Factor XI Deficiency: a study of clinical, laboratory and molecular modifiers of bleeding phenotype

Niamh Maire O'Connell

University College London

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Declaration

I, Niamh O'Connell, 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. The development of the homology models for the FXI protein domains and molecular modelling (Chapter 6) was a joint collaboration with Professor Stephen Perkins and Rebecca Saunders, Department of Structural and Molecular Biology, UCL.

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Niamh O'Connell, M.B., F.R.C.P.I., F.R.C.Path.

Thesis abstract

Factor XI (FXI) is a plasma glycoprotein that participates in the consolidation phase of blood coagulation and is important in the creation of a stable fibrin clot. Deficiency of FXI leads to an injury-related bleeding diathesis, which is notable for the variability in the bleeding tendency and the lack of a clear relationship between bleeding and FXI coagulant activity (FXI:C). A comprehensive understanding of the factors which influence the bleeding tendency in factor XI deficiency would enable a more structured evaluation of bleeding risk and would focus the treatment choices for individual patients. The purpose of this work is to comprehensively analyse physiological and genetic factors which modify the clinical phenotype of FXI deficiency. In this study, a comprehensive bleeding history was obtained from participating patients with known FXI deficiency (n=102) which was then independently scored. This clinical bleeding score was utilised to compare the following laboratory parameters between patients with and without a clinical bleeding tendency: aPTT, FXI:C, FXI:Ag, Blood group, von Willebrand factor levels, inherited thrombophilic traits, Thrombin generation (by subsampling and continuous methods) and TAFI levels (as evaluated by TAFI antigen and a clot lysis assay). The underlying genetic mutation causing the FXI deficiency was evaluated in all patients and where an existing Jewish mutation was not found, novel mutations were sought. Molecular modelling of mutations in the FXI gene was undertaken which required development of molecular models of the apple domains and serine protease domain of the FXI protein. In addition, genetic analysis of a polymorphism in a gene (ALG6) encoding a specific glucosyl transferase was undertaken due to the link between FXI deficiency and congenital disorders of glycosylation. Finally, the first clinical trial of the use of recombinant FVIIa in FXI deficiency was conducted as part of this study.

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Abbreviations

Ap	Apple domain
aPTT	Activated partial thromboplastin time
AT	Antithrombin
AUC	Area under the curve
bp	base pairs
BPL	BioProducts Laboratory
BSA	Bovine serum albumin
CLT	Clot Lysis time
CRM+	Cross-reacting material positive
CRM-	Cross-reacting material negative
CTI	Corn trypsin inhibitor
CV	Co-efficient of variation
DIC	Disseminated intravascular coagulation
ETP	Endogenous thrombin potential
FaXID	Recombinant FVIIa in Factor XI deficiency study
FFP	Fresh frozen plasma
FV	Factor V
FVL	Factor V Leiden
FVII:C	Factor V coagulant activity
FVIII:C	Factor VIII coagulant activity
FIX	Factor IX
F11	Factor XI gene
FXI	Factor XI protein
FXIa	Activated Factor XI

FXI:Ag	Factor XI antigen
FXI:C	Factor XI coagulant activity
FXIIa	Activated Factor XII
HGVS	Human Genome Variation Society
HK	High molecular weight kininogen
LA	Lupus anticoagulant
LFB	Laboratoire Francais du Fractionnement et des Biotechnologies
NCBI	National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov)
NIBSC	National Institute for Biological Standards and Control
NP	Normal plasma
OD	Optical density
PC	Protein C
PL	Phospholipid
PPH	Post partum haemorrhage
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Protein S
PT	Prothrombin time
PTA	Plasma thromboplastin antecedent
PTGM	Prothrombin gene mutation
PTCI	Potato tuber carboxypeptidase inhibitor
rFVIIa	Recombinant activated factor VII
rTF	Recombinant tissue factor
SCR	Structurally conserved region
SD	Solvent detergent

SD-FP	Solvent detergent treated frozen plasma
SNP	Single nucleotide polymorphism
SP	Serine protease
TAFI	Thrombin activatable fibrinolysis inhibitor
TAFIa	Activated TAFI
TAFIa(i)	Inactivated TAFI
TAFI-AP	Activation peptide of TAFI
TEG	Thromboelastography
TF	Tissue factor
TGT	Thrombin generation test
tPA	Tissue plasminogen activator
VWD	Von Willebrand disease
VWF	Von Willebrand factor
VWF:Ag	Von Willebrand factor antigen
VWF:RiCoF	Von Willebrand factor Ristocetin co-factor activity
VWF:CBA	Von Willebrand factor Collagen binding activity

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Chapter 1 Introduction

1.1 Introduction

Factor XI (FXI) is a plasma glycoprotein that participates in the consolidation phase of blood coagulation and is important in the creation of a stable fibrin clot.(1) Deficiency of FXI leads to an injury-related bleeding diathesis, which was first described in 1953 and has been historically termed 'Haemophilia C', 'plasma thromboplastin antecedent deficiency' and 'Rosenthal syndrome'.(2-4) The bleeding disorder is now known as 'factor XI deficiency' and is notable for the variability in the bleeding tendency and the lack of a clear relationship between bleeding and FXI coagulant activity (FXI:C).(5-7) This creates clinical difficulty in planning therapy for elective invasive procedures or in treating patients for acute injuries, since the available therapeutic agents have potential side effects. Currently, bleeding risk is predicted empirically by assessing the FXI:C level, past bleeding history and the nature of the injury or surgery. However, some patients have had no or few prior surgical procedures and it is often difficult to obtain reliable information about remote events. A comprehensive understanding of the factors which influence the bleeding tendency in factor XI deficiency would enable a more structured evaluation of bleeding risk and would focus the treatment choices for individual patients. The purpose of this work is to comprehensively analyse physiological and genetic factors which modify the clinical phenotype of FXI deficiency.

1.2 FXI protein structure and function

1.2.1 The *F11* gene and FXI protein biochemistry

The FXI gene is located at the distal end of the long arm of chromosome four.(8) This 23 kb gene consists of 15 exons and 14 introns, and encodes a 607 amino acid mature protein, which is the zymogen for a serine protease.(9) The C-terminal light chain contains the serine protease domain which is similar to that of many other coagulation factors. The N-terminal heavy chain is unusual in that it contains four tandem Apple domains, each of which has a specific role in binding co-factors, substrate, platelets or other FXI monomers.(10;11) The only other coagulation factor to possess similar tandem Apple domains is Prekallikrein.(12;13) The substrate FIX is bound by apples 2 and 3 whereas apple 3 is the main site for platelet binding.(14;15) High molecular weight kininogen (HK) circulates in a non-covalent complex with FXI and facilitates binding of FXI to activated platelets. All of the apple domains are involved in the complex formation with HK, with apple 2 playing the most important role.(16)

FXI is unique amongst coagulation serine proteases in that it exists as a homodimer, with two identical 80 kD monomers joined by both covalent and non-covalent bonds.(17) The apple 4 domains dimerise through covalent protein-protein interactions which are stabilised by the formation of a disulphide bond at Cys321, although the latter is not critical to dimerisation.(18;19) Apples 2 and 3 make contributions to dimerisation but hydrophobic interactions between the two FXI monomer apple 4 domains are critical, in particular residues Leu284, Ile290 and Tyr329.(20-23)

FXI may be activated by factor XIIa, thrombin or FXIa and the dimeric conformation is essential for activation, although the primary physiological activator remains controversial.(22;24) Cleavage occurs at Arg369-Ile370 in each monomer and the activated FXI remains in dimeric form.(17) However, an intermediate form of activated FXI has been identified in which only one of the FXI monomers has been activated (termed ½-FXIa).(25)

FIX binds to FXIa but not to the zymogen FXI as a consequence of a conformational change in the FXIa protein which allows substrate binding to occur.(21;26) FXIa cleaves FIX at two sites (Arg 145 and Arg 180) although a singly cleaved intermediate is not formed, unlike the situation during activation of FIX by FVIIa/TF.(27) Both active sites of the FXIa dimer are not required for successful cleavage of FIX.(22;25)

1.2.2 Role of FXI in coagulation

There are a number of conundrums associated with the role of FXI in the coagulation cascade. Firstly, the realisation that activated FVII (FVIIa)/tissue factor (TF) complex can activate FIX and promote coagulation via the intrinsic as well as the extrinsic systems has called into question the role of the contact factors.(28) Deficiencies of the contact factors (FXII, HK and prekallikrein) do not lead to a clinical bleeding phenotype. Despite the fact that HK and Zn^{2+} ions are important chaperones for FXI in the circulation, HK deficiencies are not detrimental to normal coagulation. The clinically important coagulation serine proteases contain a GLA domain to interact with the platelet surface which is not present in FXI.(29) Finally, the FXI protein uniquely exists as a homodimer.

Advances in the understanding of the biochemistry of FXI in recent years have explained some of these conundrums.(30) The small amounts of thrombin, which are generated in the initiation phase of coagulation, are sufficient to activate FXI, which can then activate FIX and further increase the thrombin-generating potential of the consolidation phase of coagulation.(24) Despite the fact that FXI is known as a contact factor, activated platelets, rather than negatively charged surfaces, provide a preferential surface for the activation of FIX by FXI.(31;32) The binding of FXI to activated platelets occurs via the third apple domain of FXI and platelet glycoprotein Ib/IX/V.(33;34) Prothrombin and Ca^{2+} ions can substitute for HK/ Zn^{2+}, explaining why deficiencies of HK do not lead to bleeding.(35) The dimeric structure of FXI is important for activation to FXIa and for the dual roles of platelet binding and substrate activation.(22;30)

The current understanding of the role of FXI in the coagulation cascade may be hypothesised as follows. Small amounts of thrombin, generated by the exposure of FVIIa/TF at the site of vessel injury during the initiation phase of coagulation, serve to activate platelets and FXI, possibly to the ½-FXIa intermediate. Binding of the zymogen portion of ½-FXIa to activated platelets via apple 3 and GPIb localises FXI to the surface on which the consolidation phase of coagulation occurs.(29) The apple 3 domain of the activated monomer of the FXI molecule is then available for substrate binding and activation. Although FVIIa/TF activate FIX, this pathway is rapidly inhibited by Tissue Factor Pathway Inhibitor (TFPI). The activity of the primary inhibitor of FXI, protease nexin II, is confined to the fluid phase and therefore platelet-bound FXI is able to freely activate FIX and thus provide a burst of thrombin localised to the site of vessel injury.

1.2.3 Platelet FXI

The presence of FXI activity in platelets which is distinct from plasma FXI has been described.(36;37) An alternatively spliced variant of FXI which lacked exon 5 was demonstrated in platelets and a megakaryocytic cell line and it was hypothesised that even patients with very low levels of plasma FXI could retain some haemostatic function via this alternative platelet FXI.(38;39) However, another group were unable to replicate this finding and demonstrated that platelet FXI mRNA had the same sequence as liver mRNA.(40) Subsequently, a highly sensitive real-time RT-PCR technique was used to evaluate FXI mRNA isolated from the platelets of FXI deficient individuals and again no alternatively spliced variant was detected.(41) Normally spliced FXI mRNA was detected in monocytes and granulocytes as well as in platelets and levels were similarly low in all three cell types, possibly a result of ectopic transcription.

Given current evidence, platelets do not appear to contain an alternatively spliced FXI.(42) If platelet FXI activity is present, it is likely to be derived from the product of the full-length FXI mRNA, either synthesised in the megakaryocyte or internalised from plasma.

Thrombin generation and platelet FXI

Two studies of thrombin generation in FXI deficiency have evaluated the possibility that normal platelet derived FXI may compensate for the impairment in thrombin generation seen in severe FXI deficient plasma.(43;44) In both studies normal washed platelets were added to PPP derived from the plasma of patients with a homozygous type II mutation but no correction of the impaired thrombin generation was seen. This

appears to refute the hypothesis that platelet derived FXI (at least in the in-vitro thrombin generation assay) can substitute for a deficiency in plasma FXI.

1.2.4 FXI molecular model and crystal structure

Up to 2004 there was no available molecular model or crystal structure for the FXI protein. The first crystal structure published was of a recombinant protein consisting of the catalytic domain of FXIa ($rhFXI_{370-607}$). This protein was crystallised in complex with a substitution mutant of ecotin, a pan-serine protease inhibitor.(45) The isolated catalytic domain was subsequently crystallised but neither of these crystal structures gave any indication of the interactions of the four apple domains with each other and with the protease domain.(46)

More recently, the FXI zymogen has been purified from human plasma and the crystal structure determined.(47) The four apple domains have a unique ternary packing structure which is said to resemble a flat saucer and form a platform on which the protease domain sits. When dimerised, these apple domain "saucers" are inclined at an angle of 70º and form an inverted V shape with the protease domain active site clefts pointing away. The crystal structure gives insight into the activation of the FXI protein. Thrombin binds to the Apple 1 domain via Glu66 (48) and platelet GP1b binds to the Apple 3 domain via Lys252 and Lys253.(32) In the monomer saucer structure of the apple domains, Apple 1 and 3 are diametrically opposite one another and are sterically separated by the protease domain. However, in the dimer conformation the Apple 1 domain of one monomer is in close proximity to the Apple 3 domain of the other monomer and it is likely that the FXI dimer supports ternary binding and a trans-activation mechanism.

1.3 FXI deficiency

1.3.1 Incidence of FXI deficiency

FXI deficiency is a rare bleeding disorder, first described in 1953 (2) and occurring at a frequency of one in a million in the general population. However, the condition is known to be more common among a number of well-defined populations, including all Jewish communities, Iranians and French Basques.(49-51) The Ashkenazi Jews have the highest incidence of FXI deficiency amongst the Jewish communities. The gene frequency for FXI deficiency was calculated on the basis of FXI:C levels and family history in 36 Ashkenazi Jewish probands, 114 relatives of the probands and 428 healthy Ashkenazi Jewish people and the estimated frequencies for "homozygotes" was 0.1-0.3% and for "heterozygotes" was 5.5-11%.(52) Subsequently, 1040 patients were evaluated using molecular techniques and the estimated risk for heterozygous FXI deficiency was 9% (1:11) in Ashkenazi Jews and 3.3% (1:30) in Iraqi Jews.(49) The risk for homozygous or compound heterozygous FXI deficiency was 1:450 and 1:3571 in the two groups respectively. The incidence in other communities is lower but can represent a significant minority in the population e.g. the 1% incidence of FXI deficiency in the Basque community.(51)

1.3.2 FXI coagulant activity and antigen levels

Normal levels of FXI range from 70 to 150 U/dL.(5) The lower limit of normal for FXI is higher than that seen for other coagulation factors which have a lower limit of normal of approximately 50 U/dL. The lower limit of normal for FXI of 70 U/dL was confirmed in 103 blood donors.(53)

Homozygotes or compound heterozygotes for mutations causing FXI deficiency have a severe deficiency of FXI and their FXI:C levels are less than 20 U/dL (5) although some groups quote levels of <15U/dL for severely deficient individuals.(54) In a study of 24 families, none of 16 severely deficient patients had a FXI:C level >11 U/dL (5) while in another study of 41 presumed homozygotes levels ranged from <1 to 12 U/dL.(55) In 46 severely deficient Ashkenazi Jews with disease severity confirmed by molecular analysis, FXI:C ranged from to <1 to 13.5 %.(56)

Heterozygotes for a mutation in the FXI gene have a partial deficiency of FXI. In earlier studies, partial deficiency was defined as a FXI:C of 15-49 U/dL (52), 20-61 U/dL (57) and 28-60 U/dL.(56) However this was revised to define partial deficiency as FXI levels between 20 and 70 U/dL.(5) In 62 presumed heterozygotes, FXI:C ranged from 17-72 U/dL.(55) It should be noted that some partially deficient patients can have FXI:C levels within the normal range. Twenty (49%) of 41 "obligatory carriers" had FXI:C levels above 50 U/dL and six had levels >100 U/dL.(52) Heterozygotes for mutations causing FXI deficiency had FXI levels ranging from 28 to 126% of normal.(56) Amongst 39 partially deficient patients, 29 (74%) had FXI:C levels of >50 U/dL but 6/39 (15%) patients had levels >70 U/dL.(5) In a study of 38 FXI deficient patients from Iran, patients were characterised as having severe/moderate (FXI:C < 6%) or mild FXI deficiency (FXI:C 6-30%), in a classification of FXI deficiency modelled on that used for Haemophilia A and B.(50) However, this classification is not conventionally used in FXI deficiency. Patients did not have FXI genotyping performed and it is probable that there were homozygotes or compound heterozygotes in both groups. This causes considerable difficulty in interpreting the correlation between disease severity and bleeding symptoms.

1.3.3 FXI gene mutations

FXI deficiency in the Jewish community is caused by four well-defined mutations.(58;59) It is important to note that the nomenclature commonly used to describe these Jewish mutations may be confused with the nomenclature used to describe the phenotypic expression of mutations in coagulation factor genes, where a type I mutation results in concordant reductions in protein expression and function (cross-reacting material negative, CRM-) and a type II mutation causes normal expression of a dysfunctional protein (cross-reacting material positive, CRM+). The Jewish mutations are termed Types I–IV and are the equivalent of type I (CRM-) mutations of other coagulation factor genes, characterized by low FXI coagulant activity and antigen. Type II and Type III are the two most common mutations and the mutated alleles occur with equal frequency in severely deficient patients (49% and 47% of mutant alleles respectively).(56) The Type II mutation is a nonsense mutation (Glu117Stop) in the Apple 2 domain responsible for substrate binding, while Type III is a missense mutation (Phe283Leu) in Apple 4, the domain important for dimerisation. Type I and Type IV mutations (a G to A transition at the donor splice site in intron N and a 14 bp deletion at the exon 14 / intron N splice site, respectively) both affect splicing in the catalytic domain.

People of Ashkenazi descent have a heterozygote frequency of 9% and a homozygote frequency of 0.22%.(49) Analysis of the genotypes of 125 unrelated Ashkenazi Jews with severe FXI deficiency found the most common genotype to be compound heterozygosity for the Types II and III mutations (52% of patients). Homozygosity for these two mutations was also equally prevalent (22.4% of patients for each mutation).

The frequencies of the Type I and Type IV mutant alleles were much lower at 1.2% and 0.4%.

The Type II mutation is believed to have arisen between 586 BC and 70 AD and is prevalent in all main Jewish communities, Iraqi, Sephardic and Ashkenazi Jews.(49) In contrast, the Type III mutation occurs only in Ashkenazi Jews, suggesting that it arose more recently (15th–20th centuries). The type III mutation is not found in Iraqi Jews who lived in relative isolation in Iraq until the 1950s. Comparison of the frequency of FXI intragenic polymorphisms in FXI deficient Iraqi, Sephardic and Ashkenazi Jews with unaffected Jewish individuals has confirmed a founder effect for both the Type II and Type III mutations.(60)

Genotype appears to be associated with the level of FXI clotting activity in severely deficient patients.(56) Type II homozygotes have the lowest levels and Type III homozygotes the highest (mean percentage of normal values of 1.2% and 9.7%, respectively), with intermediate levels seen in compound heterozygotes (3.3%).

1.3.4 Clinical phenotype

Classification of bleeding symptoms in FXI deficiency has been variable. One group defined patients as bleeders if they had two or more of the following symptoms: easy bruising, epistaxis, menorrhagia, postpartum bleeding, prolonged bleeding after superficial cuts or haemorrhage after surgery or dental extraction.(61) A five point classification system has also been used and in these studies, experienced haemostasis physicians were asked to use their usual criteria to assign patients to one of five

categories of bleeding - definite bleeder (1), probable bleeder (2), indeterminate (3), probable non-bleeder (4) and definite non-bleeder (5).(5;7) Concordance between two assessors was found to be 67% and receiver-operator (ROC) curves confirmed that the bleeding tendency, as assessed by the five point scale, was more sensitive than other parameters such as excessive menstrual bleeding and bleeding after tonsillectomy. Another study used pre-defined criteria for epistaxis, menorrhagia, internal bleeding, muscle haematoma, haemarthrosis and post-operative bleeding to identify significant bleeding although such criteria can be difficult to apply in a retrospective setting.(50)

Surgical and traumatic bleeding

Unlike the haemophilias, FXI deficiency is rarely manifested as spontaneous bleeding even in severely affected patients and bleeding is typically related to trauma, surgery, menstruation or childbirth.(6) Surgical procedures on areas of high intrinsic fibrinolytic activity such as the oral and nasal cavities, prostate and uterus are associated with increased rates of post-operative haemorrhage.(7;55;56) Collins et al evaluated 156 procedures in 25 severely deficient and 30 partially deficient patients who were treated prior to the widespread availability of effective treatment.(55) Patients did not receive FXI replacement or tranexamic acid. Of these 156 procedures, dental surgery accounted for 55, tonsillectomy for 31 and 70 were a heterogeneous group of other procedures. Bleeding occurred after 51% of dental procedures, 71% of tonsillectomies and 31% of other procedures. Bleeding rates were higher for severely deficient patients for dental surgery (60% versus 43%) and tonsillectomy (86% versus 59%) but were similar for "other" procedures (32% versus 31%). However, as can be seen, bleeding rates in untreated partially deficient patients ranged from 31% to 59%. In contrast in this study,

there was one episode of bleeding after 38 procedures covered with FFP and no bleeding after 45 procedures covered with FXI concentrate.

In a cohort of Iranian patients with FXI:C < 30U/dL who underwent surgery without cover, bleeding occurred in 24 of 38 patients (63%) after general surgery and 21 of 38 (55%) after dental procedures.(50) In a recent analysis of 120 unrelated patients with severe FXI deficiency (FXI:C < 15 U/dL), bleeding rates were evaluated after surgery, without the use of either FXI replacement or antifibrinolytic agents.(62) Bleeding rates were 49-66.7% when surgery was carried out in tissues with high fibrinolytic activity (e.g. oral cavity, nasal mucosa, prostate and urinary tract). This contrasted with bleeding rates of 1.5-40% in other sites. The highest rate of bleeding at non-fibrinolytic sites was for hysterectomy (40% bleeding rate in five patients) followed by 23% for 13 patients undergoing herniorraphy and 20% in five patients undergoing cholecystectomy. Bleeding rates for appendicectomy was 4.5% and for circumcision was 1.5%. No bleeding was recorded in six fracture surgeries, one knee replacement and two halux valgus repairs. Thirty-six patients had surgeries in areas with and without high levels of fibrinolytic activity and 18 had similar bleeding symptoms (i.e. whether bleeder or non-bleeder) in both surgery types. Of the remaining 18 patients, the majority (15 patients) only bled in sites with high fibrinolysis. These data were used to advocate against the use of elective FXI replacement, at least in procedures on tissues without high fibrinolytic activity.

Although, the published literature from the Israeli group suggests that bleeding rates in non treated Israeli patients are not as high as that suggested by literature from other

countries, the figures for bleeding from the oral cavity in particular are broadly in line across the studies.(63)

Bleeding and genotype

Bleeding is more common in severely deficient patients than in partially deficient patients.(7) However, evidence linking the Jewish *F11* gene mutations with bleeding tendency is limited. There is evidence to suggest that some Jewish patients with Type II mutations and very low FXI levels experience more bleeding with surgical procedures than Type III homozygotes.(56) The relationship between genotype and bleeding tendency was assessed in 20 patients known to be heterozygous for the type II mutation and compared to 41 partially deficient patients with an unknown mutation.(7) Approximately half of the patients in each group were classified as bleeders: 45% of patients heterozygous for the type II mutation and 49% of patients with an unknown mutation.(7) However, no differences in bleeding tendency were identified between the two groups of heterozygotes.

More recently, the genotype of 120 severely deficient patients did not influence the bleeding rate regardless of the site of the procedure.(62)

Bleeding related to severity of FXI deficiency

In the earliest studies of FXI deficient patients, partially deficient patients were said to rarely experience bleeding with invasive procedures and urological procedures were successfully performed without plasma cover.(3;4;54;64) Other authors have stated that "heterozygotes rarely bleed excessively following trauma".(6) However, post-operative

or traumatic bleeding was reported in 22 of 48 deficient patients from 25 kindreds, of whom nine were classified as severely deficient and 13 were classified as partially deficient.(57) The frequency of bleeding in 81 partially deficient patients was 48% (28/58 patients after exclusion of 23 patients with indeterminate bleeding histories) which is more pronounced than the other rare bleeding disorders.(7) Bleeding symptoms were compared between patients with partial FXI deficiency and relatives with a normal FXI:C. Partially deficient patients were more likely to bleed after dental extractions (51% versus 9%) and have menorrhagia (41% versus 18%). Eighteen of 30 kindreds in this study came to medical attention due to bleeding in partially deficient patients, including one case of fatal post-operative haemorrhage. A study of 45 Israeli families with FXI deficiency showed that bleeding symptoms were reported by 58% of 26 severely deficient individuals compared with 20% of 46 partially deficient patients and 9% of 47 unaffected family members.(65) Predictors of bleeding were the FXI:C (i.e. severe deficiency was more likely to be associated with bleeding than partial deficiency) and surgery involving mucous membranes.

The bleeding tendency in women may manifest as menorrhagia which was reported in 41% of partially deficient patients in one study and another report found that menorrhagia was present in 59% of a cohort of 20 women with FXI deficiency, without any relationship to FXI levels.(7;66)

Co-inherited bleeding or thrombotic disorders

In view of the lack of correlation between bleeding and FXI levels, other haemostatic modifiers such as co-inherited bleeding or thrombotic disorders have been considered. Von Willebrand disease (VWD) was diagnosed in 13% of the patients with FXI

deficiency in a small study.(61) Bolton-Maggs et al did not find an increased incidence of type I VWD but did find a correlation between von Willebrand factor antigen (VWF:Ag) levels and bleeding.(7) There was an increase in bleeding in 62% of patients with VWF:Ag less that 70 U/dL, in addition to a partial deficiency of FXI. However, the use of a VWF level of 70 U/dL to predict bleeding tendency would lead to a 25% false positive rate. Patients with blood group O had slightly lower levels of VWF:Ag and a slightly increased risk of bleeding symptoms but these differences did not reach statistical significance. FVIII:C was correlated with FXI:C in a study of 26 severely deficient and 46 partially deficient patients.(65) However, levels of FVIII and VWF were not associated with bleeding. Blood group O had a minor association with bleeding.

Co-inherited thrombophilic traits have been reported to alleviate bleeding symptoms in patients with haemophilia. Heterozygosity for the FV Leiden mutation reduced concentrate usage and the number of bleeding episodes in patients with severe haemophilia A.(67) The influence of these traits on the clinical phenotype of FXI deficient patients requires further study.

FXI deficiency in pregnancy

Unlike FVIII and von Willebrand factor, factor XI levels do not change significantly in FXI deficient women in pregnancy although some conflicting evidence of changes in FXI levels has been reported in normal women.(68-70) This may be due to differences in FXI assay reliability and technique over the time period between studies.

Obstetrical outcomes have been evaluated in 28 pregnancies in 11 women with FXI deficiency (four severely and seven partially deficient).(70) One patient had a spontaneous miscarriage. Bleeding complications were documented in 15 of 28 pregnancies (one post abortal bleed, four antepartum haemorrhages, four primary post-partum haemorrhages (PPH), six secondary postpartum haemorrhages). Postpartum haemorrhage was seen in 24% of pregnancies and included women with partial as well as severe deficiency. Transfusion of red cells was required in two cases and transfusion of plasma was needed in three cases. Elective FXI replacement was used to cover labour and delivery in five cases and no bleeding complications were reported (FXI concentrate for two caesarean sections and one vaginal delivery and plasma for two other vaginal deliveries).

In a study of 164 pregnancies in 62 severely deficient women, 19 deliveries were covered with fresh frozen plasma while the remainder received no elective FXI replacement.(71) Postpartum haemorrhage occurred in 36 cases, with transfusion of red cells required in 12 cases. Rates of PPH were higher following vaginal delivery in which FXI replacement with plasma was not given versus those in which plasma was administered (24% versus 14%). There were 18 Caesarean deliveries, of which six were covered with plasma. PPH was recorded in two caesarean deliveries in which elective FXI replacement was not given. Neither the presence of a thrombophilic trait (Factor V Leiden or the Prothrombin gene mutation) nor the occurrence of bleeding with a prior procedure influenced the likelihood of obstetrical bleeding.

FXI deficiency in the neonatal period: Circumcision

Religious circumcision is carried out on male Jewish infants at day eight of life unless there is a clear medical contra-indication e.g. jaundice. There is little prospective evidence in the literature regarding outcomes post circumcision in FXI deficient families. Many reports in the literature are anecdotal or rely on hearsay evidence from an adult patient regarding events in the neonatal period. Bolton-Maggs et al reported that four of 16 infants with levels <10 U/dL bled post circumcision, one requiring ITU admission and blood transfusion.(5) In the same study, retrospective reporting of symptoms by adults revealed that one individual with a FXI:C of 34 U/dL bled after circumcision but other patients who had no bleeding after circumcision had post-operative bleeding later in life. Reporting from the same centre, another author reported that three of 16 (19%) patients bled post circumcision, two with FXI:C <2 U/dL and one with FXI:C of 34 U/dL, the latter probably the same patient reported by Bolton-Maggs.(55) A later report detailed seven circumcisions without bleeding (7) while a study of obstetrical outcomes reported that a neonate of a FXI deficient woman bled after circumcision requiring blood transfusion. In a recent study of 120 severely deficient adult patients, 65 patients reported having had a circumcision but only one patient reported bleeding.(62)

1.3.5 Treatment of FXI deficiency

Tranexamic acid and topical fibrin glue

Bleeding in FXI deficiency is especially likely in areas of high fibrinolytic activity and, therefore, antifibrinolytic agents have been used extensively in this condition.(56;72;73) Tranexamic acid is the most frequently used agent and has the advantage that it can be

given orally as a tablet or a 5% mouthwash, as well as intravenously. Dental extraction in 19 severely deficient patients undergoing 41 dental procedures (mainly extractions) were managed successfully with tranexamic acid alone (1g QDS starting at 12 hours pre surgery and continuing for 1 week).(74) All patients had a documented bleeding tendency (14 bled with prior dental procedures, five bled following other surgery or trauma). Topical fibrin glue has also been used for dental procedures.(74;75)

Desmopression (DDAVP)

A number of case reports have suggested that small but significant increases in FXI levels occur after treatment with Desmopressin (DDAVP).(76;77) Intravenous infusion of DDAVP (0.3μg/kg) resulted in a mean increase in FXI:C of 19 U/dL (range 12-25 U/dL) in two normal individuals and patients with VWD and FXI deficiency (two patients each).(76) A mean increase of 12 U/dL (range 9-14 U/dL) was recorded in six patients with partial FXI deficiency given DDAVP at a dose of 0.3μg/kg subcutaneously.(77) However, in a study of 33 individuals with von Willebrand disease or Mild Haemophilia A, who underwent a DDAVP trial, increases in FXI:C and FXI:Ag were clinically insignificant.(78) The mean ± SD FXI:C pre DDAVP was 90.7 U/dL (±22.9) and was 92.1 U/dL (±20.9) one hour post DDAVP. Similarly FXI:Ag was 92.2 U/dL (±20.1) and 89.9 U/dL (±21.3) one hour post DDAVP. No difference was seen whether the DDAVP was administered intravenously (n = 16) or subcutaneously (n = 17). One patient in this study had a combined FVIII and FXI deficiency and the FXI:C did not increase with DDAVP. The lack of evidence of a specific increase in FXI:C with DDAVP does not preclude a more general pro-haemostatic effect.(79)

FXI replacement

In circumstances where the bleeding phenotype of the patient and/or the nature of an impending surgical procedure suggest that there is likely to be an increased risk of bleeding, the accepted method of prevention of bleeding is the replacement of FXI.(6;72;80) FXI replacement is achieved by the use of fresh frozen plasma (FFP) or FXI concentrate. Solvent-detergent treated FFP is preferred due to improved viral safety.(81) FXI concentrate has limited availability and in many countries world-wide, plasma is the only available treatment.(72;82)

Plasma

The use of plasma to replace deficient FXI and prevent bleeding during a hernia repair was described in 1955.(83) More recently, 38 procedures (including 18 dental extractions) in which FFP was used to prevent bleeding were reported.(7) Bleeding was reported after only one procedure, a uterine D&C. No further details were presented on pre and post FXI:C levels but a modest increase in FXI:C to < 30 U/dL in patients with severe FXI deficiency was subsequently referred to in a later paper by the same author.(72) Similarly, an earlier case report suggested that the average rise in FXI was 37% for each litre of plasma infused.(84)

Solvent-detergent treated plasma (SD-FP) is now recommended on the basis of improved viral safety.(81) This product was evaluated in eight patients with coagulation factor deficiencies including FXI deficiency and had a similar half-life to FFP (45 hours).(85) One commentator has expressed concern about possible variability in FXI content in SD plasma.(53) Treatment with SD-FP may lead to volume overload

and allergic reactions.(86) In a study of severely deficient coagulopathic patients, standard doses of SD plasma did not normalise FXI:C activity.(87)

FXI concentrate

FXI concentrate is manufactured in the United Kingdom by BioProducts Laboratory (BPL) and in France by Laboratoire Francais du Fractionnement et des Biotechnologies (LFB).(88;89) It is an unlicensed product, available on a named patient basis and is not freely available in many countries including the United States.(82)

The BPL product is derived from pooled donor plasma and viral inactivation consists of dry heating at 80ºC for 72 hours in the final container.(88) The initial formulation included antithrombin to inhibit FXIa (mean 102 U/mL). Haemostatic efficacy was confirmed in 30 patients in whom 31 invasive procedures were carried out. Normal levels of FXI activity were recorded in all patients and bleeding occurred in one case (a patient undergoing coronary artery bypass grafting with evidence of thoracic bleeding but no bleeding from the vein graft site). The LFB product is also manufactured from pooled donor plasma.(89) Viral inactivation consists of solvent-detergent (SD) treatment and nanofiltration and the product contains heparin (3-5 U/mL) and antithrombin (2-3 U/mL). The original paper did not include evidence of clinical efficacy but in-vitro and in-vivo animal studies of thrombogenicity did not reveal any concerns regarding toxicity.

However, a number of reports of thrombo-embolic side effects emerged after these concentrates were introduced. Of three patients who received a SD treated FXI

concentrate which is not currently in use, one patient died after occlusion of all coronary artery grafts while another developed severe anaphylaxis.(90) Subsequently, two patients receiving the LFB product showed evidence of activation of the coagulation system by in-vitro testing but no clinical thrombosis.(91) Four elderly patients with pre-existing cardiovascular disease were reported to have had thrombotic complications using the BPL product.(92) Mild activation of coagulation was described in two of three patients receiving 30 U/kg of the LFB FXI concentrate in a pharmacokinetic study prior to surgery but one of the patients developed disseminated intravascular coagulation (DIC) after a 60 U/kg dose subsequently administered for surgery.(93)

Modification of the BPL product occurred in 1993 with the addition of heparin 10 U/mL and the use of in-vitro batch testing for thrombogenicity.(82) A review of a decade of experience with the BPL FXI concentrate showed that in 129 patients receiving treatment for 179 treatment episodes, there were 10 possible thrombotic events and the majority of cases occurred in patients with pre-existing vascular disease and aged > 60 years.(94) In 1998 these data were updated and 12 probable or definite thrombotic episodes were reported for 229 treatment episodes in 161 patients.(72)

Data on the use of the LFB FXI concentrate in 56 FXI deficient patients undergoing 85 procedures were collected using a questionnaire.(82) Three cases of thrombosis were reported: two DIC with thrombosis and one pulmonary embolus. All three cases were aged more than 60 years and the first infusion was more than 40 U/kg. Recommendations on the administration of FXI concentrate now advise treating physicians to avoid doses of more than 30 U/kg, to avoid peak FXI:C levels of more

than 70 U/dL and to consider the risk-benefit ratio carefully in patients with pre-existing risk-factors for thrombosis.(80) Avoidance of the use of concomitant anti-fibrinolytic agents and monitoring of laboratory markers of thrombosis are also suggested.

With-holding upfront FXI replacement

The Israeli group have recently suggested that replacement therapy is not required for severely deficient patients undergoing certain surgical procedures including circumcision, appendicectomy and orthopaedic surgery, based on a retrospective review of clinical history and medical notes.(62) It is suggested that treatment could be reserved for patients who develop bleeding post-operatively. No data on the validity of the latter course are presented with regard to outcomes associated with such a policy (e.g. requirement for cellular blood products, wound complication rates, duration of hospitalisation). It should also be noted that the number of orthopaedic procedures reported is low (nine) and that no information about the prior bleeding history of the patients in the study was presented. A similar policy has been suggested for labour and vaginal delivery.(71)

1.3.6 FXI inhibitors

Inhibitors to coagulation factors, acquired after treatment with exogenous clotting factor concentrates, pose a major difficulty in treatment.(95) FXI inhibitors were first reported soon after the original description of FXI deficiency and by 2000, there were reports of 15 individual patients in the literature.(96-104) All reported patients had hereditary FXI deficiency and most had received plasma derived FXI replacement. The FXI inhibitors were characterised as polyclonal IgG antibodies which bound primarily to the FXI domains involved in surface binding to FXII, HK and thrombin and neutralised FXI and

purified FXIa.(98;101;105) The largest study of inhibitors in FXI deficiency evaluated 118 severely deficient Israeli patients of whom seven had developed a FXI inhibitor and an additional three patients from the UK and the US.(106) All FXI inhibitors were detected in patients with a II/II genotype who had previously been exposed to plasma. FXI inhibitors were not detected in patients with a II/III or a III/III genotype. In this cohort, seven of 21 (33%) patients who were homozygous for the type II mutation and had received plasma, developed an inhibitor versus none of the 13 patients with the same genotype who had not received plasma. It seems likely that the incidence of FXI inhibitors in the literature is an under-representation and that more comprehensive screening of at risk patients would identify additional patients. Evaluation of the biochemical effects of six FXI inhibitors isolated from patient samples in this study confirmed earlier reports that FXI inhibitors impede activation of FXI by thrombin and FXIIa and impair activation of FIX but do not impair amidolytic cleavage of small substrates by FXIa.

FXI inhibitors tend not to present as spontaneous bleeding but may be detected clinically in the peri-operative period as breakthrough bleeding and /or a worsening response to FXI replacement. Pre-operative screening should detect clinically relevant inhibitors and is recommended in patients with the II/II genotype who have previously received FXI replacement (either plasma or FXI concentrate). Inhibitor screening should also be performed in any patient who shows an unexpectedly poor response to treatment or a worsening clinical phenotype. Current evidence suggests that development of FXI inhibitors is unlikely with other Jewish genotypes but information is lacking for non-Jewish genotypes.

1.3.7 Recombinant factor VIIa

The recognition of the presence of FXI inhibitors and the development of a new coagulation factor for use in Haemophilia A and B with inhibitors, led to the early use of recombinant factor VIIa (rFVIIa) in a patient with an inhibitor to FXI.(102) Subsequently a number of case reports have appeared in abstract or journal article format which support the efficacy of peri-operative rFVIIa in patients with FXI deficiency with inhibitors.(107-111) The dose of rFVIIa used initially varied from 60 to 90 μg/kg but more recently lower bolus doses have been suggested (12 to 20 μg/kg).(110;111) Continuous infusion has been used in some cases.(107;111)

In a fluorogenic thrombin generation assay using PPP from five patients with inhibitors to FXI, rFVIIa corrected the ETP to 50% of normal at a concentration of 1 μg/mL (equivalent to a clinical dose of 45 μg/kg).(106)

1.4 Thrombin generation and FXI

1.4.1 The history of thrombin generation assays

Two versions of the thrombin generation assay were described in the 1950s but since these methods were time-consuming and cumbersome for routine use, the assay was not adopted widely.(112-114) A method of continuously measuring thrombin generation was developed which was less labour intensive, less prone to manual error and amenable to semi-automation.(115;116) With the advent of a range of suitable substrates and technology which allows rapid analysis, thrombin generation began to be applied to the clinical settings of hypo- and hyper-coagulability.(117-119) The endogenous thrombin potential (ETP) was used to describe the total amount of thrombin

generated in the test but other parameters such as lag-time and peak thrombin generated can also be measured. The continuous method was suitable for evaluation of platelet poor plasma (PPP) but the chromogenic substrates used and the defibrination process ruled out using platelet rich plasma (PRP) in a continuous method. Nonetheless, the procoagulant surface of platelets was felt to be important and thrombin generation was shown to be defective in PRP from patients with Bernard-Soulier syndrome using a subsampling method and a chromogenic substrate.(120) The development of fluorogenic substrates and a method for calculating thrombin concentrations (which is more complex than for chromogenic substrates) enabled the development of a continuous fluorogenic thrombin generation test (TGT) which was suitable for both PPP and PRP.(121;122) The presence of the substrate itself throughout the reaction does influence the amount of thrombin generated in the continuous method but choice of the correct substrate and use of appropriate calculations allow accurate assessment of thrombin generation.(123;124) Since the rediscovery of the thrombin generation assay and the development of automated technology, thrombin generation has been evaluated in Haemophilia A and B, rare bleeding disorders, and thrombophilia (reviewed in (122)).

1.4.2 Standardisation of TGT

With the emergence of the TGT as a coagulation test with widespread clinical utility, standardisation of the assay became imperative. The published data up to 2003 had used a range of TF concentrations, PL sources and concentrations, substrates, reaction volumes and reagents. In an attempt to quantify the variation and eliminate it with the use of standard protocols, a study was undertaken in four separate laboratories of samples from normal individuals (ten), two lyophilised reference normal plasmas and

four lyophilised samples from individuals receiving anticoagulants.(125) Plasma samples from these patients were double centrifuged. There was a considerable variability between four different centres when analysing the various samples using a TGT customised in each laboratory (CVs varied from 2% to 114%, mean 60%). Imprecision studies varied from 1.15% to 19.72%. Using a standardised protocol on the same samples, the intercentre CV was 18.1% and the imprecision study CVs varied from 2% to 14.5%. Of note, these studies used a low dilution of TF (Recombiplastin diluted 1 in 2 in buffer) and so may not be applicable to TGT using lower concentrations of TF.

Influence of phospholipid source

The phospholipid (PL) surface is now appreciated as the critical platform on which the initiation and propagation phases of coagulation take place. Activation of FIX by surface bound FXIa is accelerated by the presence of phospholipids.(126) The source of phospholipids varies in in-vitro coagulation testing, from synthetic phospholipid mixtures to synthetic phospholipid vesicles to PRP or the addition of exogenous washed platelets to PPP. In addition, residual platelets and platelet microparticles may be found in PPP prepared by standard methods.(127)

In studies of thrombin generation in FXI deficient plasma, there has been no direct comparison of PPP and PRP. Studies of PPP have variously used synthetic mixtures of PL (106;128) and PL vesicles.(43;119) One study of thrombin generation in FXI deficiency used PRP or exogenous normal washed platelets added to PPP in a fluorogenic assay.(44)

The concentration of PL has also varied but concentrations between 1-10μM have generally been used. In a fluorogenic assay of rFVIIa induced thrombin generation, the effect of varying the phospholipid concentration varied from 0 to 64μM was evaluated.(128) In FXI deficient plasma, an increase in the amount of thrombin generated occurred with increasing PL concentrations up to a concentration of 32μM, above which no further increase was seen. The addition of 0.2pM TF to a TGT containing PL and rFVIIa, markedly augmented the amount of thrombin generated. In the absence of PL, regardless of the addition of rFVIIa or rFVIIa/TF, no thrombin was generated.

The conclusion which may be drawn from these studies is that the addition of PL to PPP is critical. Synthetic PL mixtures and PL vesicles have been used without clear evidence of superiority of one over another. Similarly, there is insufficient evidence for the use of PRP in preference to PPP but the use of PRP is likely to most closely resemble the conditions which pertain to in-vivo coagulation.

Influence of plasma preparation on TGT

After routine centrifugation, residual platelets may remain in plasma and there is a concern that freezing and thawing of PPP may cause disruption of residual platelets and provide an additional phospholipid surface on which thrombin may form, altering the ETP. This effect in most clearly seen in TGT which do not contain added PL and the effect may be abolished by filtration through cellulose filters or ultracentrifugation.(127;129) The effects of pre- or post-thaw filtration of PPP was further investigated in a TGT using 1pM concentrations of rTF and varying concentrations of PL (0, 0.5 and 1 μmol/L).(130) In this experiment on the standardization of ETP measurements,

filtration of plasma which had been centrifuged once was necessary either before or after freezing to eliminate the effects of disrupted residual platelets which increased the ETP when plasmas were thawed. This effect was most evident in experimental conditions which did not contain added PL. Of note, the authors did not study the effect of double centrifugation prior to freezing. Platelet microparticles are present in the circulation and can provide a phospholipid source even after routine centrifugation. Ultracentrifugation removes all platelet microparticles.(131) In a study of thrombin generation associated with viable internal mammary artery sections, a chromogenic subsampling TGT, using PPP and 0.6 pM TF and no additional PL, was evaluated.(132) No difference in thrombin generation in this assay was seen between standard centrifugation and ultracentrifugation. A study which was designed to evaluate the relative effects of TF and PL in FVIII, FIX and FXI deficient plasmas also used ultracentrifugation (presumably to outrule any confounding effects of residual platelets).(128)

Influence of contact activation

Contact activation of FXII with subsequent activation of FXI has been postulated to be a possible confounding factor in ETP studies.(133) Studies of the individual factors in a synthetic model of coagulation included the addition of corn trypsin inhibitor (CTI) to ensure that contact activation was fully inhibited.(134;135) However, contact activation did not appear to occur to any significant degree in a dilute thromboplastin assay evaluating the contribution of FXI to coagulation using low concentrations of TF as the initiator.(136) No differences were seen in a chromogenic TGT in FXI deficient plasma in the presence and absence of a monoclonal antibody to FXII.(43) Inhibition of FXII

was not used in two other studies of thrombin generation in FXI deficient plasmas using chromogenic substrates.(106;119)

In a fluorogenic TGT in PRP, recalcification resulted in the onset of thrombin generation after a lag phase of 20 minutes, which was fully inhibited by CTI.(44) Subsequently, the use of CTI in a fluorogenic thrombin generation assay has been shown to abolish contact activation at concentrations of TF <15pM in PPP and PRP.(133;137) However, in a fluorogenic assay of FXI deficient plasma no contact inhibition was used and no thrombin was generated over 60 minutes in FXI deficient plasma which was recalcified.(128)

Thus, the evaluation of the effects of contact inhibition has been conflicting. In a chromogenic assay, inhibition of FXII has not influenced the thrombin generation curve in FXI deficient plasma.(43) In the fluorogenic assay, there is evidence in some (but not all) studies of contact activation requiring the use of CTI.(44;128;133)

1.4.3 The role of FXI as determined by thrombin generation assays

In in-vitro and synthetic models of coagulation, deficiencies of FXI are not associated with a reduction in thrombin generation when high concentrations of TF are used (>10pM).(43;134;136;138) However, at low TF concentrations (< 5 pM ≈ 175 pg/mL) there is impairment of thrombin generation in FXI deficient plasma.(43;134) The role of FXI in the coagulation cascade appears to be especially relevant in circumstances where the initiation phase of coagulation is prolonged. This may be due to the fact that FXI activation by thrombin occurs late in the coagulation cascade.(138) In situations

where the initiation phase is rapid (e.g. when using higher concentrations of TF) the contribution of FXI may be relatively less important.

In a further study of a synthetic coagulation system FXI was found to reverse the inhibitory effect of elevated levels of Vitamin K dependent clotting factors.(139) FIX inhibits the activation of FX by the FVIIa/TF complex due to competition by the two zymogens for the same complex enzyme and results in a prolongation of the initiation phase. Elevated prothrombin appears to be particularly relevant in the presence of a limited phospholipid concentration (2 μM) and when using phospholipids rather than platelets. This inhibitory effect of elevated FIX or prothrombin is abolished by physiological levels of FXI and by the use of platelets as the phospholipid surface. The effectiveness of FXI in reversing the inhibitory effect of elevated vitamin K dependent clotting factors may be due to the slower generation of FXa and the prolongation of the initiation phase of coagulation in this setting.

Wielders et al examined the role of thrombin in the feedback activation of FXI in a thrombin generation assay using PRP.(44) Washed platelets were obtained from normal donors and FXI deficient PRP was prepared by suspending the normal washed platelets in FXI deficient plasma (the patient from whom the plasma was obtained was a homozygote for the type II mutation and had a FXI:C < 1%). A continuous thrombin generation assay was performed using a fluorogenic substrate and coagulation was initiated with varying concentrations of thrombin (0 to 5 nM). Of note, in this assay contact activation of coagulation (with a lag time of 20 minutes) was noted when the PRP was recalcified and all assays were performed in the presence of CTI.

The hypothesis to be tested was that thrombin could promote further thrombin production in PRP in a FXI dependent manner. In this study thrombin dose-dependently increased thrombin generation in the presence but not in the absence of platelets. There was a marked reduction in the lagtime and the peak thrombin generated in FXI deficient plasma but thrombin generation was restored by adding 10% normal plasma.

The authors also evaluated the effect of collagen which was found to enhance thrombin generation, possibly due to the procoagulant effect that collagen induces via platelet glycoprotein VI and exposure of anionic phospholipids.

Activation of PRP by the addition of 2 nM or 20 nM rFVIIa was also enhanced by pre-incubation with collagen. The addition of 20 nM rFVIIa and 5 μg/mL collagen also partially restored thrombin generation in PRP containing FXI deficient plasma.

In conclusion, this study suggests that thrombin can initiate and maintain further thrombin generation in PRP but only in the presence of FXI. Furthermore, platelet derived FXI and TF are not involved in this generation of thrombin but activated platelets are crucial. Collagen enhances thrombin generation.

1.4.4 Tissue factor as initiator

Factor XI is activated by FXII in the presence of high molecular weight kininogen and a negatively charged surface.(17) However, characterisation of FXI as part of the contact activation system, whose other members are not known to be associated with a bleeding diathesis, was problematic, since patients with both severe and partial FXI deficiency

are known to have bleeding symptoms (section 1.3.4). The Josso loop was evidence for the importance of feedback activation by thrombin of FIX and explained the critical bleeding diathesis of severe FVIII and FIX deficiency.(140) The confirmation that FXI too could be activated by small amounts of thrombin provided an explanation of the role of FXI in coagulation.(141;142) The importance of FXI in the maintenance of a stable clot was shown by the demonstration that FXI is necessary for the generation of TAFI at low concentrations of TF.(143) Cawthern et al showed that at low concentrations of TF, FXI deficient plasma did show evidence of thrombin generation but at a slower rate than in normal plasma. Subsequently the importance of the phospholipid surface was well described. FXI is activated on the surface of activated platelets and platelet GP1b is the critical receptor.(144)

Concentration of TF used for initiation

Butenas et al chose a recombinant tissue factor (rTF) concentration of 5 pmol/L (approx. 175 pg/mL) when conducting studies of thrombin generation in the context of varying concentrations of essential coagulation proteins and their inhibitors.(135;145) This rTF concentration was chosen to enable generation of thrombin even when procoagulant proteins were reduced to 50% of mean plasma levels.

Wielders et al state that in normal plasma prothrombin is maximally converted at a TF concentration of approximately 2.5 pM.(118) In this paper, the authors demonstrate that the ETP is dependent on the concentration of TF up to a concentration of approximately 3 pM. Above this level ETP and prothrombin consumption are independent of the TF concentration and type of thromboplastin. Other authors have quoted TF concentrations of >5 pM as the level above which the concentration of TF ceases to influence

prothrombin consumption.(145) By using a 5pM concentration of rTF, Wielders et al sought to create a test of ETP which was independent of changes in the activity of the thromboplastin but yet would be close to the physiological situation.

Lawrie et al conducted a multicentre assessment of ETP and in their standardized assay protocol used 100 μL of Recombiplastin diluted 1 in 2 with HEPES buffered saline to initiate coagulation in 80 μL of plasma.(125) Although the concentrations of rTF are not given, such a low dilution of rTF will equate to a high concentration of rTF for initiation. Of note, this protocol was used to evaluate intra- and inter-laboratory CVs for normal plasma pools and anticoagulated plasma samples.

In PRP, rTF concentrations of 1pM were used.(121) Concentrations of 1pM and 0.6 pM have also been used in more recent studies.(130;132)

In FXI deficient plasma, Keularts showed that a difference in ETP was seen when a concentration of 7.5 pg/mL (approximately 0.21 pM) whereas no change in thrombin generating ability was seen when a TF concentration of 37.5 pg/mL(=1pM) was used.(43) Subsequently, Al Dieri evaluated seven individual FXI deficient plasmas and found a normal ETP but rTF at a concentration of 15 pM (525 pg/mL) was used.(119)

1.4.5 Thrombin generation studies in patients with FXI deficiency

Following from these initial studies which indicated that FXI was important in the generation of thrombin, particularly at low TF concentrations, the recently re-discovered thrombin generation assay has been used to quantitate thrombin generation in FXI deficient plasma. Keularts et al conducted a series of experiments on plasma from

normal volunteers and nine patients with severe FXI deficiency.(43) No information on patient bleeding symptoms was presented.

Both PRP and PPP were evaluated in a chromogenic subsampling thrombin generation assay using a 10μM concentration of phospholipid and varying concentrations of TF. In these experiments, FXI deficiency was found to influence thrombin generation only when TF concentrations were 7-10 pg/mL (the concentration found in the thromboplastin time is 10 ng/mL). At concentrations of TF higher than 10 pg/mL, thrombin generation was normal even in patients with a type II mutation and severe FXI deficiency. At a concentration of TF of 7.5 pg/mL, there was a relationship between the FXI concentration and the ETP. At 10% FXI levels, the ETP in PPP was 50% of normal. In PRP from patients with a type II mutation, there was a marked impairment of ETP at low TF concentrations which was corrected by addition of 1% PPP. Plasma from patients with a type III mutation who had a FXI:C of approximately 9% had normal thrombin generation in PRP. Of note, in these studies there was no evidence of influence of contact activation since the addition of monoclonal antibodies to FXII did not alter the results found. Additionally, plasma with severe FXII deficiency was evaluated in a similar TGT. Without TF there was little thrombin generated but with 7.5 pg/mL of TF, thrombin generation was normal. Finally, in this study washed normal platelets were added to FXI deficient PPP but no effect of platelet derived FXI was evident in the amount of thrombin generated.

A subsequent study of thrombin generation was undertaken in a variety of factor deficiencies including FXI deficiency.(119) In this study, plasma was derived from patients and importantly bleeding symptoms were documented with a specific

questionnaire. The thrombin generation assay used was a subsampling method with 4μM phospholipids and activation by 15 pM TF. The ETP was evaluated in seven patients with FXI deficiency whose baseline FXI:C ranged from <1% to 54%. In this study, the ETP was reduced by approximately 50% in patients with FXI:C <1% and was normal in patients with higher FXI:C. Patient with FXI:C <1% were also stated to have a "mild" bleeding symptoms and patients with levels >1% had no bleeding. However, the total number of patients is small and it is unclear from the paper how many of the seven patients had FXI:C <1%. The earlier Keularts study had stated that the use of TF at concentrations > 10 pg/mL(≈ 0.2pM) was not sensitive to alterations in thrombin generation in FXI deficient patients and more recently, in a synthetic model of coagulation, it has been confirmed that at a TF concentration of 10pM thrombin generation is normal when FXI is deficient. Therefore, the usefulness of this study is limited due to the use of a high concentration of TF for initiation of coagulation.

Plasma from five patients with severe FXI deficiency and inhibitors was evaluated in a fluorogenic thrombin generation assay using PPP and added phospholipids (concentration not stated).(106) No thrombin was generated on recalcification.

Thrombin generation was evaluated in 14 patients with severe FXI deficiency in a fluorogenic thrombin generation assay.(128) No information was given regarding the exact FXI:C for each patient or the presence or absence of bleeding symptoms. Phospholipids at a concentration of 4μM were used and coagulation was initiated by recalcification. No TF was used initially since the subsequent experiments were designed to evaluate the effect of rFVIIa, TF and phospholipid concentration. No thrombin generation was evident in recalcified severely FXI deficient PPP.

It is apparent from these studies, that there is an impairment of thrombin generation in patients with severe FXI deficiency at low TF concentrations or when TF is not available. If the TF concentration is >1pM, the thrombin generation is likely to be normal. None of the studies published to date have evaluated partial deficiency of FXI and no correlation between thrombin generation and bleeding symptoms has been published.

1.4.6 The effect of rFVIIa in thrombin generation studies of FXI deficiency

Thrombin generation was evaluated in five patients with severe FXI deficiency with inhibitors (all were homozygous for the type II mutation).(106) A fluorogenic substrate was used with synthetic phospholipids. Thrombin generation was initiated by recalcification and increasing concentrations of rFVIIa (0-7 μg/mL) were added. In the absence of rFVIIa, the thrombin generation curve was flat and was similar to that of patients with severe FXI deficiency without inhibitors. Increasing amounts of rFVIIa resulted in increasing amounts of thrombin generation. In all five patient samples, correction of the ETP to 50% of normal was obtained with a mean rFVIIa concentration of 1 μg/mL, equivalent to a clinical dose of 45 μg/kg.

As part of a larger study on the effect of rFVIIa on thrombin generation in patients with haemophilia, 14 patients with severe FXI deficiency were included.(128) The thrombin generation assay in this study was a fluorogenic assay in which PPP was recalcified to initiate coagulation (CTI was not used) and no exogenous source of TF was added. There was no thrombin generation over 60 minutes in FXI deficient plasmas in the presence of phospholipids alone but the addition of rFVIIa at a concentration of 1.75 μg/mL (35 nM, equivalent to a clinical dose of 80 μg/kg) restored thrombin generation.

The latter effect was reduced if either FVIII or FIX were inhibited. Increasing concentrations of phospholipid increased the amount of thrombin generated in FXI deficient plasma treated with 1.75 μg/mL rFVIIa up to a concentration of 32 μM. Adding 0.2 pM (≈10 pg/mL) TF and rFVIIa at a concentration of 0.875 μg/mL (17.5nM) to FXI deficient plasma resulted in an additive increase in the amount of thrombin generated which was more marked if the concentration of phospholipids was also increased to 32 μM from 4 μM.

1.5 Thrombin activatable fibrinolysis inhibitor (TAFI)

1.5.1 Introduction

TAFI and the fibrinolytic system

The fibrinolytic system is a critical element of haemostasis since it is pivotal to the dissolution of clot and the maintenance of a patent vascular tree. The fibrinolytic system is comprised of the inactive proenzyme Plasminogen, two Plasminogen activators (tissue plasminogen activator, tPA and urokinase-type plasminogen activator, uPA), one inhibitor of Plasminogen activation (Plasminogen activator inhibitor 1, PAI 1) and two inactivators of plasmin, α2-antiplasmin and Thrombin activatable fibrinolysis inhibitor (TAFI).(146)

The TAFI protein was identified in the mid 1990s when it was also termed Carboxypeptidase U or Carboxypeptidase R.(147-149) The term TAFI is now the recognised nomenclature and a crystal structure has recently been published for human TAFI.(150)

TAFI is a 60 kDa procarboxypeptidase glycoprotein which consists of 423 amino acid residues which, following activation, has a molecular weight of 53 kDa and consists of 401 amino acids.(147;148) The *TAFI* gene is located at chromosome 13q14.11 and has 11 exons spanning 48kb.(151) Thrombin is the main activator of TAFI, a reaction which is increased by a factor of 1250 by thrombomodulin (152), although it may also be activated by plasmin and trypsin.(153) Activation occurs by cleavage of the Arg92-Ala93 peptide bond with release of a 92 amino acid activation peptide and an active molecule TAFIa. There is a biphasic pattern of TAFIa generation during in vitro clot lysis, the first wave is mediated by thrombin and the second by plasmin.(154) The active enzyme is inactivated by a conformational change which is temperature dependent. Release of the activation peptide results in the conformational change in TAFIa which disrupts the catalytic site.(150;155) TAFIa exhibits thermal instability and has a half-life of 8 minutes at 37ºC, which increases to two hours at 25ºC.(156;157)

TAFI inactivates fibrinolysis in an indirect manner by cleaving C-terminal lysine and arginine residues from partially degraded fibrin. The removal of these residues reduces the number of binding sites for plasminogen and tPA on fibrin, and consequently the degradation of fibrin is slowed. In addition, TAFIa eliminates the conversion of Glu-Plasminogen to Lys-Plasminogen and reduces protection of plasmin from α2-antiplasmin.(158)

There are two naturally occurring variants of TAFI with either a Threonine or an Isoleucine residue at position 325.(159) These variants have different properties after activation with respect to thermal instability (differing by a factor of 2, the isoleucine

isoform being more stable) and antifibrinolytic activity (differing by 60%, the isoleucine isoform being more active).

The Km for the activation of TAFI by thrombin is far below the plasma TAFI concentration.(152) For this reason, it may be hypothesised that an increase in TAFI concentration will result in increased formation of TAFIa, with a consequent decrease in fibrinolysis and promotion of thrombosis. Experimentally, addition of increasing amounts of purified TAFI to normal plasma results in a linear dose-dependent increase in the clot lysis time (CLT).(160) Alternatively, changes in TAFI activity or rates of inactivation could be wholly or partially responsible for the influence of TAFI on fibrinolysis. It should be noted though that the effect of TAFIa on fibrinolysis shows saturation with regards to TAFIa levels. The extent of prolongation of fibrinolysis is approximately 3-4 fold at saturation TAFIa levels. Under certain conditions, TAFIa can be stabilized by competitive inhibitors and the antifibrinolytic effect can be enhanced to a 20-fold prolongation of fibrinolysis.(161) Therefore, alterations in TAFIa may attenuate but do not eliminate fibrinolysis.

A threshold effect has been demonstrated for TAFIa which is defined as the minimal TAFI activity necessary to prevent acceleration of fibrinolysis and is dependent on tPA concentration. The TAFIa threshold has been calculated at 8 U/L at a tPA concentration of 40ng/mL which is a limited percentage of the total amount of TAFI available for activation (963 U/L).(162;163)

More recently, it has been suggested that the pro-enzyme TAFI may have intrinsic enzymatic activity (164) and that this continuous and stable activity may have a role in

protecting clot from fibrinolysis after the initial burst of TAFIa which is rapidly inactivated at 37ºC.

Role of FXI in Fibrinolysis

FXI contributes to the generation of thrombin and to the inhibition of fibrinolysis. In a turbidometric assay, fibrin generation was initiated with decreasing concentrations of recombinant TF (Innovin) in plasma deficient in FXI and FXII and in the same plasma reconstituted with FXI (final concentration 0.5 U/mL).(143) While at higher rTF concentrations there was no difference in the amount of fibrin generated, less fibrin was generated in FXI deficient plasmas at rTF dilutions ranging from 3×10^4 to 3×10^5. This effect was abolished with pre-incubation of the FXI used to reconstitute the deficient plasma with a specific antibody. The effect of FXI on clot lysis was then studied by adding t-PA (final concentration 30 U/mL, chosen to produce clot lysis in 60 to 90 minutes). At the highest rTF concentration (10^3 dilution), no effect of FXI on clot lysis is seen when deficient plasma is compared with plasma reconstituted with FXI. However, at lower rTF concentrations (10^4 and 10^5 dilutions) the rate of clot lysis is increased in FXI deficient plasma. It is notable that the effect of FXI in clot lysis is seen at rTF concentrations at which no alteration in fibrin formation was noted. The possibility that the newly described TAFI protein was responsible for the effects described was mentioned in a note added in proof.

Once the TAFI molecule had been identified it was then confirmed that FXI dependent inhibition of fibrinolysis was TAFI dependent. (165) In this study, inhibition of fibrinolysis in FXI deficient plasma is restored by the addition of FXIa but this effect is completely abolished by pre-incubation with an antibody to TAFI. Fibrinolysis is

enhanced in TAFI deficient plasma which is restored by the addition of purified TAFI but this restorative effect is again abolished by pre-incubation with an antibody to FXI.

1.5.2 TAFI Antigen

Measurement of TAFI:Ag

There are a number of commercially available kits for the detection of TAFI antigen. It has been suggested that there is an association between TAFI:Ag levels and genotype for certain TAFI polymorphisms which is more marked with certain assays. This is postulated to result from variable antibody reactivity to TAFI isoforms, in particular the The325Ile (1040C/T) variant. In 92 healthy individuals, the TAFI genotype was as follows: Ile/Ile 9%, Thr/Ile 42% and Thr/Thr 49% and the corresponding TAFI levels as measured by Elisa were 0.45, 0.6 and 0.83 U/mL.(166)

TAFI:Ag levels in normal individuals

TAFI antigen levels show considerable variability but are normally distributed in the human population. In an early evaluation of TAFI:Ag using an Elisa technique and murine monoclonal anti-TAFI antibodies, the mean level was 111.4%±25.2 (compared to a normal pool) in 20 individuals with a range of 72-154%.(160) Chetaille et al evaluated 249 healthy Caucasian men and non-pregnant women, 118 black African men and 12 pregnant women using a sheep anti-TAFI IgG in a commercial kit (Milan Analytica).(167) In this study, there was no gender variation between men and women but TAFI:Ag levels were lower in African males than Caucasian males. TAFI:Ag increased with age in women but there was no alteration in pregnancy. There was no evidence of an influence by circadian rhythm or of inter-individual variation over time. In a study of 948 patients with venous thromboembolism and controls, TAFI:Ag was

measured by electroimmunoassay using rabbit anti-TAFI IgG and the lack of a gender difference in TAFI:Ag was confirmed as was an increase in TAFI:Ag with age in women.(168) In addition, in this study, women on the combined oral contraceptive pill showed increased levels of TAFI:Ag. In these early studies, results were expressed as a % of a pool of normal individuals, varying from 30 to 64 individuals. TAFI:Ag levels (using the Affinity Biologicals kit) in a series of 96 unrelated patients with severe FXI deficiency were determined to be 121.2±30.7% in patients with acute myocardial infarction and 134.3±47.2% in patients without myocardial infarction.(169)

A number of polymorphisms have been described in the promoter region of the TAFI gene and in the 3'UTR which have been associated with variations in TAFI antigen levels.(170)

TAFI levels and disease associations

In cardiovascular disease, it was unexpectedly found that cases had lower TAFI levels and a higher incidence of "lowering" TAFI polymorphisms than controls.(171)

Increased levels of TAFI:Ag have been postulated to be associated with thrombosis and in the Leiden thrombophilia (LETS) study, TAFI:Ag levels above the 90th centile, as seen in 9% of controls and 14% of patients, were associated with a two fold increase in the risk of thrombosis.(168) In addition, individuals in the LETS study with a CLT > the 90th percentile had a two-fold increased risk of thrombosis.(172) Increases in the CLT were not strongly correlated with any particular coagulation factor or TAFI and are presumed to reflect the balance of procoagulant and fibrinolytic factors.

1.5.3 TAFI activity

Measurement of TAFIa: Clot Lysis and enzymatic methods

TAFI activity can be evaluated either by performing a specific enzymatic assay or by a clot lysis assay.

The clot lysis assay is a global assay encompassing the entire fibrinolytic system. It is a plasma based assay which utilises an activator of thrombosis, usually thrombin or recombinant tissue factor and the reaction mix will generally contain a phospholipid source, calcium, tPA and buffer. Potato tuber carboxypeptidase inhibitor (PTCI) is a specific inhibitor of TAFI and may be added to the reaction mix to define the contribution of TAFI to the CLT.(154;173;174) In this way, the CLT is a surrogate measurement of TAFIa activity in a given plasma sample. Other coagulation factors and inhibitors may also be added such as recombinant factor VIIa or antibodies to FXI for example to evaluate their specific effect on fibrinolysis.(173)

The clot lysis assay requires a prolonged incubation from 80 to 220 minutes at 37ºC.(143;154;160;173;174) The clot lysis time (CLT) is measured in minutes and has been defined in two ways: as the time from the midpoint of the clear-to-maximum turbid transition to the midpoint of the maximum turbid-to-clear transition and also as the time to achieve a 50% reduction in the maximum turbidity.(143;173)

Two groups have described enzymatic assays for TAFIa.(175;176) Both use thrombin/thrombomodulin as the activator of TAFI and detect the TAFIa formed by detecting cleavage of hippuryl-Arg, either by a colorimetric reaction (175) or by HPLC.(176) Assays are run with and without PTCI to determine the contribution

attributable to TAFI versus other plasma carboxypetidases e.g. Carboxypeptidase N. A commercially available kit has been developed which is based on the enzymatic assay (Actichrome® TAFI activity assay; American Diagnostica, CT, USA).

TAFIa in normal individuals and in Haemophilia

TAFI was confirmed to play a role in the modulation of clot lysis in healthy individuals in the presence of a normal intrinsic pathway.(160) There is a linear relationship between TAFI antigen and clot lysis time when purified TAFI is added to TAFI depleted plasma. In 20 healthy volunteers, the mean clot lysis time was found to be 62 ± 11.5 minutes but there was considerable inter-individual variation (41 – 82 mins). When the clot lysis assay was repeated in the presence of a FXI antibody the clot lysis time was reduced to 38.7 ± 8.6 minutes. A significant correlation was seen between the TAFI antigen and both clot lysis time and TAFI activity levels in these individuals. The correlation between TAFI antigen and clot lysis is lost when a FXI inhibiting antibody is used. TAFI activity was also measured enzymatically after activation by thrombin/thrombomodulin and this was correlated with clot lysis indicating that the clot lysis assay is an accurate surrogate for the functional activity of TAFIa in plasma.

In pooled severe Haemophilia A plasma, clot lysis times were normal at high TF concentrations but were severely decreased at moderate to low concentrations of TF.(177) Addition of FVIII, TAFI or thrombomodulin restored clot lysis times in the presence of low concentrations of TF. Fifty-six individuals with severe Haemophilia A were studied and again, clot lysis times at baseline were decreased. CLT in the presence of PTCI were similar to the baseline CLT, showing that minimal activation of TAFI occurs in Haemophilia A plasma. Addition of physiological concentrations of

FVIII, TAFI or TM increases the CLT significantly. Notably, some individuals with Haemophilia A had normal CLT at baseline, illustrating the inter-individual variability seen. No correlation between TAFI levels and bleeding was reported.

1.5.4 The influence of rFVIIa on fibrinolysis

In haemophilic plasma, the addition of recombinant factor VIIa (rFVIIa) at concentrations above 31.3 U/mL significantly prolonged the CLT and this effect was abolished by the addition of a specific inhibitor to TAFI (PTCI).(173) It was noted that there was considerable variability in the concentration of rFVIIa required for half maximal prolongation of the CLT (median 73 U/mL, range 10.7-250 U/mL) and that the corresponding concentration of rFVIIa required to shorten the clotting time was much less (median 8.4 U/mL, range 1.7-22.5 U/mL). The effects of rFVIIa on clotting time and CLT were not correlated with each other. It is notable that the concentrations of rFVIIa required for clotting in this study are similar to those needed to promote haemostasis while the higher concentrations needed to inhibit lysis are similar to those required to maintain haemostasis.(178)

In a study of fibrin aggregation curves, initiated by thrombin and performed in a microplate, commercially purchased FXI deficient plasma had a CLT of 22.5±0.7 minutes compared to 24±2.2 minutes for pooled normal plasma.(179) The addition of rFVIIa at concentrations from 2.4, 4.8 and 9.6 μg/mL normalised the CLT. It could be argued that the use of thrombin as the initiator results in significant activation of TAFI which is non-physiological compared to the amount of thrombin that might be generated in a severely FXI deficient patient in-vivo.

1.6 Conclusion

Considerable progress has been made in the understanding of the genetics, structure and function of the FXI protein in recent years. In particular, the interaction between the FXI apple domains and its activators, co-factors and substrates has been clarified. Identification of mutations in the *F11* gene causing clinical FXI deficiency is also adding to the understanding of the function and importance of specific residues and regions.

However, the increase in knowledge has not yet translated into a better understanding of the bleeding diathesis in deficient patients. The variable and unpredictable bleeding tendency in this condition continues to present great difficulties to clinicians counselling and treating patients. Although a considerable amount of clinical data have been published on FXI deficiency, much of the clinical research was done at a time when detailed genetic analysis was not readily available, newer proteins such as TAFI were unknown and re-emergent techniques such as thrombin generation had not yet become widely utilised. Indeed there remains some controversy about the prevalence and importance of clinical bleeding symptoms in FXI deficient individuals, especially those with partial deficiency. The optimum treatment for patients requiring invasive procedures is uncertain and new products with increased viral safety are needed. The rarity of this bleeding disorder means that it can be difficult to accumulate data on large numbers of patients and clinical recommendations may be based on case reports and small case series.

In this context, a project to study the clinical and laboratory features which determine the bleeding tendency in FXI deficiency was designed. The aims of this project were to

clearly define the genotype and phenotype of a large cohort of FXI deficient patients and to try to identify modifying factors which influence the bleeding tendency using the following methods:

- Allocation of a bleeding score to all participating patients, having determined the bleeding history by the use of a standard questionnaire
- Evaluation of routine laboratory coagulation parameters including FXI:C, FXI:Ag, von Willebrand screen, blood group and thrombophilia screen
- Evaluation of the influence of TAFI by means of estimation of TAFI:Ag, TAFI:Ac and clot lysis time
- Development of a thrombin generation assay with appropriate modification for use in FXI deficient plasma
- Screening of the *F11* gene for causative mutations
- Development of a molecular model of the FXI protein to evaluate the effect of mutations on protein structure and function, since a crystal structure for the FXI protein did not exist at the time of the study
- Evaluation of the use of recombinant factor VIIa for the prevention of bleeding associated with surgery in FXI deficient patients in a pilot clinical trial.

The assessment of bleeding tendency in this study was fundamental and was utilised through the different aspects of the work to define the relationship between possible modifiers of bleeding and clinical bleeding phenotype.

Chapter 2 Standard Materials and Methods

2.1 Patient recruitment

All patients studied were aged >18 years and were registered as having FXI deficiency at the Katharine Dormandy Haemophilia Centre and Haemostasis Unit, Royal Free and University College Medical School, London, United Kingdom. The local Ethics Committee approved the study. Informed patient consent was obtained in all cases. Patient consent, bleeding history and blood samples were taken at a dedicated clinic held weekly over a six month period in 2001/2002. The patient information leaflet, consent form and data collection form along with a statement of ethical approval and clearance from the Royal Free Hampstead NHS Trust Clinical Governance Support centre are shown in Appendix 10.1.

2.2 Specimen collection

Whole blood samples were collected by venepuncture from participating patients, using minimal venous stasis. For coagulation analysis, 40 mLs of blood was drawn into citrate (0.106mol/L trisodium citrate solution, Sarstedt Monovette, Nümbrecht, Germany). The citrate samples were spun for 12 minutes at a speed of 3000rpm and a temperature of 4^0C. The supernatant plasma was separated and spun again for a further 12 minutes before being aliquoted into 1 mL Eppendorf tubes and frozen at -70^0C.(180) Prior to analysis, the plasma was defrosted at 37^0C for 5 minutes. Thawed plasmas were stored at 2-8 ^{0}C until assay and were assayed within 2 hours of thawing.

For genetic analysis, 10 mLs of whole blood was drawn into EDTA (K^{3+}EDTA, 1.6 mg/mL, Sarstedt Monovette, Nümbrecht, Germany). The EDTA sample was spun for 10 minutes. The buffy coat was aspirated with a Pasteur pipette, placed in a clean Eppendorf and frozen at -70^0C.

2.3 Bleeding history and Bleeding Score

2.3.1 Bleeding history

A detailed bleeding history was taken by a single clinician (NOC) at a dedicated clinic, using a standard data collection form (appendix 10.1). Patients were assigned a unique study number. The following details were recorded:

- Demographics (name, DOB, ethnic origin, religion, registered baseline FXI:C, blood group)
- Diagnosis of FXI deficiency (circumstances of diagnosis)
- Bleeding history (dental extraction, epistaxis, trauma, major surgery, minor surgery, circumcision)
- Medications (in particular aspirin, NSAIDs, anticoagulation)
- Family history (number of first degree relatives, description of bleeding symptoms)
- Menorrhagia, pregnancy history, post partum haemorrhage
- Treatment episodes (number and type of treatments)
- Vaccination status

In the case of bleeding episodes, the type of treatment given including transfusion, re-operation and factor replacement was recorded.

2.3.2 Bleeding score

The bleeding histories were subsequently scored independently by three clinicians with experience in haemostasis, one of whom was the clinician who had taken the original history. Patients were identified by study number only. The clinicians scoring the bleeding histories were blinded to the patients FXI:C and other laboratory results. Scores were compared to assess concordance between the three reviewers.

A five point bleeding score was used to designate bleeding tendency.(5;7) A definite bleeder was allocated a score of one and a probable bleeder was given a score of two (Table 2.1). Conversely, a definite non-bleeder was given a score of five and probable non-bleeders had a score of four. A score of three (indeterminate) was applied if the reviewer felt that the patient had not undergone sufficient haemostatic challenges on which to base a judgement or if there was conflicting evidence. In two cases, a bleeding score of three was applied where there was disagreement amongst the reviewers about the bleeding score.

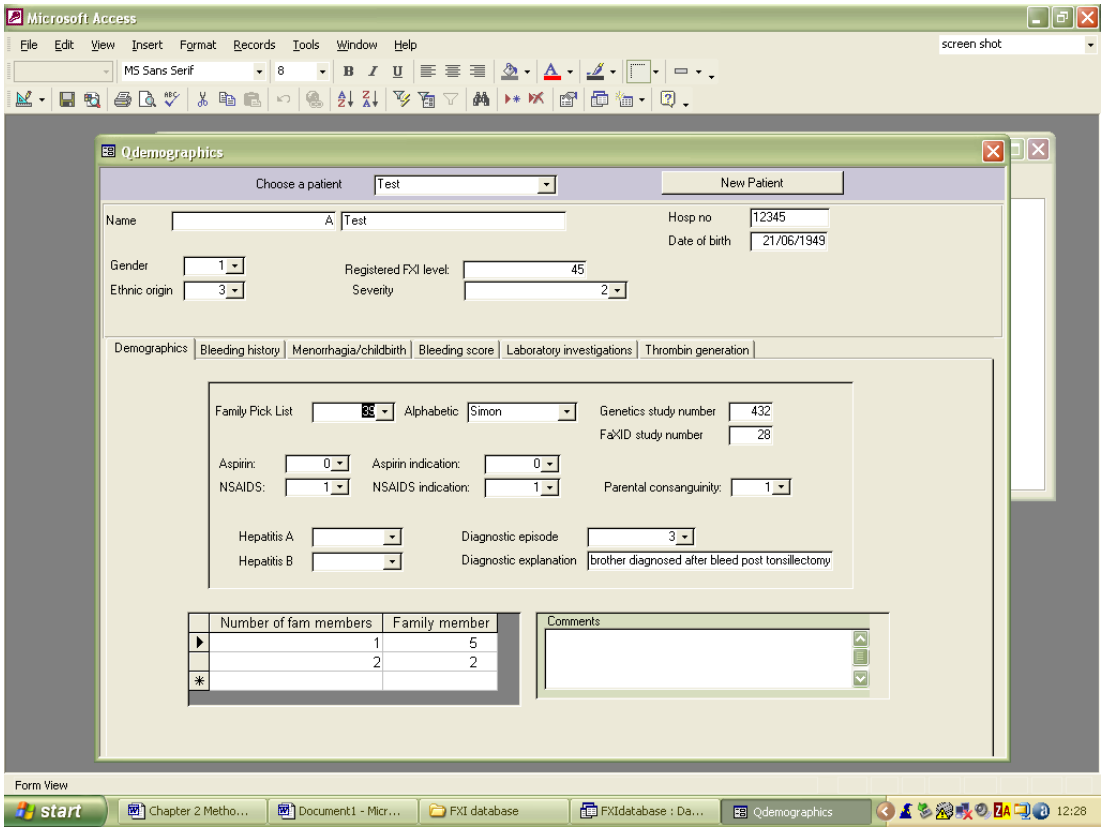
Table 2.1: Bleeding score related to bleeding phenotype (5;7).

Bleeding Score	Bleeding Phenotype
1	Definite bleeder
2	Probable bleeder
3	Indeterminate
4	Probable non-bleeder
5	Definite non-bleeder

2.3.3 Patient database

All patient information gathered in the bleeding history (fig 2.1) and patient bleeding scores (fig. 2.2) were recorded in a specifically designed, password-protected patient database (Microsoft Access® 2002). Individual data items were assigned numerical codes to aid data retrieval.

Figure 2.1: Patient demographics displayed in FXI patient database.



Subsequently, patient data derived during the laboratory analysis was added to the patient database (fig. 2.3), thus ensuring a robust, secure and easily searchable patient database.

Figure 2.2: Bleeding scores from each of three observers and consensus bleeding score displayed in FXI patient database.

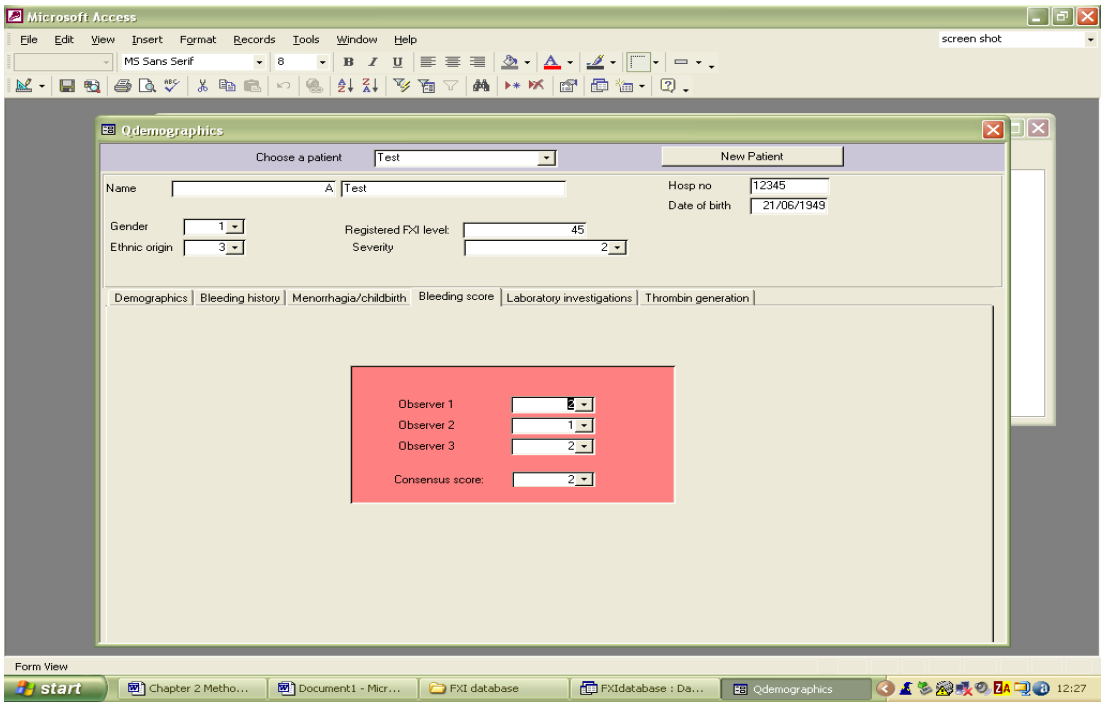
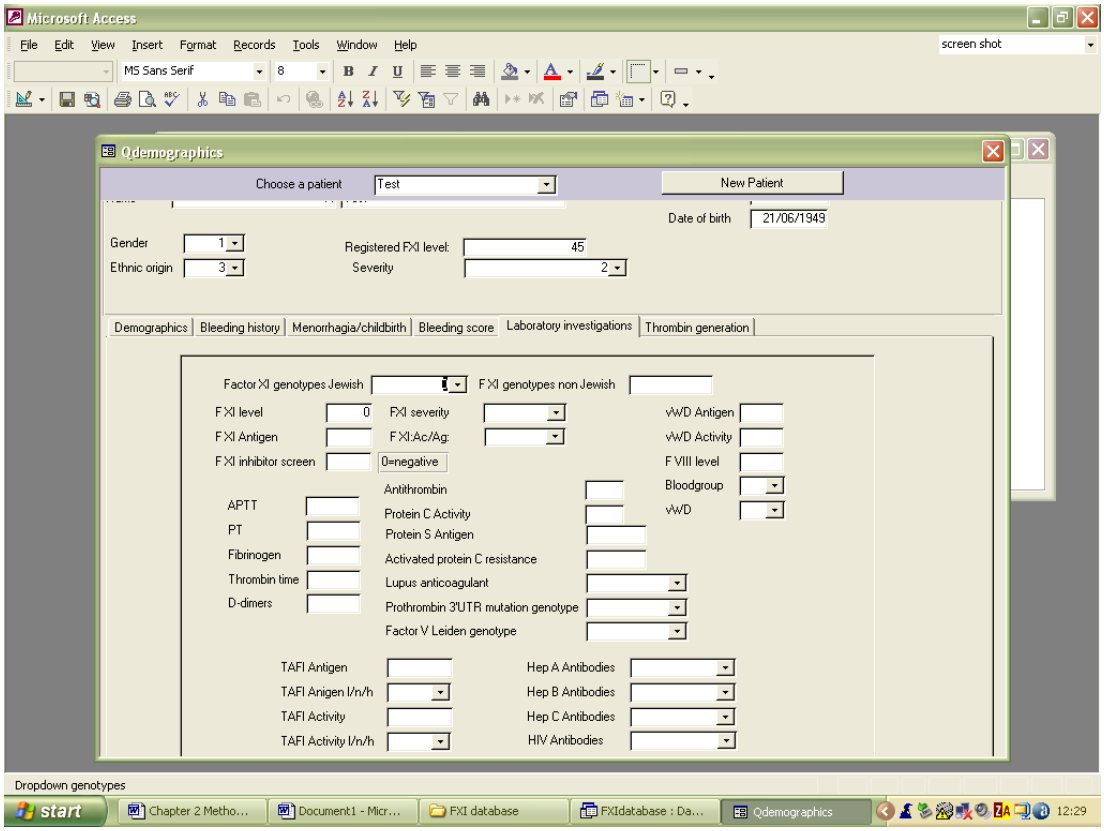
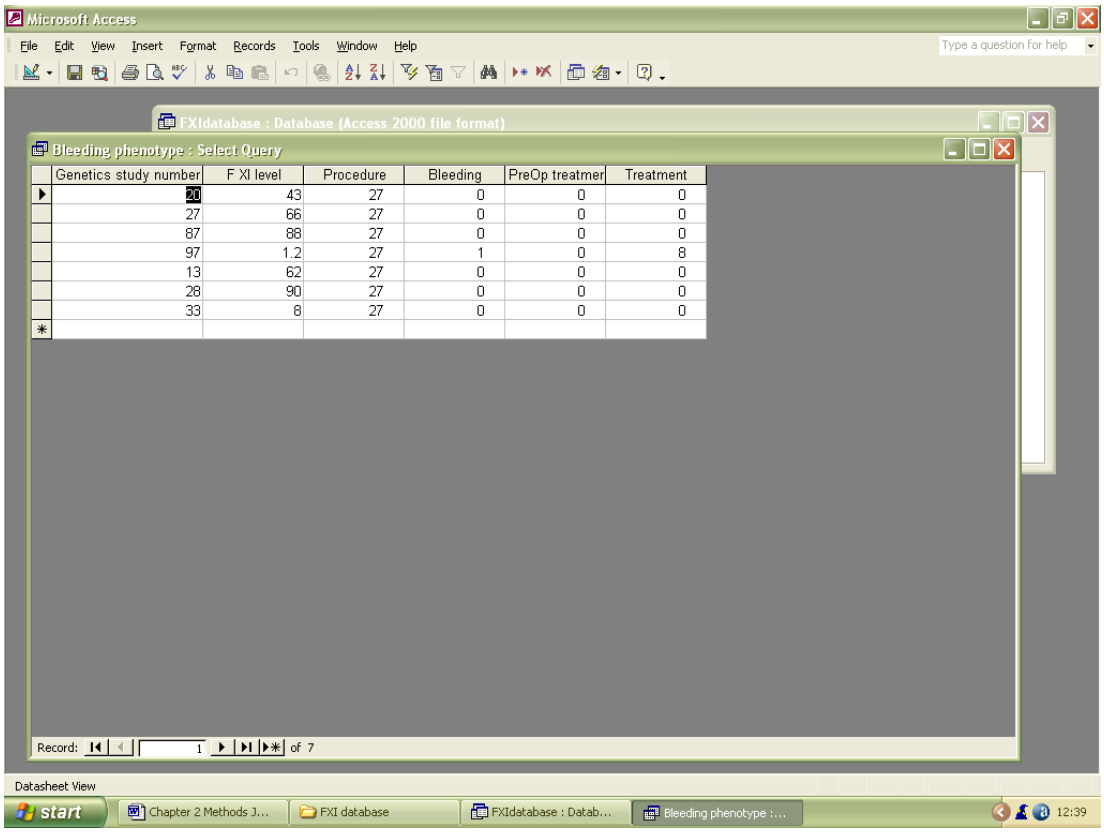


Figure 2.3: Laboratory Investigations screen, FXI patient database.



Queries regarding individual parameters were easy to run. In the example given in figure 2.4, the database is queried regarding patients undergoing combined tonsillectomy and adenoidectomy (code 27). Seven patients are identified with study number and FXI:C. None of the patients received pre-operative treatment (all code 0 in the "PreOp treatment" column). One patient suffered post-operative bleeding (code 1 in the "bleeding" column) and required a blood transfusion (code 8). Database queries were easily customised to include or exclude additional data.

Figure 2.4: A database query on patients undergoing combined tonsillectomy and adenoidectomy.



2.4 Coagulation analysis

FXI:C was measured using a one-stage activated partial thromboplastin time in three dilutions (1:10, 1:20 and 1:40) on an ACL-3000 (Instrumentation laboratories, Cheshire, UK). FXI:C was assayed against a normal pool which had been assigned a value of 100 U/dL. The activator used in the factor assay was a lyophilized silica APTT reagent (Instrumentation laboratories, Cheshire, UK) and the activation time was five minutes. A FXI deficient plasma with a FXI:C <1 U/dL was used for all dilutions, either commercially obtained (Technoclone UK Ltd, Surrey, UK) or obtained from a patient known to be homozygous for the Jewish type II *F11* gene mutation. The reference interval was obtained from analysis of the FXI:C levels of 34 individuals without a known coagulation disorder and was calculated as the range encompassing the mean ± 2SD (70-140 U/dL). The inter-assay co-efficient of variation (CV) was 6.3% for normal plasma and 4.2% for abnormal plasma.

FXI:Ag was measured using an enzyme-linked immunosorbent assay which utilised a polyclonal goat anti-human FXI IgG as the capture antibody and a peroxidase goat anti-FXI as the detection antibody (Kordia Life Sciences, Leiden, The Netherlands).

Assays for PT, aPTT, FVII:C, FVIII:C, VWF:Ag, VWF:CBA, antithrombin, protein C, protein S, APCR, lupus anticoagulant and blood group were performed according to the standard laboratory protocols in use at the time in the Katharine Dormandy Haemophilia centre Coagulation Laboratory.

Thromboelastography (TEG) was performed using a computerized thromboelastograph (Haemoscope Corp., Illinois, USA). Within 60 minutes of venepuncture, citrated whole

blood (340 μL) was added to a warmed TEG cup containing 20 μL of 0.2 mol/L calcium chloride and the pin was raised and lowered into the cup three times. The TEG analyser recorded the following parameters: the reaction time (r time), the clot formation time (k time), the alpha angle and the maximum amplitude and these parameters were presented in graphical and numerical form.

2.5 Thrombin Generation Tests

2.5.1 Materials

Common materials for all TGT

Tris buffered saline (TBS): 3.03 g Trizma base, 4.4 g NaCl in 500 mLs sterile water, pH 7.4

Bovine serum albumin (BSA): 20% solution (Sigma-Aldrich, Dorset, United Kingdom).

TBS/BSA: 1 mL of 20% BSA was added to 19 mLs TBS, (concentration 50mM Tris base, 150mM NaCl, 1% BSA, pH 7.4)

Recombinant tissue factor (rTF): RecombiPlastin, (HemosIL, Instrumentation Laboratory, Cheshire, U.K.). The Recombiplastin was reconstituted in 20 mLs of sterile water rather than the supplied diluent which contains calcium. A 10 ng/mL solution of Recombiplastin was made by further dilutions in sterile water and stored at 4 ^{0}C (total dilution 1 in 2620).

Phospholipid: Bell and Alton platelet subsitute (Diagen, Diagnostic reagents, Thame, Oxon.)

1. Subsampling method: Reconstituted with 1 mL TBS (concentration 233 μg/mL(181)) giving final concentration 4.66 μg/mL (13 μM) in the reaction mix.
2. Continuous method: Reconstituted with 4 mLs TBS giving final concentration 6.5 μM in the reaction mix.

Reptilase: (Pentapharm, Basel, Switzerland) reconstituted with 1 mL sterile water.

0.1 M calcium chloride: (Baxter, Berkshire, U.K.)

Sterile water: (Baxter, Berkshire, U.K.)

Flat bottomed microtitre plate: Nunc Maxisorp (Thermo Fisher Scientific, Roskilde, Denmark)

Water bath at 37^0C

Ice

Wooden sticks

Specific materials and working solutions for subsampling method

Chromogenic substrate: H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline dihydrochloride - S2238 (Chromogenix, Milan, Italy). 16.55mLs sterile water was added to a 25mg vial of substrate and a working solution was made by diluting this 50:50 in TBS/BSA.

EDTA buffer: 3.03 g Trizma base, 5.1135 g NaCl, 1.395 g EDTA in 500 mLs sterile water, pH 8.4

EDTA/BSA buffer: 5 mLs 20% BSA added to 95 mLs EDTA buffer (concentration 7.5mM EDTA, 50mM Tris base, 175mM NaCl, 1% BSA, pH 8.4)

rTF/$CaCl_2$: A working solution of rTF/ $CaCl_2$, to give a final concentration of 10 pg/mL in the reaction mix, was made on the day of each experiment by diluting the 10 ng/mL rTF stock solution in 0.1M $CaCl_2$ (1 in 216 dilution, total dilution approximately 1 in 565920).

50% acetic acid: glacial acetic acid mixed 50:50 with sterile water

Bovine thrombin: (Sigma-Aldrich, Dorset, United Kingdom) 1000 U/mL for standard curve

Plate reader: Dynex MRX (Dynex technologies, West Sussex, U.K.) to read at 405nm.

Polystyrene LP4 test tubes: (Western Laboratory Service Ltd, Hampshire, U.K.)

Specific materials and working solutions for continuous method

Chromogenic substrate: Methylmalonyl-L-α-methylalanyl-arginyl-paranitroanilide - CBS 00.68 (Diagnostica Stago, Reading, U.K.). The vial was reconsitituted with 1.25 mLs sterile water to give a concentration of 6 mmol/L. A working solution was made by diluting this solution 50:50 in 0.1M $CaCl_2$.

Normal plasma control: Cryocheck (Precision Biologic, Hampshire, U.K.)

rTF/TBS/BSA: A working solution of rTF in TBS/BSA, to give a final concentration of 10 pg/mL in the reaction mix, was made on the day of each experiment by diluting 5.5 µl of the 10 ng/mL stock solution in 1994.5 µl TBS/BSA.

Kinetic plate reader: Versamax tuneable microplate reader (Molecular Devices (UK) Ltd, Berkshire, U.K.)

2.5.2 Subsampling method

Thawed plasma was defibrinated by adding 20 µl of reptilase to 1mL of plasma. The plasma sample was vortexed briefly and incubated in a waterbath at 37^0C for 15 minutes. The sample was then placed on ice for 10 minutes before the clot was removed by winding onto a wooden stick. The plasma was centrifuged at 13,000 rpm for three minutes and any residual clot removed. The defibrinated plasma sample was kept on ice until assayed. Plasmas were tested in triplicate.

To assay thrombin generation by the sub-sampling method, 300 µl of plasma was incubated with 20 µl phospholipid (final concentration 13 µM) and 40 µl TBS/BSA

buffer at 37^0C for 5 minutes. Clotting was initiated by adding 100 µl recombinant tissue factor diluted in 0.1M calcium chloride (rTF/$CaCl_2$, final concentration 10 pg/mL) which had been warmed in the waterbath for two to three minutes. Exactly 30 seconds after addition of the rTF/$CaCl_2$, the first 10 µl sample was removed and added to a well of a microtitre plate containing 140 µl of an EDTA/BSA buffer to stop the reaction. Sequential 10 µl samples were removed and added to individual wells of the microtitre plate every 30 seconds for 30 minutes in the case of partially deficient or rFVIIa treated plasmas or every 60 seconds for 60 minutes in the case of severely deficient plasmas. When the plate was full or the end time had been reached, 50 µl of chromogenic substrate S2238 was added to each well and exactly 3 minutes later, 80 µl of 50% acetic acid was added to stop colour formation. The plate was then read at 405nm. The absorbance from the first well of a series of wells for an individual sample was subtracted as a blank from all raw absorbances to control for the baseline colour of the plasma. The contributions of thrombin and the thrombin-α2-macroglobulin were calculated using a macro supplied by the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, U.K.) and based on the method described by Hemker.(116) The thrombin generated for each triplicate plasma was calculated and averaged. The thrombin curve was graphed and the area under the curve (AUC) and peak thrombin calculated using a standard curve generated by assaying serial dilutions of bovine thrombin.

2.5.3 Continuous method

Plasma was defibrinated as outlined above and was kept on ice until assayed. Plasma samples were tested in duplicate and two normal plasma controls were tested in duplicate in the first and last wells of each microtitre plate.

To assay thrombin generation by the continuous method, 20 μl of Phospholipid (final concentration 6.5 μM) was added to each well of a flat bottomed plate. 80 μl of prewarmed rTF suspended in a TBS/BSA solution (final concentration of rTF 10 pg/mL) and 80 μl of test plasma were then added to the well. The plate was mixed on the plate reader and incubated at 37^0C for 2 minutes. Coagulation was initiated by adding 40 μl of substrate/$CaCl_2$ solution. The plate was mixed and the plate reader was set to commence reading at 405 nm every 30 seconds continuously for 30 minutes. The results were downloaded to an Excel spread sheet (Microsoft® Excel 2002). The contributions of thrombin and thrombin- α2-macroglobulin complex to the absorbances recorded by the plate reader were calculated using a macro (see below). The endogenous thrombin potential was calculated as the sum of the absorbances attributable to thrombin expressed as a percentage of normal plasma control.

2.5.4 Mathematical calculation of Endogenous Thrombin Potential in the Continuous Thrombin Generation Test

In the course of the thrombin generation test, thrombin is generated which converts substrate which is detected by measuring optical density However, as the experiment proceeds a portion of the thrombin generated complexes with α2-macroglobulin and this complex also converts substrate. If the contribution of the α2-macroglobulin - thrombin complex to the total optical density is not subtracted, an overestimation of thrombin generation will result. The mathematical formula to perform this calculation has been described by Hemker, both for the subsampling and the continuous methods for measuring thrombin generation.(116) The formula for the continuous method was entered onto an Excel (Microsoft® Excel 2002) spreadsheet in the form of a macro which could be applied rapidly and accurately to the raw data obtained from the

microtitre plate reader. The macro was validated against a manual calculation for conversion of the raw data to ensure accuracy of the computer generated results before it was applied to patient data.

2.6 TAFI

2.6.1 TAFI Antigen

Materials

Imulyse® TAFI Antigen kit (Biopool International, Sweden), subsequently marketed as VisuLize® TAFI Antigen kit (Affinity Biologicals Inc., Ontario, Canada) containing:

- 6 strips of 16 wells pre coated with a polyclonal sheep antibody to human TAFI
- Standard reference plasma
- Control plasma A
- Control plasma B
- TAFI deficient plasma
- 10x wash buffer concentrate
- Sample diluent
- Peroxidase labelled detecting antibody
- Tetramethylbenzidine (TMB) substrate
- 0.2M H_2SO_4 (Stop solution)
- Adhesive plate sealer

Also required were:

- Reagent grade water

- 1 channel pipettes
- 8 channel pipettes
- Laboratory timer
- Microplate reader: Dynex MRX (Dynex technologies, West Sussex, U.K.) to read at 450 nm

Method

A six point standard curve (1/100 to 1/3200 dilutions) was derived by diluting the standard reference plasma in TAFI deficient plasma. Control and test plasmas were diluted 1 in 2 in TAFI deficient plasma and then 1 in 100 in sample diluent, to give a final dilution of 1/200.

The pre-coated wells were prepared by washing three times with wash buffer (300 μL per well). Standards and samples were loaded into the wells in duplicate (100 μL per sample) and incubated at room temperature for one hour. 100 μL of sample diluent is loaded in duplicate as a "blank". The wells were emptied by inverting onto absorbent blotting paper and were washed three times. 100 μL of detecting antibody was applied to the wells and incubated at room temperature for 30 minutes. Again the wells were emptied and washed three times as before. 100 μL of TMB substrate was added to each well and the colour was developed for exactly 10 minutes at room temperature. 100 μL of 0.2M H_2SO_4 stop solution was added to the wells in the same order as the TMB substrate and the plate was read at 450nm.

A standard curve was constructed by plotting the TAFI concentration (as given on the vial of standard reference plasma) on the x axis and the mean absorbance value for each of the dilutions on the y axis of log-log graph paper. The mean absorbance values of

the control and test plasmas was read off the standard curve and multiplied by the appropriate dilution factor (2 for 1/200 dilution). The TAFI concentration obtained for the control plasmas must be within the range stated on the vial labels. The intra-assay C.V. was 5.8% and the inter-assay C.V. was 8%, as quoted by the kit manufacturer.

2.6.2 TAFI Activity

Materials

Actichrome® TAFI Activity kit (Product no. 874, American Diagnostica, Greenwich, CT, USA) containing:

- TAFIa standard
- Assay buffer
- TAFI developer
- TAFI activation reagent
- Activation stop reagent
- Activation enhancer reagent

96 well microtitre roundbottomed plate

Deionized filtered water

8 channel pipette (50-200 μl)

1 channel pipette (10-200 μl)

1.5 mL Microfuge tubes

Micro-titre plate shaker

Micro-titre plate reader: Dynex MRX (Dynex technologies, West Sussex, U.K.) to read at 490 nm

2M H_2SO_4 (sulphuric acid)

Method

Each test plasma was assayed in duplicate in the unactivated form and after activation by a thrombin/thrombomodulin complex. The difference in absorbance between the activated and the unactivated plasma represents the amount of activatable TAFI in the sample.

Each test plasma (20 µL) was first diluted in assay buffer (480 µL) in a microfuge tube and vortexed. For each unactivated plasma sample, 115 µL of assay buffer and 25 µL of diluted plasma was added to the well. For the activated plasma samples, 90 µL of assay buffer, 25 µL of diluted plasma, 10 µL of TAFI activation enhancer and 15 µL of TAFI activation reagent were added to the well. The plate was incubated at room temperature on an orbital plate shaker for 20 minutes.

During the 20 minute incubation, the TAFIa standards were prepared. Microfuge tubes were labelled A1/A2 to E1/E2. 250 µL of assay buffer and 50 µL of TAFIa standard are added to tubes A1 and A2. 150 µL of assay buffer was added to the remaining microfuge tubes. 150 µL of TAFIa standard was transferred from tube A1 to B1 and from A2 to B2. This was repeated from B1/B2 to C1/C2 and so on to E1/E2. The 150 µL removed from E1/E2 was discarded. When incubation of the plate was complete, the TAFIa standards were transferred from the microfuge tubes to the appropriate wells on the plate. 150 µL of assay buffer in wells F1/F2 was the "blank". The resulting concentrations of TAFIa standard dilutions were 2, 1, 0.5, 0.25 and 0.125 µg/mL in wells A1/A2 to E1/E2 respectively.

After incubation, 10 µL of activation stop reagent was added to the test but not the standard or blank wells. 50 µL of TAFI developer was then added to each well and the plate was incubated at 37^0C on a plate shaker for 30 minutes. 50 µL of 2M H_2SO_4 stop solution was added to each well and the absorbance was read at 490nm.

A standard curve was constructed on linear graph paper. The mean absorbance of the unactivated plasma was subtracted from the activated sample. The resulting absorbance represents the TAFI carboxypeptidase activity and the TAFI concentration was read off the standard curve and multiplied by 25 to account for the original dilution.

2.6.3 Clot Lysis Assay

Materials

$1x10^5$ dilution of recombinant TF: Innovin (Dade Behring, Milton Keynes, U.K.)

1M Calcium Chloride ($CaCl_2$)

Phospholipid source: Phospholipid vesicles, 1µM solution diluted 1:500 in Hepes buffer

Tissue plasminogen activator (tPA): (Sigma-Aldrich, Dorset, United Kingdom)

Potato tuber carboxypeptidase inhibitor (PTCI): (Sigma-Aldrich, Dorset, United Kingdom)

Recombinant factor VIIa: NovoSeven® (NovoNordisk, Bagsvaerd, Denmark)

HEPES buffer:

25mM HEPES = 2.98 g/500 mLs

137mM NaCl = 4 g/500 mLs

3.5mM KCl = 0.13 g/500 mLs

3mM $CaCl_2$ = 1.5 mLs 1M $CaCl_2$ in 500 mLs

20% bovine serum albumin: (Sigma-Aldrich, Dorset, United Kingdom)

Kinetic plate reader: Versamax tuneable microplate reader (Molecular Devices (UK) Ltd, Berkshire, U.K.)

Flat bottomed microtitre plate: Nunc Maxisorp (Thermo Fisher Scientific, Roskilde, Denmark)

Working solutions & reagents

12.75mLs of 1M calcium chloride was added to 87.25 mLs of distilled, sterile water, giving a concentration of 127.5mM. 20 µL of this solution was added to the final reaction volume of 150 µL to give a final concentration of 17mM.

The tPA vial was reconstituted by adding 5.95 mLs of distilled, sterile water to the vial containing 10 µg tPA, creating a 1680 ng/mL solution. A final concentration of 56 ng/mL was obtained when 5 µL of this solution was added to the final volume of 150 µL.

The vial of PTCI was reconstituted by adding 6.7 mLs of distilled, sterile water to the vial containing 5mg of PTCI, creating a 750 µg/mL solution. When 5 µL of this solution was added to the final volume of 150 µL, a final concentration of PTCI of 25 µg/mL was obtained.

rFVIIa was reconstituted by adding 20 mLs of distilled, sterile water to a vial of rFVIIa containing 1.2 mg of the active agent (eptacog alpha). The addition of 5 µL of this solution to the final reaction volume of 150 µL created a final concentration of 40nM.

The HEPES Buffer was made up by dissolving 2.98 g of HEPES, 4 g of NaCl, and 0.13 g of KCl in 400 mL of distilled, sterile water and by adding 1.5 mLs of 1M calcium chloride. The pH was checked and adjusted to 7.4 as necessary. The volume was made up to 500 mL with water in a volumetric flask. The buffer was stored at 4^0C. Immediately prior to use, 0.5 mL of 20% BSA was added to 100 mL of HEPES buffer. Buffer with added BSA was used within 24 hours.

Method

Test and normal plasma (a pool of 20 normal individuals) were thawed at 37^0C for five minutes, then kept on ice until assayed. All assays were performed within two hours of thawing. Note, lipaemic plasma samples were unsuitable for analysis.

To perform the clot lysis assay, 75 µL of plasma was added to 30 µL of buffer, 10 µL of phospholipid, 5 µL of tPA, 5 µL of rTF and 5 µL of PTCI or rFVIIa. If neither of the latter two reagents were to be added, an extra 5µL of buffer was added to make a total of 35µL of buffer. Samples were analysed in duplicate. The plate was mixed and incubated at 37^0C for two minutes. Finally, 20µL of $CaCl_2$ was added and the plate was mixed. The plate was read at 405nm every 2.5 minutes for 120 minutes. The plate reader was maintained at a temperature of 37^0C throughout. A lid was placed on the plate to prevent evaporation.

When the assay was complete, the Optical Density curve was reviewed and the raw data was exported to the Excel program (Microsoft®Excel 2002). The start OD and maximum OD were identified and entered onto a separate Excel worksheet. The midpoint of the clear-to-maximum turbid transition was calculated and the time that this

point was reached was obtained from the raw OD curve. Similarly, the time to the midpoint of the turbid-to-clear transition was calculated. The clot lysis time was calculated as the time interval in minutes between these midpoints (termed CLT1). The CLT obtained was the mean of two experiments. Clot lysis curves were obtained in the presence (CLT -PTCI) and absence of PTCI (CLT-buffer)and the contribution attributable to TAFI was calculated as the difference between these two results (i.e. CLT-buffer minus CLT-PTCI).

An alternative method of calculation of the clot lysis time was also used (the time from the occurrence of the maximum OD to the midpoint of the turbid-to-clear transition) and was termed CLT2.

2.7 Genetic analysis

2.7.1 Specimen collection and DNA extraction

DNA extraction was performed on thawed buffy coat samples using a kit according to the manufacturer's instructions (QIAamp DNA Blood Mini kit, Qiagen GmBH, Hilden, Germany). DNA was quantitated by running the patient samples on a 2% gel at 100v for 40 minutes or by using a spectrophotometric method.

2.7.2 Materials for PCR and sequencing

Primers: MWG-Biotech UK Ltd Milton Keynes, U.K.

PCR nucleotide mix (dNTPs): Promega, Southampton, U.K.

TAQ polymerase: Bioline, London, U.K.

Restriction endonucleases: Roche Diagnostics, East Sussex, U.K.

Sequencing PCR purification kit: QiaQuick PCR Purification kit, Qiagen Ltd, West Sussex, U.K.

Big dye terminator v1.1 Cycle sequencing kit: Applied Biosystems, Warrington, U.K.

Sterile water: Baxter, Berkshire, U.K.

NH_4 buffer: Bioline, London, U.K.

Magnesium

SeaKem LE Agarose: Cambrex BioScience, Berkshire, U.K.

NuSieve GTG agarose: Cambrex BioScience, Berkshire, U.K.

TBE buffer: Promega, Southampton, U.K

Ethidium bromide

6Xsucrose loading buffer

DNA molecular Marker V (fragment sizes 8-587bp): Roche Applied Science, East Sussex, U.K.

Techne PHC-3 thermal cycler: www.techne.com, Bibby Scientific, Staffordshire, U.K.

3100 Avant Sequencer: Applied Biosystems, Warrington, U.K.

Electrophoresis tank

Eppendorf tubes

Strip tubes (for PCR)

Microtitre plates and covers (for overnight restriction)

Pipettes: Gilson

Disposable pipette tips: CLP Ltd, Northampton, U.K.

2.7.3 General method for PCR

A PCR worksheet was completed for each PCR experiment. A bulk mix was made up in a clean PCR area consisting of the following: Sterile water 711 µL, NH_4 buffer 100 µL, 1.25mM dNTPs 100 µL, Mg2+ 24 µL, forward and reverse primers 20 µL. This mix was sufficient to assay 20 samples and each sample contained Sterile water 35.5

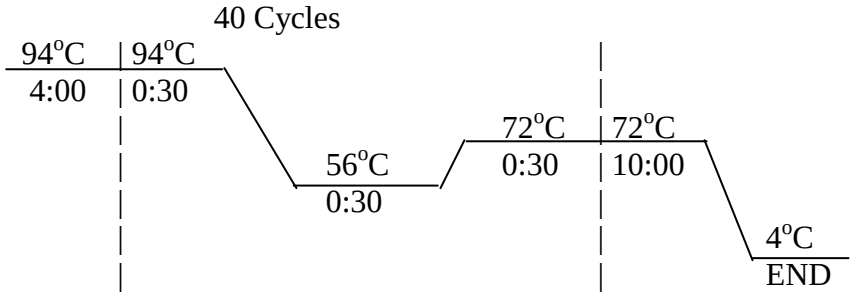
μL, NH_4 buffer 5 μL, 1.25mM dNTPs 5 μL, Mg2+ 1.2 μL, forward and reverse primers 1 μL. The bulk mix was exposed to UV light for 10 minutes on a transilluminator. Taq polymerase (5 μL) was then added to the bulk mix, ensuring each sample contained 0.25 μL. An aliquot of 48 μL of bulk mix was added to a plastic strip tube for each sample and control. Sample and control DNA (2 μL) was added to the relevant tubes and no DNA was added to the blank control. The strip tubes were placed in a Techne PHC-3 thermal cycler at the appropriate settings (see below). The PCR products were stored at 4^0C until further analysed.

To assess the PCR products after amplification, a 1.5% agarose gel was poured. An aliquot (5 μL) from each sample was added to 6Xsucrose loading buffer (2 μL) in an eppendorf tube. The sample/buffer mix was then loaded on to the gel and electrophoresed at 100v for 20 minutes. Marker V (5 μL) was placed in lanes 1 and 13 to indicate the size of the PCR products. The gel was then visualised using the UV transilluminator to confirm that a PCR product of the correct size had been amplified, that the blank control did not contain PCR product and that there were no additional PCR products.

2.7.4 Genetic analysis for thrombophilia

The factor V (FV) Leiden and prothrombin gene mutations were analysed using a multiplex PCR, according to the standard operating procedure in use in the laboratory at the time. Each sample contained sterile water 36 μL, NH_4 buffer 5 μL, 1.25mM dNTPs 5 μL, Mg2+ 1.5 μL, forward and reverse primers for FVLeiden and the Prothrombin gene mutation 2 μL, Taq polymerase 0.25 μL and DNA 1 μL.

The PCR conditions for the multiplex PCR were as follows:



The PCR products were checked as described above, then underwent restriction enzyme digestion overnight with HindIII at 37^0C. The samples were then run on a 2% gel consisting of 1% agarose and 1% NuSieve GTG agarose, at 120v for two hours. A blank and two controls (FVLeiden and Prothrombin gene mutation heterozygous and FVLeiden homozygous and Prothrombin gene heterozygous) were run with each set of patient samples. The gel was examined under UV light and photographed.

Normal (wild type samples) had bands of 241bp for the FVLeiden mutation and 345bp for the Prothrombin gene mutation. Heterozygotes had bands of 241/209bp and 345/322bp respectively and the FVLeiden Homozygote control had a band of 209bp after restriction.

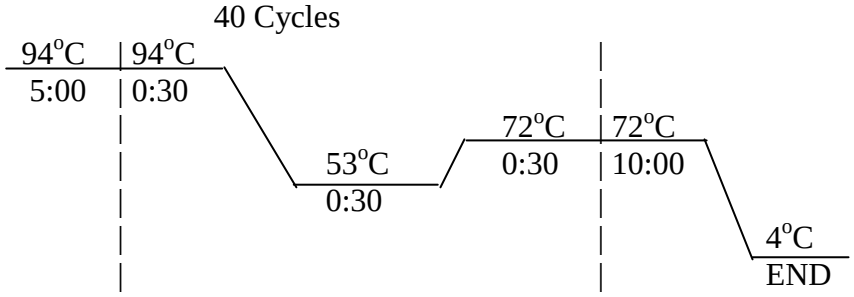
2.7.5 Genetic analysis for Jewish mutations in *F11* gene

The four Jewish mutations in the *F11* gene were analysed by restriction analysis. Exons 5 and 9 were amplified by PCR, using primers as set out in table 2.2, to identify type II and III mutations respectively. A third set of primers, which amplify the junction of exon 14-intron N, were used to identify patients with type I and IV mutations (table 2.2).

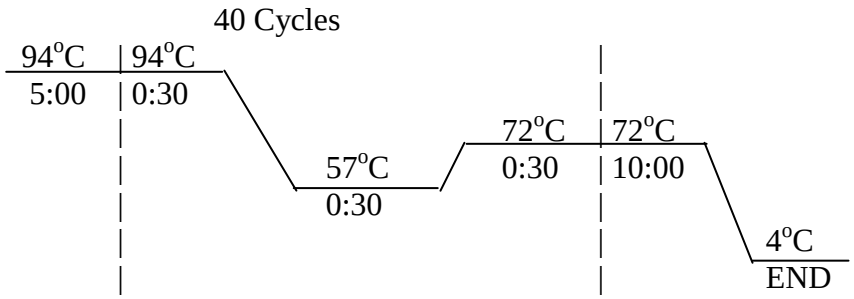
Table 2.2: Primer sequences for restriction enzyme analysis of the Jewish type I – IV mutations.

FXI Region	Primer Sequences	Product size (bp)
Exon 5	Forward 5'-gcc cct aga atc tgg aag gta-3'	458
	Reverse 5'-cgc ttt tga aat tag cca agt at-3'	
Exon 9	Forward 5'-gcc ccg agg agg ctg ata-3'	380
	Reverse 5'-cct cct ctc ccg tga agt att tt-3'	
Exon14/ Intron N	Forward 5'-tta gtg acc aac gaa gag tgc cag-3'	162
	Reverse 5'-ccc caa cgc att aag cat tcc aat-3'	

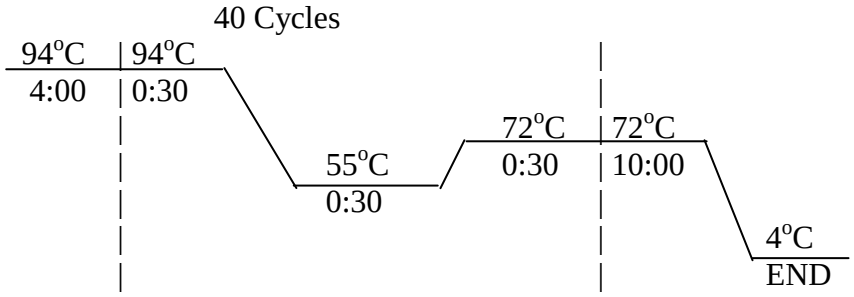
The PCR conditions for amplification of exon 5 were as follows:



The PCR conditions for amplification of exon 9 were as follows:



The PCR conditions for amplification of exon 14/Intron N were as follows:



The PCR products were digested overnight with BsmI, Sau3A and MaeIII for the type II, III and I/IV mutations respectively (table 2.3). The type I mutation abolishes a MaeIII restriction site, the type II mutation abolishes a BsmI restriction site while the type III mutation creates a Sau3A restriction site and the type IV 14bp deletion abolishes the donor splice site and an MaeIII restriction site in intron N creating two aberrant PCR products.(58;59) The samples were then run on a 2% gel at 120v for two hours for the type II and III mutations and on a 4% gel at 120v for two hours for the type I and IV mutations. The gel was examined under UV light and photographed. A blank and normal, heterozygous and homozygous controls (where available) were run with each sample. Examples of each restriction enzyme analysis are shown in figures 2.5 to 2.7.

Table 2.3: Restriction enzyme analysis for the Jewish type I-IV mutations including restriction conditions and products.

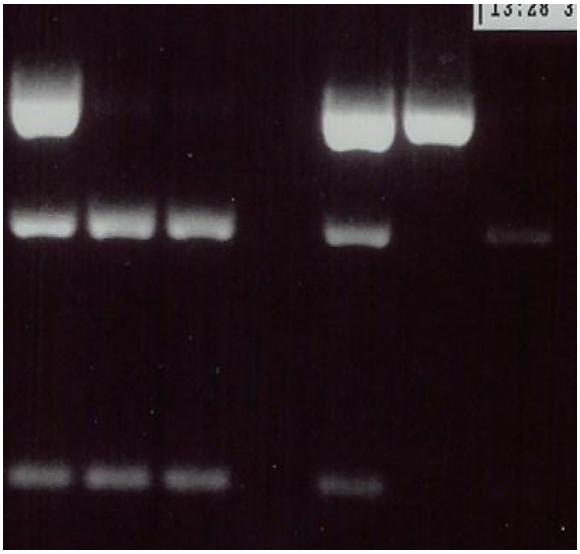
Mutation Type	Restriction enzyme(vol)	Vol. of PCR product (μl)	Conditions	Restriction products (bp)		
				Normal	HeZ*	HoZ**
I	MaeIII (1 μl)	19	55^{o}C overnight	99/60/3	159/99/60/3	159/3
II	Bsm1 (1 μl)	19	65^{o}C overnight	333/125	458/333/125	458
III	Sau3A (1 μl)	19	37^{o}C overnight	380	380/280/100	280/100
IV	MaeIII(1 μl)	19	55^{o}C overnight	99/60/3	99/60/3 & 2 aberrant bands~160bp	2 aberrant bands~160bp

*HeZ: Heterozygous for the mutation
**HoZ: Homozygous for the mutation

Figure 2.5: Restriction enzyme analysis F11 Exon 5.

Lane 1: Heterozygote patient, Lanes 2&3: Normal patients, Lane 4: Blank,

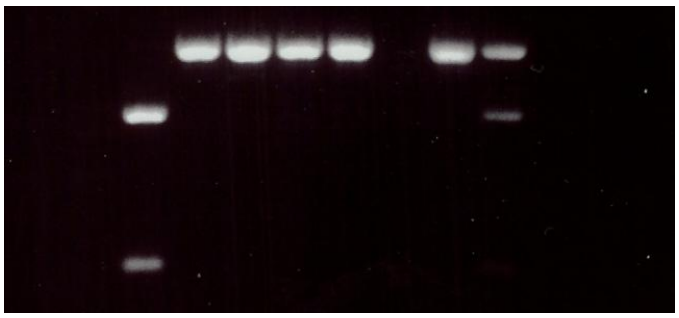
Lane 5: Heterozygote control, Lane 6: Homozygote control, Lane 7: Normal control.



Lanes: 1	2	3	4	5	6	7

Figure 2.6: Restriction enzyme analysis F11 Exon 9.

Lane 1: Homozygote patient, Lanes 2-5: Normal patients, Lane 6: Blank, Lane 7: Normal control, Lane 8: Heterozygote control.



Lanes: 1	2	3	4	5	6	7	8

Figure 2.7: Restriction enzyme analysis F11 Exon 14/Intron N.

Lane 1: DNA ladder, Lanes 2-11 and 13-18: Normal patients, Lane 12: Heterozygote patient, Lane 19: Blank, Lane 20: Normal control.

Lanes:1-20 from left to right

2.7.6 Sequencing of *F11* gene

Primer Design and PCR

Primers were designed using a combination of previously described primers and newly designed primers (see table 2.4). Annealing conditions for the PCR reactions and product size are also given in table 2.4. Primers that enabled the amplification of the exons and preceding and proceeding regions of intronic DNA were chosen. All sequence information was gathered from the NCBI database.

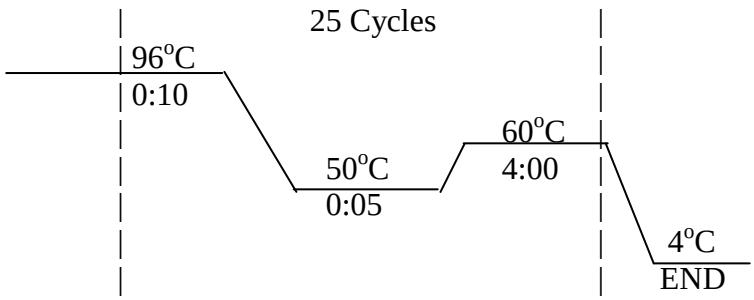
The PCR reactions were carried out according to the general method for PCR reactions given above. All samples were purified using the Qiagen QiaQuick PCR Purification kit to remove unused PCR reagents.

Table 2.4: Primer sequences, annealing temperatures, product sizes and references for sequencing PCR of the 15 exons of the F11 gene.

F11 region	Primer Sequences	Tm (^{0}C)	Product size (bp)	Ref
Exon 1	Forward 5'- att cag tgt att gag aaa gca ag-3' Reverse 5'- gct tga gag gaa tgt taa ata gt-3'	55	291	(182)
Exon 2	Forward 5'- ccc tat ttc ttg taa gtc-3' Reverse 5'- aat ggt gtg gga ttc tcc-3'	47	227	New
Exon 3	Forward 5'- atg tat gcc cag taa aat cc-3' Reverse 5'- atg tta gat gga aat gtg tt-3'	50	293	New
Exon 4	Forward 5'- gct ttc tgt gtg ctg act tt-3' Reverse 5'- cag ctg gta ttt gtt gat tc-3'	57	226	(183)
Exon 5	Forward 5'- ccc cta gat ct gga agg ta-3' Reverse 5'- cga ttc tgt ttt tca tcg ac-3'	53	253	(183)
Exon 6	Forward 5'- gca gtt gga aga ata aga cac-3' Reverse 5'- gaa tta cat cat caa gaa gtc g-3'	46	185	(182)
Exon 7	Forward 5'- tcc tga tag ctg gtg aat tg-3' Reverse 5'- gaa gat aac aaa tta tcc tta ctt g-3'	57	247	(183)
Exon 8	Forward 5'- gat ata ttt cta ctt ccc ttt tg -3' Reverse 5'- ctc agc cag aat gca gaa ct-3'	46	173	(182)
Exon 9	Forward 5'- aca tgc tga ggg agg gtc t-3' Reverse 5'- gac aag ggt gtc tgc ata ac-3'	52	245	(182)
Exon 10	Forward 5'- gca tta tgt tta tac cgt ttt g-3' Reverse 5'- cag cta tat ttt ttc aag tga c-3'	52	175	(182)
Exon 11	Forward 5'- aag ctg ctc atc aca atg ct-3' Reverse 5'- ttt cct aac atg cta gta at-3'	51	274	New
Exon 12	Forward 5'- gcc aca cac ttc aca atg tc-3' Reverse 5'- ggt cag gcc gta agt cta gt-3'	57	332	(183)
Exon 13	Forward 5'- tac agt gga aga aga gtc tc -3' Reverse 5'- tga cag ggc aga aaa ggt tc-3'	53	242	new
Exon 14	Forward 5'- tat ggt tat tct aca aac gaa c-3' Reverse 5'- caa ttt gca tat att cca ttg g-3'	46	220	(182)
Exon 15	Forward 5'- tct gag ttg atc tgt gca cc-3' Reverse 5'- tac aac gat cat aga acg gg-3'	57	398	(183)

Sequencing:

Sequencing of the PCR products was carried out on an Applied Biosystems 3100 Avant Sequencer. Each sequencing reaction was carried out using 1.5 µl of ready reaction mix (as supplied in the Big Dye Terminator v1.1 Cycle sequencing kit), 1 µl of template DNA, 3.2pmol of sequencing primer and 16.5 µl of dH2O for a final reaction volume of 20 µl. This was then placed in a thermal cycler on the following program:



Following amplification the products were purified using the following protocol. EDTA (2 µL), sodium acetate(2 µL) and 50 µl of 95% ethanol were added to each sample, mixed and left to incubate at room temperature for 15 minutes. The microcentrifuge tubes were then spun for 20 minutes. The supernatant was then removed carefully. The pellet was rinsed with 250 µL of 70% ethanol and the sample was centrifuged for five minutes and the supernatant was discarded. The samples were dried at 95^0C for one minute in the thermal cycler. The samples were resuspended in 20 µL Template suppression reagent as supplied and were transferred to sequencing tubes. The samples were rendered single stranded by placing the samples at 95^0C for two minutes. The samples were then placed on ice and loaded on to the sequencer for automated capillary sequencing.

2.7.7 ALG6 Polymorphism

The PCR and sequencing reactions for exon 10 were carried out according to the general methods given above. PCR conditions are given below. The primers are given in table 2.5.

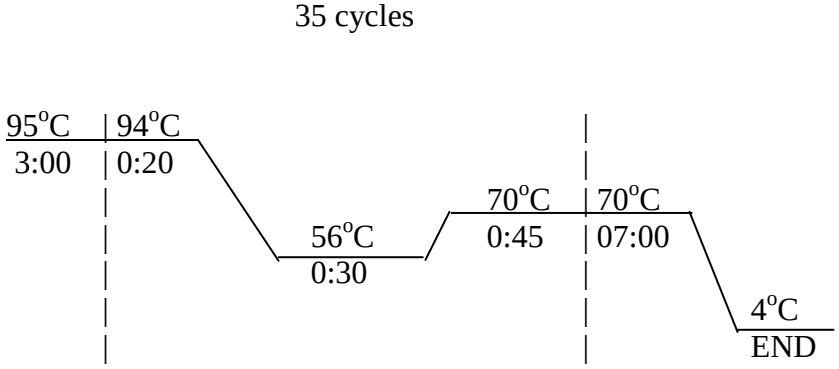


Table 2.5: Primer sequences for PCR and sequencing of exon 10 ALG6 gene.

ALG6	Primer Sequences	Reference
Forward	5'-aaa ctt aag ttg ata aat aat atg atc-3'	(184)
Reverse	5'- gtc taa cac aga agc taa gta tgg g -3'	(184)

2.8 Molecular Modelling

2.8.1 Identification of *F11* gene mutations

Mutations in the *F11* gene causing FXI deficiency were identified by searching the Human Gene Mutation database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.htmL). Additional mutations were identified by a Medline search using the following search expressions: factor XI, FXI, F11 deficiency, plasma thromboplastin antecedent (PTA), PTA deficiency, Rosenthal's syndrome. Other mutations were identified by searches of abstracts from recent international haematology and haemostasis meetings. For the

mutations that had been published in sufficient detail, phenotypic data was included, namely FXI:C in most cases, and FXI:Ag and/or bleeding phenotype in some cases. Mutations published in abstract format were grouped according to the presence or absence of phenotypic data.

2.8.2 Construction of FXI homology models

Homology models of the Apple (Ap) 1 to Ap4 and serine protease (SP) domains were constructed using INSIGHT II 98.0 modelling software with HOMOLOGY, DISCOVER and BIOPOLYMER modules (Accelrys, San Diego, CA) and the structural refinement program O (185) on Silicon Graphics workstations. Alignments of the five FXI domain sequences with their template structures resulted in the identification of the structurally conserved regions (SCRs) within these sequences, and these SCRs were built using a rigid body fragment assembly method implemented by the HOMOLOGY module. The loop regions between the SCRs were then either modelled from a database of protein fragments using the Brookhaven loop database of INSIGHT II or generated using a constrained minimization algorithm implemented by HOMOLOGY. Energy refinements using the DISCOVER module were then carried out on these initial models in order to refine the connectivity of the loop splice junctions, the loop regions and the N- and C-termini. The structure verification program PROCHECK (186) and O were used alongside the energy minimisation program of the DISCOVER module and the structure idealisation program REFMAC5 of CCP4i (187) in order to improve the connectivity, check bond angles and lengths and reduce the number of bad contacts within the five domain models.

Suitable template protein structures for modelling the FXI Ap domains (members of the PAN-module superfamily) were obtained from PSI-BLAST (188) searches of the Protein Data Bank (PDB) (189) using the PredictProtein server (http://cubic.bioc.columbia.edu/predictprotein/submit_def.htmL). The PAN-module superfamily contains the following protein domains: the N-terminal N domains of members of the plasminogen/hepatocyte growth factor family, the apple domains of the plasma prekallikrein/coagulation factor XI family and domains of various nematode proteins.(190) The HOMOLOGY module produced a structural alignment of the sequences of these known structures by constructing a Cα distance matrix for each structure and calculating the root mean square distances for elements within the matrix. CLUSTALW (191) alignments of other sequences belonging to the PAN-module superfamily (190) of the InterPro database (192) were also added to the Ap domain alignment. The Ap sequences obtained were the *E. Tenella* adhesin protein (PDB code: 1HKY) and the anti-platelet toxin from *H. officinalis* (PDB code: 1I8N_A). The N-terminal domains of human (PDB code: 2HGF), rat (HGF_RAT), mouse (HGF_MOUSE) and *Xenopus* (HGF_XENOPUS) hepatocyte growth factor, and the N-terminal domains of mouse (PLMN_MOUSE), bovine (PLMN_BOVIN), macaque (PLMN_MACMU) and human (PLMN_HUMAN) plasminogen were also used. The four closely-related Ap domains of mouse (KAL_MOUSE1-4), rat (KAL_RAT1-4) and human (KAL_HUMAN1-4) prekallikrein were directly compared with the four Ap domains of FXI (FXI_AP1-4). For completeness, two hypothetical nematode proteins with the same conserved pattern of cysteine residues were also included (YMP9_CAEEL, YSM5_CAEEL.1), as well as other PAN modules such as Microneme Antigen precursor (MIA_SARMU) and a rat macrophage stimulating protein precursor (MSP_RAT). Secondary structure assignments were included in order to correctly

assign sequence gaps to the loop regions, and to conserve the location of the cysteine residues. The secondary structure of Ap1 was predicted from the sequence alignment of the sequences specified above using the consensus result of five methods, namely the GOR III (193), PHD (194), JPred (195), PSI-PRED (196) and SAMT-99 algorithms (197-199).

Suitable templates for the SP domain were identified using BLAST (188) to search the Protein Data Bank. From this, the top 500 matches were analysed with CLUSTALW to identify five close homologues from a total of 84 unique structures using a guide tree. These were human α-thrombin (PDB code 1H8I), human protein C (PDB code 1AUT), human complement C1r (PDB code 1GPZ), boar sperm β-acrosin (PDB code 1FIZ) and human mast cell β-tryptase (PDB code 1AOL). The HOMOLOGY module was used to structurally align these five templates. The FXI SP domain was then added to this alignment using a consensus of the pairwise sequence alignments with the five templates.

The DSSP program (200) was used to assign secondary structures in both the crystal and NMR structures and the final modelled structures. Secondary structures are assigned as follows: H and G, α-helix and 3_{10} helix; E and B, β-strand; T, turn; C and S, coil and loop. Side chain solvent accessibilities in the structures and models were calculated using NACCESS (201), this being based on an implementation of the Lee and Richards algorithm using a probe radius of 1.4 Å to represent the water molecule. The results are on a scale of 0 to 9 for each residue, where 0 corresponds to 0% to 9% accessibility, 1 corresponds to 10% to 19% accessibility, and so on. Buried residues

have accessibilities of 0 or 1. Figures of the protein structures were generated using MOLSCRIPT (202) and RASTER3D (203).

2.9 Statistical Analysis

Statistical analysis for laboratory parameters was performed using SAS (SSPS, NC) or the statistics package on Excel (Microsoft® Excel 2002). For evaluation of correlation, the Pearson correlation coefficient (coefficient of variance) was calculated using Excel. The Wilcoxon two-sample test (SAS) was used for analysis of numerical data between two groups for the thrombin generation test i.e AUC and peak thrombin versus severe or partial FXI deficiency and AUC, peak thrombin and ETP versus bleeding history (bleeder or non-bleeder). The Kruskal-Wallis test (SAS) was used for analysis of numerical data for more than two groups (i.e. evaluation of bleeding tendency versus tertiles of FXI:C, VWF:AG, VWF:CBA and TAFI:Ag and versus multiple genotypes). The ANOVA analysis of variance (Excel) was used to calculate the significance of TAFI activity as measured by the CLT versus severe or partial deficiency. The Chi-squared test (SAS) was used to evaluate proportions of patients with different blood groups in bleeder and non-bleeder categories.

Chapter 3 Clinical and laboratory features of FXI deficiency

3.1 Introduction

A key feature of FXI deficiency is the variable nature of the bleeding phenotype.(6;7) It is the unpredictable nature of the bleeding tendency coupled with the known side effects of haemostatic therapy in this condition which make clinical determination of the need for treatment so difficult.(72)

Since the condition was established in the 1950s, a considerable published literature has documented the range of bleeding symptoms in FXI deficient patients (see Introduction Chapter 1 for a detailed analysis).(3;5-7;50;54-57) More recently, a number of publications have appeared which have focused on clinical manifestations of bleeding in severely deficient patients.(62;71) However, questions still remain regarding the cause of the variable bleeding tendency, the existence of a clinically relevant bleeding disorder in heterozygotes and the optimal treatment for FXI deficient patients.(50;204)

The aim of this study is to rigorously evaluate modifiers of the bleeding tendency in FXI deficiency, in order to define the nature of the variable bleeding tendency and to evaluate possible causes for the variability. A critical element of this study is the careful documentation of the prior bleeding history and allocation of a bleeding score.(5;7) The pattern of prior bleeding history is compared to previous studies, in particular in the light of more recent evidence suggesting that haemostatic cover is not always required.(62;71) The bleeding score can then be correlated with laboratory assessments of coagulation and genetic studies. In this study, the basic diagnostic tools for FXI deficiency (aPTT, FXI:C, FXI:Ag and genetic analysis) are evaluated for diagnostic sensitivity and correlated with bleeding score.

VWF:Ag and VWF:Ac, blood group and thrombophilic traits, which have been previously suggested to modify the bleeding tendency in FXI deficiency and Haemophilia, are re-evaluated in this cohort of patients, with particular reference to the bleeding score. The state of glycosylation of the FXI protein may influence activity and a preliminary analysis of a potential genetic modifier (ALG6) is undertaken.

3.2 Methods

3.2.1 Bleeding scores

As outlined in the Methods (Chapter 2) a detailed bleeding history was taken by a single clinician using a standard template. A five point bleeding score was used to designate bleeding tendency (Table 3.1).(5;7) The bleeding histories were scored independently by three clinicians with experience in haemostasis and inter-observer concordance was assessed.

A score of three (indeterminate) was applied if the reviewer felt that the patient had not undergone sufficient haemostatic challenges to make a decision or if there was conflicting evidence. In two cases, a bleeding score of three was applied where there was disagreement amongst the reviewers about the bleeding score.

Table 3.1: Bleeding score related to bleeding phenotype.(5;7)

Bleeding Score	Bleeding Phenotype
1	Definite bleeder
2	Probable bleeder
3	Indeterminate
4	Probable non-bleeder
5	Definite non-bleeder

Bleeding score consensus

In 73 cases (72%), there was either total consensus between all three reviewers (27 cases) or two reviewers were agreed and one was within one point on the scoring system (46 cases). In the latter case, the score applied was that of the two reviewers who were in agreement. In ten cases, two reviewers agreed on the score but one applied a score that was more than one point different. In this case, the outlier score was disregarded. In 19 cases (19%), all three reviewers disagreed in the score to be applied. The scores were then further reviewed by two reviewers together and a consensus score was applied in 17 cases. There were two cases where a consensus could not be agreed even after review and in these cases a score of three (indeterminate) was applied.

3.2.2 Laboratory investigations

The methods used for the evaluation of the aPTT, FXI:C, FXI:Ag, FVIII:C, VWF:Ag, VWF:CBA and thrombophilia screening are outlined in Chapter 2 Methods.
Primers, PCR conditions and the methodology employed for sequencing exon 10 of the ALG6 gene are outlined in Chapter 2 Methods.

3.3 Results

3.3.1 Participants & patient demographics

In 2001, the Katharine Dormandy Haemophilia Centre and Haemostasis Unit had 263 patients, aged 18 years or over, who had been diagnosed and registered with FXI deficiency over a time period from 1978 to 2001. All of these 263 patients were invited to participate in the study. It was not known which of the participating patients had also participated in earlier studies but it is likely that there is some overlap in patient data

between this study and earlier work.(5;55) 103 individuals attended the study clinic and 102 (39% of registered patients) agreed to participate in the study. There were individuals from 80 families and in 65 of these, single family members were tested. More than one family member participated in the study in the remaining 15 families; 10 families had two participants, three families had three participants and two families had four participants in the study. Parental consanguinity was present in two severely deficient Jewish patients and one partially deficient Portuguese patient (first cousins in two cases and a more distant relationship in one case).

Patient demographics are shown in Table 3.2. The largest ethnic group was Ashkenazi Jewish reflecting the demographics of the catchment area of the haemophilia centre. The second largest group had English heritage (16%). The remaining eight patients came from Ireland, Portugal, Turkey, Cyprus, Ghana, Bangladesh, South-east Asia and the Caribbean.

Table 3.2: Patient demographics

Patient characteristics	
Age	**Years**
Median	46.5
Range	18-84
Gender	**Number of patients**
Male	42
Female	60
Ethnic origin	
Jewish	78
English	16
Other	8

3.3.2 Bleeding phenotype

In all, 636 episodes with potential for bleeding were recorded and comprised major and minor surgery, dental procedures, trauma, bruising and epistaxis.

124 dental procedures were reported. Treatment prior to the dental procedure was given to cover 22 episodes (9 FXI concentrate, 7 plasma, 3 rFVIIa, 3 tranexamic acid) and 14 of these procedures were carried out on 11 severely deficient patients. One patient suffered bleeding post dental extraction despite prior treatment with plasma. In contrast, there were 41 reports of bleeding where prior treatment was not given and 14 of the patients involved had a severe deficiency. This gives a crude bleeding rate of 40% for dental extractions without prior treatment.

A total of 330 invasive procedures involving major or minor surgery were reported. In 55 of these episodes, pre-operative treatment was given and in two of these procedures bleeding occurred despite treatment (one severely deficient patient who received tranexamic acid cover for minor breast surgery and one partially deficient patient who bled post rhinoplasty and septoplasty despite FXI concentrate). In the surgical procedures where pre-operative treatment was not administered, bleeding was reported by patients in 85 cases (crude bleeding rate 26%). However, certain procedures like tonsillectomy are associated with bleeding in 60% of patients while untreated patients with severe FXI deficiency have a definite bleeding risk with procedures such as hernia repair and CABG. Selected procedures with pre-operative treatment rates and bleeding outcomes are given in table 3.3.

In the overall patient group the following therapies were given in the management of unexpected post-operative bleeding: cellular blood transfusion (23 episodes), FXI concentrate (18 episodes), plasma (17 episodes), FXI replacement unspecified (2 episodes) and re-operation (12 episodes).

Table 3.3: Bleeding outcomes in selected invasive procedures (all patients)

Procedure	No. of procedures	No. of pts	No. of severely deficient pts	Prior treatment	Bleeding in untreated pts (%)	Bleeding in severely deficient pts (%)
Tonsillectomy	33	33	16	0	20 (60.1)	14 (87.5)
Appendicectomy	22	22	5	0	3 (13.6)	1 (20)
Hernia repair	7	6	3	2	3 (60)	2 (67)
CABG	3	3	2	1	2 (100)	1 (50)

Male patients

Amongst 42 male patients, 23 confirmed that they had had a circumcision in the neonatal period, of whom 11 patients had severe FXI deficiency (table 3.4). Bleeding was reported after circumcision in two cases (one severe and one partial deficiency). Later in life, six patients had prostate biopsy or thermal treatment, of whom two severely deficient patients had prior treatment with FXI concentrate and plasma and did not have bleeding and four partially deficient patients had no prior treatment and one had bleeding. Additionally, two partially deficient male patients reported prostatectomy or TURP without pre-operative treatment. One patient had two such procedures and bled on both occasions while the other patient did not report bleeding.

Table 3.4: Selected invasive procedures in male patients with FXI deficiency

Procedure	No. of procedures	No. of patients	No. of severely deficient patients	Prior treatment	Bleeding
Circumcision	23	23	11	0	2
Prostate bx/ thermal Rx	6	6	2	2	1
Prostatectomy/TURP	3	2	0	0	2

Female patients

In 60 women with FXI deficiency, the invasive procedures most likely to result in bleeding were gynaecological procedures (n=28). Bleeding after gynaecological procedures was associated as might be expected with major procedures such as hysterectomy (42%) and caesarean section (29% of procedures performed without cover) but was also seen after minor procedures such as laparoscopy and resection of cysts and polyps (table 3.5). Bleeding was also reported associated with a wide variety of other invasive procedures ranging from minor procedures such as breast surgery and varicose vein surgery to major procedures such as nephrectomy and oesophagectomy.

Table 3.5: Gynaecological procedures associated with bleeding.

Gynaecological procedures	No. of procedures	No. of episodes associated with bleeding
Dilatation and curettage	23	6
Hysterectomy	12	5
Laparoscopy	17	4
Caesarean section	8	2
Cone biopsy	2	2
Resection cervical polyp	2	2
Resection vaginal cyst	2	2
D&C/hysteroscopy/laparoscopy	2	1
Oopherectomy	2	2
Hysterectomy and oopherectomy	1	1
Vaginal repair	1	1

Pre-operative treatment was given in only five cases and no bleeding was reported (FXI concentrate was given to four severely deficient patients and plasma was given to one partially deficient patient). Conversely, post-operative treatment was required in 47 episodes of bleeding, including a cellular transfusion rate of 32% of these bleeding episodes (table 3.6).

Table 3.6: Post-operative treatment required in female patients reporting bleeding after any invasive procedure (47 episodes, 4 episodes counted twice due to dual therapies, see below).

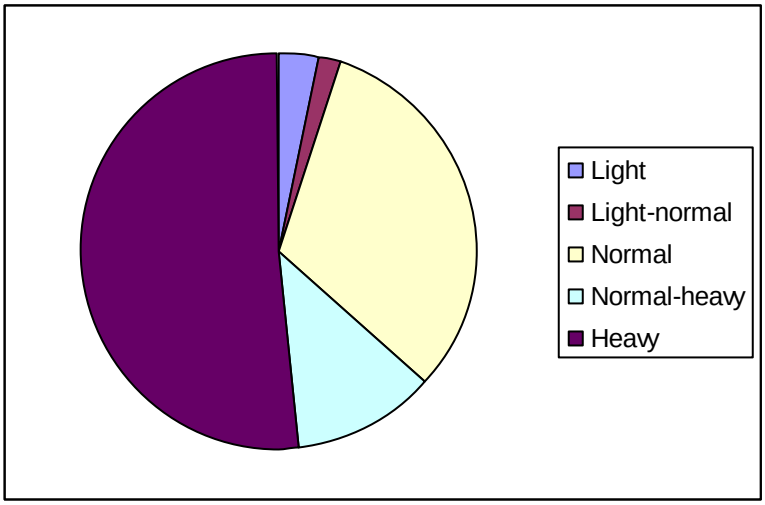
Post-operative treatment	Number of bleeding episodes treated
FXI replacement	7
Tranexamic acid	5
Transfusion*	15
Re-operation†	8
Other (suturing, packing etc)	16

**Two patients also received FXI replacement, one patient also underwent a re-operation*
†One patient also received a transfusion as above.

Of the sixty women who participated in the study, 31 (52%) subjectively reported heavy menses (fig. 3.1). Of the women who reported heavy menses, 11 were in the severely deficient category and the proportion was similar to the overall proportion of severely deficient patients in the study. Two thirds of FXI deficient women with heavy menses (20 of 31 patients) had a partial deficiency of FXI. Of the 31 women who self-reported heavy menses, 25 reported clots, 22 required double protection and 20 stated that their menses had always been heavy. Seventeen women sought medical attention for heavy

menses and of these, 13 received one or more specific treatments, most commonly the combined oral contraceptive pill (8 cases) and hysterectomy (4 cases).

Figure 3.1: Patient subjective description of menses (n=60). Numbers of patients: heavy 31, normal-heavy 7, normal 19, normal-light 1, light 2.



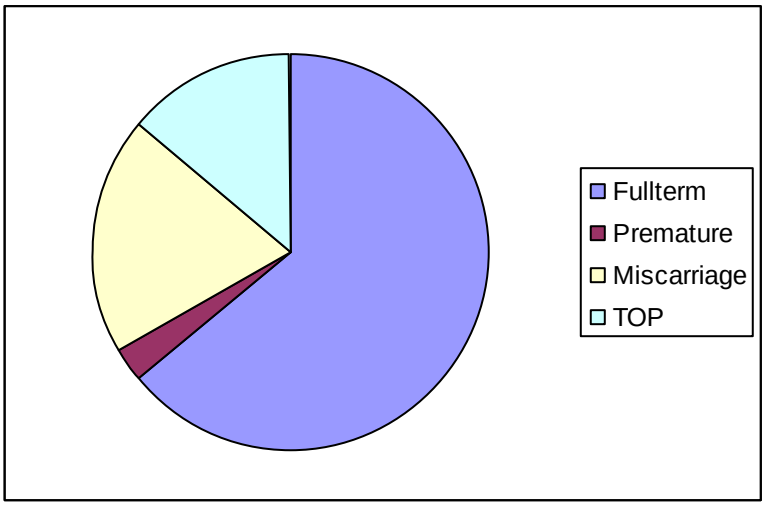
Pregnancy

Of the sixty women participating in the study, 47 had had at least one pregnancy. Of these 47 women, 14 were severely deficient and 33 were partially deficient in FXI. 134 pregnancies were recorded and comprised 98 full term deliveries, 1 premature delivery, 21 spontaneous miscarriages and 14 terminations. Only six deliveries were covered with FXI replacement prior to delivery (five full term deliveries including one caesarean section and one termination). The full term deliveries occurred in five women of whom one was severely deficient. FXI concentrate was used in three cases, plasma in two cases and FXI replacement was unspecified in one case. The severely deficient patient

had a baseline FXI:C of 8 U/mL and had two deliveries covered by plasma; one was associated with bleeding despite plasma and one was uneventful.

The mode of delivery was known in 87 full-term deliveries. Vaginal delivery was recorded for 79 pregnancies in total and bleeding was reported in 20 of these (25%). Caesarean section was recorded for eight pregnancies of which one patient received pre-operative FXI replacement (baseline FXI:C 52 U/mL) and no bleeding was reported. Seven caesarean sections were not covered with FXI replacement and bleeding occurred in two cases (28.6%), one of which required a blood transfusion.In total, bleeding occurred in 36 pregnancies (27% of pregnancies involving 26 women). Nine of these bleeding events occurred in seven severely deficient patients. The majority of bleeding episodes (23 of 36, 64%) were related to full term deliveries (fig. 3.2). The bleeding episodes were treated with blood transfusion in 7 cases, dilatation and curettage alone in 6 cases, D&C with FXI replacement in 3 cases, oral iron in one case and treatment was unknown or no treatment was reported as given in 19 cases.

Figure 3.2: Bleeding associated with pregnancy (n=36 episodes in 26 women). Numbers of patients: fullterm pregnancy 23, miscarriage 7, termination of pregnancy 5 and premature delivery 1.

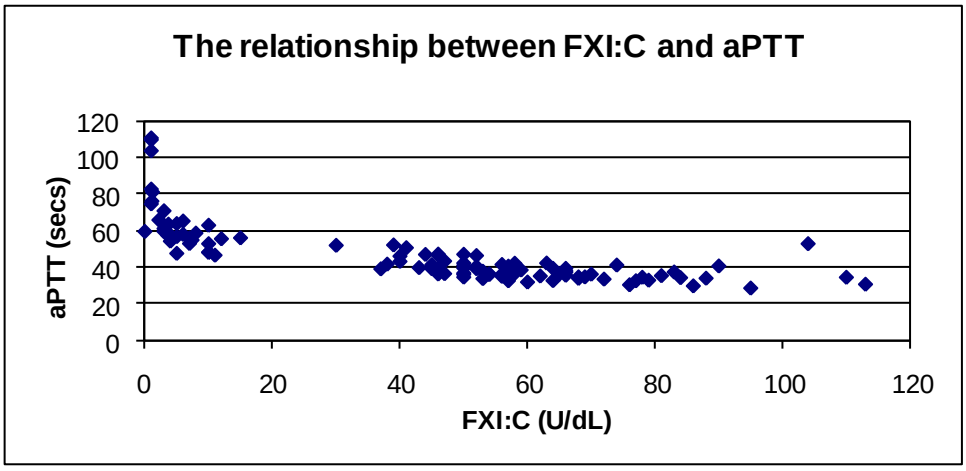


3.3.3 Laboratory investigations

Activated partial thromboplastin time

The activated partial thromboplastin time (aPTT) was performed in all patients (fig.3.3). The aPTT was within the normal range (28-38 seconds) in 37 patients (36%), of whom the FXI:C was below the normal range in 23 (23%).

Figure 3.3: The relationship between FXI:C and aPTT (102 patients).



FXI:C and FXI:Ag levels

Patients were registered with FXI:C levels ranging from <1-70 U/dL. On re-testing the FXI:C levels ranged from <1 to 113 U/dL (figure 3.4 and table 3.7). No overlap in FXI:C levels is seen between severely deficient and partially deficient patients.

Figure 3.4: Individual FXI:C levels for 102 patients registered with FXI deficiency (each dot represents one patient FXI:C measurement). Note the clear demarcation (arrow) between severely deficient patients (maximum FXI:C 15 U/dL) and partially deficient patients (minimum FXI:C 30 U/dL).

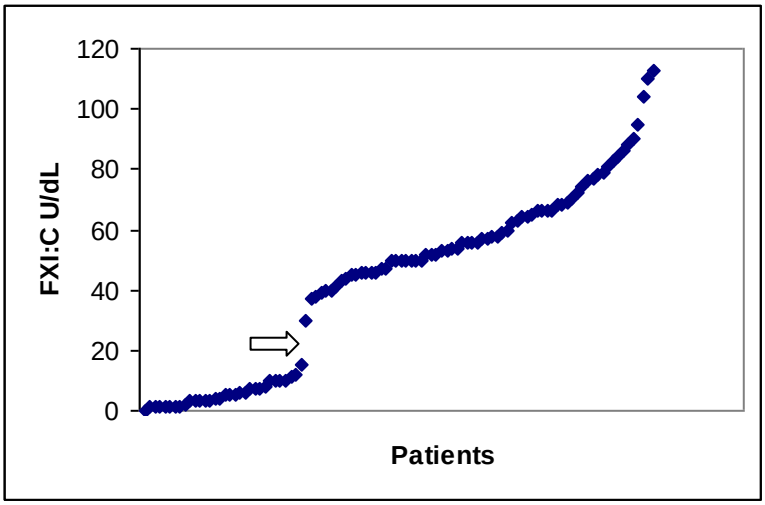


Table 3.7: FXI:C levels in 102 individuals with Factor XI deficiency.

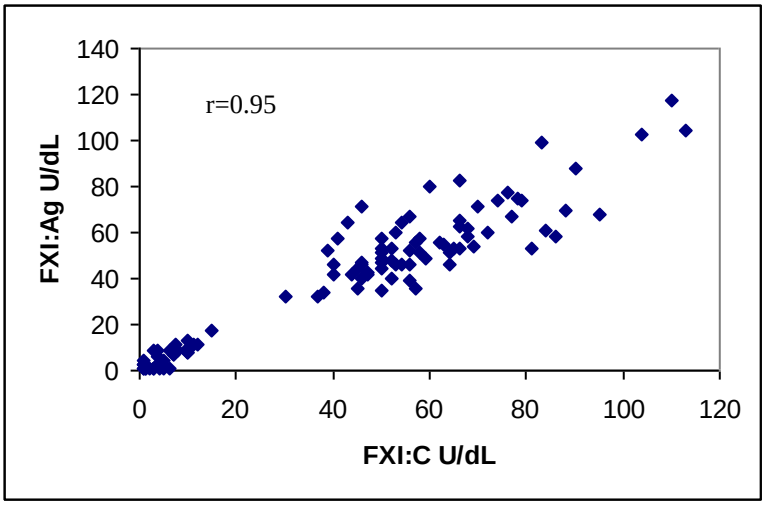
FXI levels (U/dL)	Number of patients
Severe deficiency (FXI < 20)	32
<2	10
2-5	10
6-20	12
Partial deficiency (FXI 20-70)	70
20-50	17
51-59	23
60-69	13
>70	17

Only 17 of 70 (24%) partially deficient patients had FXI:C <50 U/dL (the previously accepted lower limit of normal for FXI:C, now considered obsolete). A further 51% of partially deficient patients had FXI:C between 51 and 69 U/dL.

Normal FXI:C levels were found in 17 patients. The registered levels in these patients ranged from 42-68 U/dL. Of these 17 patients with FXI:C > 70 U/dL, six had a FXI:Ag < 70 U/dL and Jewish mutations were present in five. Of the 11 patients with normal FXI:C and FXI:Ag, Jewish mutations were present in five patients and four of the five patients had positive family histories. In these five cases reliance on coagulation assays alone for diagnosis would have resulted in incorrect genetic counselling being given.

The FXI:Ag was correlated well with the FXI:C in the majority of cases (Fig 3.5, r=0.95). One patient had a severely deficient FXI:C <1 U/dL, a normal FXI:Ag 105 U/dL and evidence of a lupus-type FXI inhibitor.

Figure 3.5: Correlation between FXI:C and FXI:Ag in 101 patients with FXI deficiency (excluding one patient with an acquired inhibitor to FXI and a FXI:C <1 U/dL and FXI:Ag 105 U/dL).



There were nine partially deficient patients with discordant results where either the FXI:C or FXI:Ag was in the deficient range but the other parameter was normal (table 3.8). In one case the normal FXI:Ag was indicative of a mutation causing secretion of a functionally defective protein (Ser576Arg) but in two other cases the normal FXI:Ag occurred in patients with confirmed Jewish mutations. In the cases of six patients with a normal FXI:C but a low FXI:Ag, Jewish mutations were present in five of them and the deficiency state would have been missed if FXI:C alone was used as the screening test.

Table 3.8: Patients with partial FXI deficiency and discordant FXI:C and FXI:Ag. Patients heterozygous for the Jewish type II mutation are indicated by II/WT and those heterozygous for the type III mutation are indicated by III/WT.

Patient study number	FXI:C	FXI:Ag	Genotype
Patients with low FXI:C and normal FXI:Ag			
005	46	71	Ser576Arg
076	60	80	III/WT
027	66	83	II/WT
Patients with normal FXI:C and low FXI:Ag			
083	72	60	II/WT
014	77	67	III/WT
009	81	53	II/WT
071	84	61	Unknown
081	86	58	III/WT
003	95	68	II/WT

There was some variation noted between the registered FXI:C and the level measured in the current study. Overall of 70 partially deficient patients, 29 had a current FXI:C which was within 15% of their registered level, two patients had levels which were more than 15% lower that the registered level and 39 patients had levels which were more than 15% above their registered FXI levels. The mean increase over baseline registered FXI:C was 49% in patients whose current FXI:C was >70 U/dL (range 17-90%).

Of 32 severely deficient patients, 13 had a current FXI:C which was within 15% of their registered level but all remained within the severely deficient FXI:C range. One patient with a registered level of 10 U/dL was found to have a current FXI:C of 62 U/dL and a FXI:Ag of 56 U/dL which was confirmed on repeat testing, indicating an error in the original sample.

Correlation between bleeding score and FXI:C

The bleeding scores in relation to phenotypic severity and FXI:C level are given in Table 3.9. A positive bleeding history (scores 1 or 2) was seen in 81% of severely deficient patients. However, five patients (16%) with FXI:C levels of <20U/dL were scored as probable non-bleeders (score 4). No severely affected patient was scored as a definite non-bleeder (score 5).

In contrast, the partially deficient patients were equally likely to be bleeders (47%) as non-bleeders (46%). The percentage of definite bleeders was higher in severely affected patients than in the patients with partial deficiency (50% versus 14%). There

was no difference in the FXI:C levels in either group between the bleeders versus the non-bleeders.

One severely affected patient and five partially deficient patients were allocated an indeterminate bleeding score, in the majority of cases because the patient had insufficient haemostatic challenges on which to determine a bleeding tendency but in two cases because of disagreement amongst reviewers.

Table 3.9: Bleeding scores versus severity of FXI deficiency and FXI:C.

Severe deficiency*					
Bleeding Score	1	2	3	4	5
No. of patients	16	10	1	5	0
% of patients	50	31	3	16	0
FXI:C U/dL					
Median	3	6.5	5	3.6	n/a
Range	<1-12	<1-15	n/a	<1-6	n/a
Partial deficiency†					
Bleeding Score	1	2	3	4	5
No. of patients	10	23	5	17	15
% of patients	14	33	7	24	22
FXI:C U/dL					
Median	51.5	52	66	64	56
Range	46-113	30-79	45-69	40-110	40-84

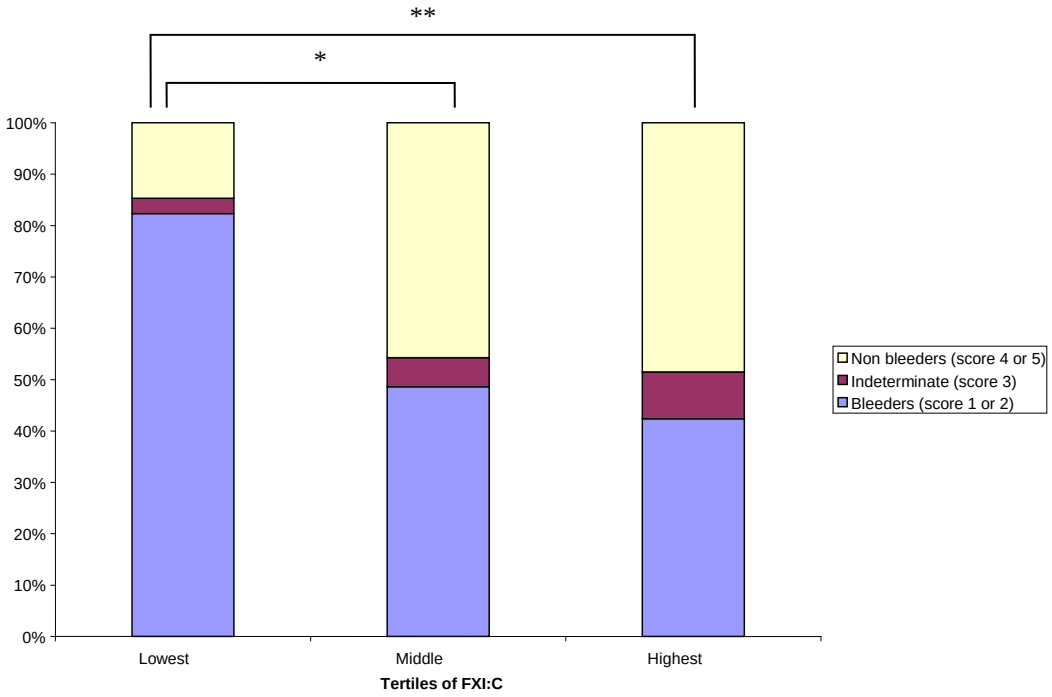
*Severe deficiency: Patients registered with FXI:C < 20 U/dL. *n*=32
†Partial deficiency: Patients registered with FXI:C 20-70 U/dL. *n*=70

The bleeding tendency is significantly associated with FXI:C but only in the lowest third of the patient group. Patients in the lowest tertile of FXI:C levels having a significantly higher incidence of bleeding scores 1 and 2 compared to patients in the middle and highest tertiles of FXI:C (fig.3.6). All but two of the patients in the lowest tertile (32 of 34 patients) had a severe deficiency of FXI (FXI:C <1-15 U/dL) and 82.3% of these patients were classified as bleeders. However, there were also five patients in this group who were classified as probable non-bleeders, indicating that FXI:C alone is not predictive of bleeding. There was no significant difference in bleeding scores between the middle and highest tertiles.

A similar relationship existed for FXI:Ag with a significant increase in patients with bleeding scores 1 and 2 in the lowest tertile: 84.8% versus 48.5% and 39.4% in the middle and highest tertiles respectively ($p<0.0001$, Kruskal-Wallis test).

Figure 3.6: The relationship between bleeding tendency and FXI:C.

Patients (n=102) were divided into three groups (tertiles) according to the FXI:C level and the proportion of patients classified as bleeders, indeterminate or non-bleeders was calculated for each tertile. Patients in the lowest tertile had FXI:C levels of <1-38 U/dL, patients in the middle tertile had FXI:C levels of 38-58 U/dL and patients in the highest tertile had FXI:C levels of 58-113 U/dL.



* p<0.0001 for the comparison between lowest and middle tertiles (Kruskal-Wallis test)

** p<0.0001 for the comparison between lowest and highest tertiles (Kruskal-Wallis test)

Von Willebrand factor and blood group

Mild type 1 von Willebrand disease was previously diagnosed in one patient entered in the study and a further patient had FVIII:C, VWF:Ag and VWF:CBA levels consistent with the disease. Both of these patients were classified as probable non-bleeders. One further patient who is a probable bleeder had borderline FVIII:C and VWF:CBA.

The relationship between VWF:Ag and VWF:CBA levels and bleeding tendency were statistically analysed in tertiles. The likelihood of having a positive bleeding history was not statistically different between the highest, intermediate and lowest tertiles of VWF:ag or VWF:CBA ($p=0.28$ and $p=0.48$ respectively, Kruskal-Wallis test). No significant difference in levels of VWF antigen or activity was seen when bleeders (score 1 or 2) were compared to non-bleeders (score 4 or 5)($p=0.5$, Kruskal-Wallis test).

Partially deficient patients were further studied to assess the ability of VWF and FVIII levels to predict bleeding, as had been previously suggested.(7) Table 3.10 shows the mean, median and range in partially deficient patients.

Table 3.10: VWF and FVIII levels in 70 partially deficient patients

	FVIII:C	VWF:Ag	VWF:CBA
Mean	97	100	101
Median	92.8	99.5	102.3
Range	34-198	49-284	46-257

The use of VWF and FVIII cut-off points of 70 IU/dL would not accurately predict bleeders in this patient group (table 3.11).(7) In only four patients were all three parameters (FVIII:C, VWF:Ag and VWF:CBA) < 70 IU/dL. Three of these were classified as probable or definite bleeders and one was classified as a probable non-bleeder.

Table 3.11: Lack of correlation of bleeding score with specific VWF and FVIII levels

	VWF:Ag <70	VWF:CBA <70	FVIII:C <70
N	12	11	9
Bleeder*	5	6	5
Indeterminate	2	0	0
Non-bleeder†	5	5	4

*Bleeder includes patients with a bleeding score of 1 or 2.
†Non-bleeder includes patients with a bleeding score of 4 or 5.

Blood group data was available in 89 of 102 patients (27 severely and 62 partially deficient patients). Blood group O was present in 44 of 89 patients (49%) and the proportion of patients classified as probable or definite bleeders was 64%. In contrast groups A and B accounted for 30% and 13% of patients respectively and the proportion of probable or definite bleeders was 48% in group A and 50% in group B. Six patients were group AB with equal numbers of bleeders and non-bleeders. The proportions of bleeders in patients with blood groups O and A was not significantly different (Chi-squared test).

Thrombophilic traits

Twelve patients had a reduced level of one or more of the natural anticoagulants (antithrombin, protein C and protein S) and two patients had a lupus anticoagulant, one of whom also had a low protein C (figure 3.7). Combined deficiencies of the natural anticoagulants were seen in three patients, which was due to liver disease in one case. Five of these 14 patients (36%) were classified as probable or definite non-bleeders, including both patients positive for a lupus anticoagulant. Five patients with low levels of natural anticoagulants were classified as definite bleeders (36%) and an additional four were probable bleeders (28%).

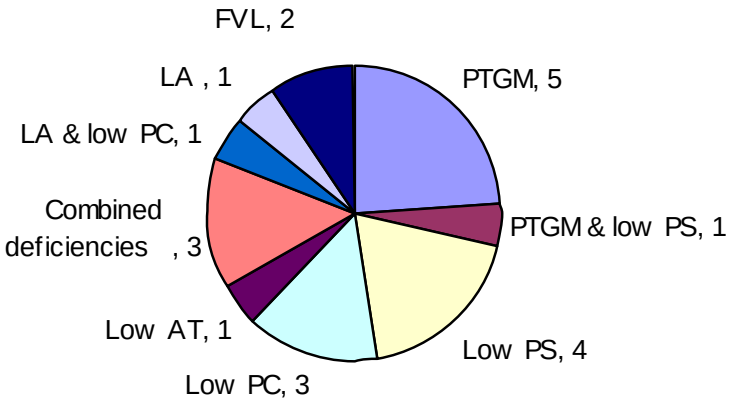
Two patients were found to be heterozygous for the factor V Leiden mutation. One was severely affected with a FXI:C of 7.4 U/dL and the second was partially deficient with a FXI:C of 50 U/dL. Both patients were scored as probable bleeders but with low numbers it is impossible to determine whether the FVLeiden is preventing a more severe bleeding phenotype.

The frequency of the prothrombin (PT) gene mutation was 6%, which is in keeping with published incidence rates in Jewish populations for this mutation.(169) Of the six patients, two had bleeding scores of 1 (definite bleeders) and FXI:C levels of 3 U/dL and 50 U/dL, the latter patient also had a low protein S. One patient (FXI:C 52 U/dL) was a probable bleeder. Two patients were probable non-bleeders with levels of 8 U/dL and 40 U/dL while the last patient had an indeterminate bleeding history (FXI:C 68 U/dL). Again, with small numbers of affected patients, it is not possible to identify a modulatory effect of the PT gene mutation.

Of a total of 21 patients with an identifiable thrombophilic trait, 14 (66.7%) of patients are classified as probable or definite bleeders. It is unlikely that these traits are modifying the bleeding phenotype to a large extent.

Figure 3.7: Thrombophilic traits in patients with FXI deficiency

Patients (n=21) with deficiencies of the natural anticoagulants: antithrombin (AT), protein C (PC), protein S (PS) or with a lupus anticoagulant (LA) or with a genetic thrombophilia: factor V Leiden (FVL) or the prothrombin gene 3'UTR mutation (PTGM).



FXI Glycosylation

A further potential modulator of the bleeding tendency in FXI deficiency is the state of glycosylation of the FXI Protein. The FXI protein has five potential N-linked glyscosylation sites as does Prekallikrein.(10;12) However, not all of these are glycosylated in FXI since the carbohydrate content is 5% whereas in Prekallikrein the carbohydrate content is 15%.(205) There is clearly potential for differences in the glycosylation pattern of the FXI protein which may be influenced by the presence of the polymorphisms in the glycosylation pathway.

Investigation of the congenital disorders of glycosylation (CDG) have revealed that a polymorphism in the ALG6 gene (T911C leading to an amino acid change F304S), which encodes a critical enzyme in glycosylation, is associated with a more severe clinical phenotype and may act as a genetic modifier.(184) In a Caucasian control group, the allelic frequency of the T911C polymorphism was 0.28. In a group of patients with congenital disorders of glycosylation, the overall allelic frequency was similar (0.31) but when patients were subgrouped according to disease severity, patients with a severe disease phenotype were significantly more likely to carry a mutant allele (allelic frequency 0.41 versus 0.21 for the mild/moderate disease phenotype). Thus, the presence of a mutant allele (whether in heterozygous or homozygous form) was associated with a worse clinical phenotype.

Of the 102 FXI deficient patients in the study, 80 DNA samples were available for analysis. Exon 10 of the ALG6 gene was sequenced and sequence alterations consistent with the F304S polymorphism were identified. The results for analysis of the F304S polymorphism are shown in table 3.12. The allelic frequency(q) was 0.24 (38 mutant

alleles of a total of 160 alleles) which is slightly less than the baseline Caucasian population. The overall distribution of the ALG6 genotypes are similar to mild/moderate phenotypes in CDG (approximately 40% with a mutant allele) rather than severely affected CDG patients, of whom 70% had at least one mutant allele. There is therefore no evidence that the polymorphism in ALG6 is over-represented in an unselected cohort of FXI deficient patients.

Table 3.12: Frequency of the F304S polymorphism in the ALG6 gene in 80 patients with FXI deficiency.

	Homozygous WT (Phenylalanine at aa position 304)	Heterozygous	Homozygous Mut (Serine at aa position 304)
Number of patients with FXI deficiency	47	28	5
% of patients with FXI deficiency	59	35	6
% of patients with Mild/Moderate CDG	61	36	3
% of patients with Severe CDG	30	60	10

3.4 Discussion

The strength of this study is the comprehensive re-evaluation of a large cohort of patients with severe and partial FXI deficiency and the ability to correlate all variables to the bleeding score. The bleeding tendency, as measured by a structured questionnaire, has been described previously in FXI deficiency.(5;7) Concordance in that study between individual assessors of the bleeding history questionnaires was 67% which compares well with the current study (72%).

A low proportion of indeterminate bleeding scores were assigned in the current study (6%) compared to 28% (43 of 152 individuals) in the earlier study. In the latter, significant proportions of patients in the indeterminate category were < 15 years of age (44%) or had never had a haemostatic challenge (35%). Allocation of a greater proportion of patients to specific bleeder or non-bleeder categories is likely to improve correlations with other laboratory and genetic parameters in this study.

Bleeding rates following invasive procedures have been reported in a number of previous studies although the exact methodologies have been variable in regards to inclusion of severe and partial deficiency and description of procedures with and without pre-operative treatment.(5;7;50;55;62) In relation to dental extraction, rates of bleeding have been recorded in two studies as 60% and 49% in severely deficient patients without prior treatment.(55;62) The rate of bleeding after dental extraction without prior treatment in the current study is 48.5% in severely deficient patients and 40% for the whole patient group. Similarly, rates of bleeding after tonsillectomy in untreated severely deficient patients in these two studies (86%,60.6%) compare to rates described in the current study (87.5% in untreated severely deficient patients). Partially

deficient patients have been reported to have rates of bleeding (without prior treatment) after tonsillectomy of 28% and 59% in the literature compared to 35% in the current study.(7;55) Therefore, bleeding rates in the current study, in relation to invasive procedures on areas of high fibrinolytic activity, are broadly in line with previously published rates in the literature.

Bleeding after invasive procedures in the oral cavity is likely to result in the need for emergency treatment including transfusion. In the current study, in untreated severely deficient patients, five required transfusion or FXI replacement after dental extraction while eight patients required transfusion and/or re-operation after tonsillectomy. The balance of risk would appear to favour pre-operative treatment in such procedures.(62) In addition, there is evidence that prior treatment with FXI replacement prevents bleeding (no bleeding in 45 procedures covered with FXI concentrate in (55).

In areas without high intrinsic fibrinolytic activity, the overall rate of bleeding is also broadly similar for procedures (31% in (55), 1-40% in (62), 26% in the current study). However, some variation in bleeding rates associated with individual procedures is evident. For example, the Israeli group has published rates of bleeding in untreated severely deficient patients after circumcision (1.5%), appendicectomy (4.5%) and hernia repair (23%) which are less than those found in the current study (8.7%, 20%, 60%).(62) While numbers may be small for individual procedures, patients who develop bleeding frequently require transfusion of cellular blood products or re-operation. In the current study, there were 23 episodes of cellular blood transfusion, 17 episodes of plasma transfusion and 12 re-operations required for management of post-operative bleeding.

The Israeli group have used the data collected from their patient group to argue for with-holding upfront treatment for surgery on areas without high intrinsic levels of fibrinolytic activity. However, the consequences of bleeding require evaluation also. These may include exposure to less virally safe cellular blood products in an emergency situation as opposed to treated FXI concentrate or plasma in an elective manner as well as risks of wound infection and an inferior wound outcome due to bleeding.

Rates of bleeding after delivery in women with severe FXI deficiency have been described.(71) For women who have not received prior treatment with plasma, bleeding rates of 24% for vaginal delivery and 16.7% for caesarean section were recorded. In women who received plasma prior to delivery, bleeding was recorded in two of 14 vaginal deliveries and none of six caesarean sections. In the current study, data were recorded on deliveries in 47 women with severe (n=14) and partial deficiencies of FXI and interestingly, the rates of bleeding in untreated patients was similar to those for severely deficient patients in Israel for vaginal delivery (25%) but higher for caesarean section (28.6%). Transfusion of plasma or red cells was required in 12 patients (35.5% of patients bleeding after vaginal delivery) in the Israeli study for the management of post-partum haemorrhage. In this study, transfusion was recorded in one of two patients bleeding after caesarean section and in six of twenty episodes of bleeding after vaginal delivery. In addition, FXI replacement was given in three episodes of bleeding. Therefore, the overall rate of emergency transfusion was 10 of 23 cases of bleeding (43%) which included patients with both severe and partial deficiency. Again, the Israeli group have pointed to the figure that 70% of severely deficient women do not require any form of transfusion for management of labour. However, risk balance analysis for upfront FXI replacement requires a review both of the rates of bleeding and

transfusion and the relative risks of adverse events for transfusion of untreated cellular blood products versus elective administration of a virally safe product.

The data presented from this study confirm that a normal aPTT is not sufficient to outrule heterozygous FXI deficiency, since approximately a third of such patients will have a normal aPTT. This finding that the prolongation of the aPTT is not found in all patients with FXI deficiency is in agreement with previous studies.(206-208) The activator and phospholipid source and concentration used in different aPTT assay reagents have a significant effect on the sensitivity of the aPTT assay for the detection of mild FXI deficiency.(209) The limit of sensitivity for prolongation of the aPTT in FXI deficient plasma with a silica aPTT activator was 57.5 U/dL in the latter study.

All patients had a re-evaluation of FXI:C which resulted in the identification of one individual incorrectly registered as severely deficient, when both the FXI:C and FXI:Ag were in the partially deficient range.

There were a relatively large number of patients whose FXI:C was higher than the registered FXI:C, even into the normal range. Some of the registered FXI:C levels are likely to have been done many years previously. The increase in FXI:C may be due to subtle changes in reagents or instrumentation in the aPTT-based FXI:C assay. Alternatively, FXI:C in these patients may have increased with age. In the Leiden thrombophilia study the mean FXI:Ag was 91.8% in 77 control individuals <30 years versus 99% in 173 control individuals aged >50 years and the average increase in FXI:Ag was 0.19% per year of age suggesting that part of the increase may be age related.(210)

Ten Jewish individuals in this study had normal FXI:C levels but on genetic testing, a *F11* mutation was confirmed. Five of these also had normal FXI:Ag levels. All of these patients had previously been registered with a lower FXI:C (< 70 U/dL), again raising the question that some current aPTT-based FXI assays may be less sensitive to heterozygous FXI deficiency.

The addition of FXI:Ag to the diagnostic test repertoire for FXI deficiency enabled identification of six individuals with normal FXI:C but low FXI:Ag (of whom five had Jewish mutations confirmed), in whom FXI deficiency might have been outruled if only FXI:C had been performed. In addition, evaluation of FXI:Ag resulted in the correct characterisation of a *F11* gene mutation as a functional defect (see Chapter 6 for further details of this mutation – Ser576Arg).

In populations with a high incidence of FXI deficiency and in patients with a confirmed family history of FXI deficiency, screening for FXI deficiency should include both the FXI:C and the FXI:Ag assays. Since coagulation assays are not be sufficient to diagnose an underlying *F11* gene mutation, genetic testing is recommended.

Although the FXI:C is not completely determinant for the bleeding tendency, the likelihood of bleeding in the lowest tertile of FXI:C (corresponding to severe FXI deficiency) is significantly higher than for patients with partial deficiency and amounts to a positive bleeding history in 82% of patients. In partially deficient patients, 40-50% of patients had a bleeding tendency but this is unrelated to whether the FXI:C falls in the middle or the highest tertile. Clearly, the presence of severe FXI deficiency is a

significant but not an absolute predictor of the bleeding tendency. In partial FXI deficiency, the absolute level of FXI:C does not appear to determine bleeding.

A very small proportion of patients had a diagnosis of von Willebrand disease in this study, in contrast to the 13% of patients diagnosed in an earlier study.(61) Earlier reports had indicated that levels of FVIII or VWF could influence the bleeding tendency and were predictive of bleeding.(7;65) In one study, a VWF:Ag less than 70 U/dL and a partial deficiency of FXI led to an increased incidence of bleeding (62% of patients).(7) In another study, FVIII:C was correlated with FXI:C in a study of 26 severely deficient and 46 partially deficient patients.(65) However, the use of a VWF level of 70 U/dL in this study did not identify a group of patients at higher risk of bleeding. Patients with blood group O in both studies had a slightly increased risk of bleeding symptoms. In the current study, there was no statistical difference in proportions of bleeders in blood group O versus blood group A although the percentage of bleeders was higher in blood group O (64% versus 48% with blood group A).

There have been a number of conflicting reports of modulation of the clinical phenotype of haemophilia by co-inherited thrombophilic traits.(67;211-214). In a reconstituted model of coagulation, the presence of the FVLeiden gene mutation in haemophilic plasma led to an increase in thrombin generation.(215) However, in this cohort of patients, despite the fact that 21% of patients had a thrombophilic trait, two thirds of these patients were still classified as bleeders. It is therefore not likely that thrombophilic traits are modifying the bleeding phenotype in FXI deficiency.

There is evidence for alterations in clinical phenotype in von Willebrand disease associated with polymorphisms in genes encoding glycosylation enzymes.(216;217) In the evaluation of children with congenital disorders of glycosylation (CDG), FXI levels are used as one of the laboratory parameters of disease severity.(218;219) In this study, evaluation of one potential genetic modifier of glycosylation did not reveal an over-representation of a polymorphism in the ALG6 gene associated with a more severe phenotype in CDG. However, the possibility that variation in glycosylation is a modifier of phenotype in FXI deficiency remains and further evaluation in this area is warranted.

In conclusion, at present the most robust predictor of bleeding in FXI deficiency is the presence of a severely reduced FXI:C (<20 U/dL). However, within this group of patients a small number will not experience severe bleeding. In patients with a partial deficiency, approximately half of the patients will have bleeding symptoms and lower levels of FXI:C, FVIII:C or VWF:Ag do not accurately predict bleeders. In addition, blood group O and the presence of a thrombophilic trait do not influence bleeding tendency to any great extent. The influence of FXI glycosylation remains to be further elucidated.

Chapter 4 Thrombin generation in Factor XI deficiency

4.1 Introduction

The detection of fibrin formed as a direct result of thrombin generation is the basis of the screening tests of coagulation: the prothrombin time(PT) and the activated partial thromboplastin time(aPTT) and others. However, these tests are limited in their ability to detect subtle defects in thrombin generation since excessive amounts of initiators are used and consequently fibrin formation occurs very early in the time course of thrombin generation.(220) At the end-point of the PT or APTT reaction, 5 to 10% of the total thrombin has been formed and the vast majority of the thrombin generated is formed after this point.(221) The conventional screening tests for coagulation effectively measure the lag-time of thrombin generation rather than the total amount of thrombin generated by a given plasma. The limitations of these tests are illustrated by the fact that the PT and aPTT are variably sensitive to hypocoagulable states and insensitive to hypercoagulable states. For example, in the patient group under investigation in this work, 23% of patients had a normal aPTT despite a low FXI:C.

Although two versions of the thrombin generation assay were described in the 1950s, the manual method was time-consuming and cumbersome for routine use.(112-114) A method of continuously measuring thrombin generation was developed which was less labour intensive, less prone to manual error and amenable to semi-automation.(115) With the advent of a range of suitable substrates and technology which enables rapid analysis, interest in the thrombin generation assay as an assay of global coagulation function has increased.(117-119)

Factor XI deficiency leads to a defect in the generation of thrombin in the consolidation phase of coagulation since FXI is activated by thrombin late in the course of thrombin generation.(142) FXIa further amplifies the generation of thrombin which serves to stabilise the clot through the activation of TAFI (thrombin activatable fibrinolysis inhibitor).(143)

The variable bleeding tendency in FXI deficient patients is as yet unexplained. The variability is not reflected in the FXI coagulant activity as measured by standard assays. A study of thrombin generation in plasma from nine severely deficient patients suggested that thrombin generation was dependent on FXI at low concentrations of tissue factor.(43) However, other studies using higher concentrations of TF had suggested that significant thrombin generation could be seen in plasma with very low levels of FXI:C (1%).(119) This study aimed to evaluate whether significant differences in thrombin generation could be seen in severe and partial FXI deficiency at low concentrations of TF and to define the relationship between thrombin generation and FXI:C levels, bleeding tendency and concomitant bleeding disorders or thrombophilic traits.

4.2 Subsampling Thrombin Generation Test

4.2.1 Method

In brief, defibrinated platelet-poor plasma was incubated with phospholipid (concentration 13 μM) and buffer. Coagulation was initiated with rTF (concentration 10 pg/mL[0.3pM]) and calcium chloride. Timed subsampling from the reaction mix into a stop solution (comprising an EDTA/BSA buffer – 7.5mM EDTA, 50mM Tris base, 175mM NaCl, 1% BSA, pH 8.4) was commenced 30 seconds after the initiation of

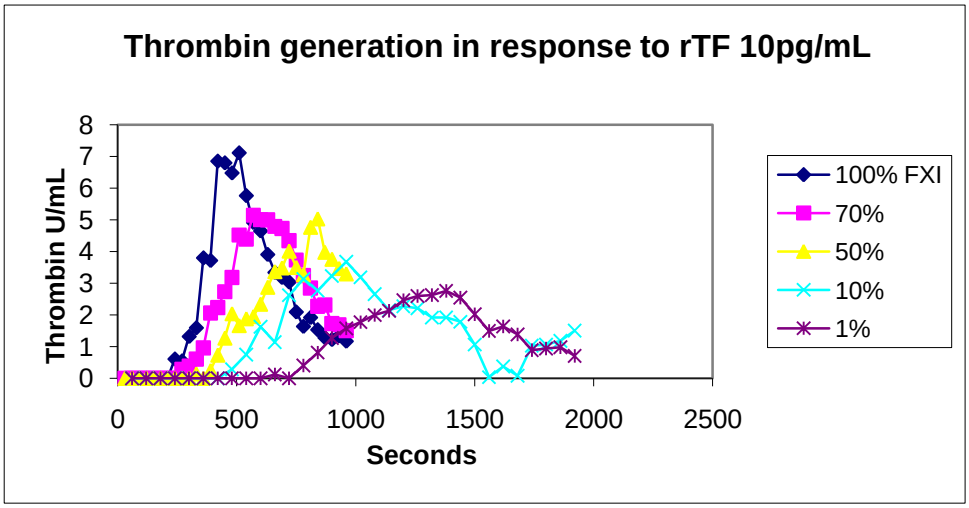
coagulation and continued every 30 or 60 seconds for 30 or 60 minutes for partially and severely deficient plasma respectively. A chromogenic substrate for thrombin (H-D-Phe-Pip-Arg-pNa.2HCl, S2238, Chromogenix) was then added to determine the amount of thrombin generated in each subsample and the colour reaction measured at 405nm. The full method is described in Chapter 2.

4.2.2 Development of the subsampling assay

Initially tissue factor (at concentrations of 10 pg/mL, 20 pg/mL and 40 pg/mL) was used to initiate thrombin generation in plasma with 100%, 50%, 25%, 10% and <1% concentrations of FXI (created by diluting normal pooled plasma into FXI deficient plasma). A dose response was demonstrable for the different FXI concentrations at 10 pg/mL with measurable thrombin generation even at FXI:C of <1 U/mL (Figure 4.1). Although the standard assay time of 15 minutes was satisfactory for normal plasma, it was too short for FXI deficient plasmas and was subsequently extended to 30 minutes for partially deficient plasma and 60 minutes for severely deficient plasma with the sampling interval increased from 30 seconds to 60 seconds.

To ensure a reliable source of TF with a specific concentration, Recombiplastin Batch number N0828542 was standardised against a lipidated recombinant human tissue factor of known concentration (American Diagnostica, product number 4500L, 50 ng TF). The concentration of this batch of Recombiplastin was calculated at 26,453.33 ng/mL. This TF source was used in all thrombin generation assays.

Figure 4.1: Dose response of varying concentrations of FXI using TF 10 pg/mL as initiator in the subsampling method.



4.2.3 Patient selection

The subsampling method was used to assay thrombin generation in plasma from 12 patients with severe FXI deficiency and 11 patients with partial FXI deficiency (Table 4.1). Random patient lists were seperately obtained for partially deficient and severely deficient patients. In the case of partially deficient patients every sixth patient from the list was chosen for thrombin generation by the subsampling method. This generated 10 patients. An additional patients plasma was assayed as part of the FaXID study (see Chapter 7) and was included. This gave a total number of patients of 11. For the severely deficient patients every fifth patient was chosen. This gave a total of six patients. A further five patients were specifically chosen because they were the only severely deficient patients with a bleeding score of 4 (probable non-bleeders). One patient had thrombin generation assayed as part of the FaXID study. This gave a total number of patients of 12.

Overall, six of the total of 23 patients had also participated in the FaXID study and therefore had plasma samples available pre and post treatment with recombinant Factor VIIa which were evaluated for thrombin generation.

Table 4.1: Patient selection for thrombin generation by subsampling method.

Patient Selection	Severe FXI deficiency	Partial FXI deficiency
Random	6	10
Bleeding score 4	5	0
FaXID trial participant	1	1
Total	12	11

4.2.4 Results

AUC: comparison between severe and partial FXI deficiency

The AUC for normal plasma using this subsampling assay varied from 2360.9 to 2910.9 U/mL. The same batch of commercial normal plasma was used in all assays. The median area under the curve for thrombin generation in severely deficient plasmas was 1574.2 U/mL with a range from 1260.4 to 3119.4 U/mL. The median area under the curve for partially deficient plasmas was 3119.5 U/mL with a range from 1402.5 to 5354.1 U/mL. The P value for the comparison between the severely deficient and partially deficient plasma is significant at 0.0062 (Wilcoxon two-sample test).

Of note, five of the eleven partially deficient plasmas had AUCs which were more than 10% above the highest value for normal plasma, raising the possibility of activation of the plasmas in the course of the assay. Only three partially deficient plasmas had AUCs

which were more than 10% below the lowest value obtained for normal plasma. Four of the severely deficient plasmas had AUCs which were in the range found for normal plasma.

Peak thrombin generation: comparison between severe and partial FXI deficiency

The peak thrombin level was also analysed and compared between patients with severe and partial deficiencies. The median peak thrombin level generated was found to be 1.95 U/mL in severely deficient plasma versus 6.5 U/mL in partially deficient plasma. The range for peak thrombin generation in normal plasma was 7.1 – 9.0 U/mL while the respective ranges for peak thrombin generation were 1.4 – 4.3 U/mL and 3.7 – 12.8 U/mL in severely and partially deficient plasmas. The p value for the comparison between severely and partially deficient plasmas was significant at <0.0001 (Wilcoxon two-sample test). All of the peak thrombin levels in the severely deficient plasmas were below the range found in normal plasma and there was minimal overlap with the peak values seen in partially deficient plasmas. Within the partially deficient group, one had a peak thrombin value which was in the range seen in normal plasma while three plasmas had peak thrombin values greater than normal plasma.

AUC and peak thrombin generation: comparison between bleeders and non-bleeders

Tables 4.2 and 4.3 show the relationship between AUC and peak thrombin levels generated, according to the bleeding history in patients with severe and partial deficiency respectively. A trend to lower AUC and peak thrombin levels is seen in bleeders in both categories of FXI deficiency but the p values were not significant by the Wilcoxon two-sample test (Fig 4.2).

Table 4.2: Relationship between bleeding history and FXI:C, AUC and peak thrombin generated in severely deficient patients evaluated for thrombin generation by subsampling method.

Severe FXI deficiency						
Bleeding history	N	FXI:C* Range U/mL	AUC† Median U/mL	AUC Range U/mL	Peak thrombin† Median U/mL	Peak thrombin Range U/mL
Bleeder††	7	3 - 12	1357.6	1260.4 – 2304.7	1.85	1.4 – 2.9
Non-bleeder¶	5	<1 - 8	1790.9	1312.9 – 3119.4	2.5	1.8 – 4.3

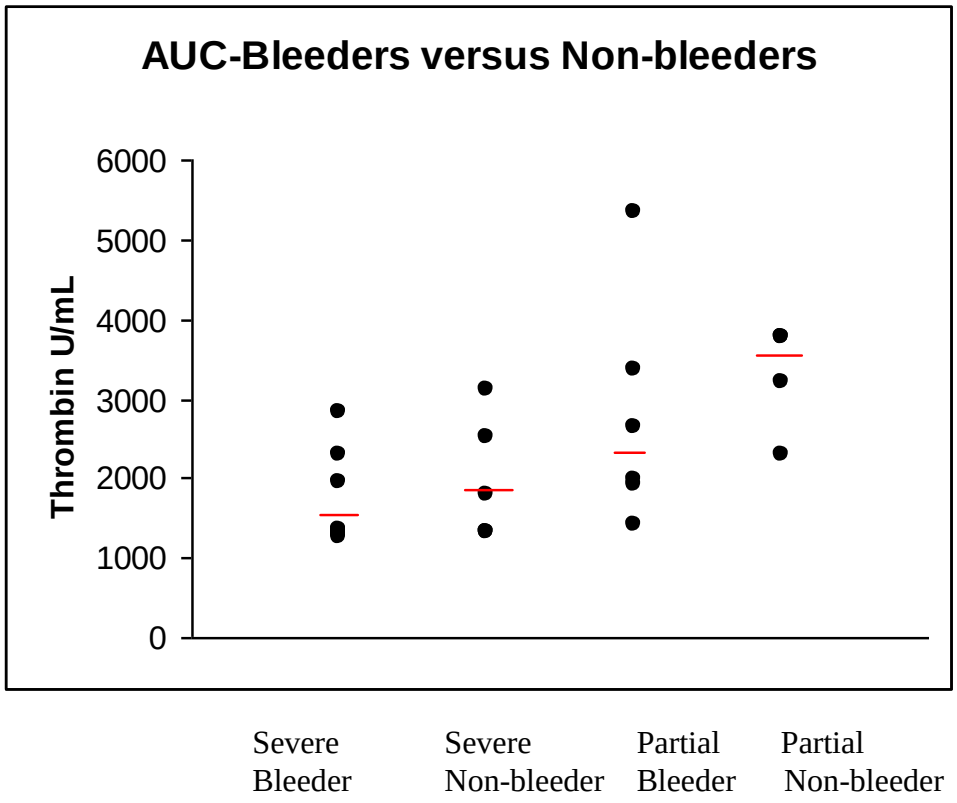
*One patient with a FXI:C of <1 U/mL had a high responding FXI inhibitor and another with a FXI:C of <1 U/mL had a lupus anticoagulant.
†p values for the comparison between bleeders and non-bleeders not significant
††Bleeder includes patients with bleeding scores 1 and 2.
¶Non-bleeder includes patients with bleeding scores 4 and 5.

Table 4.3: Relationship between bleeding history and FXI:C, AUC and peak thrombin generated in partially deficient patients evaluated for thrombin generation by subsampling method.

Partial FXI deficiency						
Bleeding history	N	FXI:C Range U/mL	AUC* Median U/mL	AUC Range U/mL	Peak thrombin* Median U/mL	Peak thrombin Range U/mL
Bleeder[†]	6	15 – 83[††]	2322.1	1402.5 – 5354.1	5.15	3.7 – 12.8
Indeterminate bleeding history	1	50	n/a	n/a	n/a	n/a
Non-bleeder[¶]	6	44 - 64	3480.6	2291.4 – 3769.9	6.55	6.2 – 8.2

*p values for the comparison between bleeders and non-bleeders not significant
[†]Bleeder includes patients with bleeding scores 1 and 2.
[††]One patient with partial FXI deficiency had concomitant liver disease, accounting for a FXI:C of 15 U/mL. If this patient is excluded, the range of FXI:C levels in partially deficient patients was 41 to 83 U/mL.
[¶]Non-bleeder includes patients with bleeding scores 4 and 5.

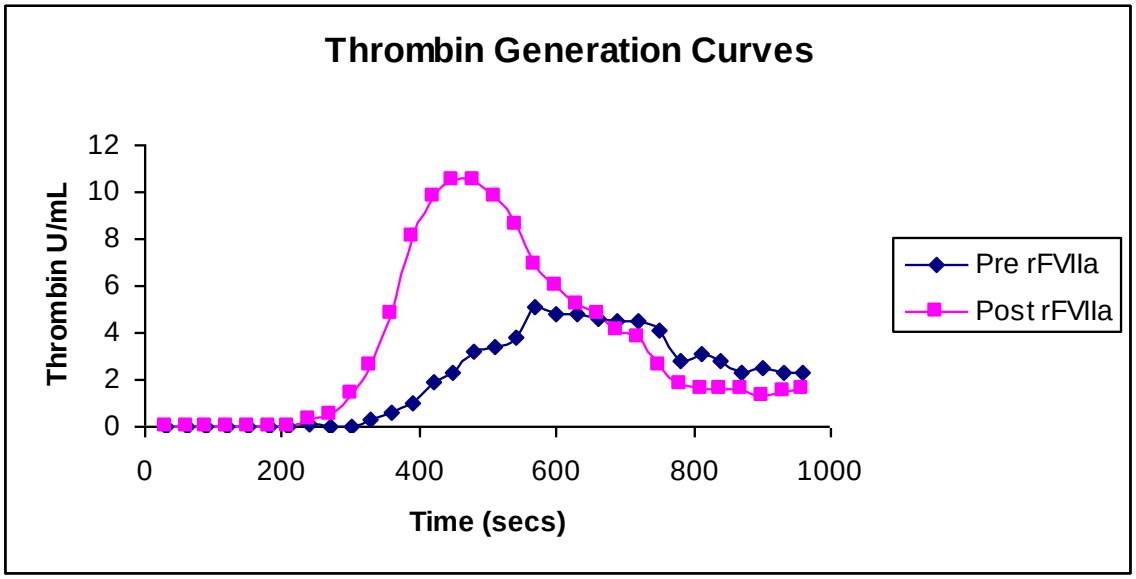
Figure 4.2: AUC of patients according to bleeding score (excluding one patient with an indeterminate score). Median values indicated by red horizontal line.



AUC and peak thrombin generation: pre and post treatment with rFVIIa

In the six patients who had received recombinant Factor VIIa the median AUC increased to 3804.5 U/mL after the administration of rFVIIa compared to 2147.55 U/mL prior to rFVIIa adminstration (normal plasma range 2360.9 – 2910.9 U/mL). Typical thrombin generation curves are shown in figure 4.3. The peak thrombin level also increased after adminstration of rFVIIa from a median of 4.9 U/mL to 11.5 U/mL (normal plasma range 7.1-9.0 U/mL).

Figure 4.3: Typical thrombin generation curves pre and post rFVIIa using sub-sampling method.



4.2.5 Conclusions from Thrombin Generation Subsampling method assays

From these preliminary studies of the subsampling Thrombin Generation method in FXI deficiency, the following conclusions were drawn:

1. Thrombin generation was measurable in FXI deficient plasma at low tissue factor concentrations.
2. Using a low TF concentration, a dose response was detectable at different FXI plasma concentrations, including in severely FXI deficient plasma.
3. The difference in thrombin generation between severely and partially deficient plasmas was significant.
4. There was a non-significant trend to lower thrombin generation in patients classified as bleeders versus non-bleeders.

However, the subsampling assay is cumbersome and the possibility of activation of some plasmas during the assay cannot be discounted. Therefore, development of a continuous thrombin generation assay was undertaken.

4.3 Continuous Thrombin generation test

4.3.1 Method

Defibrinated platelet-poor plasma was incubated briefly with phospholipid (concentration 6.5 μM), rTF (concentration 10 pg/mL) and buffer. Coagulation was initiated by adding calcium and a chromogenic substrate for thrombin (MM-MeAla-Arg-pNa.HCl, CBS 00.68, Diagnostica Stago) was added to enable continuous measurement of thrombin generation by reading the colour of the reaction mix every 30 seconds for 30 minutes. The full method is described in Chapter 2.

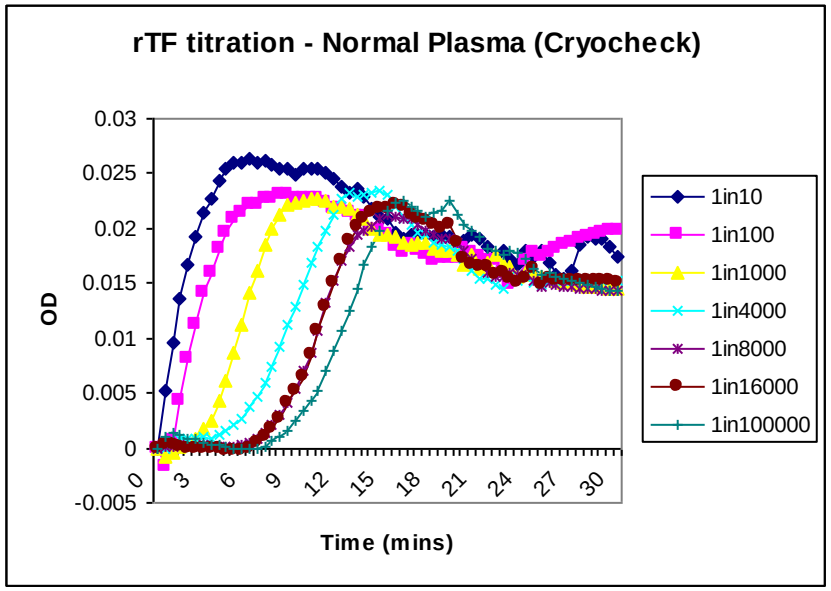
4.3.2 Development of assay

As a consequence of the limitations of the sub-sampling thrombin generation method, a continuous thrombin generation method was developed. Optimisation of published methods was required in particular because a relatively low TF concentration was used relative to previously published methods of continuous thrombin generation (10 pg/mL as in the sub-sampling assay).

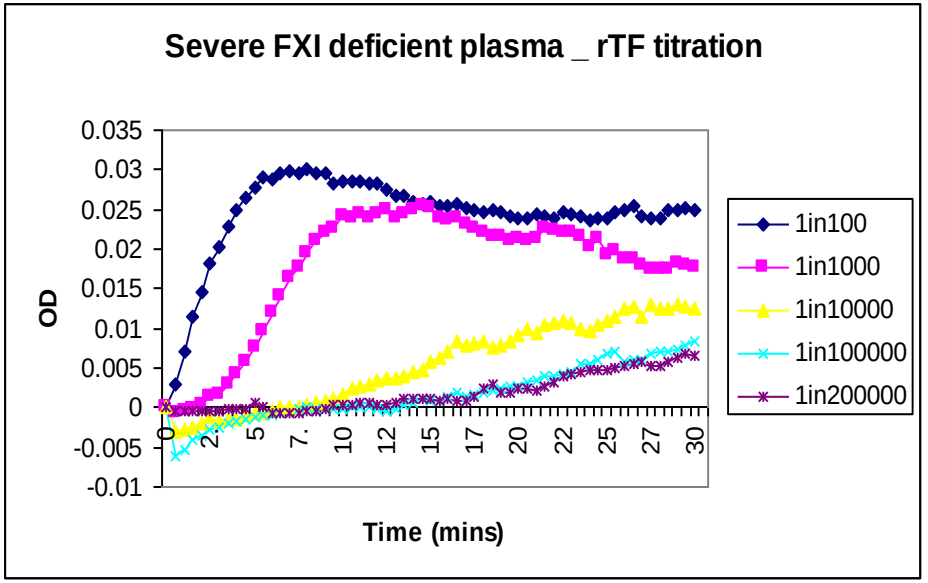
Initially, a titration of rTF from 1/10 to 1/100,000 in normal plasma was performed to confirm that the TGT would be sensitive to varying rTF concentrations (Fig. 4.4a). Next, a similar rTF titration was performed in FXI deficient plasma (Fig. 4.4b). It is notable that the ETP curves are not very different to normal plasmas at higher rTF

concentrations (i.e. 1/100 and 1/1000) dilutions and only become significantly abnormal at dilutions of 1/10,000 or more (Fig 4.4 a & b). Reaction volumes had to be adjusted since microtitre plates were being used instead of test-tubes. A phospholipid concentration of 6.5 μM in the final reaction mixture was found to be optimal and was superior to frozen PS/PC vesicles. The raw OD data represents the total amount of substrate cleaved by thrombin and the α2 macroglobulin-thrombin complex which is formed during the reaction. A computer program was developed to rapidly calculate the contribution related to thrombin alone by subtracting the contributions from the α2 macroglobulin-thrombin complex, as described by Hemker.(116)

Figure 4.4a and b: Comparison of TF titration in normal plasma and FXI deficient (FXI:C <1U/mL) plasma(Raw OD curves). With higher concentrations of TF (1/100 and 1/1000 dilutions) the steady state OD achieved for both plasmas is between 0.015 and 0.025. With lower concentrations of TF, the lag time for NP increases and the curve becomes flatter for the severely deficient plasma.



(a)



(b)

4.3.3 Reproducibility of the continuous TGT

The intra-assay co-efficient of variation (CV) for normal plasma varied from 7.1% to 16.2% (Table 4.4). The inter-assay CV for a normal pool (20 normal individuals) comparing four results (in duplicate) on four separate days was 14.9%. The reliability of the results achieved was found to be sensitive both to the manual methods used (e.g. type of pipette) and to the reagents (e.g. necessity to ensure the correct pH of the buffers for example). The ETP was calculated for the normal plasma control (Cryocheck) twice on each 96 well plate. The normal controls were assayed in duplicate in the first two wells of the 96 well plate and a second set of duplicates in the last two wells. The ETP of patient samples was expressed as a % of the average from the four normal control wells on each plate.

Table 4.4: Intra-assay imprecision studies for normal plasma in the continuous thrombin generation test.

	Cryocheck	Normal pool Expt 1*	Normal pool Expt 2	Normal pool Expt 3	Normal pool Expt 4
Mean	1.654	1.3225	1.5275	1.265	1.245
SD	0.118	0.214	0.205	0.183	0.101
CV(%)	7.1	16.2	13.4	14.5	8.1

*Expt: Experiment

4.3.4 Patients

A total of 100 patients had plasma available for analysis. Results are available for 66 patients. Patients were not selected specifically and the remaining 34 patients were not assayed or required repeat assays which could not be performed due to time constraints.

4.3.5 Results

Comparison of severe and partial FXI deficiency

At higher TF concentrations, the ETP was normal even in severely deficient plasmas. At a TF concentation of 10 pg/mL and following the optimisation steps above, a clear distinction could be made between different concentrations of FXI:C (Figure 4.5). The median ETP for all 66 patients was 57% with a range of 1-141.6% (compared to normal plasma, allocated a value of 100%). Table 4.5 shows the results for the ETP for all patients and according to the severity of the FXI deficiency.

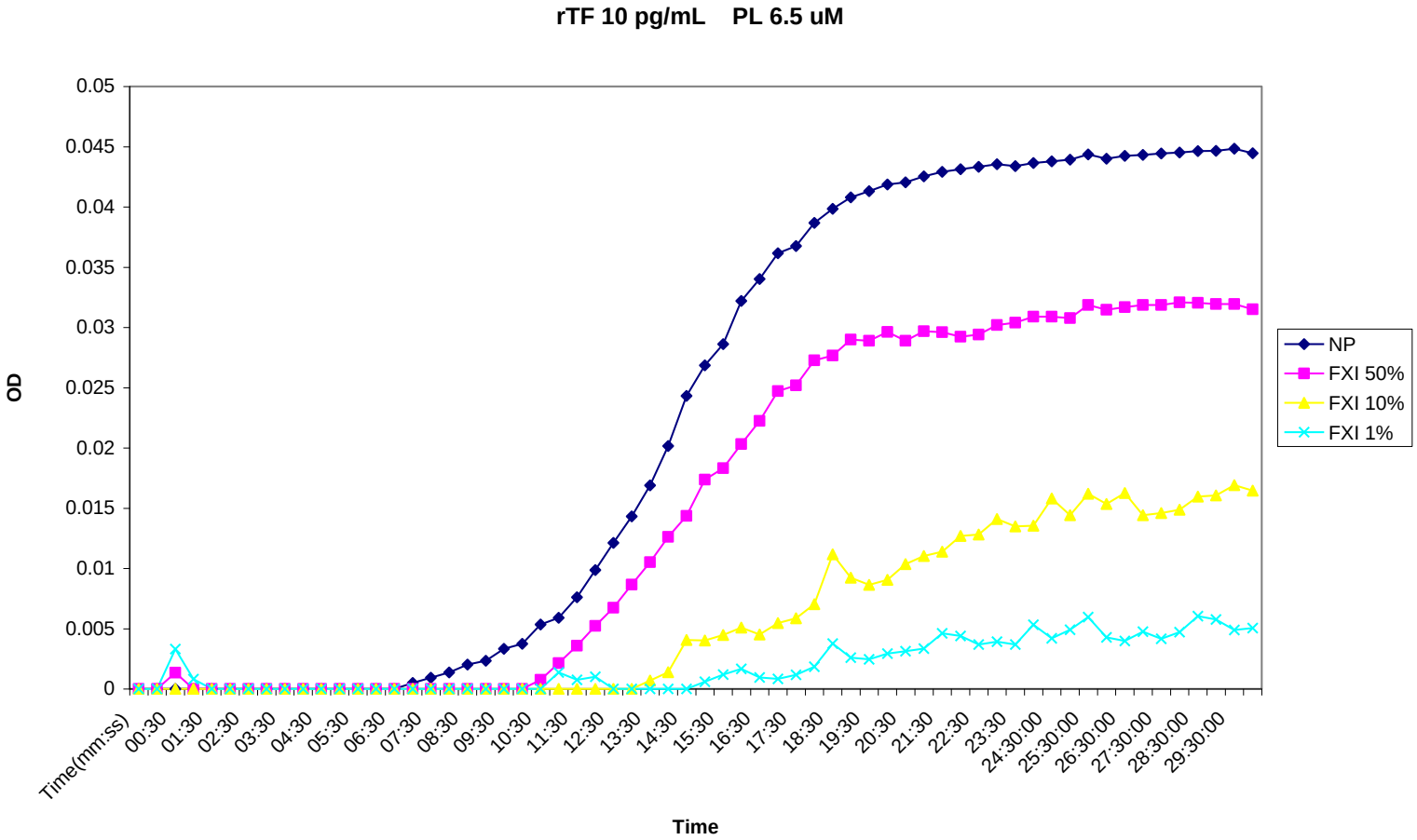
One severely deficient patient with a bleeding score of 2 had an unusally high ETP of 38.5%. Apart from this patient, two other severely deficient patients had ETPs of approximately 25%, one with a bleeding score of 1 and the other with a bleeding score of 4. The remaining nine severely deficient patients had ETPs of <20%.

Of the partially deficient patients, only 6 of 54 patients had an ETP of <30%. One patient with a bleeding score of 1 and a relatively low FXI:C of 30%, had an ETP of 8.7%. This is in the range of severely deficient patients. The other five patients had ETPs ranging from 21.4 to 29.1% and included one definite non-bleeder while the others were classed as probable (n=3) and definite bleeders (n=1).

Table 4.5: ETP results for all patients and according to severity of FXI deficiency.

	Number of patients	ETP (% Normal Plasma)			
		Median	Range	25^{th}-75^{th} percentile	5^{th}-95^{th} percentile
All patients	66	57	1-141.6	n/a	n/a
Severe FXI deficiency	12	10.9	1-38.5	6-18.6	3.1-31.6
Partial FXI deficiency	54	68.8	8.7-141.6	48.4-83.4	24.7-116.8

Figure 4.5: Titration of FXI:C in the continuous ETP method.



Correlation of Bleeding score and FXI:C with ETP in Partial FXI deficiency

A total of 54 patients with partial FXI deficiency were included. Of these, two patients had an indeterminate bleeding history (score 3) and were not included in this analysis. Therefore 52 partially deficient patients were evaluated. The results for FXI:C and ETP according to bleeding scores 1 or 2 (i.e. bleeder) or 4 or 5 (non-bleeder) are given in table 4.6. The p value for the comparison between bleeders and non-bleeders is 0.02 (Wilcoxon two-sample test).

Table 4.6: The relationship between ETP and bleeding score for partially deficient patients (ETP expressed as % of normal plasma).

Bleeding score	Number of patients	FXI:C Median (Range)	ETP* Median	ETP Range
Bleeder†	26	52 (30-113)	52.4	8.7-135.6
Non-bleeder††	26	57.5 (40-110)	77.3	24.8-141.6

*p value for the comparison of median ETP between bleeders and non-bleeders =0.02
†Bleeder includes patients with bleeding scores 1 and 2.
††Non-bleeder includes patients with bleeding scores 4 and 5.

The median and range for FXI:C between the "bleeder" versus the "non-bleeder" groups are similar. This further demonstrates the lack of relationship between the FXI:C and bleeding history. The correlation co-efficient for the relationship between FXI:C and ETP in these patients is 0.43, confirming that FXI:C is not the only determinant of ETP.

As only 12 severely deficient patients were evaluated a statistical analysis of the relationship between ETP and bleeding history was not possible. Two severely deficient patients had a bleeding score of 4 (probable non-bleeder). These two patients

had ETPs of 16.3% and 25.3% but there were also two of 10 patients in the "bleeder" category with ETPs of >20%.

Concomitant bleeding disorders and thrombophilic traits in Partial FXI deficiency

Patients were assessed for concomitant bleeding disorders. In the bleeder category, one patient had a low FVIII:C while in the non-bleeders, one patient had a low FVIII:C, two patients had von Willebrand disease and one patient had dysfibrinogenaemia. There did not appear to be a correlation between bleeding tendency and other bleeding disorders. Of the four patients with a low FVIII:C or von Willebrand disease, two had an ETP which was less than the 25th percentile for the partially deficient patient group.

Ten of 54 partially deficient patients had an abnormal thrombophilia screen. Seven of these 10 patients had ETPs above the 75th percentile for the partially deficient patient group and three of seven had an ETP above the 95th percentile. However, this trend was not clearly linked to bleeding tendency – of the seven patients with an elevated ETP, three were classified as bleeders and four as non-bleeders.

4.4 Comparison of TGT methods

Different thrombin generation methods are not easily comparable. The sub-sampling thrombin generation method described here utilises a thrombin standard and the results are expressed in units of thrombin. On the other hand, the continuous ETP method refers to a normal plasma run in parallel and given a nominal value of 100%.

There were 12 patients whose plasma was assayed by both methods, four severely deficient and eight partially deficient patients. Of the severely deficient patients, two

sets of results were broadly concordant with one another while two sets were discordant e.g. an ETP higher than the median for the group with a sub-sampling AUC lower than the median. Within the group of eight partially deficient patients, five had concordant results and three had discordant results.

4.5 Discussion

The results of the Thrombin Generation test performed by the subsampling method confirm that there is a difference in thrombin generation potential between severely and partially FXI deficient plasma. This is in contrast to a previous report that plasma from patients with severe Factor XI deficency had normal thrombin generating potential.(119) However considerably higher concentration of tissue factor was used in this study to initiate thrombin generation (15pM ≈ 525 pg/mL) It has been proven that the presence of FXI is critical when thrombin is generated using a low concentration of tissue factor as the initiator.(43) In the pivotal study by von dem Borne et al, plasma deficient in FXI and FXII was reconstituted with FXI (12.5nM). At higher concentrations of TF there was no difference in fibrin formation between plasma with and without FXI. However at dilutions of TF $>1x10^4$ a gradual increase in fibrin formation was seen in the presence of FXI as concentrations of TF decreased. In the study by Keularts et al, there was no difference in thrombin generation between normal plasma and FXI deficient plasma (FXI:C <1%) at TF concentrations of 37.5 or 300 pg/mL but a significant difference was seen at a TF concentration of 10 pg/mL. It is now evident from this and other studies that coagulation may be initiated by sub-picomolar concentrations of TF (134;136;220;222) and that the TF concentration in whole blood cannot exceed 20 fM (=0.02pM).(223)

Given this data, a low TF concentration of 10 pg/mL(≈0.27pM) was utilised in the current work with the express aim of demonstrating differences in thrombin generation: firstly between normal, partially deficient and severely deficient plasmas and secondly between patients with different bleeding tendencies but similar FXI:C levels. Using higher concentrations of TF would have obscured the role of FXI which is seen particularly at low TF concentrations. A number of TF concentrations were evaluated (10,20 and 40 pg/mL). Compared with the 10 pg/mL concentration, the 40 pg/mL concentration resulted in greater thrombin generation for severely deficient plasmas but thrombin generation in partially deficient plasmas was near normal. Since a particular aim of the study was to evaluate differences in bleeding tendency in partially deficient patients, the lower concentration was chosen. This had the consequence that longer data acquisition times were needed for severely deficient patients.

The AUC and peak thrombin generated in the subsampling TGT were also compared in patients who were classified as bleeders versus non-bleeders. There was a non significant trend for higher area under the curve and peak thrombin in non bleeders. However only small numbers of patients were tested in each category due to the laborious nature of the test. Some patients with partial deficiency had higher AUCs and peak thrombin than normal plasma raising the possibility of activation of the plasmas either at blood sampling, in storage, in the freeze-thaw process or in the assay itself.

Finally it was notable that treatment with rFVIIa normalised thrombin generation. Since rFVIIa is not providing replacement therapy in patients with FXI deficiency, it is useful to have proof that the goal of therapy, to restore thrombin generation, is achieved. In the absence of a specific laboratory marker for efficacy of rFVIIa, a thrombin

generation test may be a useful marker of efficacy and safety. There have been a number of small studies of the effect of rFVIIa on thrombin generation. In a series of five patients with severe FXI deficiency and inhibitors, thrombin generation was corrected to 50% of normal with rFVIIa at a concentration of 1 μg/mL, equivalent to a clinical dose of 45 μg/kg.(106) Subsequently, plasma from 14 patients with FXI deficiency were evaluated in a thrombin generation assay (five had inhibitors and may have been the same patients as in the earlier study).(128) In these patients, no thrombin generation was evident in the presence of phospholipids and calcium alone. However, rFVIIa at a concentration of 1.75 μg/mL (35nM, equivalent to a clinical dose of 80 μg/kg) restored thrombin generation. The latter effect was reduced if either FVIII or FIX were inhibited. The effect of rFVIIa could be augmented in FXI deficient plasma if a small amount of TF was added (0.27pM ≈10 pg/mL) or if the concentration of phospholipid were increased.

The subsampling TGT provided proof of principle that at low TF concentrations, differences in thrombin generation could be seen between normal plasma and FXI deficient plasma and between severe and partial deficiency. In addition, there was evidence of differences in thrombin generation among patients with similar FXI:C levels which might relate to their bleeding tendency.

The main difficulty with the subsampling method is that it is non-automated and extremely labour intensive. This reduces the number of patient samples that can be assayed. The accuracy of results and the reproducibility of the assay are affected by manual error. An automated or semi-automated method would allow increased numbers of patient samples to be assayed in a more robust way. Assay of larger

numbers of plasmas is important to determine whether there is a clear demarcation in thrombin generation between severely and partially deficient plasma. In addition, a larger patient group might allow identification of a relationship between thrombin generation and bleeding tendency. For these reasons, a continuous TGT was developed. The parameters chosen for the continuous TGT in this study were developed to allow subtle differences in thrombin generation to be detectable between patients with similar FXI:C levels but different bleeding tendencies rather than to give a definitive value for thrombin generation.

The continuous TGT confirms that the endogenous thrombin potential is significantly lower for severely deficient plasma than partially deficient plasma. Although the range of ETP results was markedly lower for severely deficient plasma, there were some patients in each category of FXI deficiency with values for ETP which overlapped, generally in the range 20-30% of normal plasma ETP.

There was a significant relationship between ETP and bleeding tendency in partially deficient patients. There was no relationship between the FXI:C and ETP and the median FXI:C levels were similar in the bleeder and non-bleeder categories.

The continuous thrombin generation method was semi-automated and therefore allowed for increased numbers of samples to be assayed. However, the assay process still contains significant manual steps which do not lend it easily to routine laboratory assay. The manual aspects of the assay lead to intra-assay and inter-assay CVs which are variable and in some cases higher than those normally accepted in routine laboratory

practice. The measured CVs for normal plasma are in keeping with published CVs for similar assays.(125)

Factors which may influence the thrombin generation in patients with FXI deficiency include co-existant VWD or thrombophilic traits. There were limited numbers of patients in the group with such laboratory abnormalities. The most notable trend was that patients with a thrombophilic trait had a increased likelihood of having an ETP above the 75th percentile and above the 95th percentile of the range of ETP in partially deficient patients. Increased thrombin generation has been reported in plasma from patients with haemophilia and FVII deficiency who have concomitant thrombophilia.(214;215) However, the increased ETP did not correlate with bleeding tendency in FXI deficient patients.

In patients with a partial deficiency of FXI, the conformation of the FXI dimer may influence protein function.(224) It is feasible that dimers of WT and mutant FXI may co-exist with WT-WT dimers and mutant-mutant dimers and the relative proportions of each may vary. One can speculate that relatively higher proportions of WT-WT dimers may correlate with higher thrombin generation potential and a lesser tendency to bleed. However, none of four mutations proven to cause such a "dominant negative" effect (Ser225Phe, Cys398Tyr, Gly400Val, Trp569Ser) has been identified in the patients in this study.(224;225)

Inhibition of FXIIa by corn trypsin inhibitor (CTI) has been used in some studies of a reconstituted synthetic model of coagulation to ensure that contact activation did not play a role in thrombin generation.(134;135) However, contact activation did not

appear to occur to any significant degree in a dilute thromboplastin assay evaluating the contribution of FXI to coagulation using low concentrations of TF as the initiator.(136) Other studies of thrombin generation in FXI deficient plasmas, using TF as initiator, similarly did not use an inhibitor of FXII.(43;106;119) In a study of thrombin generation in FXI deficient plasma (FXI:C <1%), thrombin generation curves were obtained in the presence and absence of a monoclonal antibody to FXII and no differences were seen.(43) Consequently, corn trypsin inhibitor was not used in the assays described in this study. Subsequently, the use of CTI in a fluorogenic thrombin generation assay has been shown to abolish contact activation at concentrations of TF <15pM in PPP and PRP.(133;137) The effects of CTI on thrombin generation in a chromogenic assay were not evaluated.

The aim of this study was to provide initial proof of principle regarding differences in thrombin generation between patients with similar FXI levels but different bleeding histories. In effect, patient samples which had been collected, stored and treated in a similar manner were compared to each other in an assay designed to optimise the chances of detecting differences. Clearly this chromogenic assay using PPP and exogenous phospholipid is not physiological but the evidence that the thrombin generation assay can differentiate patients with a bleeding tendency supports the future development of more physiological TGTs using PRP which may be applicable in the routine setting.(226)

Neither the subsampling nor the continuous chromogenic TGT assay as evaluated in this study is amenable to reliable use in a routine laboratory. However, these studies do indicate that the TGT may be able to detect subtle differences in thrombin generation

potential in plasmas with similar FXI levels. Preliminary evidence has been presented which suggests that ETP differs in plasmas from patients with different bleeding histories but similar FXI levels.

Further optimisation of the TGT in FXI deficient plasma would include evaluation of a maximally automated assay, preferably using PRP. Other issues which need to be more fully evaluated are the use of CTI and the optimal TF concentration for use in assays of severely deficient plasma.

Chapter 5 Thrombin Activatable Fibrinolysis Inhibitor (TAFI)

5.1 Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) is a recently described indirect inhibitor of fibrinolysis, as outlined in detail in the Introduction (Chapter 1). TAFI activation appears to be dependent on thrombin generated in a FXI-dependant fashion and it may be the hypothesised that FXI deficient patients will have less inhibition of fibrinolysis than normal plasma, leading to more bleeding.(143;165) Evidence in the literature suggests that variability in TAFI antigen levels, TAFI activation or TAFI activity could cause differences in the degree of inhibition of fibrinolysis, potentially leading to a variability in bleeding tendency.(160;173;177) For example, it could be postulated that a patient with a partial FXI deficiency and higher TAFI antigen or activity levels would have greater inhibition of fibrinolysis and reduced bleeding compared to a patient with a similar FXI deficiency and lower TAFI levels.

There are a number of TAFI antigen ELISAs available but individual assays have variable sensitivity for known polymorphisms in the TAFI protein. TAFI activity can be measured after activation in a commercial enzymatic assay. TAFI activity is also evaluated by the Clot Lysis assay if the plasma sample is assayed with and without Potato Tuber Carboxypeptidase inhibitor (PTCI), a specific inhibitor of TAFI.

In this study, the following questions are posed:

1. Is there evidence for variability in TAFI antigen, activity or clot lysis time (CLT) in FXI deficient patients?
2. Are TAFI antigen, activity or CLT associated with FXI:C levels or bleeding tendency?
3. Is there evidence that FXI deficient plasmas have increased fibrinolysis as measured by the CLT?
4. Does rFVIIa correct any abnormalities in the CLT?

5.2 TAFI antigen

5.2.1 Method

Briefly, the TAFI Antigen kit used was the Imulyse® TAFI Antigen kit (Biopool International, Sweden), subsequently marketed as VisuLize® TAFI Antigen kit (Affinity Biologicals Inc., Ontario, Canada). The kit utilises a polyclonal sheep antibody to human TAFI as the capture antibody and the detecting antibody is labelled with peroxidase to enable detection using a colorimetric reaction. A standard curve is generated using a standard reference plasma diluted in TAFI deficient plasma. The method is a standard ELISA method (See Methods Chapter 2).

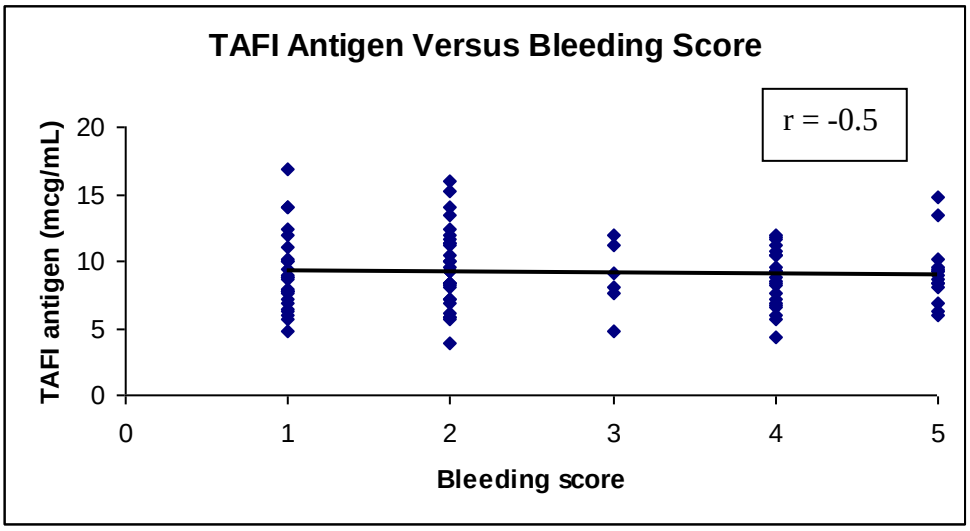
5.2.2 Results

A reference interval for the TAFI:Ag assay was obtained by analysing TAFI:Ag levels in twenty normal individuals. The mean for the normal plasmas was 9.0 ± 5.0 µg/mL, with a range of 3.8-13.4 µg/mL. The reference interval (2.5^{th}-97.5^{th} percentile) was 5-13.4 µg/mL. TAFI:Ag levels in 102 patient samples had a median and mean of 9 µg/mL and 9.1 µg/mL respectively, with a range of 3.9-16.8 µg/mL. Nine patients had

TAFI:Ag levels which were higher and four patients had levels which were lower than the reference interval. The patients with low TAFI:Ag levels had bleeding scores of 1,2,3 and 4. Three patients with increased TAFI:Ag levels had bleeding scores of 1, four had bleeding scores of 2 and two had bleeding scores of 5.

The direct relationship between TAFI antigen and bleeding score for 102 patients is shown in Figure 5.1. There is no correlation between TAFI antigen level and bleeding score and the correlation coefficient is -0.5. The relationship of TAFI:Ag in the highest, intermediate and lowest tertiles to bleeding score was assessed by the Kruskal-Wallis test. The distribution of bleeding scores was not significantly different between the tertiles of TAFI:Ag levels (p=0.06).

Figure 5.1: TAFI antigen levels in µg/mL from 102 patients with FXI deficiency versus bleeding score. Bleeding score of 1: definite bleeder, 2: probable bleeder, 3: indeterminate, 4: probable non-bleeder, 5: definite non-bleeder.



5.3 TAFI Activity (Enzymatic Method)

5.3.1 Introduction

TAFIa is known to be a very labile protein and prone to rapid degradation at room temperature (estimated t½ of TAFIa is two hours at 25ºC). In assaying TAFIa, particular care was taken regarding specific incubation temperatures and times mandated in the assay kit instructions. Likewise, plasmas for analysis were rapidly thawed in a waterbath, then placed in an ice bath until required for assay. All assays were carried out promptly. Plasmas were thawed only once.

5.3.2 Method

TAFI activity was assessed using an American Diagnostica Actichrome TAFI kit as described in the Methods Chapter 2 and briefly summarised here. In essence the kit is an assay of "activatable" TAFI, based on an assay described in the literature.(176) All plasmas were assayed twice. One set of duplicate plasma samples was activated with a specific TAFI activator - a thrombin/thrombomodulin complex, (termed the activated plasma sample) while the second plasma sample was assayed without activation (termed the unactivated plasma sample). After addition of the activation reagent, an exact 20 minute incubation at room temperature was undertaken and then an activation stop reagent was added to all samples, followed by addition of a TAFI developer which results in a colorimetric reaction. The colour reaction was stopped after a further 30 minutes and the absorbance was read at 490 nm. The TAFI carboxypeptidase activity was derived from the subtraction of the absorbance of the unactivated plasma sample from the activated plasma sample. The concentration of TAFI was then calculated from a standard curve generated from TAFI standards included in the kit.

5.3.3 Results

Despite following the specific kit instructions, the TAFI standard did not perform as expected. The absorbances obtained from the TAFI standard were much lower than the kit instructions and published data would suggest. The absorbances derived from the TAFI standard ranged from 0.224 to 0.441 when patient samples ranged from 0.38 – 0.93. A second vial of the standard was re-assayed to confirm this finding and again low ODs were obtained of 0.52 and 0.578. The kit insert implied that the top dilution of the standard might have an OD of approximately 1.4. The manufacturer was contacted. The manufacturers suggested that the standard may have been damaged in transit and a new kit was provided with the same lot of standard. This time the top standard gave an OD of 0.257 and was similar to the first kit standard. A normal plasma pool had an OD of 0.5785 and patient samples ranged from 0.4175 to 1.122.

The manufacturer was again contacted and another kit was received. The standard was from a different lot from that received previously. A series of dilutions of a normal plasma pool were also included on the plate so that results could be calculated as a % of normal even if the TAFI standard was unsatisfactory. On this occasion the top standard had a corrected OD of 0.587. Nine of 15 test samples had ODs which were outside the standard curve. Eleven of 15 test samples had TAFI levels which were more than 50% of normal when calculated against the normal plasma pool. When calculated against the TAFI standard, all 15 test samples had TAFI levels which were more than the quoted normal range in the kit insert.

A laboratory normal range was constructed by assaying 17 individual normal plasma samples and a plasma standard curve was constructed by including four dilutions of a normal plasma pool.

In regards to the unsatisfactory performance of the TAFI standard, multiple email communications with the manufacturers ensued. However, a definitive explanation for the poor performance of the standard could not be provided. A possible explanation was that the standard was being damaged in transit.

A researcher (J.Antovic, personal communication), based at the Karolinska Institut in Stockholm, Sweden, who presented data on TAFI activity using the American Diagnostica kit at the 2003 ISTH meeting was contacted. This researcher did not use either of the two batches used in this study but using two other batches, the top standard gave an OD of 1.1 with the first batch and 2.5 with the second batch. These ODs are in keeping with the ODs expected. Other researchers in the Netherlands are believed to have reported problems with variation in the standard curve with this particular assay.

The performance of the kit TAFI standard was not satisfactory. The reason for the poor performance was not clear but may have been related to storage conditions in transit. The assay involves multiple reagent addition steps and strict incubation steps so there is also the possibility that the standard or the plasma test samples were being activated or degraded inadvertently. However, it is noted that the kit instructions regarding storage, thawing and assay methodology were followed meticulously. Ultimately, the kit in question was determined not to be sufficiently reliable or robust. Since the aim was to

determine the concentration of functional TAFI present in the sample, it was decided to develop a functional clot lysis assay.

5.4 Clot Lysis Assay

5.4.1 Introduction

The clot lysis method is a surrogate method for evaluation of TAFIa.(154;160) Coagulation is activated in plasma by the addition of thrombin or recombinant tissue factor and fibrinolysis is promoted by the addition of tPA. In this system, the plasma components of fibrinolysis are measured in a global assay encompassing all of the proteins required for both thrombin generation and fibrinolysis. Since plasma contains a number of proteins with carboxypeptidase activity, potato tuber carboxypeptidase inhibitor which is a specific inhibitor of TAFI is added to tandem plasma samples. The contribution of TAFI to the clot lysis time (CLT) is defined by the difference between the CLT with and without PTCI.

5.4.2 Method

The clot lysis assay is a determination of the lysis time of a tissue factor-initiated clot by exogenous tPA. The method used was described by Mosnier in 2001 and used in a number of studies of clot lysis by the same group.(172;173;177) In brief, plasma is added to a reaction mix containing a phospholipid source, tPA (56ng/mL) and buffer. Coagulation is initiated with rTF (10pg/mL) and $CaCl_2$ (17mM). The reaction mix is incubated at 37^0C and the plate is read every 2.5 minutes for 120 minutes to detect changes in turbidity (See Methods Chapter 2). All plasmas are analysed in duplicate and the CLT is the average of both results. PTCI (final concentration 25µg/mL) was

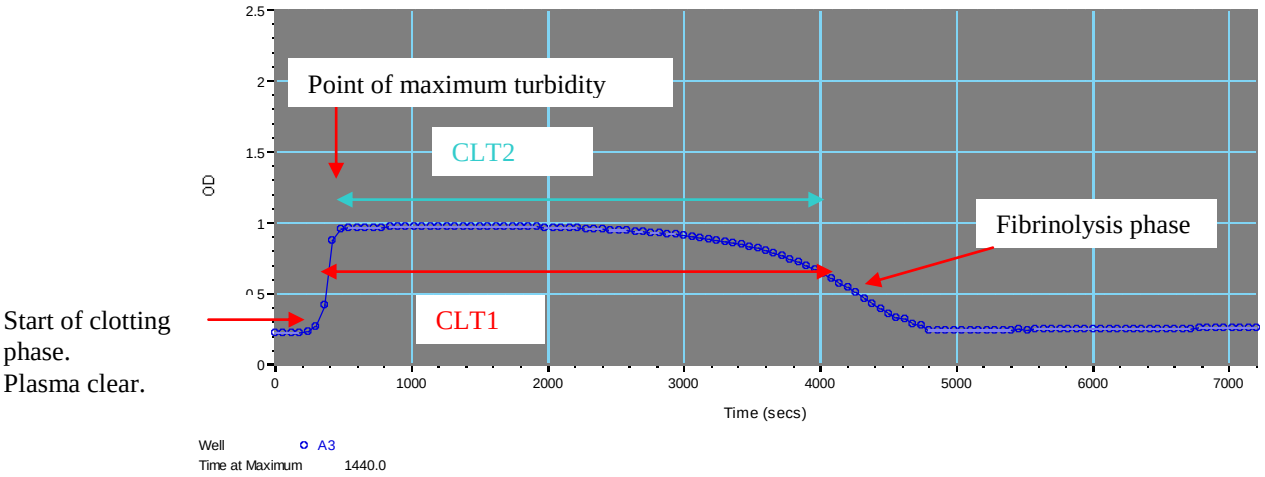
added to an identical plasma reaction mix, in tandem, to enable the contribution of TAFI to fibrinolysis to be calculated.

Recombinant tissue factor was used to initiate coagulation because the purpose of the assay is to analyse the influence of FXI-dependent thrombin generation on subsequent fibrinolysis and the addition of exogenous thrombin is likely to confound the results. Since the influence of FXI is seen at low tissue factor dilutions, a $1x10^5$ dilution of rTF was utilised for all clot lysis assays.(43;143) Concentrations of rTF are similar to those used in the thrombin generation assay. Clot lysis times were evaluated with and without the addition of PTCI to assess the contribution of TAFI.

Ex vivo spiking of a selection of patient plasmas with rFVIIa (final concentration 40nM) was utilised to evaluate the effect of this compound on fibrinolysis in FXI deficient plasmas.

For the purpose of evaluating the role of TAFI in fibrinolysis in FXI deficient patients in this study, the TAFI:CLT refers to the contribution attributable to TAFI (i.e. the difference between the CLTs obtained in the presence and absence of PTCI: CLT+PTCI and CLT-PTCI). Two methods to calculate CLT are used in the literature, which will be termed CLT1 and CLT2 here (figure 5.2). CLT1 is calculated as the time between the midpoint of the clear-to-maximum turbid transition and the midpoint of the turbid-to-clear transition.(173) CLT2 is calculated as the time from the occurrence of the maximum OD to the midpoint of the turbid-to-clear transition.(165;174) Both CLT measurements are given in units of time (minutes).

Figure 5.2: Clot lysis assay: optical density changes with time and calculation of CLT1 and CLT2.



CLT1: Time from the midpoint of the clear-to-turbid transition to the midpoint of the turbid-to-clear transition

CLT2: Time from the point of maximum turbidity to the midpoint of the turbid-to-clear transition

5.4.3 Results

Analysis of Patient samples and Normal Pool

Plasma samples from 102 patients were available for analysis (Table 5.1). Samples from 53 patients were either not assayed (*n*=19) or results were not available for technical reasons (*n*=34). In particular, lipaemic samples were unsuitable for analysis due to a high baseline optical density. Samples with FXI:C levels <2 U/dL did not generate a satisfactory curve, and were not analysed. Samples with > 20% difference between duplicate samples were deemed unacceptable for analysis.

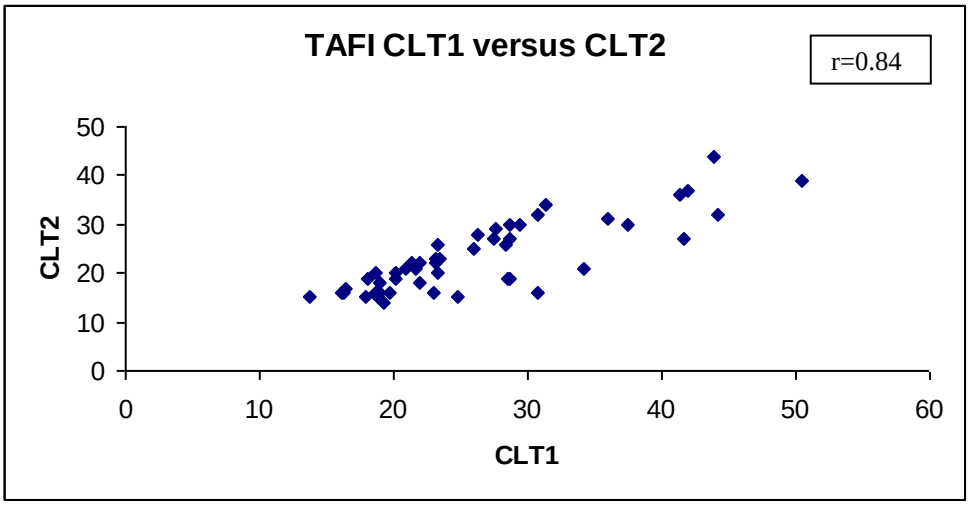
Samples from 49 patients fulfilled the acceptance criteria for results and are reported. Of 167 individual Clot lysis curves, 82% of duplicate curves were within 10% of each other and 94% were within 15% of each other. The within-assay variation is 5-16%. The inter-assay coefficient of variation for the normal pool was 18%.

Table 5.1: Summary of analysis of 102 patient samples for CLT.

Summary of CLT assays	No. of patient samples	Notes
Total patient samples	102	
Patient sample not assayed	19	No sample available 11
		FXI <2 U/dL 8
Sample assayed but no	34	Lipaemic sample 11
result		Error in curves 23
CLT results available	49	
Severe FXI deficiency	9	Range FXI:C 3-11 U/dL
Partial FXI deficiency	40	Range FXI:C 40-110 U/dL

There is a clear relationship between CLT1 and CLT2 with a correlation co-efficient of 0.84 (Figure 5.3). Since the shape of the OD curve obtained may influence the time to maximum OD from which the CLT2 is calculated and since there is a close relationship between both measurements, it was decided to conduct further analysis in relation to CLT1 only.

Figure 5.3: A comparison of the analysis of the contribution of TAFI to the Clot Lysis curve, measured by two methods (n=49) – CLT1 (midpoint of the clear-to- maximum turbid transition to the midpoint of the maximum turbid-to-clear transition) and CLT2 (time to occurrence of the maximum OD to the midpoint of the turbid-to-clear transition).



CLT

The CLT without the addition of PTCI was analysed in 49 patients and in a normal plasma pool (representative curve, Figures 5.4). The addition of PTCI considerably shortened the time to complete lysis since TAFI is specifically inhibited (figure 5.5).

Figure 5.4: Optical density curve representing the turbidity changes in the normal pool, analysed without PTCI (CLT-PTCI).

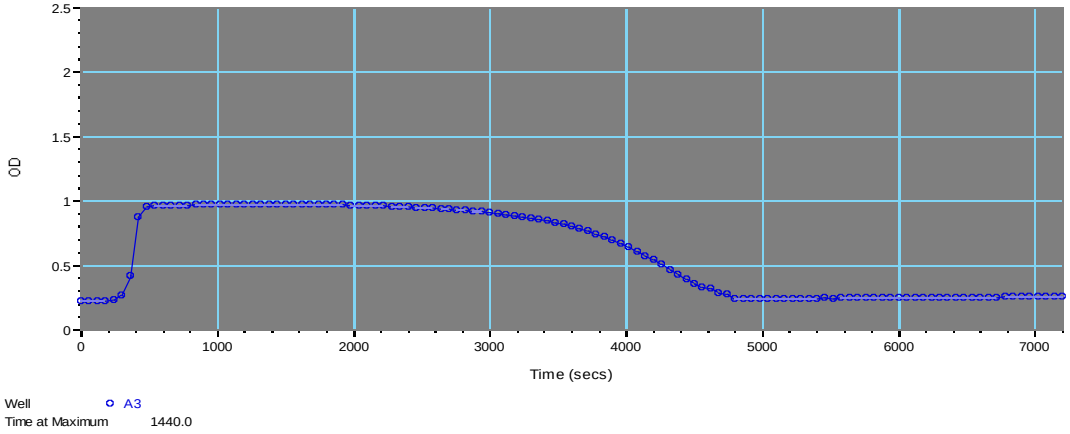
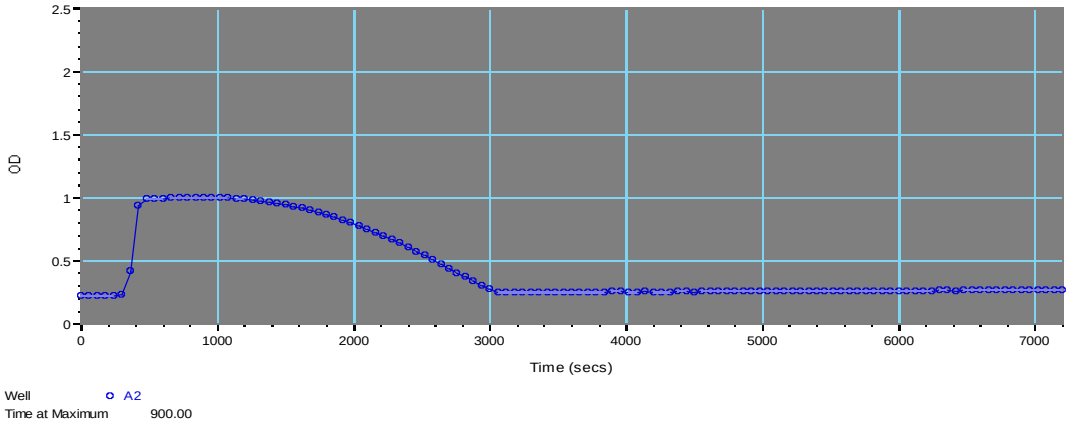


Figure 5.5: Optical density curve representing the turbidity changes in the normal pool, analysed with PTCI (CLT+PTCI). Note the CLT is considerably shorter that the CLT-PTCI.



The Normal Pool (20 individual samples) had a mean CLT of 61±22.4 minutes. The median CLT in the 49 FXI deficient patients was 59.2 minutes with a wide range (37.7-104.2 minutes). Surprisingly, there was no difference in CLT-PTCI between patients with severe or partial FXI deficiency (Table 5.2).

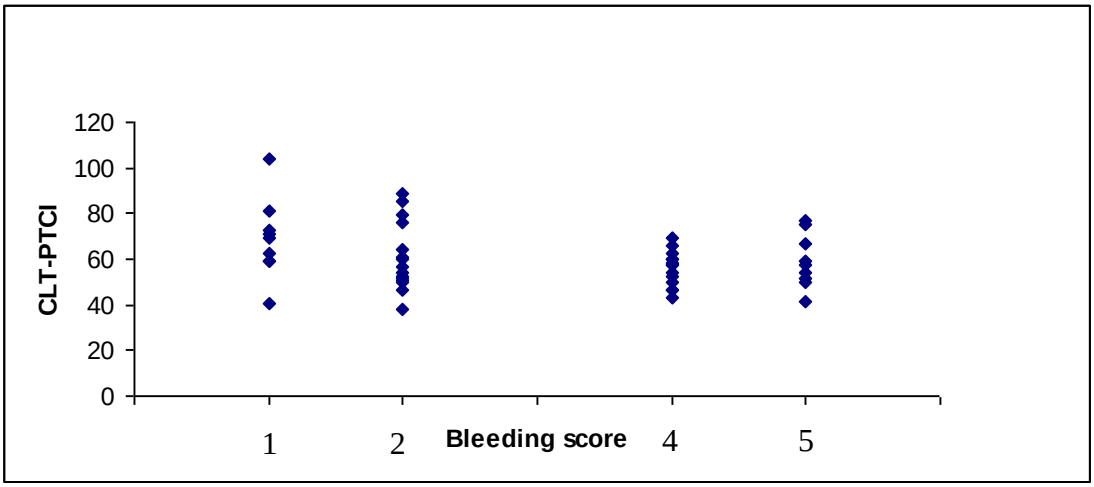
Table 5.2: Comparison of CLT in patients with severe and partial FXI deficiency. CLT-PTCI represents the clot lysis time in the absence of PTCI. The contribution of TAFI to the CLT is termed TAFI:CLT and is derived by the subtraction of the CLT in the presence of PTCI from the CLT-PTCI.

	Severe FXI deficiency	Partial FXI deficiency
Number of patients	9	40
CLT-PTCI*		23
Median	59	59
Range	43.5-76.8	37.7-104.2
Interquartile range	57.6-72.5	51.1-65.8
TAFI:CLT†		
Median	29.4	23
Range	16.1-43.9	13.8-50.5
Interquartile range	26.3-34.2	19.2-28.7

*p=0.8, ANOVA single factor analysis †p=0.12, ANOVA single factor analysis

There was no correlation between the TAFI:Ag and the CLT-PTCI (correlation co-efficient 0.14). Non-bleeders tended to have a lower and less variable CLT-PTCI than bleeders but this was non-significant (p=0.16, Figure 5.6).

Figure 5.6: Relationship between CLT-PTCI and patients with Bleeding Score 1 and 2 (definite and probable bleeders) and Bleeding Score 4 and 5 (probable and definite non-bleeders). Note: n=47, two patients with bleeding score 3 excluded.

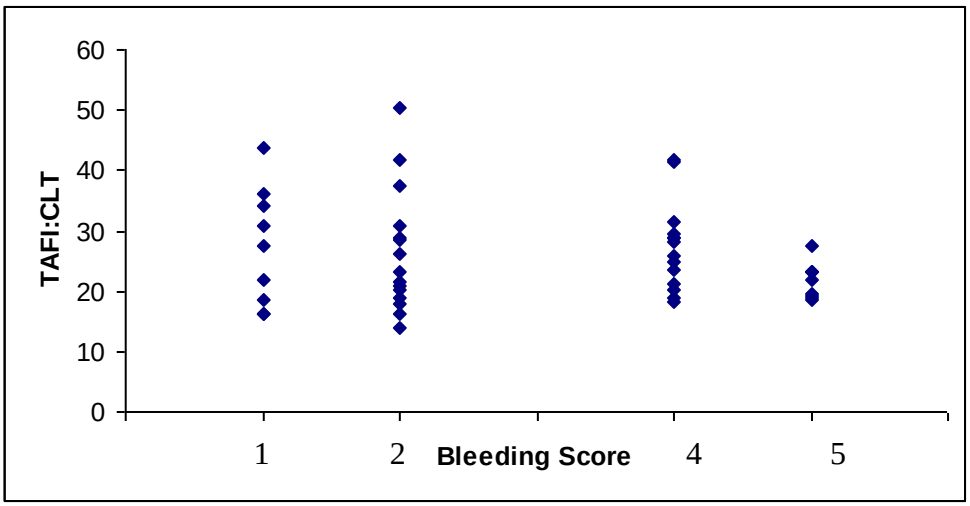


p=0.16, ANOVA single factor analysis

TAFI activity as calculated by the CLT

The contribution related to TAFI is calculated by analysing the CLT with and without PTCI. The TAFI:CLT is the difference between the two measurements. The TAFI:CLT was not correlated to the TAFI:Ag (correlation coefficient 0.1) nor to the FXI level, p=0.12 (see table 5.2). There was no significant difference in TAFI:CLT between bleeding and non-bleeding patients (Figure 5.7, p=0.4).

Figure 5.7: The contribution of TAFI to the CLT (TAFI:CLT) related to the bleeding scores of the patients (n=47, two patients with bleeding score 3 excluded).

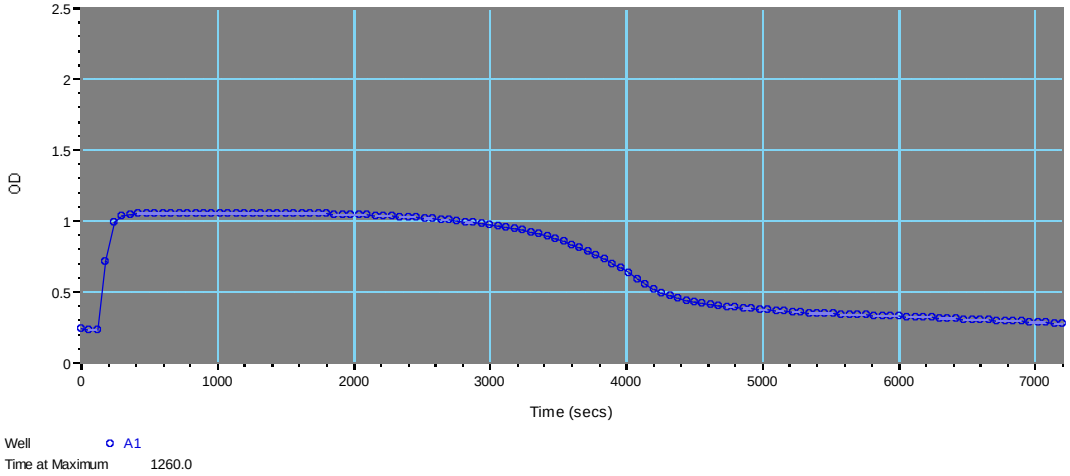


p=0.4, ANOVA single factor analysis

Influence of rFVIIa on CLT

The addition of rFVIIa prolonged the CLT in the majority (68% of samples) but not in all samples (representative curve shown in figure 5.8). The mean change was a 5% increase in CLT in the presence of 40nM of rFVIIa. Thirty-two of 47 patients had an increase in CLT with the addition of rFVIIa, ranging from 0.5-41%. The increase in CLT was more than 10% in 11 patient samples. Fifteen patient samples had a decrease in the CLT by 2-14%.

Figure 5.8: The Optical Density curve of the normal pool, analysed in the presence of rFVIIa (40nM).



5.5 Discussion

The majority of patients in this study had normal TAFI:Ag levels and only 13 patients had levels outside of the reference interval (nine patients had higher TAFI:Ag levels and four patients had lower TAFI:Ag). Of those 13 patients, there was no observed relationship with bleeding tendency. When TAFI:Ag was assessed in relation to bleeding tendency across all 102 patients there was no correlation identified. The conclusion therefore is that TAFI:Ag is not significantly variable in patients with FXI deficiency and levels do not appear to vary according to bleeding tendency. Of note, the TAFI:Ag kit used is associated with the detection of lower TAFI levels in patients with the Thr325Ile polymorphism. This could be postulated to result in an under-estimation of TAFI levels in those individuals and a weakening of the relationship between increased TAFI levels and protection from bleeding. However, the absolute level may not be the only factor involved in protection from bleeding as the Ile isoform

of TAFI may result in lower TAFI levels but more inhibition of fibrinolysis due to its increased activity and stability.(159)

TAFI activity as measured by a commercial kit was found not to be sufficiently reliable in assaying stored samples from FXI deficient patients. The main problem was the performance of the supplied TAFI standard which meant that the patient samples could not be assigned a value for the TAFI activity level. Despite attempts to rectify this problem with new standards (from both the same and different "lots"), the standard continued to perform sub-optimally and a clot lysis assay was developed to evaluate TAFI activity instead.

The CLT has been validated as a surrogate assay for TAFIa in plasma.(160) The reported inter-assay and intra-assay CVs are 3.8% and 7.6% and 4% and 3.4% which are lower than those seen in the current study.(160;172)

The addition of PTCI enables the contribution of TAFI to be calculated although it is recognised that many other proteins involved in thrombin generation and fibrinolysis influence this assay.

The CLT results obtained for normal plasma in this study are very similar to those reported in the literature. The mean CLT was 61 minutes (range 38.8-135.2 minutes) in 469 control subjects analysed as part of the Leiden Thrombophilia study.(172) In that study, CLT increased with age and were slightly higher in men. Mosnier et al reported a mean CLT for normal plasma of 62 ± 11.5 minutes (calculated by the CLT2 method)

which is in keeping with the result for the normal pool in this study (61±22.4 minutes).(160) However, the reduction in CLT seen with the addition of a neutralising antibody to FXI in that study (to 38.7 ± 8.6 minutes) is not replicated in plasma samples from patients with FXI deficiency. Of note, however patients with FXI:C < 2U/dL were excluded from the CLT assays since it was found that reproducibility of the assay at low FXI:C levels was not acceptable. In addition, thrombin was used as the activator of clotting the Mosnier study and it has been suggested that higher levels of thrombin are needed to activate TAFI.(149) This raises the possibility that using a TF mediated CLT, the amounts of thrombin generated are too low to activate TAFI.

In a study of clot lysis in plasma from patients with severe Haemophilia A, the same investigators described a Clot Lysis assay, in which coagulation was initiated by varying dilutions of Innovin ($1x10^3$ to a $1x10^7$) and the CLT was measured using the CLT1 method.(177) Of note, the CLT in normal plasma was approximately 60 minutes at all dilutions of TF. In the presence of an inhibitory antibody to FXI, the CLT was normal in the presence of higher concentrations of TF (i.e. $<1x10^5$ dilutions but the CLT was significantly reduced when the concentration of TF was lower. The CLT in patients with severe Haemophilia was investigated using a TF dilution of $1x10^5$. In these patients CLT were considerably lower than the normal pool and were similar to CLT in the presence of PTCI. The CLT was increased to normal levels by addition of TAFI or small amounts of FVIII (1U/mL). There was no correlation between TAFI levels and the severity of bleeding.

TAFI:Ag levels were found to correlate with CLT only in the presence of FXI in normal patients and in the presence of FVIII in individuals with severe Haemophilia. (160;177) The lack of correlation of TAFI:Ag and CLT in patients with severe FXI deficiency is therefore not surprising but individuals with partial FXI deficiency have residual thrombin generation via the intrinsic pathway and would therefore be expected to have a correlation between TAFI:Ag and CLT.

Individuals in the Leiden Thrombophilia study with a CLT > the 90th percentile had a two-fold increased risk of thrombosis.(172) Interestingly the correlation between CLT and individual coagulation and fibrinolytic proteins was weak. There was an association between increasing TAFI levels and increasing CLT but the contribution of TAFI to the overall CLT was small. Likewise, increases in FXI levels were very weakly associated with increasing CLT. Surprisingly, there was also no correlation between ETP and CLT considering that in earlier studies the amount of thrombin generated determined the amount of TAFI activation which occurred.(165) Therefore, it appears that the CLT is governed by the balance of procoagulant and fibrinolytic factors, rather than by any one factor.

Regarding the effect of rFVIIa on FXI plasma, a study of CLT initiated with thrombin found that there no significant difference between the CLT of normal plasma versus FXI deficient plasma (24±2.2 minutes compared to 22.5±0.7 minutes).(179) The addition of rFVIIa at a concentration of 9.6 μg/mL prolonged the CLT by two minutes. The contribution of TAFI (as measured by comparing the CLT before and after the addition of PTCI) appeared to increase slightly with higher doses of rFVIIa. These

results in conjunction with the results of the current study appear to indicate either that the CLT is insensitive to variations in fibrinolytic activity in patients with FXI deficiency or that differences do not occur. CLT assays do not confirm the theory that rFVIIa down-regulates fibrinolysis in a TAFI dependent manner.

Chapter 6 FXI gene mutations and molecular model

6.1 Introduction

The mutations commonly causing FXI deficiency in persons of Jewish heritage have been well described.(58;59) The mutations have been termed type I to IV but all are characterised by low FXI coagulant activity and antigen levels. The type II (Glu117stop) and type III alleles (Phe283Leu) are most common while type I and IV mutations (splice site mutation and deletion in intron N respectively) are rare. While the allele combinations have been shown to influence mean FXI:C levels in homozygotes and compound heterozygotes, a link between genotype and bleeding phenotype has not been demonstrated.(56) In patients with partial FXI deficiency, the presence of individual alleles appears not to influence either the mean FXI:C levels or the bleeding phenotype.(7;56)

The FXI gene is 23 kb long and contains 15 exons and 14 introns which are translated to an 18 amino acid signal peptide and a 607 amino acid mature protein.(9) The first exon encodes the 5' untranslated region and the second encodes the signal peptide. Exons III-IV, V-VI, VII-VIII and IX-X encode for four apple (Ap) domains, also known as PAN modules (Ap1 to Ap4 respectively: Figure 6.6(a)). These Ap domains show sequence homology with each other and with the Ap domains of PK.(10) The Ap domains of FXI also show sequence similarity and disulphide bridge conservation characteristic of other Ap/PAN modules such as EtMIC5, a adhesin from *Eimeria tenella*.(227) Exons XI-XV contain the coding sequence for the serine protease domain (SP: Figure 6.6(a)).(9) The SP domain is homologous to other members of the trypsin

family of serine proteases. Both FXI and PK have five potential N-glycosylation sites (three are shared), but the overall carbohydrate content of FXI is 5% whereas in PK it is 15%.(10;12)

Uniquely amongst the coagulation factor serine proteases, and in contrast to PK, FXI circulates as a dimer.(17) The two identical FXI monomers are linked by non-covalent interactions between the Ap4 domains and by a Cys321-Cys321 disulphide bond, although the latter is not critical for dimerisation.(18-20;228) Dimerisation appears to be essential for secretion of FXI and may be essential for cleavage of its physiological substrate factor IX (FIX).(27;229) FXI can be activated by thrombin or by activated FXII, or may undergo auto-activation.(230) Activation results in cleavage of the scissile bond at Arg369-Ile370 to form a heavy chain containing the Ap domains and a light chain containing the SP domain with the catalytic triad at His413, Asp462 and Ser557. Both monomers are cleaved during activation, and the dimer structure is retained after activation.(17)

Recently there has been a rapid increase in reports of FXI mutations (www.factorXI.org). The majority of these are missense mutations. The Ap3 and SP domains contain the largest number of these mutations, and mutations have now been reported for all the FXI exons except the untranslated exon I. The functional analysis of these mutations ranges from simple measurement of FXI:C with or without FXI antigen (FXI:Ag) to detailed expression studies of mutant FXI. Measurement of FXI:C and FXI:Ag enables the phenotypic classification of mutations as either Type I/ cross-

reacting material negative (CRM-) with low FXI:Ag and low FXI:C or Type II/ cross-reacting material positive (CRM+) with normal FXI:Ag and low FXI:C.
In this study, 101 patients had DNA available for analysis, of whom 77% were of Jewish origin. Since some patients may have an unsuspected Jewish heritage, the mutational analysis strategy was to firstly screen all patients for the four Jewish mutations by restriction enzyme analysis. Patients without an identifiable Jewish mutation or with inconclusive results by restriction enzyme analysis, proceeded to have the entire FXI gene sequenced to identify possible mutations. Suspected polymorphisms were compared to known SNPs in the NCBI SNPs database.

While experimental study of the mutant protein in a mammalian expression system is the gold standard for the interpretation of mutations to distinguish between disease-causing mutations and neutral polymorphisms, it was not possible to conduct such experiments for all reported mutations. For many other coagulation proteins, three-dimensional structural models are essential in order to predict the impact of a given point mutation on the protein structure and its consequence for disease. For FXI, this was not possible up to 2006 because of the nonavailability of a crystal structure. Between 1998 and 2002, publication of the crystal and NMR structure determinations for Ap domains in an adhesin protein EtMIC5 from the protozoan *Eimeria tenella*, a leech anti-platelet protein, and human hepatocyte growth factor made homology modelling of the FXI Ap domains possible for the first time.(231-233) Crystal structures for almost one hundred SP domains were available in the Protein Data Bank, and some of these, such as that of β-tryptase, provided close structural templates for modelling the SP domain in FXI. Accordingly, homology models were built for the

Ap1 to Ap4 and the SP domains in FXI. This resulted in the first three-dimensional structural analysis of mutations causing FXI deficiency.

6.2 Methods

6.2.1 DNA extraction

For genetic analysis, DNA was extracted from the buffy coat of whole blood using a kit according to the manufacturer's instructions (QIAamp DNA Blood Mini kit, Qiagen GmBH, Hilden, Germany). The DNA samples were stored at -70^0C until use.

6.2.2 Screening for Jewish Mutations

To screen for the Jewish mutations, DNA samples were thawed and specific primers were used to amplify exons 5 and 9 by PCR to identify type II and III mutations respectively. A third set of primers which amplify the junction of exon 14-intron N were used to identify patients with type I and IV mutations. The PCR products were digested overnight with BsmI, Sau3A and MaeIII for the type II, III and I/IV mutations respectively and the samples were then run on a 2% or 4% (for the type I and IV mutations) gel at 120v for two hours. The gel was examined under UV light and photographed. A blank and normal, heterozygous and homozygous controls (where available) were run with each sample. Full details of primers, PCR amplification conditions and restriction enzyme analysis are found in Chapter 2 Methods.

6.2.3 Sequencing of *F11* gene

Primers and PCR and sequencing conditions for sequencing of all 15 exons of *F11* are also given in Chapter 2, section 2.7.6. Sequencing of the PCR products was carried out on an Applied Biosystems 3100 Avant Sequencer. The cycle sequencing reactions using Big Dye Terminators v1.1© were carried out using the recommended protocol.

6.2.4 Molecular modelling

Identification of FXI mutations

As described in chapter 2, all mutations in the *F11* gene causing FXI deficiency published at the time, were identified by searching the Human Gene Mutation database, Medline and hand searching of meeting abstracts. A particular emphasis was placed on gathering as comprehensive a set of phenotypic data as possible. This data set included FXI:C in most cases, and FXI:Ag and/or bleeding phenotype in some cases. Mutations published in abstract format were grouped according to the presence or absence of phenotypic data.

Construction of FXI homology models

A detailed description of the construction of the homology models of the Ap1 to Ap4 and SP domains is given in chapter 2, section 2.8. In brief, INSIGHT II 98.0 modelling software with HOMOLOGY, DISCOVER and BIOPOLYMER modules (Accelrys, San Diego, CA) and the structural refinement program O (185) on Silicon Graphics workstations were used to construct the models. Structurally conserved regions (SCRs) were identified within the five FXI domain sequences and their template structures

following alignments of members of the PAN-module superfamily, in particular the *E. Tenella* adhesin protein (PDB code: 1HKY) and the anti-platelet toxin from *H. officinalis* (PDB code: 1I8N_A). Five close homologues for the serine protease domain were similarly identified: human α-thrombin (PDB code: 1H8I), human protein C (PDB code: 1AUT), human complement C1r (PDB code: 1GPZ), boar sperm β-acrosin (PDB code: 1FIZ) and human mast cell β-tryptase (PDB code: 1AOL).

The SCRs were built using a rigid body fragment assembly method and the intervening loop regions were then either modelled from a database of protein fragments using the Brookhaven loop database of INSIGHT II or generated using a constrained minimization algorithm implemented by HOMOLOGY. Energy refinements were done using the DISCOVER module and the structures were verified using PROCHECK (186) and O, the energy minimisation program of the DISCOVER module and the structure idealisation program REFMAC5 of CCP4i (187). The DSSP program (200) was used to assign secondary structures as follows: H and G, α-helix and 3_{10} helix; E and B, β-strand; T, turn; C and S, coil and loop. Side chain solvent accessibilities in the structures and models were calculated using NACCESS (201), on a scale of 0 to 9 for each residue, where 0 corresponds to 0% to 9% accessibility, 1 corresponds to 10% to 19% accessibility, and so on. Buried residues have accessibilities of 0 or 1.

6.3 Results of screening for the Jewish mutations in the *F11* gene

6.3.1 Genotypes for the Jewish mutations

Screening for the four Jewish mutations by restriction enzyme analysis was done in 101 patients (with a suitable DNA sample), of whom 78 were of Jewish heritage. One of the four mutations found in Jewish populations was confirmed in 77 of 101 patients. The breakdown of genotypes according to the severity of the FXI deficiency is given in Table 6.1.

The Jewish mutations were found in 30 of 32 severely deficient patients. Half of all severely affected Jewish patients were compound heterozygotes for the type II and III mutations whereas homozygosity for either the type I or II mutations were equally common (20% and 27% of severely deficient patients with Jewish mutations respectively). Neither of the two rarer disease causing alleles (types I and IV) was found in this group.

Of the remaining two severely deficient patients, one was of English, non-Jewish heritage and has an unknown mutation. The other is Jewish but has a lupus anticoagulant. The latter patient had a positive lupus-type FXI inhibitor screen and a normal FXI:Ag 105 U/dL, suggesting that this patient does not have a true FXI deficiency.

Amongst the partially deficient patients, the type II and III alleles were equally common but there were also two patients from one family who were heterozygous for the type I mutation.

Table 6.1: Genotype of 101 patients with FXI deficiency.

Genotype	Severe deficiency* N=32	Partial deficiency* N=69
II/II	6	0
II/III	15	1
III/III	8	0
I/WT	0	2
II/WT	0	25
III/WT	1	19
Total Jewish mutations	30	47
Negative for Jewish mutations	2	22

*Number of patients

6.3.2 Bleeding phenotype related to genotype (Jewish mutations)

Amongst patients with a confirmed Jewish mutation, there was a significantly higher incidence of bleeders in patients with severely deficient genotypes compared to patients with partially deficient genotypes (p=0.005) as shown in Table 6.2. No significant difference in bleeding phenotype was seen between genotypes II/II, II/III and III/III in severe FXI deficiency or between genotypes II/WT and III/WT in partial deficiency (p=0.49 in both cases, Kruskal-Wallis test).

Table 6.2: FXI genotype (Jewish mutations) and relationship to FXI:C and bleeding phenotype.

FXI deficiency	Genotype	Number of patients	FXI:C Mean (range)	Bleeder (%)	Non-bleeder (%)	Indeterminate (%)
Severe	II/II	6	1 (<1-1.2)	6 (100)	0 (0)	0 (0)
	II/III	15	4.3 (1.1-10)	11 (73)	3 (20)	1 (2)
	III/III	8	9.4 (7-12)	7 (87.5)	1 (12.5)	0 (0)
	Total*	29		24 (83)	4 (14)	1 (3)
Partial	I/WT	2	47, 50	1 (n/a)	1 (n/a)	0 (n/a)
	II/WT	25	62 (37-110)	14 (56)	10 (40)	1 (4)
	III/WT	19	62.4 (41-90)	7 (37)	9 (47)	3 (16)
	Total**	46		22 (48)	20 (43)	4 (9)

** excluding one patient with chronic liver disease ** excluding one patient with a compound heterozygote genotype but a partially deficient FXI:C level*

6.3.3 Discrepant FXI:C and genotype

Two discrepant results were found between FXI:C levels and genotype. One of the severely affected patients was heterozygous for the type III mutation but this patient also had chronic liver disease, unrelated to her FXI deficiency. It is probable that the FXI:C level was lowered to the severely deficient range by an acquired defect in liver synthesis in addition to the genetic defect. A patient with FXI:C levels of 50 U/dL was found to have the genotype of a compound heterozygote. This finding is unexplained and sequencing of exons V and IX is required to clarify the genotype.

6.4 Results of sequencing the *F11* gene

6.4.1 Patients with Jewish heritage but lacking the Jewish *F11* gene mutations

Three of 24 patients without evidence of the Jewish mutations on restriction enzyme analysis, had definite Jewish heritage. It is likely that these patients do not have a true inherited FXI deficiency. One Jewish patient has a concomitant lupus anticoagulant and a normal FXI:Ag as mentioned above, raising the possibility that the FXI:C levels are erroneously low. Another has Gauchers disease and was not on enzyme replacement treatment when first diagnosed with FXI deficiency and thrombocytopenia. Both the FXI:C and the platelet count have normalised on enzyme replacement and no further bleeding symptoms have been reported. The last Jewish patient with a negative screen for the Jewish mutations has had the FXI:C and FXI:Ag rechecked and both were >100 U/dL, raising a doubt over the accuracy of the original diagnosis. In conclusion, the three Jewish patients in this group are not believed to have a true FXI deficiency.

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6.4.2 Screening for mutations in the *F11* gene in 21 patients without a Jewish mutation or heritage

A total of 21 patients have a definite FXI deficiency but no identifiable Jewish mutation and no known Jewish heritage. One of the 21 patients has a severe deficiency (FXI:C 7.4 U/dL) and the remainder are partially deficient. The strategy to identify mutations in the FXI gene in 21 patients without an identifiable Jewish mutation consisted of direct sequencing of the 15 exons and exon-intron borders of the FXI gene. Numbering of cDNA is taken from RefSeq # NM_000128.2, where +1 corresponds to the A of the ATG translation initiation codon. Mutation names are given in the traditional format with respect to the mature protein. The recommended numbering where codon 1 is the ATG primary translation start is also given and is identified by a "p." prefix.

Missense mutations: Genotype and Phenotype

Missense mutations were identified in six patients (table 6.3). Three had previously been reported in the literature.

Table 6.3: Missense mutations identified in 21 non-Jewish patients with confirmed FXI deficiency (references are as published at the time of this study, see discussion for subsequent publications). Ap and SP denote apple and serine protease domains.

Mutation name	HGVS nomenclature	Nucleotide substitution	Exon	Protein domain	FXI:C	FXI:Ag	Ref
Pro48Leu	p.Pro66Leu	c.197C>T	3	Ap 1	52	40	No
Gly155Glu	p.Gly173Glu	c.521G>A	6	Ap 2	43	64	(234;235)
Glu297Lys	p.Glu315Lys	c.943G>A	9	Ap 4	38	34	No
Glu380Lys	p.Glu398Lys	c.1192 G>A	11	SP	30	32	No
Cys398Tyr	p.Cys416Tyr	c.1247 G>A	11	SP	57	36	(236)
Ser576Arg	p.Ser594Arg	c.1782C>A	15	SP	46	71	(237)

Patient characteristics of six missense mutations identified

Four of six patients gave their ethnic origin as English with one patient of Bangladeshi origin and one of Portuguese origin as indicated below.

Pro48Leu

A missense mutation was identified in exon 3. A change from C to T at nucleotide 197 leads to substitution of a leucine residue for a proline at amino acid 48 (Pro48Leu, p.Pro66Leu). This patient had a FXI:C of 52 U/dL and a FXI:Ag of 40 U/dL. The patient was registered as a probable bleeder. The FVIII:C was borderline at 50 U/dL while the VWF:Ag was 57 U/dL and the VWF:Ac was 58 U/dL. The patient's blood group was group O. The thrombophilia screen was negative and TAFI antigen was normal. Thrombin generation was assayed by the continuous method and was 29% of that found in normal plasma.

Gly155Glu

Substitution of A for G at nucleotide 521 (figure 6.1 and 6.2) results in a change from Glycine to Glutamate at amino acid 155 in exon 6 (Gly155Glu; p.Gly173Glu). The patient had a FXI:C of 43 U/dL and a FXI:Ag of 64 U/dL. The patient was a definite non-bleeder. The von Willebrand screen and thrombophilia screens were negative. TAFI antigen levels were normal and thrombin generation tests were not performed.

Figure 6.1: Gly155Glu forward sequence (heterozygosity for G and A at position 521 is shown at the red arrow).

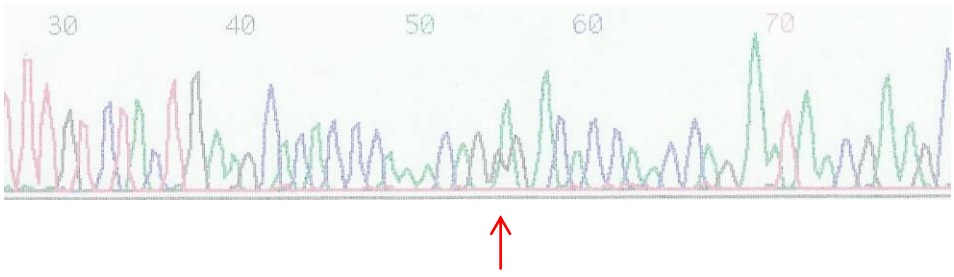
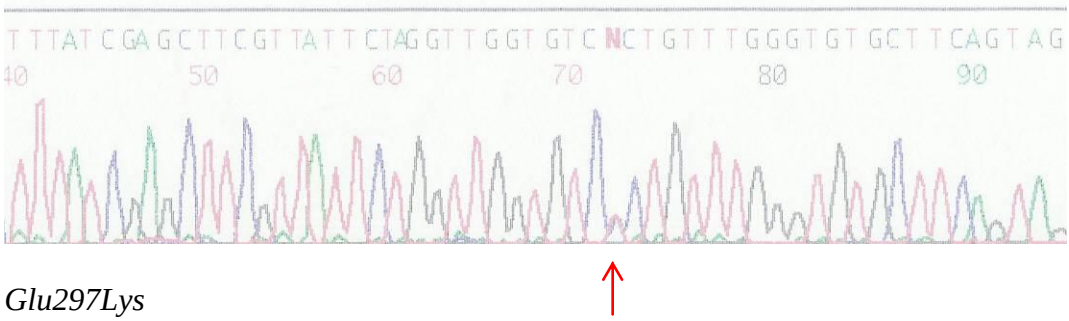


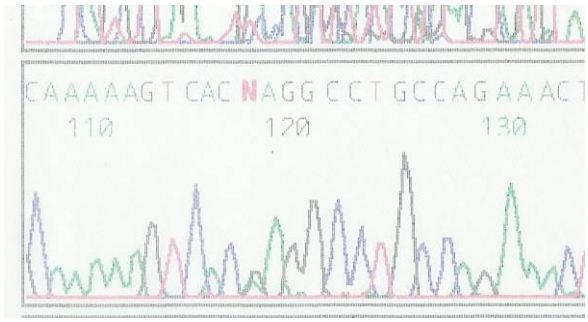
Figure 6.2: Gly155Glu in reverse sequence showing heterozygosity for C and T at the arrow.



Glu297Lys

In exon 9, a change in amino acid at position 297 from Glutamate to Lysine results from a G to A transition at nucleotide 943 (figure 6.3). The patient is a definite bleeder with a FXI:C of 38 U/dL and a FXI:Ag of 34 U/dL. The von Willebrand and thrombophilia screens were negative and TAFI antigen levels were normal. Thrombin generation was assayed by the continuous method and was 28% of that found in normal plasma.

Figure 6.3: Heterozygosity for Glu297Lys showing the dual G and A signals in forward sequence.



Glu380Lys

A Bangladeshi patient with a FXI:C of 30 U/dL and a FXI:Ag of 32 U/dL had a missense mutation identified in exon 11. Glu380Lys results from substitution of A for G at position 1192 and causes the replacement of a glutamic acid residue with a lysine in the serine protease domain. The patient had a negative von Willebrand screen and a borderline Protein C level of 66 U/dL (reference interval 70-140 U/dL) but the thrombophilia screen was otherwise negative. The TAFI level in this patient was the

lowest of any of the patients in the study and the ETP as measured by the continuous thrombin generation assay was similarly very low at 8.7% of normal. The bleeding score for this patient was 2 (probable bleeder).

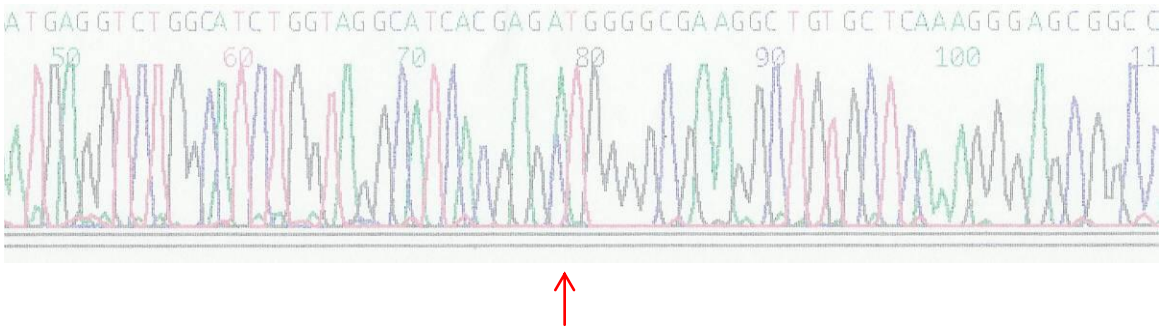
Cys398Tyr

A substitution of A for G at position 1247 of exon 11, causing replacement of a Cysteine at position 398 with Tyrosine, was found in a patient of Portuguese origin with a FXI:C of 57 U/dL and a FXI:Ag of 36 U/dL. The patient had a negative von Willebrand screen and a normal thrombophilia screen. TAFI levels were normal and the ETP as measured by the continuous method was 74.2% of normal. In keeping with the ETP, the bleeding score registered for this patient was 5 (definite non-bleeder).

Ser576Arg

A missense mutation was identified in exon 15 in another patient. Substitution of A for C at nucleotide 1782 (figure 6.4) results in a change from Serine to Arginine at position 576 (Ser576Arg, p.Ser594Arg). The patient had a FXI:C of 46 U/dL and a FXI:Ag of 71 U/dL. The patient was a probable bleeder. The von Willebrand screen revealed a borderline FVIII:C and VWF:Ac at 50 U/dL with a VWF:Ag of 64 U/dL. Of note, the patient was blood group O. The thrombophilia screen was negative and TAFI antigen was normal. Thrombin generation tests were not performed.

Figure 6.4: Heterozygosity for the Ser576Arg mutation – dual signals for A and C at position 1782(forward signal).



Synonymous Polymorphisms

Six synonymous polymorphisms were identified in six patients (Table 6.4). Three patients had two distinct polymorphisms present. Four of these polymorphisms were reported on the NCBI SNP database at the time of the study and two were not.

Table 6.4: Synonymous polymorphisms detected in 21 patients during screening of the F11 gene.

Amino acid (+ signal peptide)	Nucleotide	Triplet	Reported
Asp 125 (143)	602	GAC>GAT	NCBI
Ala 181 (199)	771	GCT>GCG	No
Ala 202 (220)	833	GCC>GCT	No
Asp 551 (569)	1704	GAC>GAT	NCBI
Arg 586 (604)	1985	CGG>GAA	NCBI
Glu 595 (613)	2012	GAG>GAA	NCBI

6.5 Molecular modelling of 42 mutations in the *F11* gene

6.5.1 *F11* gene mutations

A total of 42 mutations in the FXI gene were identified from databases, literature and abstracts, 25 of which were located in the Ap domains and 17 in the SP domain. Phenotypic information was available for 28 of the 42 mutations (Table 6.5). The remaining 14 mutations were mostly obtained from abstracts, from which detailed phenotypic characterisations were not possible (Table 6.6).

Table 6.5 FXI mutations with phenotypic and expression data(continued on next page).

Domain	Mutation site	Heterozygotes			Homozygotes			Expression in mammalian cells (% WT)			Reference
		No. of patients	FXI:C (U/dL or %)	FXI:Ag (U/dL or %)	No. of patients	FXI:C (U/dL or %)	FXI:Ag (U/dL or %)	FXI:C Medium	FXI:Ag Medium	FXI:Ag Lysate	
Apple 1	Cys38Arg	8	18-45	.[¶]	1	<1	.	<1	<1	74	(51)
Apple 2	Gly155Glu[‡]	3	41; 43; 44.3	64	0	.	.	.	.	.	(234;235)[**]
Apple 3	Phe221Ser*	1	1	1	0	.	.	9	<5	100	(238)
	Gln226Arg[†]	1	42-55	70	0	.	.	.	100	.	(239)
	Cys237Tyr[§]	2	<1	.	0	.	.	<1	3	92	(51)
	Ser248Asn[†]	2	42-55; 67-72	70; 80	0	.	.	.	100	.	(239)
	Lys252Ile[±]	1	4-4.5	.	.	.	.	.	34	104	(240)
Apple 4	Phe283Leu	Multiple	67	.	Multiple	9.7	.	.	8	20-34 (mRNA)	(56)
	Leu302Pro	.	.	.	.	.	.	.	decreased secretion	.	(241)
	Thr304Ile	.	.	.	.	.	.	.	decreased secretion		(241)
	Arg308Cys	1	41-45	32.7	0	.	.	.	.	.	(237)
	Glu323Lys	.	.	.	.	.	.	.	decreased secretion	.	(241)
	Gly336Arg[¶¶]	1	<1	2.5	.	.	.	.	.	.	(242)
	Gly350Ala[§§]	1	1	44	.	.	.	.	.	.	(242)

Domain	Mutation site	Heterozygotes			Homozygotes			Expression in mammalian cells (% WT)			Reference
Serine Protease	Thr386Asn	1	108	.	2	2	Decreased	.	.	.	(243)
	Cys398Tyr	2	40-50	.	1	<1	.	.	No secretion	.	(236)
	Gly400Val	Several	20-40	.	0	.	.	.	2	40-60	(244)
	Ala412Val	1	48	38.6	0	.	.	.	.	.	(237)
	Phe442Val	1	47	50	0	.	.	.	.	.	(183)
	Gly460Arg	2	42; 51	.	0	.	.	.	No secretion	.	(234;245)
	Tyr493His[††]	2	2; 1	.	0	.	.	12	12	31	(51)
	Trp501Cys	2	36; 40	36; 32	1	1.6	<1	.	.	.	(246)
	Lys518Asn[‡‡]	2	1	12	0	.	.	.	.	.	(247)
	Pro520Leu	1	34-48	.	0	.	.	.	100	.	(236)
	Gly555Glu	0	.	.	1	<1	100	<1	82	.	(51;248)
	Trp569Ser	Several	20-40	.	0	.	.	.	2	40-60	(244)
	Thr575Met[±±]	1	1	42	.	.	.	.	.	.	(242)
	Ser576Arg	2	27; 46	22.9; 71	0	.	.	.	.	.	(237)[**]

[¶]No data available
[‡] Two reports may relate to a single patient.
*Compound heterozygote with Lys535Ter. The expression data was presented in the poster resulting from (238).
**N. M. O'Connell, unpublished data
[†]Index patient compound heterozygous for Glu226Arg and Ser248Asn.
[§]Compound heterozygote with Glu117Ter
[±]Compound heterozygote with Cys128Stop
[¶¶]Compound heterozygote with Arg210Stop
[§§]Compound heterozygote with Cys581Stop
[‡‡]Compound heterozygote with G324G splicing mutation
[††]Compound heterozygote with Cys38Arg
[±±]Compound heterozygote with Gln88Stop

Table 6.6: FXI mutations published with insufficient phenotypic data.

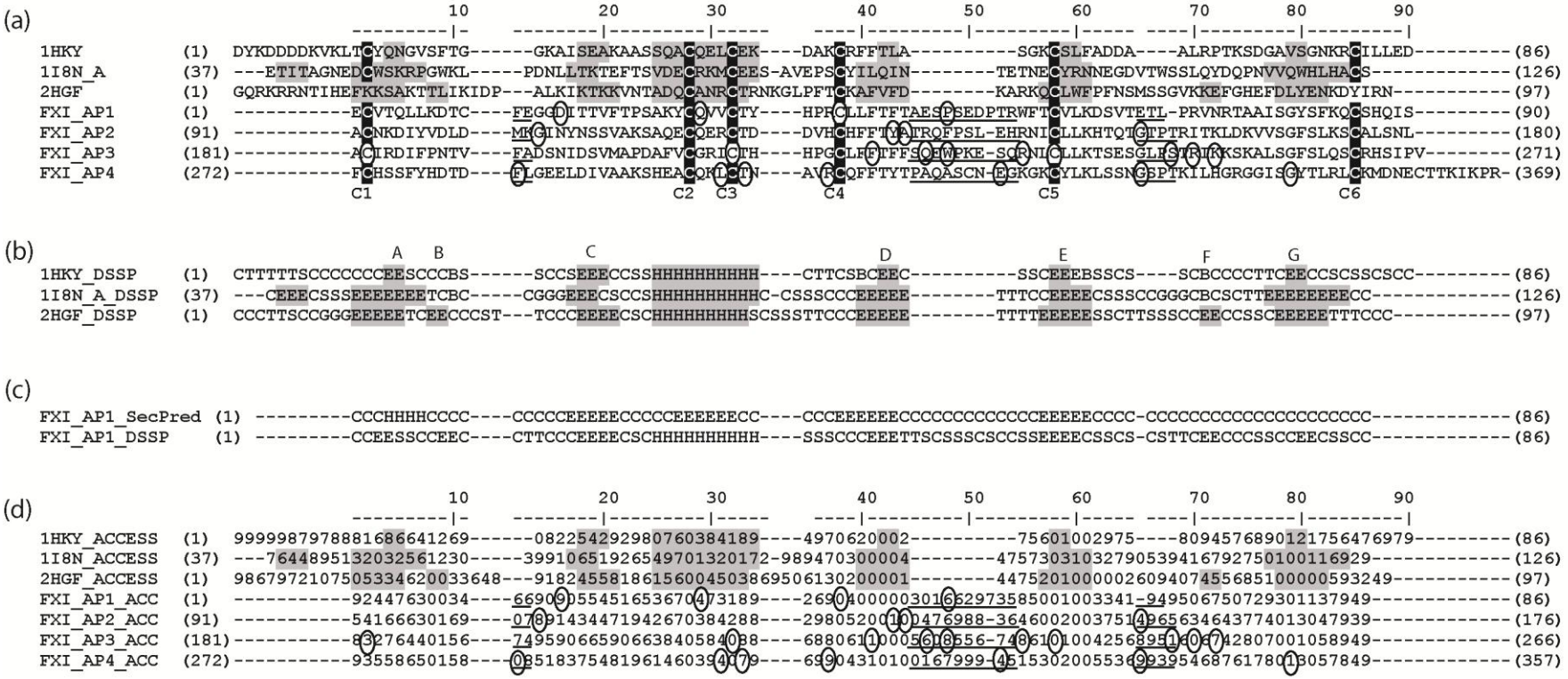
Domain	Mutation site	Reference
Apple 1	Asp16His	(241)
	Gln29His	(245)
Apple 2	Gly104Asp	(234)
	Tyr133Ser	(245)
	Ala134Pro	(245)
Apple 3	Cys182Tyr	(245)
	Cys212Arg	(238;245)
	Trp228Cys	(182)
	Arg234Ile	(245)
	Arg250Cys	(245)
Apple 4	Gly350Glu	(229)
SP	Thr389Pro	(245)
	Thr475Ile	(183;245)
	Ile600Ser	(245)

6.5.2 Construction of Ap domain models

The four Ap domains in FXI show 24% to 38% sequence identity with one another. In order to model these four Ap domains, three crystal and NMR structures that were homologous to the four Ap domains in FXI (Figure 6.5(a)) were identified from database searches. These were the ninth PAN module of an adhesin protein, EtMIC5, from the protozoa *E. tenella* (PDB code: 1HKY) (227;231), the N-domain of human hepatocyte growth factor (PDB code: 2HGF) (233), and an anti-platelet toxin from *H. officinalis* (PDB code: 1I8N).(232) A total of 23 other Ap sequences were obtained as described in Chapter 2 Methods, section 2.8.2. Even though these three structures shared low sequence identity of 8% to 13% with one another, and 11% to 22% sequence identity with the four Ap domains in FXI, they exhibit two or three conserved disulphide bridges and a conserved secondary structure consisting of a five-stranded β-sheet (β-strands C, E, D, G and A) flanked by a single α-helix on one surface and a distorted two-stranded β-sheet (β-strands B and F) on the other surface (Figure 6.6(b)). Although the Cys residues C1 and C6 at the amino and carboxy termini of the FXI Ap domains are not conserved in all the Ap domain sequences, they are present in two of the structural templates, those of EtMIC5 and the anti-platelet toxin. Superimposition of these three structures permitted reliable sequence and structure alignments and models to be made for the Ap1 to Ap4 domains (Figure 6.5(a)). The 1HKY template had the highest sequence identity (15-22%), and modelling based on this template required only three insertions and no deletions, which is fewer compared with the other two structural templates. All four resulting Ap models shared a short two-residue insertion at F12-E13 (Ap1), M102-K103 (Ap2), F192-A193 (Ap3) and F283-L284 (Ap4). The Ap1 model also has two insertions of ten residues (A45-W55) and three

residues (E66-L68), whereas the Ap2, Ap3 and Ap4 models have an insertion of nine residues at T133-H143 (Ap2), S225-Q233 (Ap3) and P316-G324 (Ap4) and another insertion of four residues at G155-T158 (Ap2), G245-S248 (Ap3) and G336-T339 (Ap4).

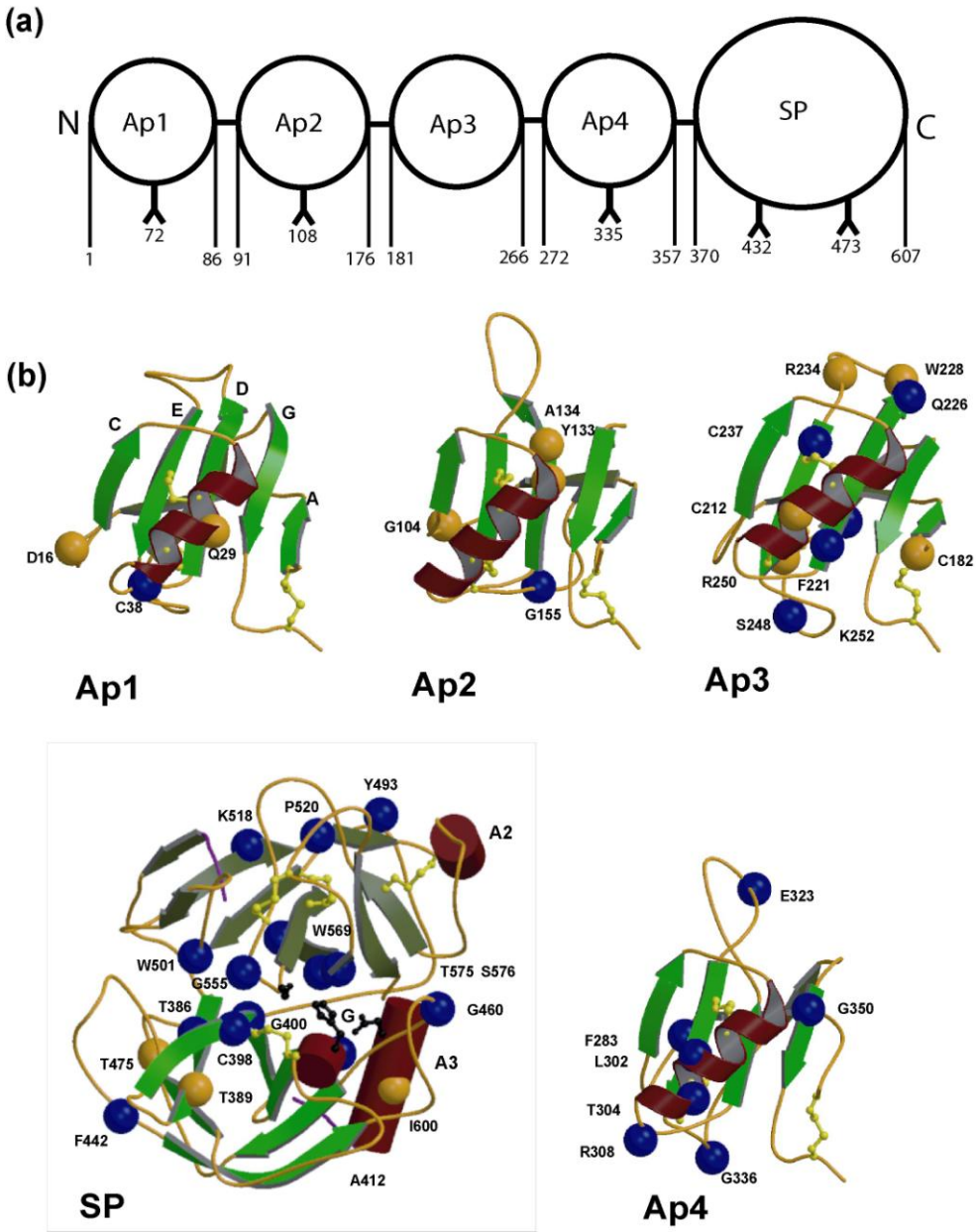
Figure 6.5: Multiple sequence alignment of the Ap and PAN module superfamily.



(a) The Ap and PAN sequences are shown. Conserved cysteine residues are highlighted in black and labelled C1 to C6. The secondary structures are highlighted by shaded boxes. The three insertions are underlined. The FXI mutations in Ap1 to Ap4 are highlighted within black ellipses. Sequences are numbered with respect to FXI_AP1. (b) The secondary structure assignments using DSSP are shown for the 1HKY, 2HGF and 1I8N structures. The shading follows that in (a). H, α-helix; E, β-sheet. The strands are lettered A to G to correspond to those seen in Figures 6.6(b). (c) The consensus secondary structure prediction for Ap1 calculated from 30 aligned sequences is compared with the DSSP analysis of the final Ap1 homology model. (d) The NACCESS sidechain accessibilities are given for each side chain of the 1HKY, 2HGF and 1I8N structures and for each of the four Ap homology models. Mutations in Ap1 to Ap4 are highlighted within black ellipses. The shading corresponds to the secondary structures of (a).

All three insertions were confined to loop regions in the 1HKY structure as desired for optimal modelling (figure 6.5(a)). The unpaired Cys321 sidechain is on the surface of the Ap4 domain as required in order that it can form the FXI dimer. The Cys11 residue of Ap1 is also surface exposed and can form a disulphide bridge.(11) The sidechain accessibilities between the three known structures and the four models showed good concordance in Figure 6.5(d). The putative N-glycosylation sites at Asn72 (Ap1), Asn108 (Ap2) and Asn335 (Ap4) all show varying degrees of solvent exposure in the models, suggesting that oligosaccharide chains can be attached to all these. Glycosylation at Asn72 and Asn108 has been confirmed (249) while the lack of observed glycosylation at Asn335 in Ap4 may be attributable to the dimerisation of the Ap4 domains as this occurs on the same face of the structure as Cys321 in the Ap4 model. The four models all satisfied validation using PROCHECK.

Figure 6.6: Domain structure of FXI.



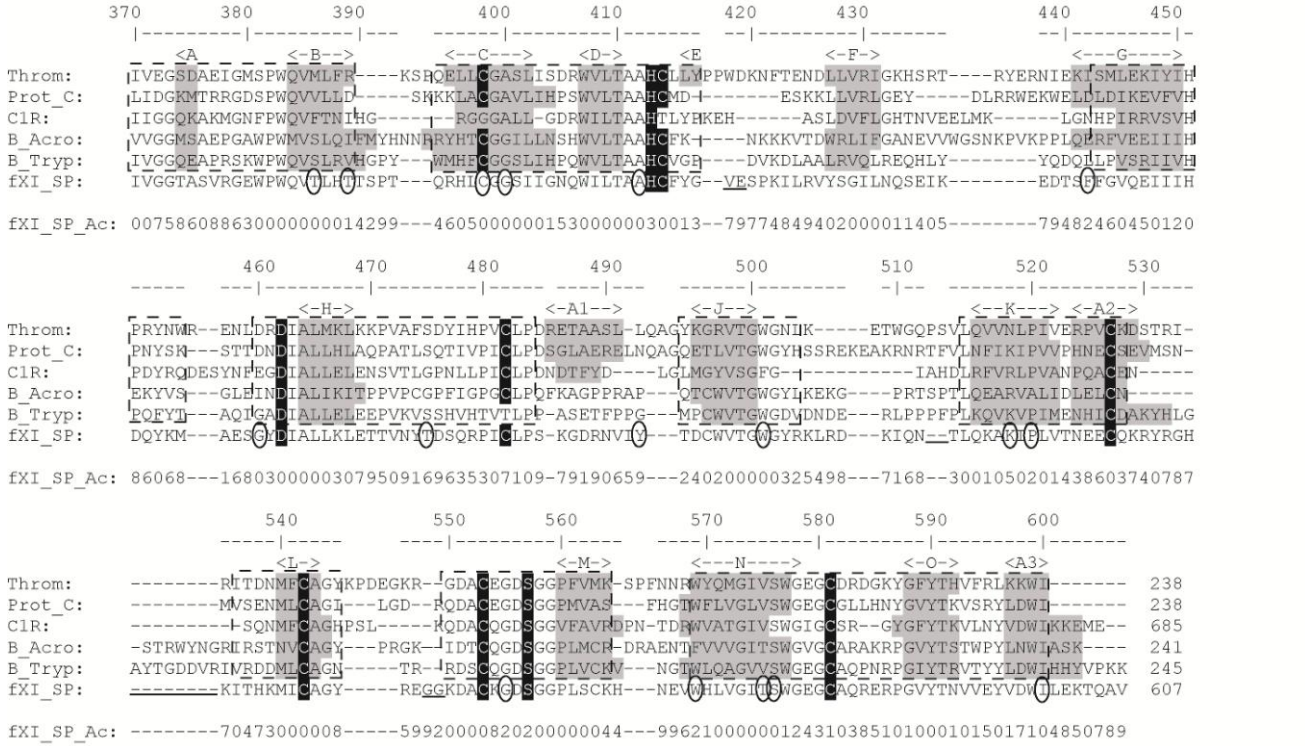
(a) Cartoon of the FXI domains to show the four tandem apple domains (Ap1-Ap4) and the serine protease domain (SP). The residue numbers at the beginning and end of each domain are indicated, and the five putative glycosylation sites are highlighted by Y symbols, of which four have been confirmed (see text).

(b) Secondary structure cartoons of the FXI domains to show their α-helix (red) and β-sheet (green) structures. The β-strands of the Ap domains are indicated by letters C, E, D, G and A. The α-helices of the SP domain are indicated by A2 and A3, and its 3_{10} helix is denoted by G. The catalytic triad in the SP domain is highlighted in black at the interface between the two subdomains shown above and below the triad. Disulphide bridges are shown in yellow. Mutations with phenotypic data are shown in blue, and those without phenotypic data are shown in orange.

6.5.3 Construction of SP domain model

In order to model SP domains in the coagulation proteases, classic SP domains such as those in chymotrypsin and trypsin have been used.(250) To improve the choice of an appropriate template, 84 unique SP structures were identified in the Protein Data Bank and five close templates were identified using CLUSTALW analysis. These five templates were used for the structural alignment with the SP domain of FXI (Figure 6.7).

Figure 6.7: Structural alignment of the sequences for the five closest matches to the SP domain of factor XI.



The sequences are labelled as follows: Throm, human thrombin; Prot_C, human Protein C; C1R, human complement C1r; B-Acro, human β-acrosin; B-Tryp, human β-tryptase; fXI_SP, human Factor XI. Regions identified as structurally conserved by the INSIGHT Cα distance matrix are highlighted by dashed boxes. The seven most conserved cysteine residues and three catalytic residues (His413, Asp462, Ser557) are highlighted in black. The secondary structure was assigned using DSSP and indicated by the arrowed ranges above the grey shaded boxes in the alignment. The 14 conserved β-strands are labelled from A to O and the three α-helices from A1 to A3. The four insertions and deletions between FXI-SP and its closest template β-tryptase are underlined. Sequences are numbered relative to FXI_SP. Mutations are highlighted with black ellipses. The NACCESS sidechain accessibilities (fXI_SP_Ac) are given for each side chain of the final SP model.

The 14-stranded β-sheet secondary structure is well conserved as a double Greek key supersecondary motif. The closest template to the FXI SP domain in terms of insertions and deletions was 1A0L, this being the crystal structure of human β-tryptase (251) with a high sequence identity of 40%. Interestingly, three of the remaining four templates corresponded to two other coagulation proteases (human α-thrombin and protein C) and one complement protease (human complement C1r). The model of the FXI SP domain built from the 1A0L template possessed a conserved core of 224 residues, with insertions and deletions only affecting 14 residues. These 14 residues corresponded to two small insertions at surface loop regions (V418—E419 and G548-G549) and two-residue and eight-residue deletions at the surface loop regions between N512-T513 and between H534-K535 respectively. These changes corresponded to surface loops of the SP structure as desired. The putative N-glycosylation sites at Asn432 and Asn473 are both located at the back of the FXI SP domain on the opposite face to that of the catalytic triad, and either can be solvent exposed, suggesting that oligosaccharide chains can be attached. Glycosylation at both of these sites has been confirmed.(249) The SP model was validated using PROCHECK.

6.5.4 Structural interpretation of 28 missense mutations in the *F11* gene

The structural effects of the 28 mutations in the FXI gene that are associated with known phenotypes (Table 6.5) are listed in Table 6.7.

In the Ap domains, protein misfolding is predicted to be the underlying mechanism of FXI deficiency in six of 14 mutations. Of the remaining eight mutations, two are predicted to affect the functional role of the Ap domains in that they are located at known binding sites, four mutations appear to affect the dimerisation site in FXI, one

mutation appears to affect the solubility of FXI and one mutation appears to be a non-disease causing polymorphism.

In the SP domain, protein misfolding is predicted to be the underlying mechanism of FXI deficiency in eight of 14 mutations. Of the remaining six mutations, four mutations are predicted to affect the ability of FXI to proteolytically cleave its substrate and two mutations appear to cluster in one structural region with functional significance but their phenotypic characterisation is impaired by compound heterozygosity.

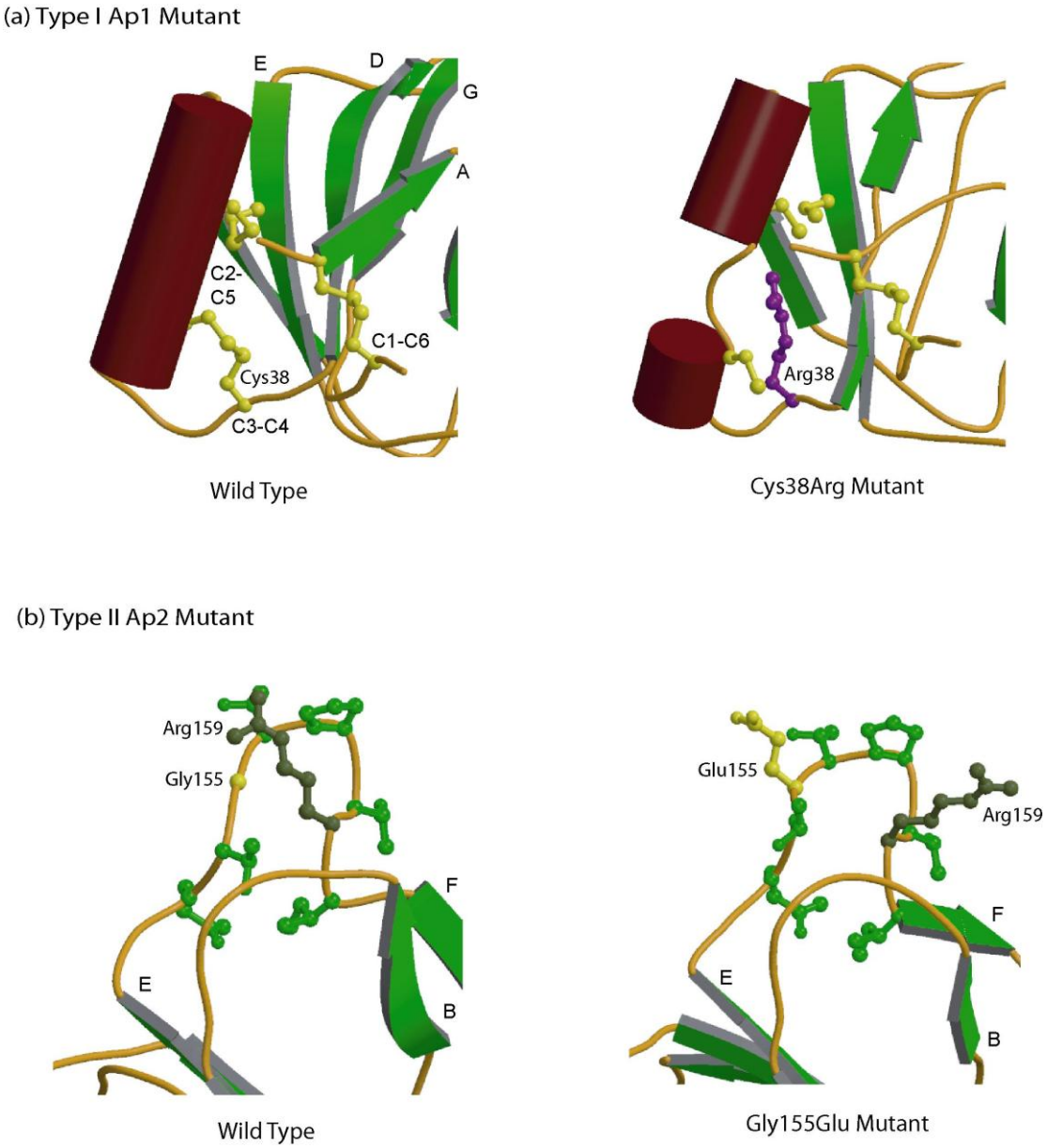
Table 6.7: Structural features of the FXI mutations with known phenotypes. (*CH: Compound heterozygosity).*

Domain	Mutation	DSSP	Accessibility	Type	Predicted Effect
Apple 1	Cys38Arg	C	0	I	Structural; Disrupts disulphide bridge
Apple 2	Gly155Glu	C	4	II	Functional; Disturbs proposed FXIa binding loop
Apple 3	Phe221Ser	E	1	I	Structural; Buried residue
	Gln226Arg	C	6	CH	Polymorphism
	Cys237Tyr	E	0	I	Structural; Disrupts disulphide bridge
	Ser248Asn	C	1	II	Functional; Replaces platelet binding site
	Lys252Ile	E	7	CH	Structural: May affect solubility
Apple 4	Phe283Leu	C	0	I	Functional/Structural; May prevent dimerisation
	Leu302Pro	H	4	I	Structural; Disrupts helix formation and disulphide bridge
	Thr304Ile	H	7	I	Structural; Disrupts disulphide bridge
	Arg308Cys	C	9	I	Structural; Disrupts disulphide bridge
	Glu323Lys	B	4	I	Functional/Structural ; May prevent dimerisation
	Gly336Arg	S	9	CH	Structural; May prevent dimerisation
	Gly350Ala	C	1	CH	Structural; Buried residue; may affect dimerisation
SP	Thr386Asn	E	0	I	Structural; Buried residue
	Cys398Tyr	E	0	I	Structural; Disrupts disulphide bridge
	Gly400Val	E	0	I	Structural; Buried residue
	Ala412Val	G	0	I	Structural; Disrupts secondary structure
	Phe442Val	S	2	I	Structural; Disrupts correct folding pathway
	Gly460Arg	S	0	I	Structural; Buried residue
	Tyr493His	C	9	CH	Functional; At surface near N-terminal Ile370
	Trp501Cys	S	0	I	Structural; Buried residue; Extra Cys residue
	Lys518Asn	E	5	CH	Functional; At surface near N-terminal Ile370
	Pro520Leu	C	2	II	Functional; At surface near N-terminal Ile370
	Gly555Glu	T	2	II	Functional; Perturbs active site
	Trp569Ser	E	2	I	Structural; Disrupts correct folding pathway
	Thr575Met	E	0	CH/II	Functional; Perturbs active site
	Ser576Arg	E	0	II	Functional; Perturbs active site

Ap mutations with known Type I (CRM-) phenotypes

There are three disulphide bridges in the Ap domains (C1-C6, C2-C5 and C3-C4). In four mutations with known Type I phenotypes, the integrity of the C3-C4 bridge is disrupted (Cys38Arg, Leu302Pro, Thr304Ile and Arg308Cys: Table 6.7 & Figure 6.8(a)). Energy minimisation of the Cys38Arg mutation in the Ap1 domain suggested that, with the disulphide bridge broken and the introduction of the larger bulkier side chain of Arg38, the α-helix was broken and the small loop at the C-terminal of the helix changed its conformation to accommodate Arg38. This interpretation is supported by the phenotypic data, which demonstrated that there was significant intracellular accumulation of the FXI protein but negligible secretion. The Leu302Pro mutant after energy minimisation also showed a similar effect, where the substitution of proline in the middle of the α-helix next to the C3-C4 bridge caused the α-helix to break. The Thr304Ile substitution replaced a hydrophilic side chain with a bulky hydrophobic one next to the C3-C4 bridge. This appeared to alter the hydrophobic nature of the helix which may be sufficient to cause it to twist to form contacts with the hydrophobic β-strand C of the central β-sheet, altering the C3-C4 loop conformation. The Arg308Cys substitution introduced a new Cys residue adjacent to the C3-C4 bridge of the Ap4 domain, suggesting that the correct disulphide pairing may not be formed.

Figure 6.8: Molecular views of selected mutated residues in two Ap domains.



(a) The wild type Ap1 domain model is depicted with its three cysteine disulphide bridges highlighted in yellow (left). These are labelled C1-C6, C2-C5 and C3-C4. One of these Cys residues is replaced by Arg (blue) in the Type I Cys38Arg FXI mutant (right). Note that the secondary structure is altered after energy minimisation.

(b) The wild type Ap2 domain model is depicted to show a face-on view of its substrate binding loop (left). The replacement of Gly155 by Glu in the Type II mutant (right) altered the β-sheet and loop structure after energy minimisation.

In two other Ap mutations, the C2-C5 disulphide bridge was disrupted. The Cys237Tyr mutation results in loss of the C5 sidechain and failure to form the C2-C5 disulphide bond. Phe221Ser is likely to perturb protein folding since it involves a fully buried residue on β-strand D, close to the C2-C5 bridge that is conserved in many other Ap sequences and exemplified for the Ap1 domain in Figure 6.8(a). A third mutation Lys252Ile (compound heterozygous) in Ap3 is distant from any disulphide bridge in this domain, but is associated with reduced secretion of FXI implying that this is a Type I disorder. It is possible that the introduction of a surface hydrophobic Ile252 sidechain at the protein surface adjacent to the exposed Ile251 sidechain may affect its solubility.

Impaired FXI dimerisation has been confirmed in one type I Ap4 mutation (Phe283Leu) and may be implicated in four other type I mutations in Ap4 (Glu323Lys; Gly336Arg; Gly350Ala; Gly350Glu). Phe283 is on a surface exposed loop between residues Thr281 and Leu288. This protein loop forms the second side of a cleft with Cys321 on the first side. Cys321 stabilises the dimer by forming a disulphide bridge with Cys321 of the opposite FXI monomer, although this Cys321 interchain bond is not required for dimer formation.(18;19) Glu323Lys is adjacent to Cys321. The surface charge reversal caused by the Glu323Lys substitution within this cleft may perturb the association of two FXI molecules. The Gly336Arg mutation (compound heterozygous) in Ap4 appears to result in impaired secretion.(242) Gly336Arg occurs adjacent to the nonglycosylated glycosylation site at Asn335 (Figure 6.6(a)). As this region of Ap4 may be implicated with FXI dimerisation, this may offer an explanation of the effect of this mutation. The effect of the Gly350Ala mutation (compound heterozygous) in Ap4 on secretion is unclear (242), but the Gly350Glu mutation at the same position

corresponds to a severe defect in dimer formation.(18;224) Since Gly350 occurs within the five-stranded β-sheet in a buried position on β-strand G, the introduction of either an Ala or a Glu sidechain may disrupt the folding of Ap4, and in turn the ability of Ap4 to mediate FXI dimerisation.

Ap mutations with known Type II (CRM+) phenotypes

Only two Type II mutations were identified in the Ap domains (Gly155Glu, Ser248Asn), for which the phenotypic data (Table 6.5) gives some evidence of a functionally defective protein. The Gly155Glu mutation in the Ap2 domain occurs at an exposed position in the Ap2 substrate binding loop between β-strands E and F, which was identified from functional studies.(252) Energy minimisation suggested that the localised loop conformation changed and altered the sidechain orientation of residues such as Arg159 (Figure 6.8(b)). Given the absence of a sidechain in Gly155, further modelling showed that this localised change in loop conformation is invariant of the position of the new Glu155 sidechain. The Ser248Asn mutation in the Ap3 domain occurred on the corresponding substrate binding loop by analogy with the Ap2 domain (Figure 6.5). Ser248 is functionally important for platelet binding (14;35) and substitution is presumed to affect the conformation of the substrate binding loop. Expression studies confirm a decreased affinity of FXI-N248 for platelet binding and impaired activation by thrombin in the presence of activated platelets.(253)

The phenotypic and modelling data suggest that Gln226Arg is a non-disease causing polymorphism in the Ap3 domain. Gln226 is located on a large surface loop insertion that is present in all FXI Ap domains, but not in the template structures. It is fully

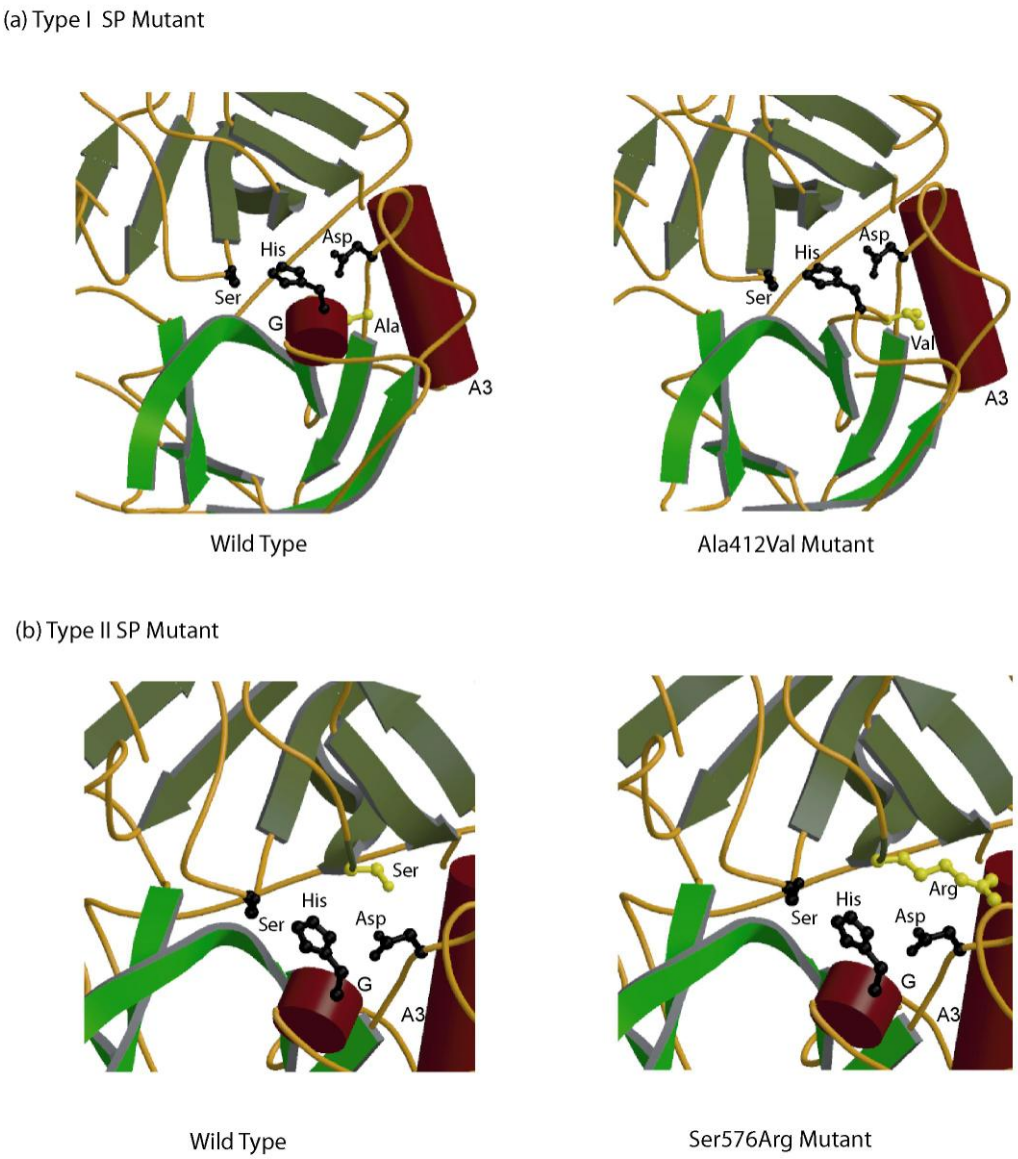
solvent exposed on the modelled structure and the loop is remote from the substrate binding region. Substitution with Arg is not predicted to result in structural or functional disruption. Compound heterozygosity for Gln226Arg and Ser248Asn was confirmed in the index patient, but FXI:C was consistent with only a partial deficiency of FXI.(239) Expression of recombinant FXI-R226 was similar to recombinant wild-type FXI while the catalytic efficiency of FXIa-R266 was also similar to wild-type FXI, suggesting a non-disease-causing polymorphism.(253) The patient's mother also had a partial deficiency of FXI and was heterozygous for Ser248Asn only.

SP mutations with known Type I (CRM-) and Type II (CRM+) phenotypes

Eight of the 14 SP mutations with known phenotypes are classified as Type I and all these occur in residues with reduced sidechain solvent accessibilities. The Ala412Val mutation provides one example of this. As Ala412 is adjacent to the catalytic triad (Figure 6.9(a)), it was first thought that this mutation has altered the conformation of the active site, so corresponding to a Type II phenotype. However the FXI SP model indicated that the fully buried Ala412 sidechain pointed away from the triad into a tightly packed protein core. Figure 6.9(a) showed the FXI SP model after the substitution with the bulkier Val412 sidechain and energy minimisation. The minimisation removed the 3_{10} helix to which the catalytic His413 was attached. This indicated that the mutation reduced the stability of the SP fold, while not affecting the formation of the active site at the catalytic triad. This interpretation is consistent with the Type I phenotype, in which both the activity and antigen levels are low.

Most of the other Type I mutations in the SP domain (Thr386Asn, Gly400Val, Phe442Val, Gly460Arg, Trp501Cys, Trp569Ser; all show low solvent accessibility and are buried) can be interpreted in a similar fashion to that for Ala412Val and appear to relate to protein packing or the folding pathway. For example, Gly460 is close to the catalytic triad His413-Asp462-Ser557, however phenotypic data shows that it is a Type I mutation (Table 6.5). Energy minimisation for the Gly400Val mutation supported this analysis, demonstrating that the protein core structure was clearly disrupted. One mutation (Cys398Tyr) breaks a disulphide bridge between Cys398 and Cys414, hence disrupting the folding of the SP domain. The Trp501Cys mutation introduces an additional Cys residue that may perturb the correct disulphide bridge formation during the folding of the SP domain.

Figure 6.9: Molecular views of selected mutated residues in the SP domain.



(a) The catalytic triad of the SP domain model is depicted in black at the centre, with the Type I Ala412Val mutant in yellow. Note that the secondary structure is altered after energy minimisation. (b) The close-up view of the catalytic triad of the SP domain model is depicted in black at the centre, with the Type II Ser576Arg mutant shown in yellow. Note that the secondary structure is NOT altered after energy minimisation.

Two Type II mutations in the SP domain (Gly555Glu and Ser576Arg) correspond to instances where partially buried small side chains are replaced by large bulkier side chains. Both these are structurally located near to the catalytic triad, and are seen to affect the active site. For Gly555Glu, Schmidt et al showed that the Glu sidechain in the mutant may impair the accessibility of the substrate for the active site cleft.(250) For Ser576Arg, Figure 6.9(b) showed that Ser576 partially projects into the substrate binding cleft formed between the two subdomains of the SP domain. The replacement by Arg576 is expected to block the binding of substrate to the cleft. This interpretation was supported by energy minimisation which showed that no conformational changes had occurred in the SP domain (Figure 6.9(b)). The Thr575Met mutation is also compound heterozygous with Gln88stop.(242) Thr575Met is considered to be a Type II mutation because its antigen level is halved (consistent with Gln88stop being a Type I mutation) and it is adjacent to the Ser576Arg mutation which has a Type II phenotype, both being close to the catalytic active site cleft. In the case of Pro520Leu, which appears to be a type II mutation on the basis of expression data, direct interference with the substrate binding cleft is not implicated since Pro520 is distant from the catalytic triad. However, because the model suggests that Pro520 is near Ile370 at the N-terminus, which is inserted into the SP domain during activation, and Pro520 defines the rim of the pocket into which Ile370 is inserted during activation, it was considered possible that this mutation may block the development of catalytic activity in FXI. Pro520 is conserved in Factor X and Factor VII, and the mutation of Pro520 in both these other proteins also caused Type II phenotypes.(254-256) The equivalent of Pro520 is also conserved in chymotrypsinogen and occupies similar positions in the crystal structures of chymotrypsinogen and chymotrypsin, indicating that its location is

invariant with the activation state of the protease. Two mutations (Tyr493His, Lys518Asn) are compound heterozygous, hence the phenotype of both these are unclear. It was interesting that both these residues form a surface cluster with Pro520Leu near the N-terminus of the SP domain, as this suggested that these may have a similar effect as the Pro520 mutation. Reduced secretion and intracellular level of the Tyr493His construct in expression studies implies that there is also a significant structural element to this mutation.

Mutations with no phenotypic data

A total of 14 FXI mutations are associated with insufficient phenotypic data at the time of this study (Table 6.6), accordingly the interpretation of these must be less certain. These are included in order to display the distribution bias of all mutations throughout the AP and SP domains, which is seen to be nonspecific (yellow spheres in Figure 6.6(b)).

Of the 11 mutations in the Ap domains, five involve directly or indirectly the conserved disulphide bridges (Gln29His, Cys182Tyr, Cys212Arg, Trp228Cys, Arg250Cys), hence these will probably involve protein misfolding and Type I phenotypes. The Gln29His substitution occurs at the centre of the α-helix next to Cys28-Cys58, and energy minimisation suggested that the charged His sidechain appeared to be close to the N-terminal charged Glu1 sidechain. This mutant is apparently not secreted but data are limited. The Asp16His and Gly104Asp mutations are aligned in the same region with the Phe283Leu mutation in Ap4 (Figure 6.5(a)), both are fully exposed on a surface loop (Figure 6.5(d)), and it was not possible to interpret these mutations further. The

Tyr133Ser and Ala134Pro mutations are both buried together on β-strand D at the start of the substrate binding loop in the Ap2 domain (Figure 6.5), and may affect FXI activity. Limited data for Tyr133Ser suggests that the FXI:C level in one patient was 38% and that secretion of the FXI mutant protein was reduced. The Arg234Ile mutation replaces a hydrophilic exposed charged residue with a hydrophobic one.

Of the three mutations in the SP domain, Thr389Pro is a buried residue on β-strand B, so misfolding may be implicated as the causative effect of this residue change. The Thr475Ile mutation removes one of the five N-glycosylation sites (Figure 6.6(a)), although the loss of the glycosylation site does not appear to be responsible for the deficient phenotype.(257) The Ile600Ser mutation is located on the C-terminal α-helix A3 at a buried position (Figure 6.7), hence this may affect the packing of this helix against the SP domain core.

6.6 Discussion

Factor XI deficiency in the Jewish population is caused by four well described distinct mutations (termed Types I-IV). The Jewish mutations are characterized by low FXI coagulant activity and antigen (i.e. cross-reacting material negative, CRM-).(56;59) The two most common mutations are a nonsense mutation in Apple 2 (Glu117Stop) and a missense mutation in Apple 4 (Phe283Leu). These two mutated alleles occur with equal frequency in severely deficient patients (49% and 47% of mutant alleles respectively) and result in a heterozygote frequency of 9% and a homozygote frequency of 0.22% in persons of Ashkenazi descent. (49;56) The Type I and Type IV mutations affect splicing in the catalytic domain (a G to A transition at the donor splice site in intron N and a 14 bp deletion at the exon 14 / intron N splice site, respectively) and allele frequencies are 1.2% and 0.4%. A founder effect has been confirmed for patients with the Type II and Type III mutations by comparison of FXI intragenic polymorphisms in FXI deficient Iraqi, Sephardic and Ashkenazi Jews with unaffected Jewish individuals.(60)

Factor XI deficiency is a rare bleeding disorder in other populations and the incidence in general populations is generally given as one per one million population. However, a number of other mutations have been reported at higher frequencies in distinct populations e.g. 1% in the French Basque population for Cys38Arg.(51) A founder effect was confirmed for the latter mutation and subsequently for a number of other mutations in specific populations (Cys128Stop in British patients, Gln88Stop in French patients).(242;258)

The unique conformation of the FXI protein as a dimer also causes a further specific "dominant negative" effect in certain mutations in the heterozygous state.(224;225) In these mutations, the FXI:C in heterozygotes is less than expected (usually <25 U/dL). This has been postulated to be caused by dimerisation of the mutated FXI with wild-type FXI intracellularly and prevention of secretion of wild-type FXI dimers in quantities normally expected in heterozygotes. Mutations for which this effect has been confirmed in expression studies are Ser225Phe, Cys398Tyr, Gly400Val and Trp569Ser. One of these mutations is located in Apple 3 and three in the serine protease domain.

Where conventional screening fails to identify a causative mutation, deletion of the whole gene may be considered which is masked in the heterozygous state by the normal allele. Two of 39 patients were found to have a whole gene deletion as the causative mutation of factor XI deficiency as the result of unequal homologous recombination between flanking Alu repeat sequences.(259)

A number of sequence alterations were identified in the study population. The Pro48Leu change had been unreported at the time of the study. Both Proline and Leucine are hydrophobic residues and the location of the residue is on a surface exposed loop region between two β sheets. This amino acid change has subsequently been identified as a non-synonymous polymorphism and hence is not likely to be the causative mutation in the case of the reported patient.(260)

The Gly155Glu mutation in exon 6 was previously reported in abstract form in 2000.(235) The patient was non Jewish and had a FXI:C of 44.3 U/dL. This patient did not have a bleeding history. This mutation was also reported by the same group in a non Jewish patient with a FXI:C of 41 U/dL.(234) Glycine 155 is found in the second

apple domain of FXI at an exposed position in a substrate binding loop. Although there was a discrepancy between the FXI:C and FXI:Ag in the patient under study here, subsequent analysis of this residue in a consensus apple structure showed that mutations in the aligned Glycine residues in Apples 3 and 4 causes a concordant decrease in FXI:C and FXI:Ag.(260) Expression studies would be useful to determine the precise effect of this mutation on protein secretion and function.

Glu 297 is located in the apple 4 domain in a random coil area of the structure and replacement of the acidic Glutamate residue with a larger basic Lysine residue would be expected to disrupt the structure of the coil region. Subsequent to this study, the Glu297Lys mutation has been reported in a number of French patients with FXI deficiency.(261;262) Patients in the latter studies who were heterozygous for this mutation had FXI:C levels of 38 to 58 U/dL and concordant FXI:Ag. This is in keeping with the FXI:C and FXI:Ag described in the UK patient in this study (38 and 34 U/dL respectively). Secretion was confirmed to be reduced to 4.5% of normal when the mutated protein was expressed in BHK cells but dimerisation was reported to be normal.(263)

The Glu380Lys mutation in the serine protease domain has not been reported to date. The replacement of Glu by Lys replaces a medium sized acidic residue with a large basic residue. The residue is exposed. The residues surrounding Glu380 have had disease causing mutations reported e.g. Arg378Cys (264), Trp381Leu (265) and Pro382Leu (261;266) although one non-synonymous polymorphism has been reported in the region (Trp381Arg ,www.factorxi.org).

The Cys398Tyr mutation had been reported in FXI deficient patients in abstract form at the time of this study (see table 6.3) and heterozygous patients were reported to have FXI:C levels of 40-50 U/dL similar to the patient reported here (57 U/dL). Subsequently, a heterozygous patient was reported with concordant FXI:C and FXI:Ag (225) and the mutation was reported the homozygous, heterozygous and compound heterozygous states.(263;266) This mutation has been reported to cause a dominant negative effect.(225) However, the patient in this study had a well preserved FXI:C of 57 U/dL although the FXI:Ag was somewhat lower (36 U/dL). The FXI:Ag would be in keeping with the dominant negative type effect but the FXI:C in this patient and in other heterozygotes reported by Gailani (FXI:C40-50 U/dL) and Mitchell (FXI:C 58 U/dL) would not. (236;237) Structurally, the Cys398Tyr mutation breaks a disulphide bridge between Cys398 and Cys 414.(260)

The Ser576Arg mutation was described in a patient with FXI:C of 27 U/dL and a FXI:Ag of 22.9 U/dL.(237) No clinical details were given for this patient. The patient reported in table 6.3 had a FXI:C of 46 U/dL but a normal FXI:Ag of 71 U/dL suggesting normal secretion of the protein. Serine 576 is found in the catalytic domain of the FXI protein and partially projects into the substrate binding cleft. The replacement of Ser576 with Arg is expected to block binding of the substrate but is not expected to result in conformational change.

This study resulted in the first detailed homology models for the structure of the FXI domains based on appropriate template crystal and NMR structures. The accuracy of the Ap domain models was demonstrated by the following: (i) DSSP analyses showed that the major secondary structure elements seen in the NMR structure of 1HKY were

present in the four Ap models after extensive energy refinement, (ii) there was general agreement between the DSSP secondary structure assignments and the consensus secondary structure predictions (Figure 6.5(c)), (iii) there was high agreement between the sidechain solvent accessibilities of the experimental and modelled structures (Figure 6.5(d)), (iv) the models were stereochemically satisfactory according to PROCHECK. The low sequence identities were as expected for small homologous β-sheet proteins, as illustrated by the 8% to 12% sequence identities seen between the three Ap structures.(267) The accuracy of the SP domain model was demonstrated using similar techniques. For all five domain models, the cores of these protein models were expected to be well predicted, while their accuracy was expected to diminish in the regions of insertions and deletions in loops at the protein surfaces. This was sufficient to locate the positions of individual amino acids, while the positions of their sidechains may not be as well determined. It was additionally not possible to predict with certainty the effect of mutations on the interactions between domains.

Previously, models for the four Ap domains had been presented using an *ab initio* molecular dynamics and energy minimisation techniques.(14;35;252;268-270) As the models presented here were based on experimentally solved homologous structures, the predictions were felt to be significantly more accurate. The major difference from the *ab initio* predictions is that these earlier models had predicted that three anti-parallel β-strands formed a continuous binding surface, with many important residues being mapped to this area. In distinction, the homology models predicted that this same region also contains three β-strands E, F and G (Figure 6.5(b)), but only two of these (E and G) participate in the five-stranded β-sheet (Figure 6.6(b)). Part of this β-sheet is

buried, while the remainder contributes to two large surface exposed loops with two distorted β-strands B and F beneath the five-stranded β-sheet.

The structural analysis of the mutations in the four Ap domains of FXI gave insight into the effects governing both Type I (CRM-) and Type II (CRM+) mutations, where either its folding, substrate binding or dimerisation is altered. The Ap domain comprises a small Cys-rich structure whose fold is primarily stabilised by its disulphide bridges.(271) This makes it susceptible to protein misfolding if these disulphide bridges are disrupted.

The majority of the 42 modelled mutations mapped to the Ap domains were classified as Type I (CRM-), where phenotypic data (Table 6.5) showed a low concordant FXI:C and FXI:Ag implying a problem with expression or secretion of the protein or its rapid degradation. The relatively frequent occurrence of mutations that affect the C3-C4 bridge indicates the importance of this disulphide bridge for the overall fold of the Ap domain.

Of the type II (CRM+) mutations affecting the Ap domains, only Ser248Asn had been previously well defined.(239;253;272) In the case of Gly155Glu, there is a discrepancy in reported FXI:Ag levels. Two patients appear to have a Type I disorder with concordant FXI:C/FXI:Ag levels (234;235) while a third has a higher FXI:Ag than FXI:C (table 6.3). The homology model was used to investigate this mutant and supported a Type II functional defect in the Glu155 mutant.

Dimerisation in FXI occurs due to covalent and non-covalent interactions between the Ap4 domains.(11;18-20;228) The structural modelling has implicated two mutations to

be on the surface of Ap4 in the region where more extensive interactions between the homodimers may occur and close to Cys321 which forms the covalent link. Of these two mutations, Phe283Leu is one of the most frequent ones causing FXI deficiency in the Jewish community.(49;56) This interpretation is supported by expression studies that indicated a reduction in secretion of the mutant Leu283 (approximately 8% of WT FXI) and an intracellular accumulation of mutant FXI monomers.(18;229)

Detailed analyses of the structural effect of mutations on the SP domain had not been possible prior to the existence of the homology model. The eight mutations associated with Type I (CRM-) phenotypes affect sidechains that are either fully buried or have low solvent accessibility (Table 6.7). The FXI homology model predicts protein misfolding in these cases, leading to rapid degradation and/or impaired secretion. Four other mutations have Type II (CRM+) phenotypes, and also correspond to low sidechain accessibility, with three of them being close to the catalytic binding site. Confirmation that the substrate binding cleft is obstructed by the mutant residue was observed in the homology model. In addition, the model suggests a mechanism for the functional defect produced by Pro520 (and by extension, the type II/CRM+ mutations seen in FX and FVII). While a definitive structural explanation for the defect in two further mutants (Tyr493His, Lys518Asn) reported in compound heterozygosity was not possible, the model shows that both mutations form a surface cluster with Pro520, and these may indicate a region of functional significance.

As with all molecular homology models, the FXI Ap and SP models are an essential adjunct to clinical and experimental data, but need to be interpreted with caution. Nonetheless, in the absence of a crystal structure for the FXI dimer, these domain

models were of use to investigators seeking to characterise new FXI mutations. The development of a curated database of known FXI mutations, together with the FXI homology models, meant that all investigators had access to the models and could determine the structural effects of novel mutations (www.factorXI.org).(273) Crystal structures for the catalytic domain of FXI in complex became available after this study was completed and on publication of the crystal structures for the complexed FXI catalytic domain, dimeric FXI in 2006 and the A4 domain in 2007, the database was updated to allow modelling of mutations on the crystal structure.(21;45-47)

Comparison of the homology models and the crystal structures was undertaken once the latter were available.(260) The structural alignment between the FXI crystal structures and the template structures (EtMIC5 and human β-tryptase) correlated well with the sequence alignment used to generate the homology models. Additionally, the root mean square deviations in the α-carbon positions between the five homology models and the FXI crystal structure were within accepted norms. There were small differences in the insertion loops and N and C termini of the Ap domains and of course, the homology models could not give information regarding Ap packing with the secreted FXI protein. The serine protease domain was shown to have reproducible secondary structures between the available crystal structures and the homology model. Within the Ap domains, the B-F β-sheet was shown to have the most variability when the homology model and crystal structures were compared, in contrast to the central five-stranded β-sheet and α-helix which were more conserved.

Of the 28 mutations with known phenotype which had been modelled on the homology models, interpretations were correct in 26 and only two mutations had been incorrectly

assigned to predicted loops (Phe283Leu and Gly350Ala). This occurred because the predicted β-strands in the crystal structure were longer than those in the homology models. In addition, 25 of 28 solvent accessibility assignments were correct in the homology models. Therefore, it may be concluded overall that the homology models were accurate and reliable in the interpretation of mutations causing FXI deficiency. However, given the limitations of all homology models (which were discussed on publication), the current crystal structures are the gold standard for interpretation of the structural effects of mutations in the *F11* gene.

Chapter 7 Prevention of peri-operative haemorrhage in FXI deficiency: FXI concentrate and rFVIIa

7.1 Introduction

The variable bleeding tendency in patients with FXI deficiency creates considerable difficulty in managing surgical bleeding in particular. At present, the decision to give FXI replacement, an antifibrinolytic agent or no treatment is an empiric one and is dependent on the prior bleeding phenotype. The level of FXI coagulant activity also plays a role in the decision to treat, since patients with FXI:C < 20 U/dL have a increased likelihood of bleeding although this is not universal even at low FXI levels. Bleeding in FXI deficiency is related to invasive procedures, especially those involving tissues with high fibrinolytic activity.(6;7;62)

Bleeding complications can be avoided by the use of FXI replacement (55) but at the risk of certain adverse events. In the case of FXI replacement with plasma, there is concern regarding fluid overload, allergic reactions and FXI levels may not be normalised.(86) With FXI concentrate, concerns exist around thrombo-embolic complications.(72;92;94) As has been discussed in the Introduction (Chapter 1), FXI concentrates have been associated in the literature with thrombo-embolic complications. In particular, risk factors for thrombosis include age >60 years, pre-existing cardiovascular disease and administration of doses of FXI concentrate > 30U/kg. The FXI concentrate produced by BioProducts Laboratory (BPL) in the United Kingdom always contained antithrombin (at a mean level of 102 U/mL). After 1993 and following case reports of thrombotic complications with other FXI concentrates, the BPL FXI concentrate was modified to include heparin (10 U/mL) and in-vitro batch

testing for thrombogenicity was undertaken.(72) A review of experience with FXI concentrate was reported in the 1998 paper and 12 probable or definite thrombotic episodes were reported for 229 treatment episodes in 161 patients. No further evaluation of the modified product has been published.

Since FXI concentrate is manufactured in the United Kingdom and France and is available on a named patient basis only, this therapy is not widely available world-wide.(88;89) Transfusion transmitted infection, including emerging pathogens, remains a consideration for all patients receiving blood products even where viral inactivation steps are undertaken.(274)

An alternative treatment option is required for FXI deficient patients. Ideally, treatment should be recombinant and capable of replacing the role of FXI in augmenting thrombin generation in the consolidation phase of coagulation.(143;165;173) Recombinant factor VIIa (rFVIIa; NOVOSEVEN®, Novo Nordisk, Bagsvaerd, Denmark) fulfils these requirements, in particular because it generates a burst of thrombin on the surface of the activated platelet. (275-277)

The use of rFVIIa in FXI deficiency was first reported in a patient with an inhibitor to FXI in 1990 when rFVIIa was used to prevent surgical bleeding after orchidectomy.(102) Subsequently, a small number of case reports in patients with and without inhibitors have confirmed that rFVIIa is effective in preventing surgical haemorrhage (See Table 7.1).(107-109) In all of the above case reports, haemostatic efficacy of rFVIIa was reported and no adverse events were documented.

Table 7.1: Dose and dose schedule of rFVIIa in FXI deficiency with and without inhibitors prior to 2001.

Case report	Patient	FXI:C (U/dL)	FXI:Inh (BU)	Surgery	Bolus dose rFVIIa (μg/kg)	Continuous infusion and dose	Tranexamic acid
Hedner (102)	Male (no other details)	N/A*	Present. Titre N/A	Orchidectomy	60 q 3h x 3 days	No	No
Lawler (107)	62 year old female	<1	48	Cataract extraction	90 x 1 dose	Yes 20 μg/kg/hr x 24 hours	No
Musso (109)	38 year old female¶	<1	N/A	Laparotomy	60 x 8 doses over 48 hours	No	Yes (5g/d)
Musso (109)	38 year old female¶	<1	N/A	Resection of breast mass	60 x 5 doses over 36 hours	No	Yes (5g/d)
Billon (108)	75 year old female†	5	38	Pleural aspiration	30 x 2 doses over 4 hours	No	No

*N/A: Not available

¶ One patient treated twice due to history of excessive bleeding following surgery covered with FFP.

†Acquired FXI inhibitor secondary to chronic myelomonocytic leukaemia

The aim of this chapter is to audit the use of FXI concentrate in practice as a haemostatic agent to prevent surgical bleeding and to evaluate rFVIIa as an alternative treatment to prevent peri-operative haemorrhage in patients with FXI deficiency.

7.2 Audit of the use of FXI concentrate in FXI deficiency

7.2.1 Methods

The use of the modified BPL FXI concentrate between 1996 and 2001 was evaluated in a retrospective audit of the medical records of 296 patients registered with FXI deficiency. The need for FXI replacement had been determined by the treating physician. In line with the policy of the Katharine Dormandy Haemophilia Centre, FXI concentrate was dosed according to UKHCDO guidelines: i.e. the dose of FXI concentrate was not more than 30 U/kg and the peak FXI:C was maintained < 100 U/dL.

All patients who had received FXI concentrate in the period September 1996 to September 2001 were identified from the Haemophilia centre database. A structured data collection instrument was used to extract data on the use of FXI concentrate to cover invasive dental or surgical procedures.

7.2.2 Results

Seventy surgical episodes were identified in 43 patients (Table 7.2). The median age was 41 years (range 1-91 years). Baseline FXI:C levels ranged from <1 to 58 U/dL and 20 patients (47%) had FXI:C in the severely deficient range. The seventy surgical

procedures comprised 19 major, 38 minor and 13 dental surgeries. Five patients undergoing dental procedures received concomitant tranexamic acid mouthwash.

Table 7.2: Patient characteristics of 43 FXI deficient patients who received FXI concentrate over a five year period 1996-2001.

Patient Demographics		
Total number of patients		43
Males		19
Females		24
Age (years)	Median	41
	Range	1-91
Baseline factor XI levels (U/dL)		
Severe deficiency (factor XI < 20 U/dL)		20
Partial deficiency (factor XI 20-70 U/dL)		23
Type of surgical procedure		
Major		19
Minor		38
Dental		13
Total		70

Patients received a median of one dose of FXI concentrate with a range of 1-5 doses (Table 7.3). The median dose administered was 1065 units (range 200-2640). The median post-infusion FXI:C was 65 U/dL and only one level > 100 U/dL was recorded. Haemostatic efficacy was documented in 66 of 70 surgical procedures (94%).

Table 7.3: Factor XI concentrate - Dose and post infusion levels.

Number of doses	
Median	1
Range	1-5
Total dose per episode (IU)	
Median	1065
Interquartile Range	750-1950
Post infusion factor XI level (U/dL)	
Median	65
Interquartile Range	58.3-78

A total of seven adverse events were documented but none were thrombotic events (table 7.4). No thromboembolic events were seen despite the fact that five patients aged > 70 years were treated and that seven angiography procedures were performed in individuals with ischaemic heart disease.

Four episodes of bleeding were recorded (6% of procedures); two resolved with further treatment with FXI concentrate and two resolved with surgical or local measures. Arterial punctures seem especially associated with bleeding but it should be noted that one patient had sub-optimal FXI:C levels and another had a surgical perforation. Three non-haemorrhagic adverse events were documented and none was serious or persistent. The development of FXI inhibitors was observed in two patients with severe FXI deficiency.

Table 7.4: Adverse events associated with factor XI concentrate.

Bleeding complications	Surgical procedure	Baseline factor XI level (IU/dL)	Post infusion factor XI level (IU/dL)	Outcome
Bilateral inguinal haematomata	Angioplasty	12	44	Resolved with 3 further doses FXI concentrate
Scrotal bruise/ subconjunctival haemorrhage	Insertion of endovascular stent	37	60	Resolved with a further dose FXI concentrate
Pericardial tamponade due to arterial perforation	Angioplasty	36	67	Resolved with serial pericardiocentesis and coronary artery stenting
Dental socket ooze	Extraction of 4 wisdom teeth	32	56	Resolved on packing with gauze
Adverse events				
Hypotension	Arthroscopy (cancelled due to reaction)	3.5	N/A	Resolved with IV hydrocortisone and chlorpheniramine
Bilateral parotid swelling	Hysterectomy/ nephrectomy	1	N/A	Resolved spontaneously
Paraesthesiae	Myringotomy	38	62	Resolved spontaneously
Inhibitor development				
BU = 7.6	Arthroscopy	1.5	38	Inhibitor screen negative 11/2001
BU = 1.9	Cataract extraction	<1	38	N/A

N/A = not available

7.2.3 Discussion

This five year evaluation of the use of a modified FXI concentrate confirms its efficacy and safety. A 94% haemostatic efficacy rate was recorded and no thrombotic complications were documented. FXI concentrate was successfully administered to five elderly patients (>70 years) and to patients with a history of cardiovascular disease although caution must be exercised in these categories of patients and a careful risk-benefit analysis done. This audit confirms that bleeding is effectively prevented by FXI concentrate. Thrombotic complications appear to be rare when guidelines regarding infusion of FXI are implemented.

7.3 The use of recombinant factor VIIa to prevent peri-operative haemorrhage in FXI deficiency

7.3.1 Methods

Between November 2001 and December 2002, consecutive patients with FXI deficiency with a planned major, minor or dental procedure were recruited to the study. Treatment with rFVIIa was offered to patients who, in the opinion of their treating clinician, would require haemostatic cover with FXI concentrate (i.e. the presence of a previous history of bleeding, procedure likely to result in bleeding, procedure with potential to result in bleeding in a critical area). Patient inclusion criteria were age > 18 years and severe (FXI:C <20 U/dL) or partial (FXI:C 20-70 U/dL) FXI deficiency or FXI deficiency with persistent high responding inhibitors. Exclusion criteria were known hypersensitivity to rFVIIa, uncontrolled disseminated intravascular coagulation, a recent thromboembolic event (within six weeks) or treatment with any haemostatic agent within 48 hours of the first dose of rFVIIa.

As a consequence of a thrombo-embolic adverse event after six months of patient recruitment, subsequent patients with a history of cardiovascular disease or with current angina or intermittent claudication were excluded. Informed consent was obtained in all cases and the protocol was approved by the Local Research Ethics Committee.

The first dose of rFVIIa was given immediately pre-operatively. A total of two doses (90 μg/kg) four hours apart was given to patients undergoing minor or dental procedures. For major procedures, rFVIIa at a dose of 90 μg/kg was given two hourly for the first twenty-four hours and four hourly for the second twenty-four hours. Oral tranexamic acid was given post-operatively for seven days, once rFVIIa treatment had finished for minor and dental procedures and once the patient was taking oral intake for major procedures.

The primary endpoints were the clinical assessment of haemostasis and the recording of adverse events. Clinical haemostasis ratings were graded as effective, partially effective or ineffective.(278) Secondary end-points were the use of additional haemostatic agents and the requirement for more than a two unit red cell transfusion. All endpoints were evaluated immediately post-operatively in all cases. Further evaluations were performed four hours post-operatively in patients undergoing minor or dental procedures and eight, 24 and 48 hours post-operatively in patients undergoing major procedures.

Pre-operatively, blood was collected into citrate tubes (0.106mol/L trisodium citrate, SARSTEDT Monovette, Nümbrecht, Germany) for analysis of the prothrombin (PT) time, d-dimers and factor VII coagulant activity (FVII:C). The coagulation tests were

repeated 15 minutes after the first dose of rFVIIa and pre and post the two or four hour dose in the case of major or minor/dental surgery respectively. Patients undergoing major surgery also had these coagulation tests repeated pre and post the 24 and 48 hour doses.

Thromboelastography (TEG) and a thrombin generation assay were also performed in a subgroup of samples where plasma was available(see Chapter 2 Methods).(116;279) The thrombin generation assay was performed on plasma which had been double spun and stored at -80^{0}C until assayed. Briefly, plasma was thawed and defibrinated with Reptilase, coagulation was initiated with recombinant tissue factor at a concentration of 10pg/mL and calcium. Subsampling from the test sample was performed at 30 second intervals into a stop solution containing EDTA and thrombin generation was determined by adding the chromogenic substrate S2238 and reading the absorbance at 405nm. Calculations of peak thrombin and area under the curve (AUC) were performed with reference to a standard curve generated using dilutions of bovine thrombin (starting concentration 1000 U/mL).

7.3.2 Results

Fourteen patients were recruited to the study and fifteen surgical or dental procedures were performed. Patient characteristics including severity of FXI deficiency, prior bleeding history and procedure type are given in Table 7.5. One patient (patient 14) had a high responding inhibitor to FXI. Inhibitors to FXI were not detected in the remaining patients.

Table 7.5: Patient demographics and procedures.

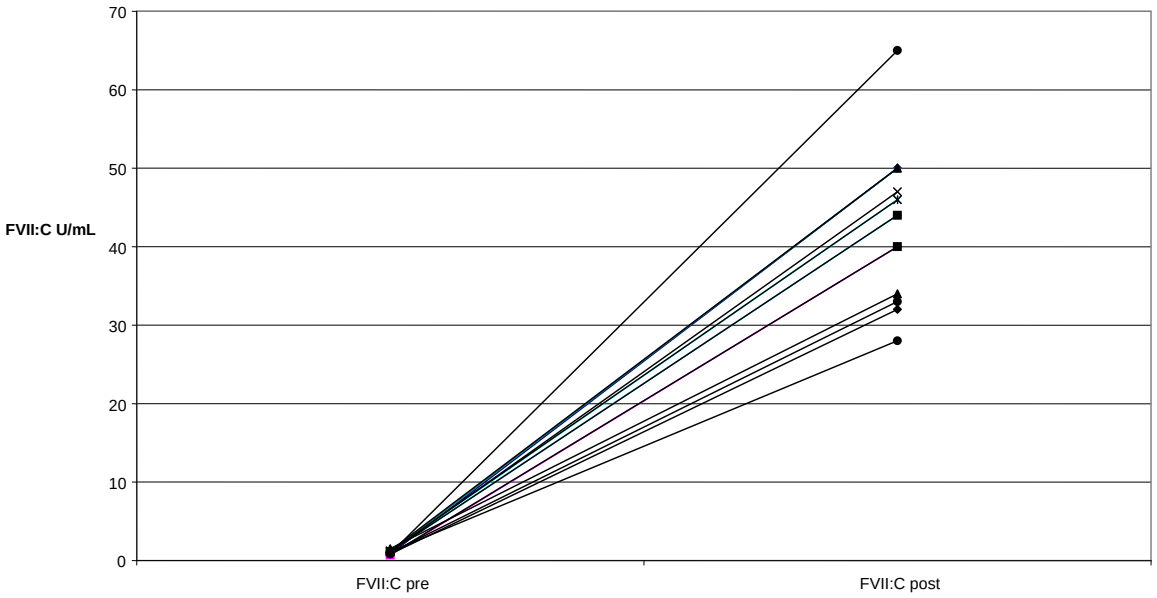
Patient	Age	Gender	Baseline FXI:C	Bleeding history	Procedure	Classification of procedure
1	24	Male	9	Yes	Molar tooth extraction	Dental
2*	43	Female	44	Yes	Removal of titanium implant	Dental
3	42	Female	56	Yes	Repair of common extensor origin (elbow)	Major
4	77	Male	48	Yes	Bilateral herniorraphy	Major
5	31	Female	44	Yes	Endometrial ablation/bilateral salpingectomy/diathermy	Major
6*	43	Female	44	Yes	2 dental titanium implants	Dental
7	77	Male	3	Yes	Hydrocoele repair	Major
8	38	Male	39	No (dental extractions)	Colonoscopy and biopsy	Minor
9	20	Female	45	Yes	Bone biopsy	Minor
10	25	Female	56	No (dental extractions)	Breast Symmetrisation	Minor
11	37	Male	58	Yes	Vasectomy	Minor
12	43	Female	42	Yes	Extraction 3 impacted wisdom teeth	Dental
13	62	Male	10	Yes	Extraction 1 impacted molar tooth	Dental
14†	65	Female	1	Yes	Total hip replacement	Major
15	59	Male	11	No (tonsillectomy, circumcision)	Extraction 1 wisdom tooth & 1 molar tooth	Dental

*Two procedures undertaken in the same patient. † This patient had a FXI inhibitor.

Haemostasis was classified as effective at all time points in all patients. No patient required an additional haemostatic agent or surgical intervention for unexpected haemorrhage and no red cell transfusions were given. One female patient, who underwent breast symmetrisation, had a decrease in haemoglobin from 11.4 g/dL pre-operatively to 9.5g/dL post-operatively and was given post-operative iron supplementation.

The prothrombin time shortened from a mean of 13.6 to 8.2 seconds (reference interval 12.9-13.9 seconds) after the first dose of rFVIIa and remained below the lower limit of the reference interval at all time points in all patients. FVII:C increased after administration of rFVIIa but there was considerable inter-individual variation (Figure 7.1). The median FVII:C level post the first dose of rFVIIa was 44 U/mL with a range of 28-65 U/mL. The trough level prior to the next dose was >6 U/mL in all cases with a range of 11-26 U/mL in major cases and 7-13 U/mL in minor cases.

Figure 7.1: FVII:C levels pre and post first dose of rFVIIa for all 15 procedures. All pre FVII:C levels were within the normal range (0.5-1.5 U/mL). Note that some patients had identical post rFVIIa FVII:C levels (three patients: 50 U/mL, two patients: 46 U/mL, two patients: 44 U/mL, two patients: 40 U/mL).



The thromboelastogram was evaluated pre and post rFVIIa in nine patients (four severely deficient, five partially deficient). All patients had a prolonged k time and a reduced angle pre rFVIIa while six of nine patients had an abnormally prolonged r time (Table 7.6). The TEG parameters corrected in all cases following therapy with rFVIIa.

Table 7.6: Thromboelastography (TEG) and thrombin generation test (TGT) results pre and post the first dose of rFVIIa.

Test	TEG						TGT			
Parameter (Normal)	r time (14-23s)		k time (3-7s)		Angle (31-49)		AUC (2910.9 U/mL)		Peak Thrombin (9.0 U/mL)	
Patient	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	32	14.9	10.7	4.8	18.5	37.5	.	.	.	.
2	22.5	7.6	9.2	3	20.5	53.5	3365.2	3829.9	11.3	11.4
3	28.6	9.9	24.8	3.8	9	44.5	1990.4	3313.8	5.1	10.5
4	23.9	9.2	8.7	2.4	25	49.5	1402.5	4303.4	4.7	11.6
5	16.9	11.4	11.6	5.8	18.5	44	.	.	.	.
9	.	.	.	.	.	.	3884.4	4614.1	12.1	13.8
12	20.8	11.8	7.3	3.6	30	46	.	.	.	.
13	28	11.3	10.1	3.1	21	59	.	.	.	.
14	55.1	10.7	12.7	2.4	20.5	62	2304.7	3779.2	2.9	16.6
15	36.4	16.8	12.6	5.9	19	39.5	1333.9	2832.5	1.8	10.1

Six patients (two severely deficient, four partially deficient) had thrombin generation in plasma measured before and after the first dose of rFVIIa (Table 7.5). The median AUC pre-rFVIIa was 2147.55 U/mL (AUC of pooled normal plasma 2910.9 U/mL) which increased to 3804.55 U/mL after rFVIIa. Peak thrombin increased from a median of 4.9 U/mL to a median of 11.5 U/mL after rFVIIa (pooled normal plasma 9.02 U).

Three adverse events were documented during the study. A 77-year-old male patient (patient 7, Table 7.4), with a history of a myocardial infarction eight years previously, developed clinical and radiological evidence of a cerebrovascular infarct after the 17th of 19 planned doses of rFVIIa and died three days later. The FVII:C levels obtained pre and post the first, second and 24 hour rFVIIa doses were similar to other patients in the study on the same dosing regimen. The maximum FVII:C level obtained was 73 U/mL post the second dose of rFVIIa. D-dimers became elevated in this patient at 24 hours post-operatively, as occurred in all patients undergoing major procedures. A 31-year-old female patient (patient 5, Table 7.4) with a strong history of atopy developed periorbital rash and itching associated with mildly elevated liver function tests. These symptoms resolved after discontinuation of treatment. Further investigations suggested an allergy to polysorbate 80, an excipient of rFVIIa. A 42-year-old female patient (patient 3, Table 7.4) developed a mild local phlebitis, which resolved with conservative therapy.

7.3.3 Discussion

There is no recombinant FXI replacement product under development at the present time. The efficacy and safety of rFVIIa is well established in the management of acute bleeds and invasive procedures in patients with severe FVIII or FIX deficiency and inhibitors. Inhibitors to FXI have been well described and are associated with severe FXI deficiency and exposure to plasma-derived FXI.(106) There was an early report of the use of rFVIIa (60 μg/kg three hourly for three days) in a patient with FXI deficiency and a FXI inhibitor undergoing an orchidectomy for testicular haemorrhage.(102) A number of case reports subsequently documented the use of rFVIIa in FXI deficiency and inhibitors.(107-109) Doses used ranged from 30-90 μg/kg. At the time that this study was designed and conducted, there were no other available data on the use of rFVIIa in patients with FXI deficiency. The dose of 90 μg/kg used in this study was chosen since there was extensive experience in prevention of bleeding in haemophilic patients undergoing surgical procedures at this dose.(280) There is also laboratory evidence that the haemostatic dose of rFVIIa is >70 μg/kg.(178)

Tranexamic acid has been used safely with rFVIIa in severe haemophilia with inhibitors. In this study, the additional use of tranexamic acid was deemed necessary since the period of cover with rFVIIa was limited to the immediate post-operative period and tranexamic acid was used to prevent late bleeding.

Thromboembolic adverse events are an uncommon but recognised adverse event associated with therapeutic use of activated clotting factors.(281) In common with other reports, the patient affected by a cerebrovascular accident in this study had pre-existing risk factors. Following this experience the protocol was modified and

subsequent patients were excluded if risk factors for thromboembolic disease were present. Patients with FXI deficiency do not appear to be inherently protected from arterial thrombosis by their coagulation factor deficiency.(169) Therefore, rFVIIa should be used with caution in such patients. More recently, reports have suggested that much lower doses of rVIIa, analogous to those used in FVII deficiency, may be effective in achieving haemostasis in FXI deficiency.(110;111) In the first of these case reports, a single dose of 12 µg/kg was sufficient to arrest a postpartum haemorrhage while in the second report, the use of bolus doses of 29 µg/kg and 18 µg/kg and continuous infusion rates of 1.1-3.6 µg/kg/hr was reported to prevent surgical bleeding during two separate spinal procedures in a single patient. These alternative dosing schedules merit further study in larger groups of patients.

Since the mechanism of action of rFVIIa is not replacement of a deficient coagulation factor, the optimal monitoring method is unclear.(282-284) Levels of FVII:C reach supra-physiological levels after therapeutic doses of rFVIIa and in vitro data suggest that haemostasis occurs if FVII:C levels are maintained at > 6 U/mL for several hours.(178) Clinical studies have revealed considerable inter-individual variation in FVII:C levels and the peak level of FVII:C is now felt to be the critical factor in promoting normal clot structure and maintaining haemostasis in patients with haemophilia.(285;286) In this study, all patients studied achieved peak FVII:C levels of >25 U/mL after one dose of rFVIIa. Trough levels were >6 U/mL, both with two hourly and four hourly bolus dosing (data not shown), which correlated with satisfactory haemostatic efficacy in all cases. Lower target FVII:C levels may be effective in patients with FXI deficiency as documented in a recent case report.(111)

Defective thrombin generation in FXI deficient patients causes inadequate activation of thrombin activatable fibrinolysis inhibitor (TAFI) and a consequent increase in fibrinolysis.(287) This correlates with the clinical observation of increased bleeding in areas of high fibrinolytic activity in patients with FXI deficiency.(62) In patients with severe haemophilia A, in-vitro studies have demonstrated that rFVIIa inhibits fibrinolysis via TAFI and the concentration of rFVIIa required shows considerable individual variation, mirroring the variability seen in FVII:C in-vivo.(173) Generation of thrombin at normal or supra-normal levels post therapy with rFVIIa is suggested by the normalisation of the TEG parameters in patients in this study and by the marked increase in the AUC and peak thrombin levels in the thrombin generation assay. Increased thrombin is postulated to lead to enhanced activation of TAFI with consequent downregulation of fibrinolysis. More recently, in a fluorogenic thrombin generation assay using PPP from five patients with inhibitors to FXI, rFVIIa corrected the ETP to 50% of normal at a concentration of 1 μg/mL (equivalent to a clinical dose of 45 μg/kg).(106)

7.4 Conclusion

Haemostatic therapy is indicated in some patients with FXI deficiency having invasive procedures, especially those with FXI:C in the severely deficient range, those undergoing surgery in areas with high fibrinolytic activity and patients with a history of surgical bleeding. The decision regarding haemostatic cover is also influenced by evaluation of the side-effect profile of individual treatment modalities.

The results of the audit of FXI concentrate confirm that this is an effective treatment and that thrombotic side effects are less common than previously reported, probably due to changes to the manufacturing process and implementation of treatment guidelines. However, it cannot be excluded that certain patients at higher risk of thrombo-embolic complications were not given FXI concentrate due to the presence of pre-existing risk factors.

In the pilot study of rFVIIa in combination with post-operative tranexamic acid, haemostatic efficacy was excellent in all patients. The use of a recombinant product to prevent surgical bleeding in FXI deficiency is especially desirable in younger patients, children and pregnant women, in view of emerging risks of transfusion including new variant Creuzfeldt Jakob disease.(288;289) However, one fatal thrombo-embolic event occurred in a patient whose only risk factor was a remote history of coronary artery disease. This patient received cover with rFVIIa at a dose of 90 μg/kg two to four hourly for 48 hours and was receiving concomitant tranexamic acid orally. Lower doses of rFVIIa have been effective in clinical case reports and a concentration of rFVIIa equivalent to a dose of 45 μg/kg normalised thrombin generation in-vitro.(106;110;111) In addition to a reduction in dose, a reduction in the duration of cover with rFVIIa should also be evaluated. Tranexamic acid could be used to provide haemostatic cover after rFVIIa cover has been discontinued but probably should not be used with rFVIIa in FXI deficient patients. Caution is required when considering either FXI concentrate or rFVIIa in patients with pre-existing risk factors for thrombosis including atherosclerosis. This group of FXI deficient patients continues to present a therapeutic dilemma to clinicians when undergoing invasive procedures.

Chapter 8 Discussion

8.1 Introduction

Factor XI deficiency is classified as a rare bleeding disorder.(80) However, when registry data is evaluated FXI deficiency is one of the more common of the rare bleeding disorders recorded, accounting for approximately 37% of rare bleeding disorders registered with the World Federation of Haemophilia global survey and the International Rare Bleeding Disorders database.(290) Many affected patients are concentrated in geographic areas associated with ethnic groups with a high allele frequency for *F11* gene mutations causing FXI deficiency. While important information may be gleaned from individual cases or small case series, only a few research centres worldwide are located in geographic areas with the potential to recruit larger numbers of FXI deficient patients. The Katharine Dormandy Haemophilia centre and Haemostasis unit is located in such a geographic area and researchers based here have made significant contributions to the published literature in FXI deficiency.(5;7;55;64;241) The purpose of the research work conducted for this thesis was to further develop and advance the clinical research programme in FXI deficiency at the Royal Free & University College Medical School.

The aim of the work presented in this thesis was

- to comprehensively document the bleeding tendency in a cohort of patients with FXI deficiency
- to accurately diagnose the FXI deficiency in those patients by evaluating the FXI:C, FXI:Ag and the causative genetic mutation where possible
- to investigate factors which may influence the bleeding tendency in FXI deficiency (including those previously published i.e. VWF, those which had

recently been identified i.e thrombophilic traits and TAFI and those which had not been comprehensively evaluated in FXI deficiency i.e. Thrombin generation)

- to develop a molecular homology model for the FXI protein (in the absence of a crystal structure) and to model known and novel FXI mutations
- to evaluate a novel treatment (rFVIIa) for the prevention of surgical bleeding in FXI deficiency

8.2 Summary of thesis findings

8.2.1 Bleeding tendency, diagnosis of FXI deficiency and other coagulation tests

The 102 participating patients in this study represent a large cohort of patients with FXI deficiency. In particular, two thirds of the patients included in this study are partially deficient in FXI which is an under-represented group in the literature.

The bleeding tendency was recorded using a standardised data collection form (appendix 10.1). The bleeding score was assigned independently by three clinicians experienced in Haemostasis and a consensus score was obtained (81% agreement overall). The source data, on which the bleeding score was determined, was recorded in an Access (Microsoft ® 2001) database to allow additional evaluation of bleeding related to haemostatic challenges. At the time of this study, the bleeding score described had previously been used in patients with FXI deficiency.(5) Subsequently, the importance of a standardised bleeding score has been recognised and disease specific bleeding questionnaires and scores have been developed.(291-294) The bleeding score used in this study has not been compared to the more recently developed scores, nor have the recently developed scores been validated in FXI deficiency.

The evaluation of the patients participating in this study included the reassessment of the FXI:C levels in these patients and comparison to their registered levels. FXI:C levels in the normal range were found in 17 patients who had previously been registered as FXI deficient. Of these 17 patients, 10 patients were confirmed to have a causative Jewish mutation for FXI deficiency (eight had known Jewish heritage, the others were English and Turkish respectively) emphasising that in a minority of patients, carrier status for this condition will be present even in the presence of normal FXI:C and FXI:Ag levels. This has implications for genetic counselling in individuals with normal FXI:C levels. The possibility of changing levels with age or with changes in assay techniques should be considered in previously diagnosed patients whose current FXI levels are normal and consideration should be given to genetic confirmation in these cases.

In this study, FXI:Ag was documented for all patients, which had not been done previously. Indeed, there had been a view that there were no CRM+ mutations causing FXI deficiency and that all FXI deficiency was caused by a failure of secretion of the mutated protein.(295) This has been demonstrated not to be true, both by the identification of patients with a CRM+ phenotype within this study (Gly155Glu, Ser576Arg) and by the identification of 11 CRM+ mutations in the *F11* gene, mainly within the SP domain.(260) In this study, there was good concordance between FXI:C and FXI:Ag (r=0.95). However, one severely deficient patient was documented to have a normal FXI:Ag and a lupus type inhibitor causing a severe reduction in FXI activity and this patient does not have a true inherited FXI deficiency. Nine partially deficient patients had discordant results, in five of these cases FXI:C was normal, FXI:Ag was low and a Jewish mutation was confirmed. Therefore, accurate and comprehensive

diagnosis requires the analysis of FXI:Ag as well as FXI:C in patients with suspected FXI deficiency.

Genetic mutations causing FXI deficiency were identified in 83 patients in this study, of a total of 98 patients with definite FXI deficiency who were screened. Of 21 patients with a definite FXI deficiency and without the presence of a Jewish mutation, six were found to have a mutation in the *F11* gene and novel mutations were found in two patients (Glu297Lys and Glu380Lys). One patient had a mutation identified which was subsequently shown to be a non-disease causing polymorphism (Pro48Leu, NCBI dbSNP ID rs5968). Therefore, there are 16 patients, in this cohort, in whom a mutation has not been identified. Of these, 11 are of English descent and the C128X mutation has been outruled in all of them.(258) Further sequencing work is being undertaken to clarify whether a mutation is present in these 16 patients.

In regards to factors influencing the bleeding tendency, the firmest association was confirmed to be the presence of a severe deficiency of FXI in this study, with 82.3% of severely deficient patients being classified as bleeders. A number of other factors were evaluated to determine whether an effect on bleeding tendency was evident (including VWF, FVIII, blood group, thrombophilic traits). No relationship is proven between the levels of VWF:Ag or VWF:CBA and bleeding tendency and the use of VWF and FVIII cut off levels of 70 IU/dL was not predictive of bleeding in contrast to earlier studies.(7;61;65) There was no significant difference in proportions of bleeders between the common blood groups. A slightly higher proportion of patients with thrombophilic traits were identified as bleeders (63.5%) thus making it unlikely that

thrombophilic traits were attenuating the bleeding tendency, as had been reported for severe Haemophilia.(67;211-214)

Evaluation of one potential genetic modifier of glycosylation did not reveal an association with an increased tendency to bleed but considering the evidence for alterations in clinical phenotype in VWD and the association between congenital disorders of glyscosylation and FXI deficiency, this is a research area which would benefit from further study.(216-218)

It should be noted that no studies of platelet function were done as part of this work and therefore, it is possible that some patients with a significant bleeding tendency have an underlying, undiagnosed inherited or acquired platelet function defect (diagnosed in 23.2% of patients under investigation for mucocutaneous bleeding in one study).(296) However, it is difficult to accept that such conditions would account for the bleeding tendency in the 50% of partially deficient patients in this study who are classified as bleeders.

8.2.2 Thrombin generation assays

Thrombin generation has been recently re-evaluated in disorders of bleeding and thrombosis as researchers have identified the need for a more "global" test of haemostatic function. Since the FXI:C level in partially deficient patients is not correlated with bleeding tendency, the determination of global thrombin generation as measured by the ETP offers an alternative means of identifying those 50% of partially deficient patients who are likely to bleed with invasive procedures. The TGT test, like the CLT described above, is a dynamic test and results vary depending on

concentrations of reagents used, in particular the type and concentration of the initiator of coagulation. Consequently, the earliest reports of thrombin generation in FXI deficient plasma stated that thrombin generation was normal.(119) However, this study and subsequent studies have shown this is not the case if lower concentrations of TF are used to initiate coagulation.(43) In this study, a significant difference in ETP was demonstrated between partially deficient patients with a history of bleeding (median ETP 52.4%) versus partially deficient patients without a bleeding history (median ETP 77.3%). Concomitant low levels of FVIII, VWF or fibrinogen or conversely the presence of a thrombophilic trait were not clearly linked to the variations in ETP between bleeders and non-bleeders.

There are limitations to the TGT used in this study. A chromogenic, semi-automated method was used but more recently, a calibrated automated fluorogenic thrombin generation assay (Thrombinoscope BV, the Netherlands) has been developed and allows more standardisation of the method. Timed pipettes, the use of a calibration standard and a specific software program enabling calculation of thrombin activity results in lower CVs.(297)

The use of PPP was required for this study as patients attended a dedicated clinic for clinical review and blood draw some months in advance of the laboratory analysis of the plasma samples. However, analysis of PRP may be expected to give a more comprehensive evaluation of global thrombin generation and would include the contribution of platelets, which are known to bind FXI via specific residues in apple 3.(34)

The specific inhibitor of the contact pathway, CTI, was not used in the TGT described in this study. Available data at the time showed that contact activation did not occur to a significant degree in a dilute thromboplastin assay and that no differences were seen in the thrombin generation curves obtained in the presence and absence of a monoclonal antibody to FXII in severe FXI plasma.(43;136) The use of CTI has been recommended in a fluorogenic TGT but has not been evaluated in a chromogenic assay.(137) More recently, studies have suggested that the requirement for CTI may be dependent on the concentration of TF used to initiate thrombin generation with CTI not needed at TF concentrations greater than 0.5pM.(298;299)

In a recent study, thrombin generation has been evaluated in 24 patients with FXI deficiency (9 severely deficient, 15 partially deficient) in a fluorogenic assay using PRP in the presence of CTI.(300) Results show that lag-time, thrombin peak and thrombin velocity but not ETP are correlated with FXI:C levels and with bleeding tendency independent of FXI activity. Of note, in this study only 9 patients had a documented bleeding tendency and only three of these had a partial deficiency of FXI. In the latter study, all patients were documented to have a normal prothrombin level. In the cohort of 102 patients under study here, prothrombin levels were not specifically assayed and could be a determinant of ETP, therefore in future studies of thrombin generation, prothrombin levels should be assayed and consideration given to assaying other relevant coagulation factors (both pro- and anticoagulant) in addition to FXI.

8.2.3 TAFI

The discovery of the TAFI molecule generated significant interest in the late 1990s as TAFI represents a clear link between the coagulation and fibrinolysis systems.(152) The role of TAFI is of particular interest in patients with FXI deficiency since it has been long recognised that bleeding is especially likely to occur after invasive procedures to areas with high intrinsic fibrinolytic activity.(63) TAFI activation has been demonstrated to be dependent on thrombin generated in a FXI dependent fashion.(165) The hypothesis generated was that alterations in TAFI antigen or activity could account for some of the variation in bleeding tendency seen in FXI deficient patients. In this study, there was no link identified between TAFI antigen and bleeding tendency although a possible limitation is the use of an elisa TAFI:Ag kit which gives a lower TAFI:Ag level in patients with one or two Isoleucine alleles at position 325, as a consequence of the TAFI polymorphism Thr325Ile. The TAFI-Ile325 isoform has a longer half-life and greater antifibrinolytic activity than the Threonine isoform.(301) So while the lower TAFI:Ag levels might be expected to lead to an increased bleeding tendency, the greater antifibrinolytic effect of the Ile isoform might be expected to be associated with a decreased bleeding tendency. Notably, the lower TAFI levels in patients homozygous for Isoleucine at position 325 have been recently shown to confer a lower risk of arterial thrombosis compared to patients homozygous for Threonine.(302)

A commercially produced TAFI activation kit was assessed and found not to be fit for purpose and therefore TAFI activity was assessed indirectly using a well-described clot lysis assay, which has been shown to correlate with both TAFI:Ag and TAFI activity in normal individuals.(160) While the relationship between CLT and TAFI:Ag levels is

lost when a specific inhibitor to FXI is added, this experimental situation may be expected to mirror severe FXI deficiency rather than partial FXI deficiency. In partial FXI deficiency, thrombin generation is not completely abolished even at low thrombin concentrations and therefore the CLT should correlate to TAFI:Ag. However, in this study, no variation in CLT was identified between bleeders and non-bleeders with FXI deficiency (9 severely deficient and 38 partially deficient patients). In addition, the CLT was not correlated with TAFI:Ag.

Of note, the CLT was initiated with low concentrations of TF. It is possible that not all of the patient's TAFI was activated in this particular CLT since activation is thrombin dependent and that the use of thrombin or higher concentrations of TF as the initiator would allow more complete activation of TAFI which could then be correlated with bleeding tendency. The concentration of TF used to initiate clot lysis has been shown to be a critical factor in determining whether normal or reduced CLT are obtained in severe Haemophilia A plasma.(177)

Alternative genotype independent elisa assays have been developed to measure the intact TAFI molecule (TAFI), the activation peptide (TAFI-AP) and the inactivated TAFI protein (TAFIa(i)).(303) Using these elisas, TAFIa(i) levels were shown to be higher in patients with cardiovascular disease (both coronary artery disease and ischaemic stroke) and there were different patterns of association between the various measures of TAFI depending on the underlying disease (for example, intact TAFI levels were higher than controls and the Ile isoform was protective in coronary artery disease only).(302) Specifically in ischaemic stroke, the activation peptide (TAFI-AP) showed the strongest association with all subtypes of ischaemic stroke.(304) It should be noted

that FXI deficiency appears to protect against ischaemic stroke but not against myocardial infarction and analysis of the different TAFI elisas has the potential to shed some light on the two disease processes.(169;305) Overall, a definite link between TAFI and bleeding tendency in FXI deficiency has not been proven but clearly there are many contentious issues in the appropriate tests required to evaluate the true impact of TAFI on bleeding and thrombosis. This is a research area which requires further evaluation.

8.2.4 Molecular modelling of protein structural effects caused by *F11* mutations

The development of molecular models for the four apple domains and the serine protease domain of FXI was a significant advance in this study.(306) Prior to the development of these models, rudimentary *ab initio* models had been used which were not accurate enough to give reliable information about the structural effects of mutations on the FXI protein.(14;34;252;268-270) The other significant advance was the universal access to these models and to the comprehensive database of FXI mutations which had been generated as part of the project.(273) The web-based curated database (www.factorXI.org) continues to be a widely used and quoted resource for researchers in the field of FXI deficiency.

The homology models were limited by the lack of availability of a crystal structure so that all interpretations were necessarily guarded in certain respects. The most obvious limitation was the lack of information on how the four apple domains and the serine protease packed together, both as monomer and dimer. In addition, while the core of the protein models was well-predicted, prediction of the insertions and deletions in loop

regions and the positions of the sidechains of amino acids was expected to be less accurate. The publication of the crystal structure provided both the means to accurately model the protein effects of mutations in the *F11* gene but also allowed an evaluation of the three-dimensional arrangement of the FXI protein domains in their dimeric conformation.(47) The pre-existing molecular homology models were compared to the crystal structure and analysis confirmed that the homology models were very accurate with small differences found in the surface insertion loops and N- and C- termini of the apple domains, as was predicted.(260) The web-based FXI database now uses the crystal structure to predict structural changes in the FXI protein caused by missense mutations. The residual limitation is that modelling is relevant only to missense mutations and evaluation of the effects of other sequence changes on protein expression and function requires alternative approaches. Although, genetic mutations in the *F11* gene have not been shown to cause variation in bleeding tendency, knowledge of the effect of the mutation on the protein structure does allow understanding and explanation of CRM- and CRM+ phenotypes.

8.2.5 Prevention and treatment of bleeding in FXI deficiency

The variable bleeding tendency in FXI deficient patients is well-recognised and is seen in severely and partially FXI deficient patients in the United Kingdom. In fact, the maximum variation in bleeding tendency in this study was seen in partially deficient patients, with half of these patients exhibiting a bleeding tendency. The severely deficient patients were almost all designated as bleeders. As such, the prevailing practice in the UK would be to replace FXI in severely deficient patients when undergoing major surgery and to consider replacement in partially deficient patients, depending on personal bleeding history and the nature of the proposed invasive

procedure.(80) This contrasts with the published approach by the Israeli group.(62;71) In Israel, partially deficient patients do not have replacement of FXI and alternative bleeding disorders are sought in patients with a bleeding history. It is felt that such patients do not have a significant bleeding tendency (U.Seligsohn, personal communication). It seems unlikely that there are true differences in bleeding between Israeli patients heterozygous for the type II or III mutations versus UK residents with Ashkenazi Jewish heritage and the same genetic mutations. An alternative explanation may be that there is a cultural difference in the acceptance of bleeding associated with invasive procedures.

Severely deficient patients in Israel do not routinely receive FXI replacement as cover for major surgery, as stated in a recent publication.(62) There is agreement that the highest risk of bleeding occurs with invasive procedures on tissues with high fibrinolytic activity. The haemostatic level of FXI is felt to be 30-45 U/dL in Israel (U. Seligsohn, personal communication) and was said to be 30 U/dL in a small case series of 5 severely deficient patients undergoing urological surgery whereas restoring FXI:C to 70 U/dL would be normal practice in the United Kingdom if replacement is advocated.(54;80) Finally plasma is used as FXI replacement in Israel whereas virally inactivated FXI concentrate obtained by plasma fractionation is generally recommended for replacement in the United Kingdom, unless there is a thrombotic contra-indication.(81)

With-holding of FXI replacement is advocated by Israelis but it is not likely to prove acceptable in many countries to expose patients to the morbidity of bleeding, surgical wound complications and infection/poor healing and possible requirement for plasma or

other cellular blood components which represent a higher risk for transfusion transmitted infection than a virally inactivated factor concentrate. In addition, it is questionable whether patients will accept a bleeding risk of up to 40% for surgery on sites without high fibrinolytic activity and in some jurisdictions, such bleeding rates may be medico-legally unacceptable.(62) Management of labour and delivery is particularly challenging in this regard. Performing a Caesarean section in the United Kingdom in a woman with severe FXI deficiency without FXI replacement is not likely to be deemed standard practice although the Israeli group have published data on Caesarean sections without FXI replacement in 12 of 18 severely deficient women.(71;80) Two of the twelve women (16.7%) not covered with plasma were reported to have had post-partum bleeding in the latter study, compared to 24% of vaginal deliveries not covered with plasma.

Bearing in mind these differences in clinical practice globally, the final part of this study evaluated haemostatic cover for invasive procedures in FXI deficient patients. Initially an audit was performed of 70 surgical episodes in 43 patients treated with FXI concentrate over a five year period. Of these treatment episodes, 19 were classified as major procedures and of the patients treated, 20 (46.5%) had a severe FXI deficiency. No thrombo-embolic events were recorded although five patients were aged >70 years and seven angiography procedures were conducted in patients with suspected ischaemic heart disease. Overall, the results of this five year audit of FXI concentrate usage provide reassurance in regards to the lack of thrombotic complications even in patients with possible cardiovascular disease.

However, the fact that FXI concentrate is a plasma derived coagulation product continues to pose a small but real risk for transmission of viral or novel infections. In certain patient groups (e.g. children, expectant women) it may be desirable to avoid plasma products where possible. Consequently, the use of rFVIIa to prevent bleeding after invasive procedures was evaluated since there was evidence of efficacy in FXI deficiency in case reports in the literature.(102;107-109) While rFVIIa proved very effective and haemostasis was effective in all patients, a possible confounding factor was the universal use of tranexamic acid with rFVIIa. Since rFVIIa has a short half-life and since it was being used for a very limited time (two doses for minor procedures and three days for major procedures) there was a concern regarding late bleeding. The rationale for the use of tranexamic acid therefore was to provide cover for the short half-life of rFVIIa and to prevent delayed bleeding. It may have been preferable to start the tranexamic acid after the rFVIIa administration was complete or to exclude it altogether. There were three adverse events of which two were minor but one (a stroke in an elderly man with a remote history of cardiovascular disease) resulted in the death of the patient. Subsequently, there has been some interest the use of a reduced dose of rFVIIa and it has been suggested that lower doses may be sufficient in FXI deficient patients.(110;111) Lower doses have been successfully used recently in FXI deficient patients with inhibitors.(307;308) Further work is required in-vitro and in-vivo to confirm the optimal dose of rFVIIa in FXI deficiency.

8.3 Future work

Arising from the work presented in this thesis, a number of areas warrant further investigation in the elucidation of the variable bleeding tendency in FXI deficiency. Firstly, no real-time platelet studies were possible in the patient cohort under study due

to the design of the study. Given that there is still some debate regarding the underlying cause of bleeding in the 50% of partially FXI deficient patients with a bleeding history, a comprehensive evaluation of platelet function in the patients already characterised in this study could be undertaken. Since the patients in this study have already had a full bleeding history and have had other possible confounding factors evaluated (e.g. VWF, FVIII, thrombophilic traits, TAFI), measurement of platelet function parameters such as platelet aggregometry, platelet flow cytometry, platelet nucleotides or other research tests could be correlated relatively easily with bleeding tendency. The theory that patients with partial FXI deficiency bleed due to underlying platelet disorders could then be proven or refuted.

While TAFI antigen and activity levels were not shown to be correlated with bleeding tendency in the assays undertaken as part of this study, there is no consensus at this time regarding the optimal TAFI assay. Alternative TAFI assays, for example evaluation of the activation peptide or the inactive form of TAFI, may prove more relevant measures of TAFI activation in-vivo in the future.

Thrombin generation assays are perhaps the most promising area of future study in FXI deficiency. In this regard, evaluation of thrombin generation in PRP is recommended and the use of a well-standardised and optimally controlled method is advocated. In particular, such assays may yet hold the key to identifying those partially deficient patients with a bleeding tendency who may need haemostatic cover for invasive procedures. This is especially important since many newly diagnosed FXI deficient patients have not had unsupported invasive procedures in areas of high fibrinolytic activity such as dental extraction or tonsillectomy, as a consequence of improved dental

health and changing medical practice. In these patients, the bleeding history is "indeterminate" and an informative laboratory assessment of bleeding tendency would significantly improve clinical decision making regarding the need for haemostatic cover.

Recombinant factor VIIa remains a promising haemostatic agent for patients with FXI deficiency who wish to avoid the use of plasma products. Further ex vivo evidence for haemostatic levels of rFVIIa is required and evaluation of changes in thrombin generation are an appropriate assay for ex-vivo investigation. Subsequently, further in-vivo studies should be done, using lower doses of rFVIIa, with or without concomitant tranexamic acid.

Finally, the link between congenital disorders of glycosylation and FXI deficiency is an intriguing one and further assessment of the glycosylation of the FXI protein in these patients may yield insight into the function of abnormally glycosylated FXI. Potential modifiers of FXI glycosylation could then be evaluated in FXI deficient patients

8.4 Conclusion

In conclusion, the aims of this thesis to comprehensively evaluate the bleeding tendency in a cohort of FXI deficient patients was achieved. A number of potential modifiers of the bleeding tendency were outruled and thrombin generation was identified as a laboratory test with the potential to discriminate between bleeders and non-bleeders with FXI deficiency. Two novel mutations in the *F11* gene were identified in patients participating in this study. The first accurate homology models were published along with a comprehensive structural interpretation of all known missense mutations. A novel treatment (rFVIIa) was evaluated in clinical study setting in a case series of

patients with FXI deficiency. A number of areas have been identified for future study, including evaluation of platelet function, newer assays for TAFI, thrombin generation in PRP, glycosylation of the FXI protein and optimal dosing of rFVIIa.

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10 Appendices

10.1 Research documentation

10.1.1 Patient information leaflet and patient consent form for Factor XI genetic study

INFORMATION FOR PATIENTS

A STUDY OF THE GENETIC FACTORS WHICH INFLUENCE BLEEDING IN PEOPLE WITH FACTOR XI DEFICIENCY

Version 1: 28/09/01

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box1365, London N16 0BW.

Thank you for reading this.

What is factor XI deficiency?

Factor XI (eleven) is an important clotting protein which is present in blood. It helps to create a clot if you cut yourself or if you are having an operation.

Factor XI deficiency is an inherited condition whereby the levels of factor XI are low in the blood. This can lead to bleeding after surgical operations, dental extractions, childbirth or after accidental injury. However, some people have no or very little bleeding even though their factor XI level is low. We would like to be able to predict which patients have a bleeding tendency and need treatment for surgery and which patients do not bleed so that we do not treat them unnecessarily.

We believe that if we had more information about the types of genetic mutation which cause factor XI deficiency we would be able to predict the bleeding tendency even in someone who has never had an operation before.

What is the purpose of the study?

The purpose of the study is to collect information from patients with factor XI deficiency about whether they have had any bleeding problems and to take a blood sample for laboratory analysis.

Why have I been chosen?

We are inviting patients registered with Factor XI deficiency in the Katharine Dormandy Haemophilia Centre and Haemostasis Unit to attend one clinic visit at the centre.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

You will be seen at the outpatient clinic on a Monday afternoon. There will be a brief interview to document any bleeding problems you may have had. Then, the blood sample will be taken. It should not take more than one hour.

What are the side effects of taking part?

Taking a blood sample usually does not cause any problems. Rarely, people may feel faint or develop a bruise over the vein. Nurses who are specialists in bleeding disorders will take the blood samples.

What are the possible benefits of taking part?

At the end of the study, we hope to be able to predict whether people will bleed or not regardless of their level of factor XI. However, we won't know until the end of the study if we will be successful. In the meantime you will continue to receive treatment according to the national guidelines for treating factor XI deficiency. Knowing the mutation which causes factor XI deficiency may benefit you in the future but we cannot guarantee this.

What if something goes wrong?

Any adverse event that occurs during this study will be reported to Professor Lee and to the Local Research Ethics Committee.
If you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Your records may also be looked at by people regulatory authorities to check that the study is being carried out correctly. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?
The results will be published in an internationally renowned journal of bleeding disorders. Copies of the article will be available in the Katharine Dormandy Haemophilia Centre. Participants will not be identified in any report/publication.

Who is organising and funding the research?

The research is organised by the doctors at the Katharine Dormandy Haemophilia Centre and Haemostasis Unit. The research is funded by a Clinical Research Fellowship grant from the Royal Free hospital NHS Trust and charitable grants to the Katharine Dormandy Haemophilia Centre and Haemostasis Unit.

Who has reviewed the study?

The study has been reviewed by the Royal Free Hospital and Medical School Local Research Ethics Committee.

Contact for Further Information

Dr. Niamh O'Connell, Clinical Research fellow or Professor Lee, Haemophilia Centre Director will be happy to answer any questions you may have about factor XI deficiency or rFVIIa treatment.

Phone: 0207 830 2068

Fax: 0208 830 2178

Email: n.o'connell@rfc.ucl.ac.uk

We would like to thank you for taking time to read this information leaflet and for considering participation in this study.

You may keep a copy of this Patient Information Sheet and your signed consent form.

CONSENT FORM

Title of Project:

A study of genetic factors which influence the bleeding tendency in patients with factor XI deficiency.

Name of Researchers:
Professor Christine A. Lee / Dr. Niamh O'Connell

	Please initial box
1. I confirm that I have read and understand the information sheet dated..... (version.....) for the above study and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
4. I agree to take part in the above study.	☐

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Researcher	Date	Signature

10.1.2 Patient data collection form

FACTOR XI GENETIC STUDY

DATE: **HISTORY TAKEN BY:**

PATIENT DEMOGRAPHICS:

NAME	
MRN	
DOB	
ETHNIC ORIGIN	
RELIGION	
BASELINE FXI LEVEL	
BLOOD GROUP if recorded	

DIAGNOSIS OF FACTOR XI DEFICIENCY:

Indicate circumstance of diagnosis:

BLEEDING HISTORY:

	Yes/No	Bled? Yes/No	Tfn?	Factor Repl?	Description
Dental Extraction					
Bruising					
Epistaxis					
Trauma					
Major Surgery					
Circumcision if male					
Minor surgery					

MEDICATIONS:

NB: record any use of ASA/ NSAIDs/ Anticoagulants

FAMILY HISTORY:

Other first degree relative affected?	Yes No
Number of relatives affected	
Type of bleeding problem:	
Dental Major surgery Minor surgery Epistaxis Trauma Menorrhagia PPH	

Attach pedigree: Yes No

FEMALE PATIENTS:

Menorrhagia?	
Age of menarche	
Ever used? COCP	
POP	
IUD	
Periods (subjective)	Light Normal Heavy
Clots	Yes /No
Double protection	Yes /No
Housebound during menses	Yes /No
Were menses *always* heavy?	Yes /No
Ever anaemic due to menorrhagia?	Yes /No
If yes, given treatment?	Iron Blood transfusion
Seen by a doctor for menorrhagia?	Yes /No
Given treatment?	COCP Progestagen T.Acid Analgesia Other
Abortion/Miscarriage	Yes /No
Gestation	
Bleeding	Yes /No
Treatment given?	ERPC/D&C Factor Conc….FFP….TA…other
Post partum haemorrhage?	Yes /No
Mode of delivery (VD/VD+forceps/vac/epis/ CS)	
Epidural	Yes /No
Transfusion?	Yes /No
Number of units transfused	
Treatment given?	
Factor XI concentrate FFP Tranexamic Acid Other	

TREATMENT EPISODES:

Number of treatment episodes	
Treatment used:	
Factor XI concentrate	
rFVIIa	
FFP	
Tranexamic acid	
Fibrin glue	
Other	

Vaccination? **Hep A** **Hep B**

Date:

Retested today?

10.1.3 Ethics approval number and Trust clearance for Factor XI genetic and clinical phenotype study

Royal Free Hampstead NHS
NHS Trust

Clinical Governance Support Centre
Royal Free Hospital
Pond Street
London NW3 2QG
Tel:020 7830 2816
Fax:020 7830 2233
zoe.spyvee@rfh.nthames.nhs.uk

Dr Niamh O'Connell
Haemophilia Centre
Royal Free Hampstead NHS Trust
Pond Street
Hampstead
London
NW3 2QG

08 October 2001

Dear Dr O'Connell

Factor XI deficiency: A comprehensive study of genetic mutations, protein structural abnormalities and clinical phenotype

Project ID: 5664 **(Please quote on ALL correspondence)**

Ethics ID: 5664

Following the approval of your ethics application and registration form your project has been fully registered with the R&D department. I would be grateful if you would inform me of any changes regarding funding, project status e.t.c

Should you have any queries please quote the ID number.

You may proceed with your project.

Yours Sincerely,

Zoe Spyvee
Research and Development Officer

10.1.4 Recombinant factor VIIa (FaXID) information leaflet and consent form

INFORMATION FOR PATIENTS

TRIAL TO ASSESS THE USE OF RECOMBINANT FACTOR VIIA TO TREAT AND PREVENT BLEEDING IN PEOPLE WITH FACTOR XI DEFICIENCY

Version 2.3: 23/10/01

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is factor XI deficiency?

Factor XI (eleven) is an important clotting protein which is present in blood. It helps to create a clot if you cut yourself or if you are having an operation.

Factor XI deficiency is an inherited condition whereby the levels of factor XI are low in the blood. This can lead to bleeding after surgical operations, dental extractions, childbirth or after accidental injury.

The treatment of such bleeding is to give an injection of factor XI concentrate to stop the bleeding. The factor concentrate is derived from blood donations and is tested and treated to prevent transmission of any infections. However, there is still a tiny risk of transmission of an infection with this product. In addition, factor XI concentrate leads to clotting in some people.

What is the drug that is being tested?

A new product to stop bleeding, recombinant factor VIIa (rFVIIa) was developed initially for the treatment of patients with haemophilia A and B who have inhibitors. It is highly effective in treating bleeding in those patients without any significant side effects. It has been used successfully all over the world for 12 years in patients with haemophilia.

Recently, rFVIIa has been used successfully in 2 patients with factor XI deficiency undergoing surgery.
rFVIIa is a recombinant product which means that it is not made from blood donations. No human proteins are involved in the manufacturing process. It should therefore be safer than the factor XI concentrate. In addition, it is not associated with clotting problems in most people.

What is the purpose of the study?

We feel that rFVIIa is a better treatment for patients with factor XI deficiency than factor XI concentrate and therefore we are offering it as first line treatment. In order to learn more about how this treatment works in patients with factor XI deficiency, we need to gather information about this treatment and the patients receiving it, e.g. baseline levels of factor XI, types of surgery, results of monitoring tests, any side effects. This study is designed to gather that information by offering this treatment to patients with factor XI deficiency who are having an operation, dental extraction or are having a baby over the next two years.

Why have I been chosen?

Patients registered with Factor XI deficiency in the Katharine Dormandy Haemophilia Centre and Haemostasis Unit who are having a planned surgical procedure or dental extraction or who are having a baby or who have bleeding after an accident are being invited to participate in this study. We hope that approximately 30 patients will participate in this study over two years.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

You will be seen at the outpatient clinic to plan your surgery, dental extraction or the birth of your baby, as usual. You will be given this information sheet and if you agree to participate, we will arrange to give you rFVIIa treatment at the time of the operation or birth instead of Factor XI concentrate. The rFVIIa treatment is given by injection into a vein like other clotting factor concentrates. The number of injections of rFVIIa needed depends on the type of operation you are having and your doctor will discuss this with you in advance. We will be monitoring the amount of blood loss during and after your procedure as usual. If there is more bleeding than anticipated, some extra injections of rFVIIa may be needed or we may need to give you another form of treatment such as factor XI concentrate. We will inform you if we think these extra treatments are necessary. The study ends once you are discharged from the hospital after your procedure.

What tests are involved in this study?

Routine blood tests are taken before the operation to check the blood count, kidney and liver function, baseline clotting tests and virology tests including tests for hepatitis and HIV. These would be taken whether you participate in the study or not. In addition, specific tests to monitor the rFVIIa treatment will be taken before the procedure and at 2, 24 and 48 hours after the procedure. These are to make sure that you are receiving the correct dose of rFVIIa for you. If we change the dose, we will check the blood tests again.

What do I have to do?

You will be given a medication to take orally for 7 days after the procedure called Tranexamic acid. This is a common medicine used in people with factor XI deficiency and other bleeding disorders to stop bleeding. It can be used alone or in combination with clotting factor concentrates

What are the alternatives for diagnosis or treatment?

There are two alternative treatments used to prevent or treat bleeding in patients with factor XI deficiency, factor XI concentrate and fresh frozen plasma. Both of these products are made from human blood donations. They are both tested and treated to prevent transmission of infectious diseases but there is still a tiny risk that a donation might be infected.

What are the side effects of taking part?

rFVIIa treatment has been used in many people with haemophilia and is very safe. Rarely, patients can develop clotting test abnormalities and clotting (e.g. heart attacks or strokes) but this is less common than with factor XI concentrate. Other rare side-effects include abnormalities in liver blood tests, allergy to the drug, high blood pressure, skin rash, fever, headache and nosebleeds.

What are the possible benefits of taking part?

The primary aim is to enable you to have your operation or baby as safely as possible. We hope to prove conclusively that rFVIIa is effective and safe in patients with factor XI deficiency who are having operations or who have an accident causing bleeding. The information we get from this study may help us to treat future patients with factor XI deficiency better.

We hope that the treatment will help you. However, this cannot be guaranteed.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

What happens when the research study stops?
You will continue to be treated with the clotting factor treatment that is considered to be most effective and safe according to national and international best practice.

What if something goes wrong?

Any adverse event that occurs during this study will be reported to Professor Lee and to the Local Research Ethics Committee. A decision will be made after considering the facts whether the adverse event was likely to have been caused by the drug and whether it is likely to happen in other patients. Any procedures or tests that are needed to prevent this problem recurring will be taken, including stopping the study if necessary.

If you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Compensation for any injury caused by taking the study medication will be in accordance with the guidelines of the Association of the British Pharmaceutical Industry (ABPI). Copies of these guidelines are available on request.

Your right at law to claim compensation for injury where you can prove negligence is not affected.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Your records may also be looked at by people from the company who manufacture rFVIIa or regulatory authorities to check that the study is being carried out correctly. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?
The results will be published in an internationally renowned journal of bleeding disorders. Copies of the article will be available in the Katharine Dormandy Haemophilia Centre. Participants will not be identified in any report/publication.

Who is organising and funding the research?

The research is organised by the doctors at the Katharine Dormandy Haemophilia Centre and Haemostasis Unit. The company who manufacture rFVIIa are donating the study drug and providing assistance in monitoring the study.
The research is funded by a Clinical Research Fellowship grant from the Royal Free hospital NHS Trust and charitable grants to the Katharine Dormandy Haemophilia Centre and Haemostasis Unit.

Who has reviewed the study?

The study has been reviewed by the Royal Free Hospital and Medical School Local Research Ethics Committee.

Contact for Further Information

Dr. Niamh O'Connell, Clinical Research fellow or Professor Lee, Haemophilia Centre Director will be happy to answer any questions you may have about factor XI deficiency or rFVIIa treatment.

Phone: 0207 830 2068 Ext. 5237

Fax: 0207 830 2178

Email: n.o'connell@rfc.ucl.ac.uk

We would like to thank you for taking time to read this information leaflet and for considering participation in this study.

You may keep a copy of this Patient Information Sheet and your signed consent form.

Patient Identification Number for this trial:

CONSENT FORM

Title of Project:
Trial to assess the use of recombinant factor VIIa to treat and prevent bleeding in people with factor XI deficiency

Name of Researchers:
Professor Christine A. Lee / Dr. Niamh O'Connell
Phone: 0207 830 2068 Ext. 5237

Please initial box

1. I confirm that I have read and understand the information sheet dated..... (version.....) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

5. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research.
   I give permission for these individuals to have access to my records. ☐

6. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

10.1.5 Ethics approval number and Trust clearance for rFVIIa (FaXID) study

Royal Free Hampstead NHS
NHS Trust

Clinical Governance Support Centre
Royal Free Hospital
Pond Street
London NW3 2QG
Tel:020 7830 2816
Fax:020 7830 2233
zoe.spyvee@rfh.nthames.nhs.uk

Dr Niamh O'Connell
Haemophilia Centre
Royal Free Hampstead NHS Trust
Pond Street
Hampstead
London
NW3 2QG

08 October 2001

Dear Dr O'Connell

Recombinant factor VIIA in the management of surgery and acute bleeding in patients with factor XI deficiency

Project ID: 5501 **(Please quote on ALL correspondence)**

Ethics ID: 5501

Following the approval of your ethics application and registration form your project has been fully registered with the R&D department. I would be grateful if you would inform me of any changes regarding funding, project status e.t.c

Should you have any queries please quote the ID number.

You may proceed with your project.

Yours Sincerely,

Z Spyvee

Zoe Spyvee
Research and Development Officer

10.2 List of peer reviewed publications related to thesis

(Paper reprints are filed after the appendices section)

- Recombinant factor VIIa to prevent surgical bleeding in FXI deficiency.

  N. M. O'Connell, A.F. Riddell, G.Pascoe, D.J.Perry, C.A.Lee

  Haemophilia. 2008 Jul; 14(4): 775-81.

- Structural interpretation of 42 mutations causing factor XI deficiency using homology modelling.

  N. M. O'Connell, R. E. Saunders, C. A. Lee, D. J. Perry and S. J. Perkins.

  Journal of Thrombosis and Haemostasis 2004; 3: 127-38.

- The factor XI deficiency database: an interactive web database of mutations, phenotypes and structural analysis tools.

  R. E. Saunders, N. M. O'Connell, C. A. Lee, D. J. Perry and S. J. Perkins.

  Human Mutation 2005; 26(3):192-8.

10.3 List of published abstracts related to thesis

- Chromogenic and fluorogenic thrombin generation assays in factor XI deficiency: correlation with bleeding tendency. O'Connell N.M., Riddell A., Lee C.A., Perry D.J. Journal of thrombosis and Haemostasis 2005; 3(Suppl.1):P2038.
- The Factor XI Deficiency Database: An Interactive Web Database of Mutations, Phenotypes and Structural Analysis Tools. O'Connell NM, Saunders RE, Lee CA, Perry DJ, Perkins SJ. Journal of Thrombosis and Haemostasis 2005; 3(Suppl.1):P0248.
- Thrombin generation and correlation with bleeding tendency in inherited FXI deficiency. N.M. O'Connell, J.H. McIntosh, A.Riddell, G. Pascoe.A. Griffioen, C.A. Lee D.J.Perry. British Journal of Haematology 2004; 125(Suppl.1):44.
- Homology modelling of mutations causing factor XI deficiency using related crystal structures and bioinformatics. N.M. O'Connell, R.E. Saunders, C.A. Lee D.J.Perry, S.J.Perkins. British Journal of Haematology 2004; 125(Suppl.1):36.
- Prevention of surgical bleeding with recombinant factor VIIa in patients with factor XI deficiency. N.M. O'Connell, G. Pascoe. A.F. Riddell, S.A. Brown, D.J.Perry, C.A. Lee. Blood 2002; 100(11): 697a.
- A modified factor XI concentrate is safe and effective in patients with factor XI deficiency. N.M.O'Connell, D.J.Perry, S.A.Brown, C.A.Lee. Haemophilia 2002; 8, 505-6.
- Recombinant factor VIIa in the prevention of surgical bleeding in patients with factor XI deficiency. N.M. O'Connell, G. Pascoe. A.F. Riddell, S.A. Brown, D.J.Perry, C.A. Lee. British Journal of Haematology 2002;117 (Suppl.1): 82.

10.4 List of other publications and invited reviews related to FXI deficiency

- Screening for factor XI deficiency amongst pregnant women of Ashkenazi Jewish origin. Kadir RA, Kingman CE, Chi C, O'Connell NM, Riddell A, Lee CA, Economides DL. Haemophilia. 2006 Nov;12(6):625-8.
- Factor XI deficiency. Niamh M. O'Connell. Seminars in Hematology 2004, 41(1), Suppl.1:76-81.
- Prevalence, causes, and characterization of factor XI inhibitors in patients with inherited factor XI deficiency. Ophira Salomon, Ariella Zivelin, Tami Livnat, Rima Dardik, Ron Loewenthal, Ophelia Avishai, David M. Steinberg, Michael H. Rosove, Niamh O'Connell, Christine A. Lee, and Uri Seligsohn. Blood 2003;101 4783-4788.
- Factor XI deficiency – from molecular genetics to clinical management. Niamh M. O'Connell. Blood Coagulation and Fibrinolysis 2003; 14(1), S59-S64.