeak values of samples with neutralised FVIII separated as per bleeding phenotype (s; min, 25th percentile (Q1), median, 75th percentile (Q3), max); trigger = 1 pM TF

Although there is a suggestion of prolongation of the ttPeak with worsening of the bleeding phenotype, the difference between categories was not statistically significant.

Table 6-5 Spearman correlations between free and total TFPI levels and ttPeak in samples with no FVIII activity; all TF concentrations and including normalised data (NR) for 2 pM TF samples

Correlations

			free TFPI	total TFPI	ttPeak anti FVIII 2 pM
Spearman's rho	free TFPI	Correlation Coefficient	1.000	.549*	.356
		Sig. (2-tailed)	.	.012	.124
		N	20	20	20
	total TFPI	Correlation Coefficient	.549*	1.000	.523*
		Sig. (2-tailed)	.012	.	.018
		N	20	20	20
	ttPeak anti FVIII 2 pM	Correlation Coefficient	.356	.523*	1.000
		Sig. (2-tailed)	.124	.018	.
		N	20	20	20
	NR - ttPeak anti FVIII 2 pM	Correlation Coefficient	-.096	.146	-.674**
		Sig. (2-tailed)	.686	.539	.001
		N	20	20	20
	ttPeak anti FVIII 1 pM	Correlation Coefficient	.321	.426	.787**
		Sig. (2-tailed)	.167	.061	.000
		N	20	20	20
	ttPeak anti FVIII 0.5 pM	Correlation Coefficient	.145	.194	.739**
		Sig. (2-tailed)	.542	.412	.000
		N	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Similarly to the LT results, there were strong correlations between total TFPI levels and ttPeak values in samples with the anti-FVIII antibody triggered with 2 pM TF (0.523; p = 0.018). The values were nearing significance for ttPeak and total TFPI in the samples triggered with 1 pM.

6.5.5.4 ETP

Measurement of ETP was limited at the lower concentrations of TF. The results for the samples with the anti-FVIII antibody triggered with 2 pM TF were examined. No significant difference was found between bleeding phenotypes, nor was there any correlation found between TFPI levels and ETP.

6.5.5.5 Rate and peak ratios

The thrombin generation curves for all patients were examined with the addition of the anti-FVIII antibody alone and with the combination of antibodies (anti-FVIII and anti-TFPI) (for examples of bleeding phenotypes 1 and 3 see Supplementary material 6.7 Figures 6–23 – 6–32). The addition of the anti-TFPI antibody improved the thrombin generation results for all patients.

Bearing in mind the inter-assay variability (particularly with the previous data relating to the use of Innovin[6]), other means of analysing the raw data were considered (in addition to normalisation of the 2 pM data) that would enable more robust comparison of patient data. The rate of thrombin generation was determined in addition to the ratio of peaks between the samples with FVIII neutralised and samples with both antibodies added.

Rate = Peak / (ttPeak – LT)

Peak ratio = Peak (anti-FVIII antibody) / Peak (combination of antibodies)

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[6] Inter-assay coefficients of variation for healthy volunteer (NRS 8) parameters: LT 19%; ETP 21%; peak 14%; ttPeak 13%

Statistical analysis was then undertaken to see if there was any difference in these parameters between the different bleeding phenotypes.

Rate

The rates of thrombin generation for samples with just the anti-FVIII antibody and the combination of antibodies were compared between bleeding phenotypes and no statistical significance was found (data not shown). The data were subsequently analysed to see whether there was any correlation between free or total TFPI levels and rate. Spearman's correlation was performed (due to the fact that some data were non-parametric and there were outliers). There were no significant findings for free TFPI and the rate parameters, however there was a strong degree of correlation between total TFPI levels and the rate of thrombin generation in the samples with the anti-FVIII antibody triggered with 2 pM TF ($\rho(18) = - 0.544$; $p = 0.013$; see Table 6-6). Thus, the higher the total TFPI levels, the lower the rate of thrombin generation in those samples without FVIII activity.

Table 6-6 Spearman's rank order correlation for free and total TFPI and rate parameters

Correlations

			Free TFPI	Total TFPI
Spearman's rho	Free TFPI	Correlation Coefficient	1.000	.549*
		Sig. (2-tailed)	.	.012
		N	20	20
	Total TFPI	Correlation Coefficient	.549*	1.000
		Sig. (2-tailed)	.012	.
		N	20	20
	Rate - anti FVIII 2 pM	Correlation Coefficient	-.322	-.544*
		Sig. (2-tailed)	.166	.013
		N	20	20
	Rate - anti FVIII 1 pM	Correlation Coefficient	-.190	-.248
		Sig. (2-tailed)	.423	.292
		N	20	20
	Rate - anti FVIII 0.5 pM	Correlation Coefficient	-.156	-.099
		Sig. (2-tailed)	.510	.677
		N	20	20
	Rate - combination 2 pM	Correlation Coefficient	-.041	-.165
		Sig. (2-tailed)	.863	.486
		N	20	20
	Rate - combination 1 pM	Correlation Coefficient	-.166	-.338
		Sig. (2-tailed)	.484	.145
		N	20	20
	Rate - combination 0.5 pM	Correlation Coefficient	.277	-.089
		Sig. (2-tailed)	.237	.710
		N	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Peak ratio

Peak ratio results were initially analysed by bleeding phenotype (see Figures 6-18 – 6-20). There was a notable outlier in the 'as expected' group data sets triggered with 1 pM and 0. 5 pM TF and this was excluded for the data analysis. One-way ANOVA was then performed to assess whether the differences between groups were statically significant (see Table 6-7).

As the between groups comparison was statistically significant for the 1 pM and 0.5 pM groups, post hoc analysis was performed in the form of independent t testing (see Table 6-8). When a Bonferroni correction was applied (making $p < 0.017$ statistically significant), there was no significant difference between the groups (including between the 'less severe than expected' and 'more severe than expected' groups).

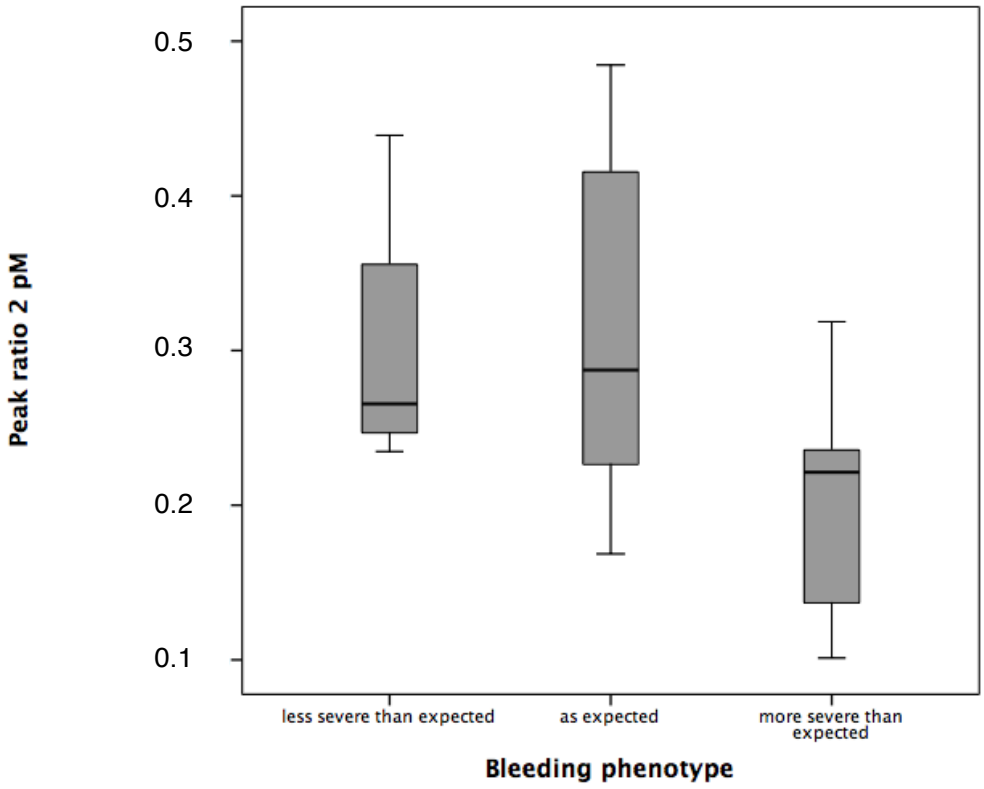


Figure 6-18 Peak ratio data (PPP + anti-FVIII)/(PPP + anti-FVIII and anti-TFPI) displayed by bleeding phenotype (min, 25th percentile (Q1), median, 75th percentile (Q3), max); trigger = 2 pM TF

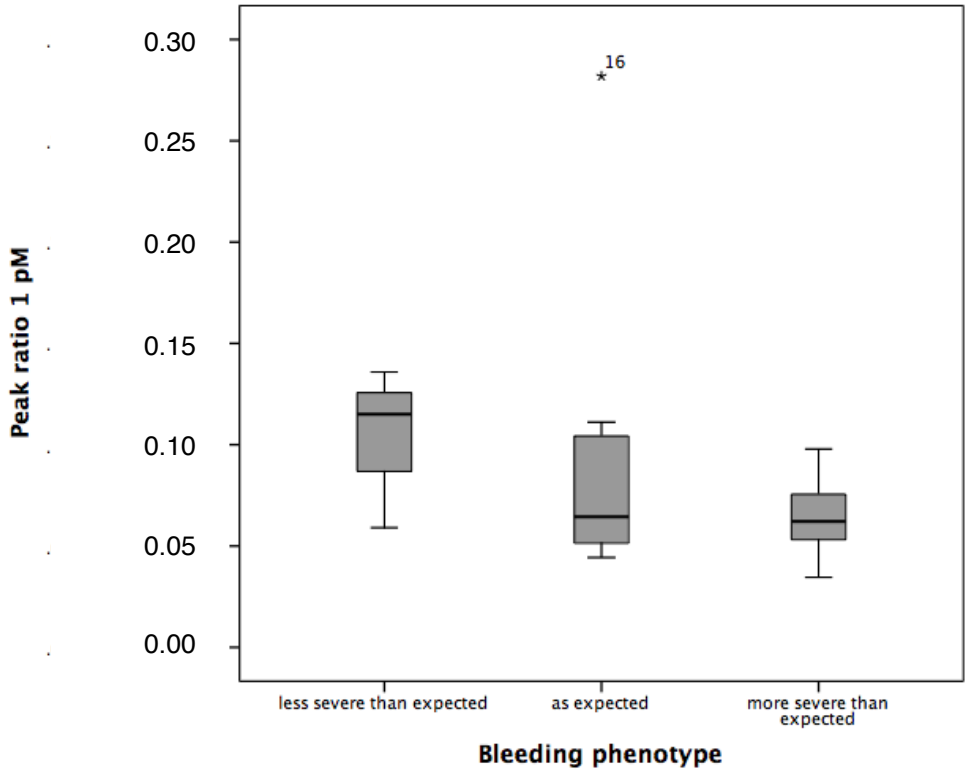


Figure 6-19 As above but trigger 1 pM TF

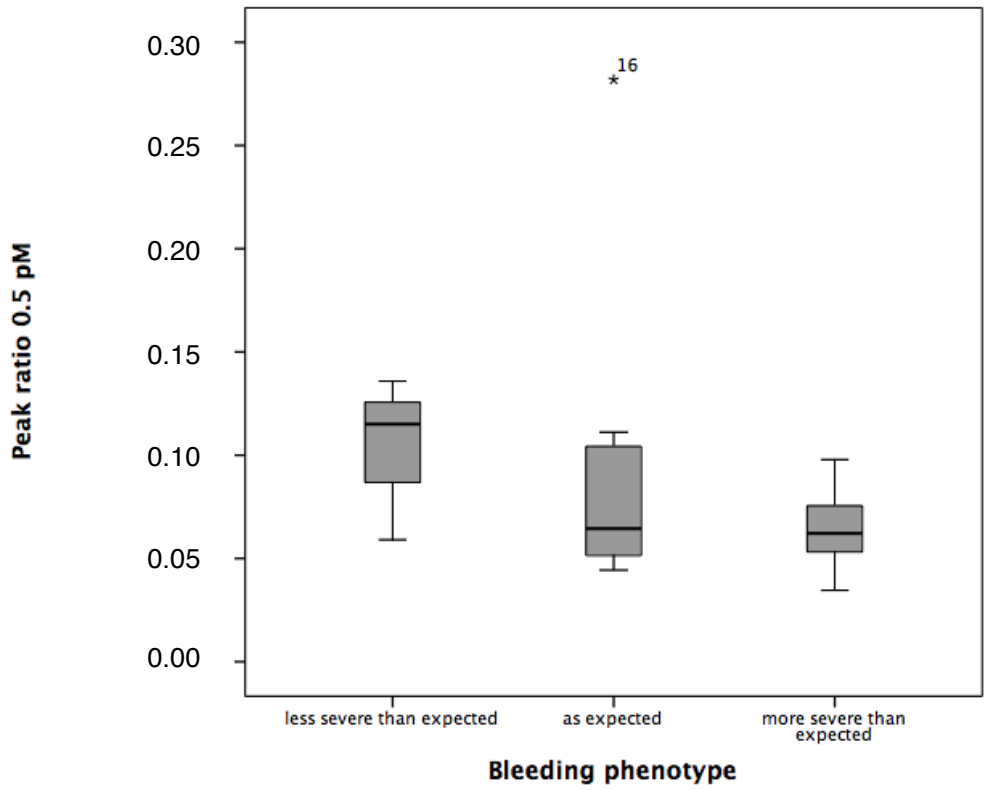


Figure 6-20 As above but trigger 0.5 pM TF

Table 6-7 One-way ANOVA for differences in peak ratio between bleeding phenotypes (outlier removed)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Peak ratio 2 pM	Between Groups	.034	2	.017	2.026	.164
	Within Groups	.134	16	.008		
	Total	.168	18			
Peak ratio 1 pM	Between Groups	.005	2	.002	3.703	.048
	Within Groups	.010	16	.001		
	Total	.015	18			
Peak ratio 0.5 pM	Between Groups	.005	2	.002	3.703	.048
	Within Groups	.010	16	.001		
	Total	.015	18			

Table 6-8 Independent t test results - less severe versus more severe phenotype (Bonferroni correction – p value significant if < 0.017)

	Levene's Test for Equality of Variances		**Independent t test**		
	F	**Sig.**	**t**	**df**	**Sig. (2-tailed)**
Peak ratio 2 pM	0.141	0.718	1.768	8	0.115
Peak ratio 1 pM	0.726	0.419	2.476	8	0.038
Peak ratio 0.5 pM	0.726	0.419	2.476	8	0.038

Further analysis was performed to see if there was a correlation between free and total TFPI levels and peak data from the samples just with the addition of the anti-TFPI antibody. No associations were found for free TFPI, however there was a strong relationship between total TFPI levels and the normalised peak values for the samples treated with the anti-TFPI antibody alone ($\rho(17) =$

0.728, $p < 0.0005$; see Table 6-9). Thus, the higher the total TFPI level, the higher the peak value when the TFPI was neutralised – a logical finding. In addition, the total TFPI levels also correlated negatively to a moderate degree with the peak ratio triggered with 2 pM TF ($\rho(17) = -0.495$, $p < 0.027$). This meant that the higher the total TFPI, the greater the difference between the peaks and, thus, the smaller the ratio (see Figure 6-21).

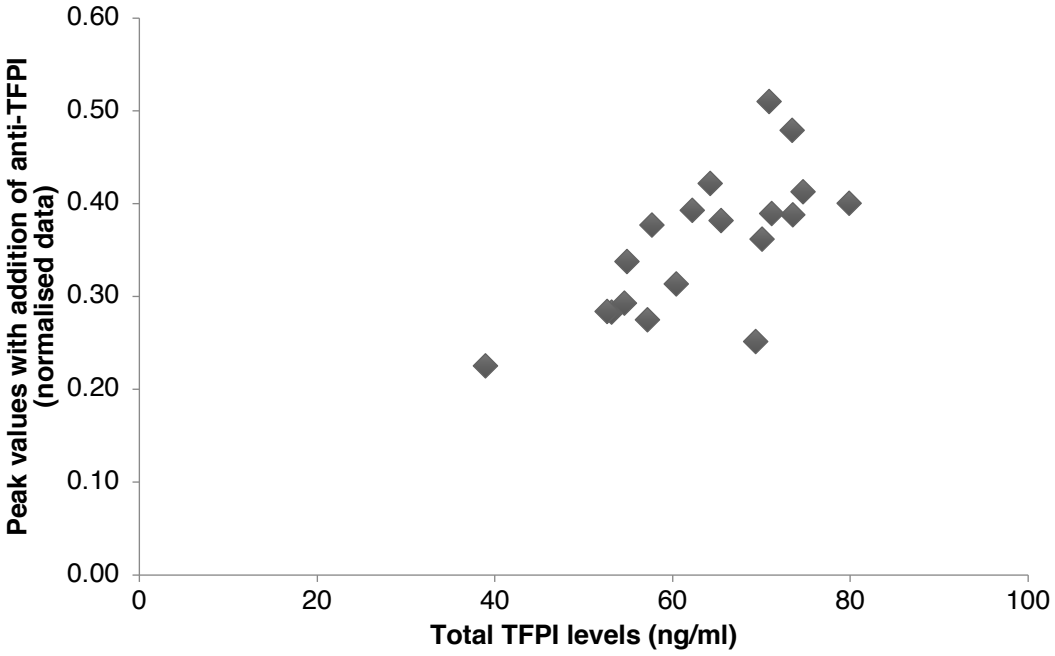


Figure 6-21 Plot of total TFPI levels against peak values (normalised against healthy control) of samples with addition of anti-TFPI antibody. The higher the total TFPI level, the greater the TGA Peak once the neutralising antibody was added.

Table 6-9 Spearman's correlation between free and total TFPI and TGA peak data; peak anti-TFPI = peak value with the addition of anti TFPI antibody; NR – Peak anti-TFPI = normalised data (NR); Peak ratio 2 pM = peak ratio between samples with addition of anti-FVIII antibody and samples with both antibodies, triggered with TF 2 pM

Correlations

			free TFPI	total TFPI	Peak-anti TFPI
Spearman's rho	free TFPI	Correlation Coefficient	1.000	.549*	.037
		Sig. (2-tailed)	.	.012	.881
		N	20	20	19
	total TFPI	Correlation Coefficient	.549*	1.000	.044
		Sig. (2-tailed)	.012	.	.858
		N	20	20	19
	Peak-anti TFPI	Correlation Coefficient	.037	.044	1.000
		Sig. (2-tailed)	.881	.858	.
		N	19	19	19
	NR - Peak anti TFPI	Correlation Coefficient	.422	.728**	-.570*
		Sig. (2-tailed)	.072	.000	.011
		N	19	19	19
	Peak ratio 2 pM	Correlation Coefficient	-.255	-.495*	.182
		Sig. (2-tailed)	.278	.027	.455
		N	20	20	19

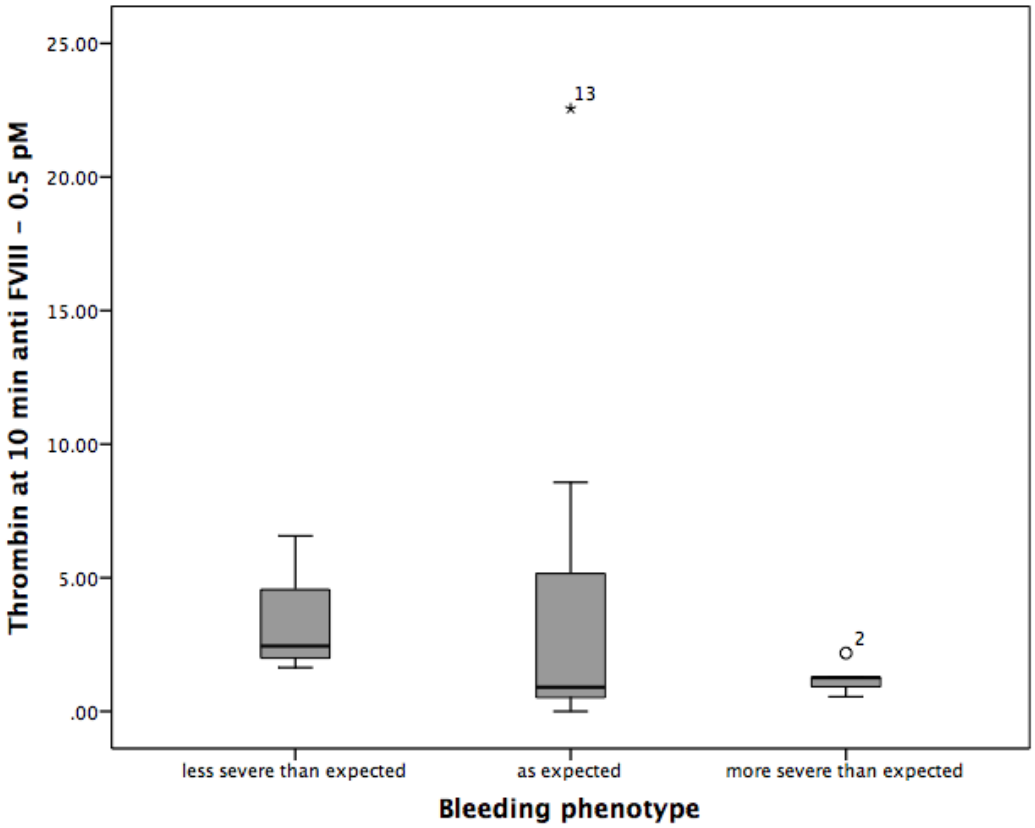
*. Correlation is significant at the 0.05 level (2-tailed).

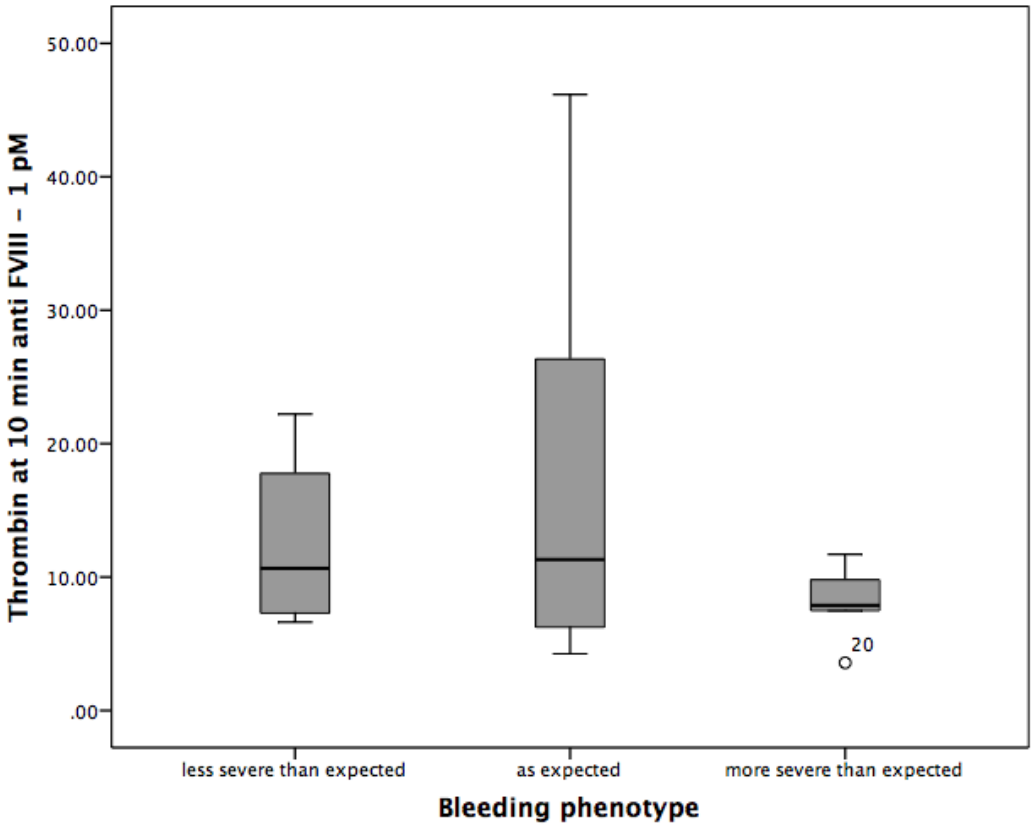
**. Correlation is significant at the 0.01 level (2-tailed).

6.5.5.6 Data at 10 minutes

Part of the interest in running the samples at a range of TF concentrations was to see if there were differences in sample activation i.e. a threshold effect. After assessing the TGA curves, it was decided to assess thrombin values at 10 minutes.

None of the results – either comparing between phenotypes or assessing for a correlation with free or total TFPI – were statistically significant. However, Figure 6-22 depicts the raw results (thrombin nM) in the form of box plots for the different categories. The lower concentrations (0.5 and 1 pM) appear more sensitive to differences with the results from those with the more severe phenotype being less variable and lower (especially at 0.5 pM). This sensitivity is lost at 2 pM with a fairly even spread of results.


Thrombin at 10 min anti FVIII – 0.5 pM
25.00
20.00
15.00
10.00
5.00
.00
13
2
less severe than expected
as expected
more severe than expected
Bleeding phenotype


Thrombin at 10 min anti FVIII – 1 pM
50.00
40.00
30.00
20.00
10.00
.00
20
less severe than expected
as expected
more severe than expected
Bleeding phenotype

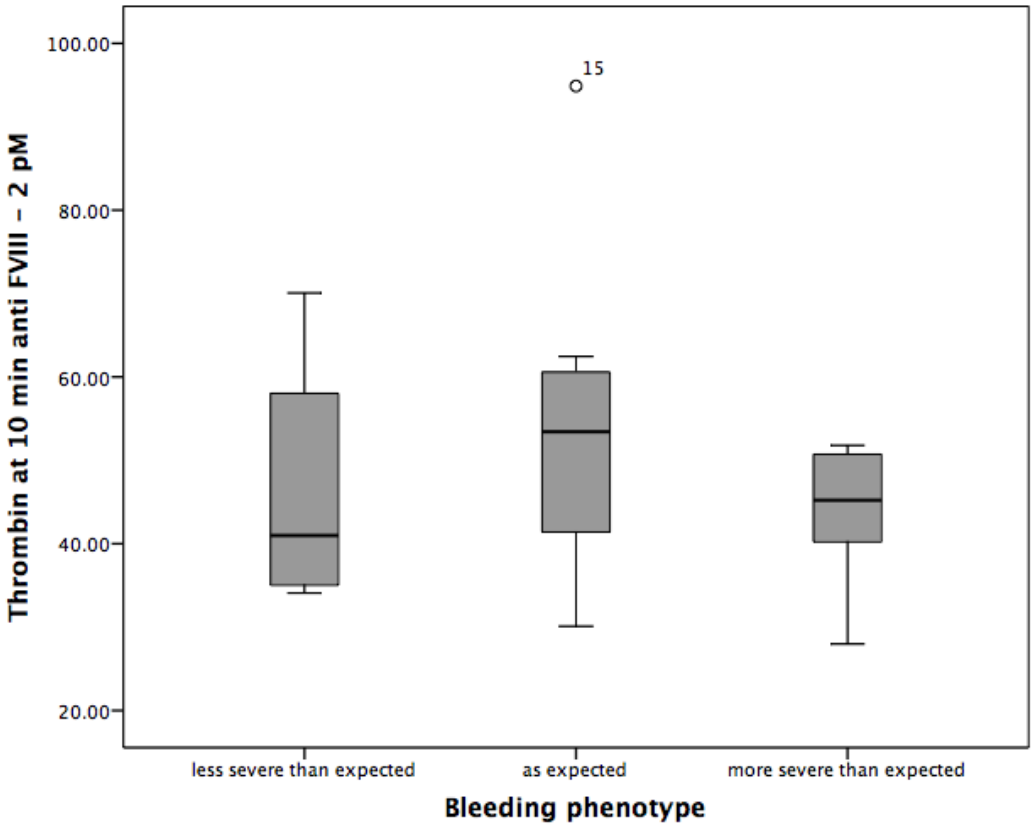


Figure 6-22 Comparison of raw thrombin results (nM) at 10 minutes triggered with increasing concentrations of TF

6.5.6 TGA parameters in the absence of FVIII – correlation between TF, FVII, FVIIa and FVIIa.AT complex

In light of the findings from the cross-sectional study, the results of the other study assays were examined with relation to the TGA findings. The samples of interest were those treated with the anti-FVIII antibody (baseline thrombin generation in the absence of FVIII) including normalised data, rate, peak ratio and thrombin value at 10 minutes. Again, this was performed by Spearman correlation in light of the non-parametric distribution and outliers. There were no significant correlations found for FVIIa or FVIIa.AT complex.

Table 6-10 Spearman correlation results for TF and FVII and TGA parameters; samples treated with anti-FVIII antibody; significant results in bold/shaded, lightly shaded area = other results nearing significance

All samples with anti-FVIII antibody	TF		FVII	
	Corr. coefficient	p value	Corr. coefficient	p value
LT 2 pM	-0.11	0.964	**0.514**	**0.020**
NR – LT 2 pM	0.101	0.672	-0.204	0.388
LT 1 pM	0.160	0.499	0.432	0.057
LT 0.5 pM	0.247	0.294	0.395	0.085
Peak 2 pM	-0.212	0.369	-0.279	0.233
NR Peak 2 pM	-0.086	0.719	-0.157	0.507
Peak 1 pM	-0.258	0.273	-0.264	0.260
Peak 0.5 pM	-0.318	0.172	0.014	0.955
ttPeak 2 pM	0.145	0.543	0.441	0.051
NR - ttPeak 2 pM	-0.025	0.917	-0.273	0.245
ttPeak 1 pM	0.126	0.596	0.413	0.070
ttPeak 0.5 pM	0.142	0.550	0.271	0.247
Rate 2 pM	-0.181	0.444	-0.310	0.183
Rate 1 pM	-0.185	0.434	-0.302	0.196
Rate 0.5 pM	-0.221	0.348	-0.043	0.857
Peak ratio 2 pM	-0.045	0.850	-0.271	0.247
Peak ratio 1 pM	**-0.517**	**0.019**	0.018	0.940
Peak ratio 0.5 pM	**-0.517**	**0.019**	0.018	0.940
Thrombin (pM) at 10 min 2 pM	-0.191	0.419	-0.326	0.160
Thrombin (pM) at 10 min 1 pM	-0.224	0.356	**-0.585**	**0.009**
Thrombin (pM) at 10 min 0.5 pM	-0.357	0.123	-0.359	0.120

FVII levels were found to be predictive of bleeding phenotype in the cross-sectional study with lower levels predictive of a 'less severe' phenotype (see Chapter 5). There was a strong positive (and statistically significant) correlation between FVII and the LT values from the samples triggered with 2 pM (0.514; p

= 0.020). The lower concentrations of TF had values nearing significance, perhaps reflecting the limited numbers of patients in the study. Thus the higher the FVII, the longer the LT – a finding consistent with the previous findings. The results for ttPeak also showed a similar pattern, but did not quite reach significance. There was no correlation with ETP, Peak, rate or peak ratio (between samples with neutralised FVIII with and without the anti-TFPI antibody).

There was also a strong correlation between FVII and the thrombin value at 10 minutes (-0.585; p = 0.009). This was an inverse relationship, i.e. the higher the FVII level, the lower the thrombin result. This would also fit with the above findings.

The only significant correlation found for TF was a strong inverse correlation between TF and the peak ratio using the 1 pM and 0.5 pM TF trigger (-0.517; p = 0.019). So the higher the TF, the lower the ratio between peaks i.e. the greater the difference between the peak results.

6.6 Discussion

Phenotypic heterogeneity is increasingly recognised in patients with severe haemophilia. This sub-study formed part of a larger study trying to identify factors that may contribute to bleeding phenotype. 20 patients with haemophilia A (moderate and severe) with a mix of underlying genetic mutations and phenotypes were selected from the larger study. PPP thrombin generation was then conducted with combinations of neutralising antibodies targeting FVIII and TFPI. The TGA was run in triplicate with a range of TF concentrations (2 pM, 1

pM, 0.5 pM) to assess for differences in sensitivity of the assay. The neutralising anti-FVIII antibody was utilised in order to abolish any residual endogenous or exogenous FVIII activity as the majority of patients were receiving prophylaxis. The anti-TFPI antibody was used to see if this could help in understanding the contribution of TFPI to the suppression of thrombin generation, as previous work has found lower levels in patients with haemophilia B as well as also correlating with severity of the disease in both A and B [Tardy-Poncet *et al.*, 2011]. These findings were not replicated in the initial cross-sectional study.

When TGA was conducted on samples with no residual FVIII (i.e. with the addition of an anti-FVIII antibody), 30% of patients had peak values within the lower end of the normal range. When results were compared between samples with and without the anti-FVIII antibody that had one-stage clotting assay and chromogenic assay FVIII results of < 1 IU/dL, patients with no functional product could be identified by curves that were superimposed (including patients both with the intron 22 inversion and other mutations).

Addition of the anti-TFPI antibody – the efficacy of which was demonstrated both by comparison with samples from a patient treated with an anti-TFPI monoclonal antibody as part of a clinical trial and free and total TFPI ELISAs – had a universally positive effect in augmenting thrombin generation to within the normal range.

Free and total TFPI levels were compared to the TGA parameters in the samples with neutralised FVIII. This demonstrated that total TFPI correlated strongly with the LT, ttPeak and the Peak with increasing levels associated with

prolongation of the velocity parameters and reduction in the peak, findings in keeping with the physiological role of TFPI. In addition, the rate of thrombin generation in the absence of FVIII also correlated strongly with total TFPI indicating that the higher the levels of total TFPI, the more reduced the rate of thrombin generation. Interestingly, there were no significant correlations with free TFPI i.e. the plasma TFPI component not bound to phospholipids.

When the individual TGA parameter results from plasma with neutralised FVIII were compared with bleeding phenotype, the LT and ttPeak were prolonged in those with a 'more severe than expected' phenotype. The peak values were correspondingly lower in this category. The TGA therefore demonstrated sensitivity to patients with a greater propensity to bleed.

Upon assessment of correlation between levels of the extrinsic pathway components studied in the cross-sectional study and the TGA parameters, increases in FVII levels were shown to correlate strongly with prolongation of the LT as well as lower thrombin levels at 10 minutes, findings that were consistent with that of the multinomial regression analysis performed in the cross-sectional study that found that higher FVII levels predicted a more severe phenotype.

Do these findings agree with the literature? Alterations in the levels of various clotting factors and their influence on thrombin generation has been studied previously in a synthetic plasma system [Butenas *et al.*, 1999]. This assessed variation in pro- and anti-coagulants between 50% and 150% (i.e. 50-150 IU/dL) in various combinations. Changes in the level of FVII were found to have a negligible effect on the initiation phase, peak thrombin level or overall amount of

thrombin generated. However, these samples were triggered with higher levels of TF (5 pM) that may have influenced the sensitivity of the assay to variation and also differs from this study in that the role of FVII or other factors was not assessed in the pathological state of complete absence of FVIII.

Thrombin generating capacity in plasma from patients with haemophilia A and B has also previously been examined ([Dargaud *et al.*, 2005]. PPP samples from an initial 20 patients were triggered with 5, 2.5 and 1 pM TF (all containing 4 μM phospholipid) and 1 pM TF was chosen for further experiments. 34 patients with haemophilia A and 12 with haemophilia B were studied with levels of FVIII/FIX ranging from 1-37 IU/dL. There was a significant correlation found in the patients with haemophilia A between FVIII level and ETP, peak and ttPeak. The sample size for the patients with haemophilia B was too small to detect significant correlations. The authors state that all parameters were clearly abnormal in patients with haemophilia. They also considered bleeding phenotype by a measure of radiological indicator (Pettersson score), patients receiving prophylaxis after recurrent serious bleeding episodes and those with a history of spontaneous bleeds (> 30 episodes). Regardless of FVIII level, they found a reduction of ETP to < 50% of normal in patients with a severe phenotype. However, there were patients with both haemophilia A and B that were the exception and had a reduced ETP but milder phenotype. The authors did emphasise the impact of residual white cells and platelets on thrombin generation and considered double centrifugation the most important pre-analytical variable.

Another group performed TGA for 23 individuals with haemophilia A (from 9 families) with additional assessment of pro-thrombotic risk factors. The patients were evaluated in terms of phenotypic severity and incorporated a range of mild, moderate and severe patients. It was not clear whether the patients were receiving prophylaxis. The findings of samples triggered with 1 pM TF demonstrated sensitivity of the assay to mild, moderate and severe phenotypes (although the FVIII levels of the individuals are not documented). The samples were then spiked with exogenous FVIII concentrate and this demonstrated that increasing FVIII levels corresponded with increased thrombin generation. No impact was found from prothrombotic mutations (such as FV Leiden mutation) on bleeding phenotype. The authors did find that there was considerable variability in TGA results in patients with undetectable FVIII activity, however the assay did not discriminate between different family members with different bleeding phenotypes [Beltran-Miranda *et al.*, 2005].

A further study has looked at the ETP in PRP from patients with severe haemophilia A and B [Siegemund *et al.*, 2003]. Samples were run with adjusted and unadjusted platelet counts. It is not clear what final concentration of TF was used as a trigger. The authors found a linear relationship in increase in ETP with an increase in platelet count to a mean of 100 x 10^9/L, beyond which there was a negligible effect. Mean residual FVIII/IX levels were between 2-3 %. They found that the influence of platelets decreased as the FVIII or FIX level increased. There was also a difference in mean values between A and B, with lower mean ETP results in patients with haemophilia B.

Salinas et al. used the thromboelastogram (TEG) and TGA to compare patients with haemophilia A with and without inhibitors – based on the hypothesis that patients without inhibitors (even with undetectable FVIII levels) have some activity and those with inhibitors have none (an assumption that is incorrect as some mutations lead to complete absence of functional product of the F8 gene). TGA was performed on PPP with the PPP Low reagent (~ 1 pM TF concentration). Samples from healthy volunteers and patients with haemophilia were also spiked with a polyclonal goat anti-FVIII antibody to replicate the presence of an inhibitor. They demonstrated that TEG demonstrates reduced clot kinetics in patients with inhibitors compared to those without and they suggest that this is due to residual low levels of FVIII in the non-inhibitor patients (this could be abolished by spiking with an anti-FVIII antibody). They were not able to discriminate between inhibitor and non-inhibitor patients with the TGA [Salinas *et al.*, 2015].

It must be borne in mind that the study conducted by the author only assessed the results of thrombin generation in PPP and thus only gives information about part of the haemostatic process and critically does not reflect the *in vivo* role of platelets and the vascular endothelium (the latter incorporates a significant proportion of the TFPI compartment, in addition to the platelet component). The study was designed in light of findings suggesting a role for TFPI in the severity of haemophilia and the expectation was perhaps that more would be gleaned about patients with a less severe bleeding phenotype. However, the results of both the cross-sectional and the TGA studies have perhaps cast more light on

those with a more severe phenotype and have found unexpected associations between FVII, FVIIa as well as levels of total TFPI and phenotype and TGA results.

Interestingly, work has been done looking at the impact of FVII on thrombin generation with the use of FVIIa in patients with haemophilia in mind [Van't Veer *et al.*, 2000]. This was done in a reconstituted pro-coagulant model using purified coagulation factors. A solution was composed containing relipidated TF, FV, FVIII, prothrombin, FIX, FX, FVIIa (at physiological levels) and varying concentrations of FVII. The authors found that increasing the concentration of FVII extended the lag phase and depressed the rate of thrombin generation. In the absence of FVIII, FVII significantly inhibited the TF initiated thrombin generation by 100 pM/L FVIIa. The absence of FVII resulted in a 5-fold enhancement of maximal thrombin generation. Increasing the concentration of FVIIa was able to overcome the inhibitory effect of its zymogen and this was also the case when TFPI was added to the system. They also found that the presence of FVII delayed the generation of FXa. These findings lend weight to the findings of this study.

Upon endothelial injury, both FVII and FVIIa can bind to exposed TF. However, of the resultant complexes, the FVIIa.TF complex is about 100-fold more active that the FVII.TF complex. There are therefore two competing reactions [Nemerson, 1983]. At baseline, when there are only low levels of TF, this is principally bound by the inactive FVII forming a low activity complex. Given that there are lower levels of FVIIa, FVII acts as a functional inhibitor of the FVIIa.TF complex until the levels of FVIIa rise to swamp the inactive zymogen. This

requires exposure and release of sufficient TF to signal significant tissue damage.

Thus, the results of this study have confirmed the sensitivity of the TGA to bleeding phenotype in patients with moderate and severe haemophilia A. It has demonstrated a marked impact of the neutralisation of TFPI with normalisation of peak results. Total TFPI levels correlate strongly with TGA parameters indicating that the higher the levels, the more suppressed the thrombin generating capacity. This perhaps underlines the importance of phospholipid in effective haemostasis (given the lack of correlation with free TFPI). Also, raised FVII levels correlate with prolonged LT and decreased thrombin results at 10 minutes. These findings are consistent with the model studied by van't Veer at al.

Thus higher FVII levels may compete with FVIIa and paradoxically suppress the generation of FXa and thrombin generation. This hints at the competitive interplay between substrates at a basal level of activation of haemostasis in the absence of the cofactor FVIII. The mechanisms driving the differing levels of FVII and FVIIa as well as TFPI levels in these patients are, as yet, unclear. However, with further study, they may provide valuable baseline information for clinicians on the predicted bleeding phenotype or patients and could thus be used to design individualised prophylaxis protocols.

6.7 Supplementary material – TGA data

Bleeding phenotype 3 – more severe than expected

Neutralising FVIII antibody alone and with addition of anti-TFPI antibody

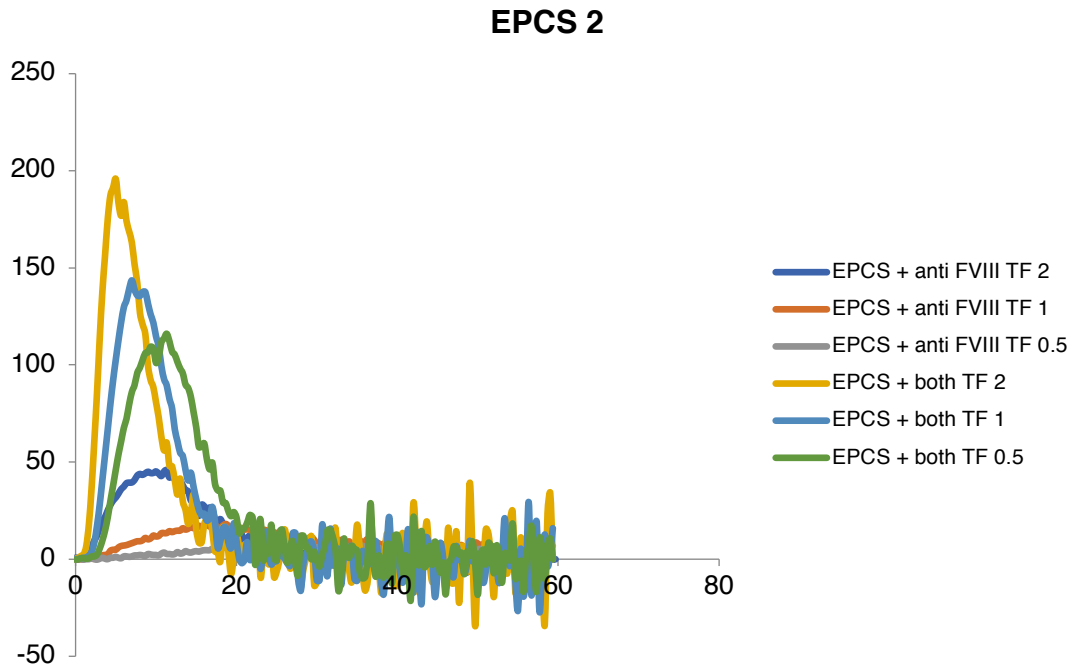


Figure 6-23 Patient samples with anti-FVIII antibody alone and combination of anti-FVIII and anti-TFPI antibodies; bleeding phenotype 3

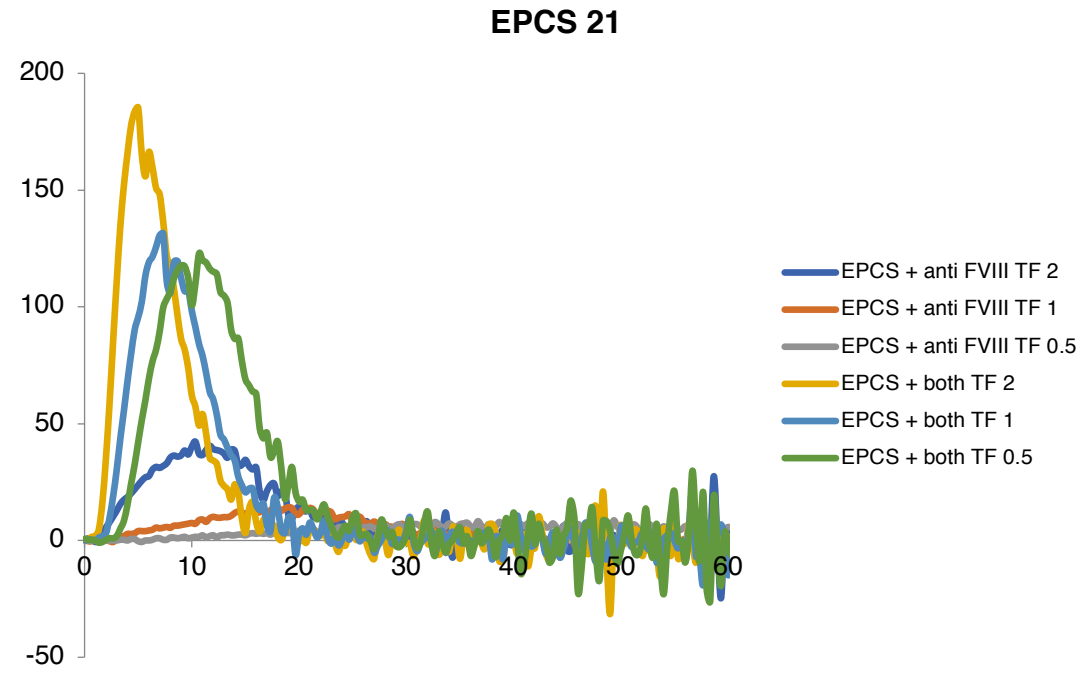


Figure 6-24 As above; bleeding phenotype 3

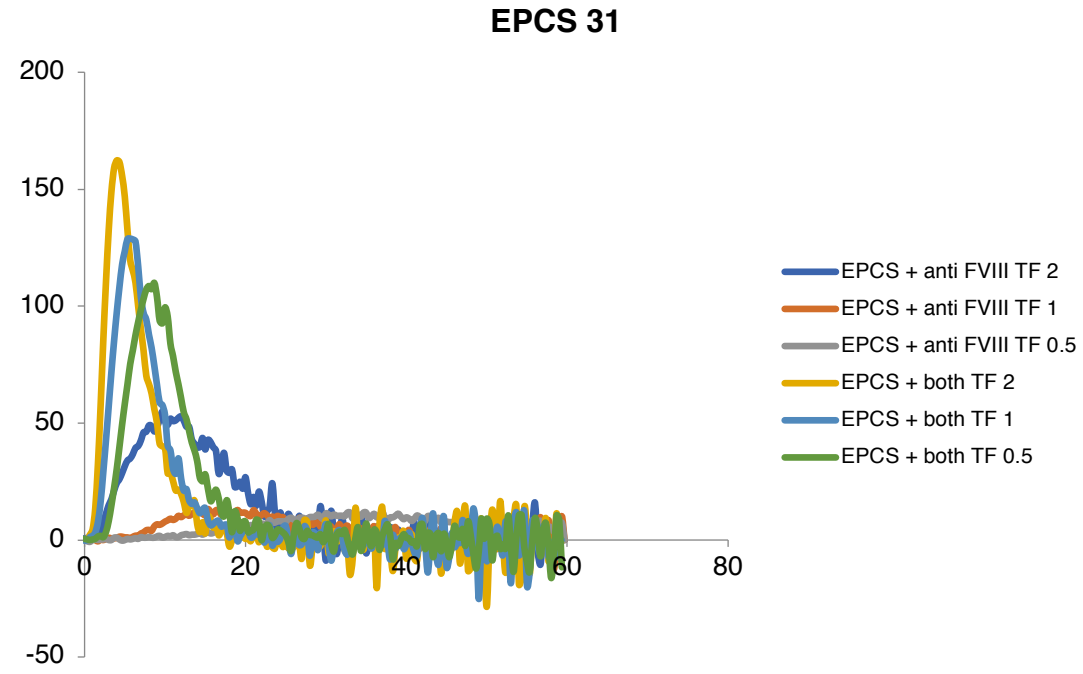


Figure 6-25 As above; bleeding phenotype 3

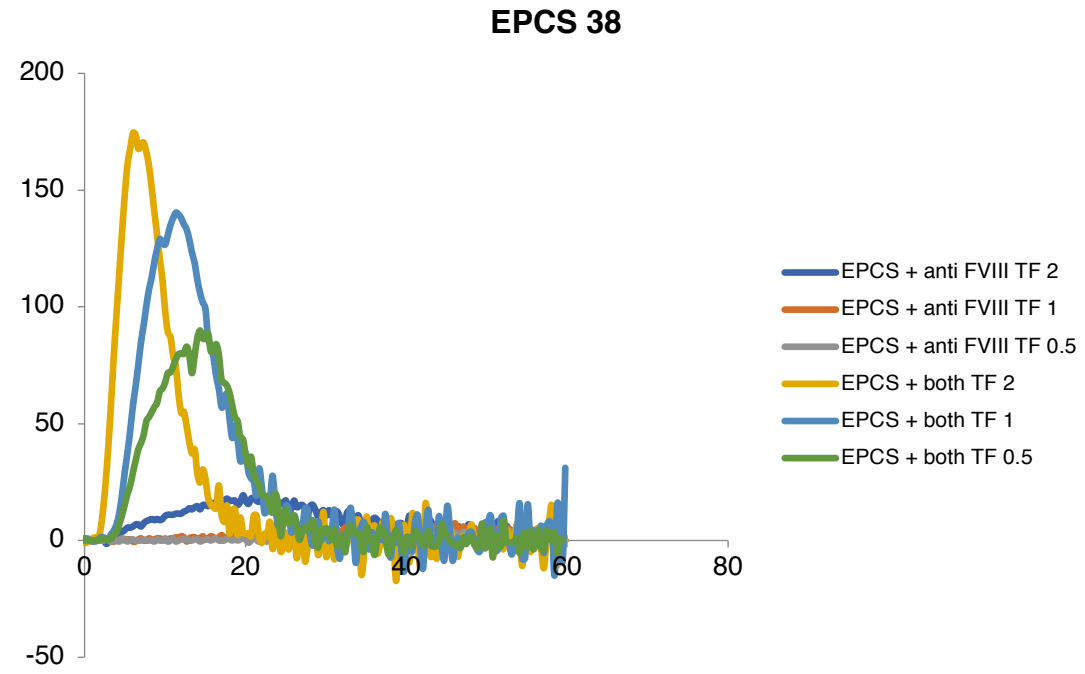


Figure 6-26 As above; bleeding phenotype 3

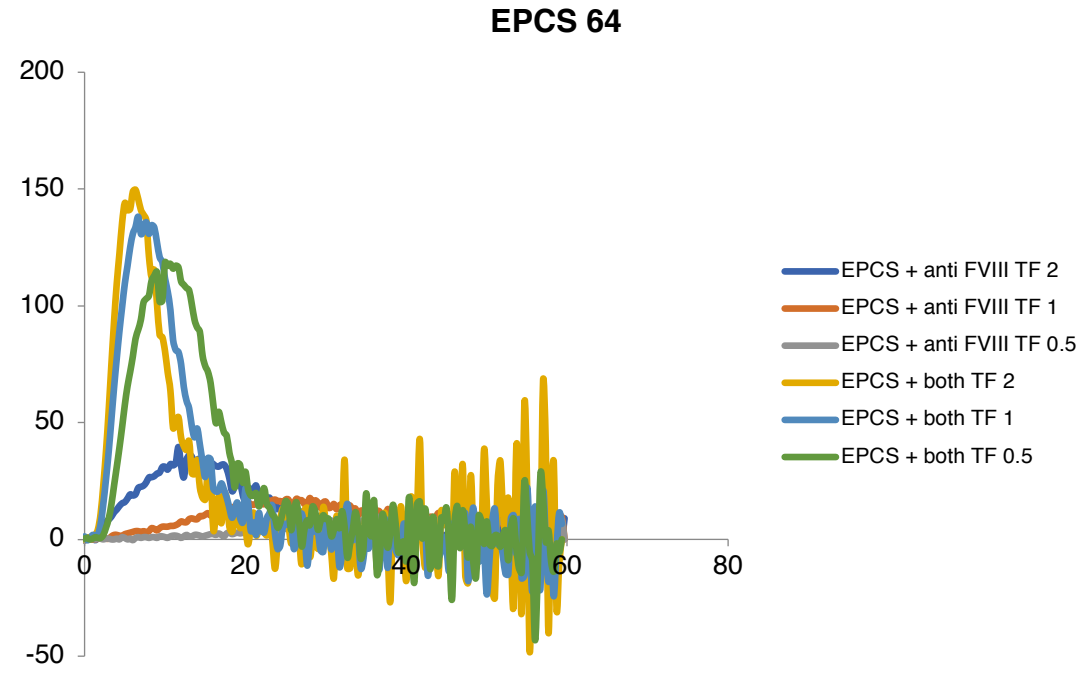


Figure 6-27 As above; bleeding phenotype 3

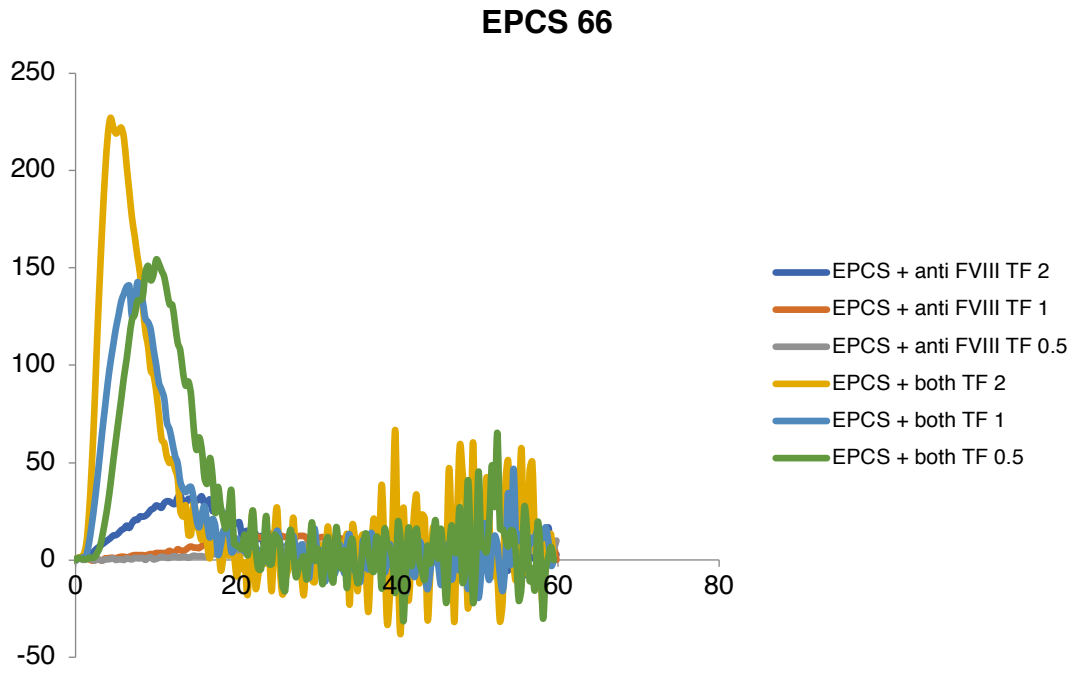


Figure 6-28 As above; bleeding phenotype 3

Bleeding phenotype 1 – less severe than expected

Neutralising FVIII antibody alone and with addition of anti-TFPI antibody

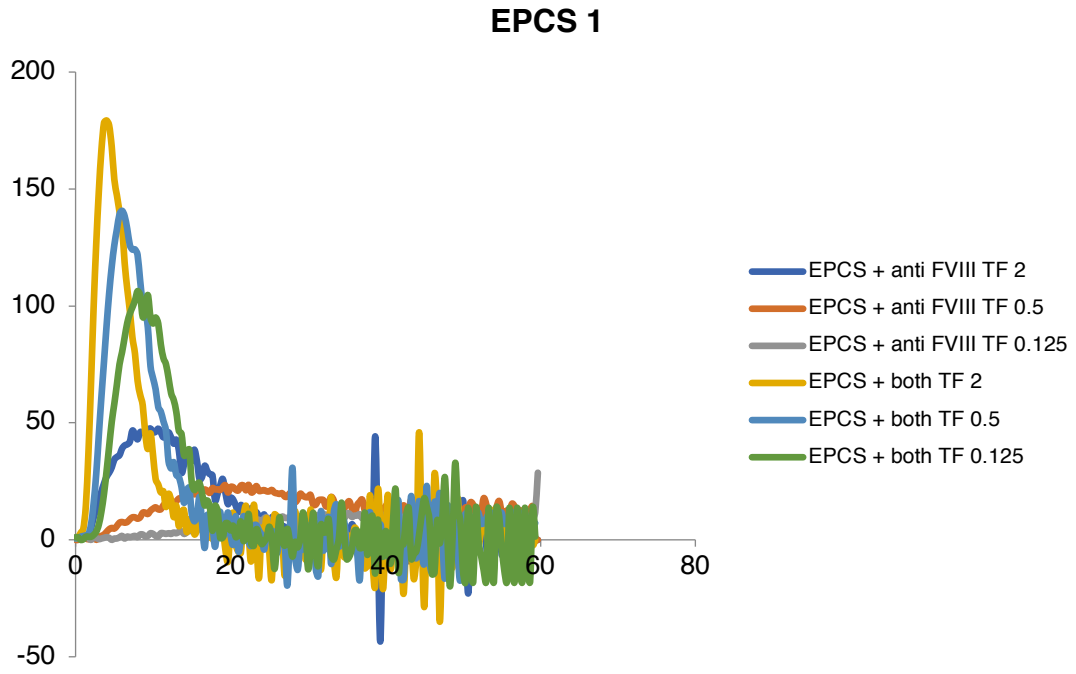


Figure 6-29 As above; bleeding phenotype 1

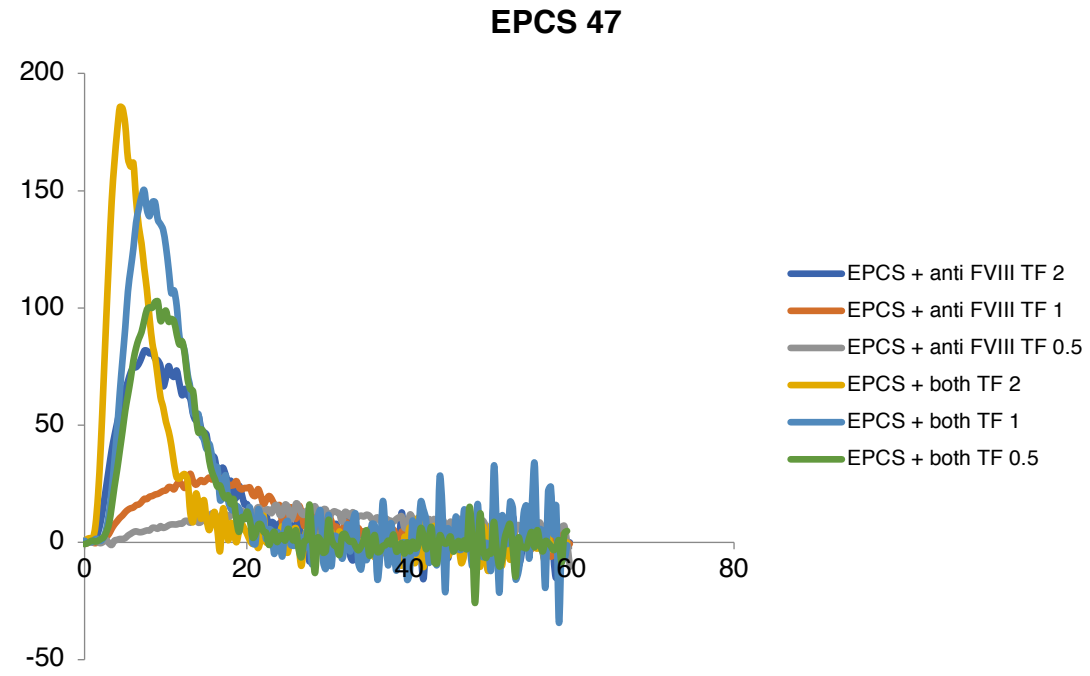


Figure 6-30 As above; bleeding phenotype 1

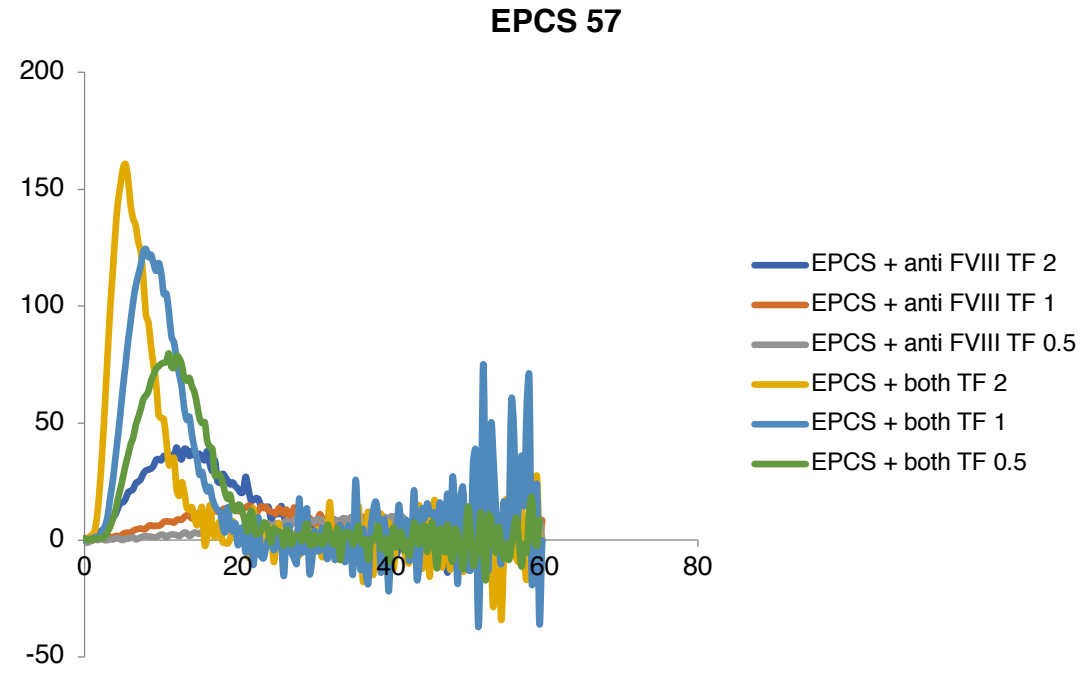


Figure 6-31 As above; bleeding phenotype 1

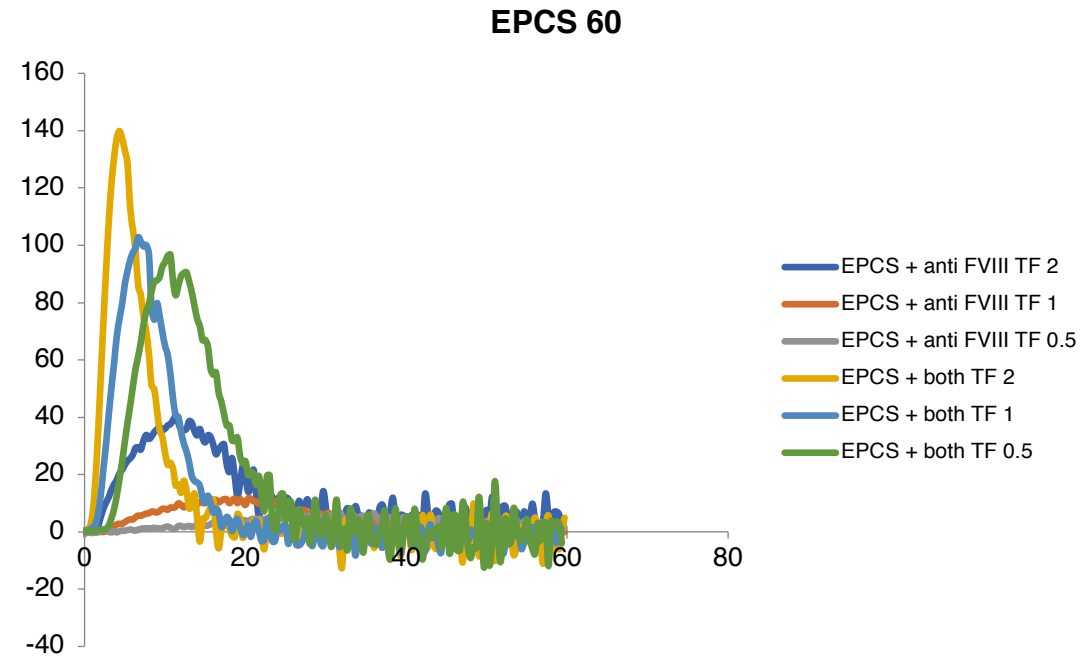


Figure 6-32 As above; bleeding phenotype 1

6.8 Supplementary material - calculations

Calculations involving anti-FVIII antibody

Sheep polyclonal neutralising anti-human FVIII antibody

Activity = 2278 BU/mg for both lots used in study

Aiming to add similar volumes of antibody to plasma aliquots; control samples run with addition of equal volume of buffer (TBS/1%BSA)

Lot 1: 25.9 mg/ml

1 mg = 1000 μl/25.9 mg = 38.6 μl

1:10 dilution i.e. 228 BU/mg/38.6 μl

600 μl and 200 μl aliquots of plasma

$C_1V_1 = C_2V_2$ *C = concentration; V = volume*

(600 μl x 20 BU)/228 BU = 52.6 μl added to 547.4 μl plasma[7]

(200 μl x 20 BU)/228 BU = 17.5 μl added to 182.5 μl plasma[8]

Lot 2: 9.6 mg/ml

1 mg = 1000 μl/9.6 mg = 104.17 μl

1:5 dilution i.e. 455.6 BU/mg/104.17 μl

600 μl aliquot – 70.5 μl added to 529.5 μl plasma

[7] 80 μl buffer added to 520 μl plasma

[8] 20 μl buffer added to 180 μl plasma

200 μl aliquot – 23.5 μl added to 176.5 μl plasma

Calculations involving anti-TFPI antibody

Sheep polyclonal anti-human TFPI antibody

No activity data available

9.3 mg/ml

Aiming for final concentration of 0.2 mg/ml in assay

1:5 dilution i.e. 1.86 mg/ml

(600 μl x 0.2 mg)/1.86 mg = 64.5 μl added to 535.5 μl plasma

(200 μl x 0.2 mg)/1.86 mg = 21.5 μl added to 178.5 μl plasma

Calculations for Innovin dilutions

Preparation of 2 pM, 1 pM and 0.5 pM final concentrations of TF using Innovin as the TF source

TF concentration for batch used for all assays calculated to be ~8000 pM using ACTICHROME® TF assay (see Chapter 3: Supplementary material 3.8)

1:10 dilution, followed by a 1:4 dilution

Reagents diluted in OBS pH 7.4 buffer

Final concentration in well – 2 pM (15 μl 1:4 dilution + 10 μl phospholipid + 225 μl OBS = 250 μl in total, 20 μl then added to well)

2 doubling dilutions of 1:4 dilution

i. 1 pM (15 μl first 1:2 dilution + 10 μl phospholipid + 225 μl OBS = 250 μl in total)
ii. 0.5 pM (15 μl subsequent 1:2 dilution + 10 μl phospholipid + 225 μl OBS = 250 μl in total)

Pt + buffer	Pt + anti-FVIII	Pt + anti-TFPI	Pt + both Ab	2 pM	
Pt + buffer	Pt + anti-FVIII	Pt + anti-TFPI	Pt + both Ab	1 pM	
Pt + buffer	Pt + anti-FVIII	Pt + anti-TFPI	Pt + both Ab	0. 5 pM	
HV + buffer	HV + anti-FVIII	HV + anti-TFPI	HV + both Ab	2 pM	
Pt calibrator	NP3 PPP Low				
HV calibrator	NP3 calibrator				

Table 6-11 Layout of 96 well plate for study thrombin generation assay; samples run in duplicate; different TF concentrations high-lighted. NP3 = in house normal plasma pool run as QC

7 Conclusion

This thesis has explored the potential of the thrombin generation assay and it's application to real-world haemostatic challenges. This test has not yet achieved the transition into the specialist diagnostic laboratory, but it can provide useful information to aid in elucidation of abnormalities in coagulation in addition to potentially guiding treatment.

There are three principal questions that need to be considered in order to determine whether any assay is fit-for-purpose. Firstly, the impact of pre-analytical variables needs to be determined, understood (such that results can be interpreted with this in mind) and minimised via standardisation of process, reagents and analysers. Secondly, analytical variables such as quality control and reproducibility need to be optimised. Lastly, the test needs to be able to deliver results that are interpretable, informative and can be used to improve patient care and outcomes. This thesis has sought to examine all three of the above with respect to the thrombin generation assay.

The initial chapters of the thesis aim to create context, review the modern thrombin generation assay with reference to standard coagulation assays and explain the mechanisms underpinning the assay. The work set out in subsequent chapters has studied the three factors described above. Firstly, after scrutiny of the technique and the supporting mathematical calculations, the protocol was reviewed and refined in order to minimise the impact of established pre-analytical variables. Secondly, a comprehensive set of normal reference ranges was generated for platelet-rich and platelet-poor samples

using two tissue factor reagents and a range of tissue factor concentrations. In order to achieve a reliable quality and normalisation control, work was performed to compare different normal pooled plasmas. Lastly, the assay was used to study both hyper- and hypo-coagulable states in order to confirm the sensitivity of the assay to these as well as determine whether results can be obtained that have clinical relevance.

The results of the normal reference ranges are notable for their wide range; this is a feature shared with many assays including coagulation factors. The TGA is an assay that can be performed on both platelet poor and platelet rich plasma and the results are significantly influenced by platelets as would be predicted by the cell-based model. The statistical analysis of the results of the samples from normal volunteers highlights that the platelet count of the PRP should be standardised (i.e. adjusted) if the aim is to study the impact of platelet function rather than platelet number. In addition, the phospholipid component of platelets (both from intact platelets and platelet microparticles and debris) is an important factor when assessing hypocoagulable states such as that seen in severe haemophilia. Although double centrifugation of plasma samples is sufficient when studying samples from volunteers and, by inference, patients with hypercoagulable states, the data suggest that triple centrifugation is required when using low TF concentrations in patients who are hypocoagulable. There is also an impact of freezing PPP samples resulting in statistically significantly differences between fresh and thawed samples using some of the triggers and so the author would recommend that either one or the other approach should be adopted for studies. The TF trigger itself is variable depending on the

source. Commercial preparations do not declare the TF concentration in each batch. However, if a 'standard' concentration is used (i.e. ~ 0.5 pM for PRP, 1 pM or 5 pM for PPP), then there is likely to be a better degree of reproducibility (with a correspondingly low coefficient of variance) if the same batch of a commercial preparation is utilised.

With respect to the wide range in normal results, although there are reasonable arguments that can be made regarding potential weaknesses in study design, it is interesting to compare so-called 'normality' with abnormal coagulation. This is particularly the case for the results from patients with haemophilia, some of whom demonstrably have no functional product of the F8 gene. Despite this, there is still a degree of baseline thrombin generation that – in some individuals – overlaps with the lower range of normality found in healthy volunteers. This would suggest that there is a variable degree of baseline activity even in the absence of a functioning tenase complex that can provide a surprising degree of coagulation (perhaps using bypassing circuits that usually play a background role in fully functioning haemostasis).

The hypercoagulable state of patients with deep venous thrombosis was studied with the aim of assessing the potential reversibility of LMWHs and pure anti-Xa synthetic pentasaccharides using prothrombin complex concentrates and protamine sulphate. After confirming sensitivity of the assay to pro- and anticoagulants, this proof-of-principle study demonstrated successful reversal (maximal with the combination of both agents) in both *in vitro* spiked plasma samples and *ex vivo* spiked patient samples. The data demonstrated that if the reversal agents (used in combination) were used on plasma samples with an

anti-Xa of less than 0.6 anti-Xa units/ml, or greater than 4 hours since administration of LMWH, then the TGA parameters could be returned to within the normal range. The data would suggest that there is an alternative to the use of PCC alone in patients who are bleeding on LWMH or who require an invasive procedure; however, this would require a clinical study to confirm the laboratory findings.

Lastly, the heterogeneity of bleeding phenotype in patients with moderate/severe haemophilia was studied. A cross-sectional study of basal levels of TF, FVII, FVIIa, TFPI (free and total) and FVII.AT complex in the absence of overt bleeding demonstrated interesting differences between the samples from patients with haemophilia A and those with haemophilia B. Although patients with both haemophilia A and B had lower circulating levels of TF, patients with haemophilia B had statistically significantly lower levels of FVII, FVIIa and FVII.AT complex. Although previous authors have shown lower levels of FVII in this cohort previously, this appears to be the first time this has been demonstrated for FVIIa and FVII.AT (that correlates strongly, as might be expected, with FVII). A reasonable argument might be that the lower levels of FIX in the patient samples (and thus the impact on the levels of the tenase complex) would impact on the activity such that the FVIIa clotting assay would be impaired. However, the patient samples were all diluted in normal plasma that contained normal levels of FIX (and FVIII) that should have provided sufficient normalisation of FIX levels for the assay (not dissimilar from the traditional 50:50 mix). Additionally, data from the literature would also seem to refute this. These data would appear to be real and require further elucidation.

They do suggest an important role for FVII and FVIIa in haemostasis – a theme that is repeated in the latter parts of the study.

The study set out to examine the particular role of TFPI in further detail (in light of clinical studies using neutralising antibodies as a novel therapy in patients with haemophilia) with the hope of further understanding phenotypic heterogeneity between patients with similar factor levels. Total TFPI (incorporating free TFPI in addition to the phospholipid-bound component) did correlate with the results of the TGA in patients with moderate and severe haemophilia A. Thus, unsurprisingly, the higher the level of total TFPI, the greater the degree of normalisation seen in the TGA parameters upon addition of a neutralising anti-TFPI antibody. In addition, higher levels correlated with a prolongation of the LT and ttPeak as well as a reduction in the rate of thrombin generation and lower peak values. These data did not tally with those from a previous study that suggested that lower *free* TFPI levels correlate with a milder bleeding phenotype; however, it would seem that these results have shown instead that higher levels of *total* TFPI correlate with a more severe bleeding phenotype (as assessed by clinicians). The study does therefore confirm the physiological role of TFPI as a natural anticoagulant.

Statistical analysis of the data from the patients with haemophilia A demonstrated that high FVII levels were predictive of a more severe bleeding phenotype whereas, in contrast, high FVIIa levels were predictive of a less severe bleeding phenotype. Work to examine the thrombin generation profiles of 20 of these patients using neutralising FVIII and TFPI antibodies subsequently showed that the assay was sensitive to very low levels of FVIII

and appeared to be able to be used to discern the patients with no functional antigen. As well as results in patients that overlapped with the lower end of the normal range, a more severe bleeding type showed some correlation with prolongation of the LT and ttPeak and well as reduction in the peak.

Restoration of thrombin generation into the normal range was striking upon addition of a neutralising anti-TFPI antibody. Comparison of results from the cross-sectional study with the thrombin generation parameters interestingly showed that increasing FVII levels correlated with an increase in LT and a decrease in the thrombin value at 10 minutes. These findings relating to FVII seem somewhat paradoxical but do replicate previous work performed using a reconstituted pro-coagulant model with purified coagulation factors with the suggestion that the FVII antigen may compete with FVIIa for binding to TF. As is now well recognised, FVII and FVIIa are present in the circulation (the latter at about 1/100th the concentration of the former). However, when FVII predominates in the presence of low levels of TF, the FVII.TF low activity complex will functionally inhibit generation of significant amounts of thrombin until the FVIIa levels increase as a result of increased exposure of TF.

Thus, although the study set out to look at the particular role of TFPI, FVII and FVIIa emerged as important components demonstrating correlation with TGA parameters as well as being predictive of bleeding phenotype. This hints at the complexity of haemostatic process and the complex interaction between complexes of different activities and the competing pro- and anti-coagulant roles of different zymogens that requires further study and elucidation. No one factor has emerged as a potential marker of bleeding phenotype, but the

baseline TGA results in the absence of FVIII (endogenous and exogenous) has shown potential in being able to predict severity. Similarly, the presence of higher levels of total TFPI has been demonstrated to correlate with a more severe bleeding phenotype.

Global haemostatic assays are gaining increasing prominence in clinical practice and the next generation of TEG and ROTEM machines are designed to minimise sample handling. In addition, newer versions of the calibrated automated thrombogram are automated with a view to wider use outside the research setting. The viscoelastic assays are used in settings such as major haemorrhage, understanding the coagulopathy of trauma and liver transplant. They do also play a role in specialist haemophilia comprehensive care centres, particularly when assessing more complex patients such as patients with haemophilia with inhibitors requiring bypassing agents for surgery. The results from the studies contained within this thesis provide additional evidence for the clinical utility of the thrombin generation assay in guiding patient care. As the technology and automation progress, there is a realistic chance that the thrombin generation assay may become part of the diagnostic array in the specialist haemostasis laboratory.

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