SOME LABORATORY AND CLINICAL ASPECTS OF HAEMOPHILIA A

by

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ABSTRACT

The hereditary bleeding disorder haemophilia A is caused by a deficiency or functional defect of coagulation factor VIII (FVIII). FVIII replacement therapy enables effective treatment of bleeding episodes. However, the care for these patients remains complex, particularly from challenges arising from the development of inhibitors (allo-antibodies) to infused FVIII. To further advance current practice in the management of Haemophilia A, a better understanding of the molecular pathogenesis of the disease is required, including the study of functional epitopes and immunogenicity of the FVIII molecule. In addition, the evaluation of coagulation deficiency by the effect of FVIII deficiency on the thrombin generation profile in a thrombin generation assay may provide novel insights into the usefulness of the thrombin generation assay (TGA) in the characterisation of, in particular, the milder form of Haemophilia A. In contrast to severe haemophilia where haemophilic osteoarthropathy is common in multiple joints (particularly knees, ankles, shoulders and elbows), we have observed that in our patients with mild haemophilia arthropathy occurs only in the ankle joints, and is often disabling. We considered that the incidence of ankle arthropathy in the milder form of haemophilia A should be properly studied.

The inhibitors are often directed against several dominant epitopes in the A2 and C2 domains of the FVIII molecule. We undertook the expression of recombinant FVIII peptides with the amino acid sequence of the C1 and C2 domains in mammalian cell expression systems. We were successful in expressing the C1 and C2 peptides in COS-7 cells. However the peptides were mostly confined to the cell pellet. The small amounts excreted into the supernatant were insufficient for further work that was planned to examine the ability of the peptides to neutralise FVIII inhibitors. We also obtained a murine monoclonal anti-FVIII antibody by immunising mice with recombinant human FVIII and performing fusion experiments with harvested spleen cells and myeloma cells. The monoclonal antibody did not inhibit factor VIII function, indicating that it may be against a non-functional epitope of factor VIII. The recombinant C1 or C2 peptides bound to the murine polyclonal anti-FVIII antibodies (post-immunisation mouse serum) in Western blot, but not to the purified monoclonal antibody.

To evaluate the thrombin generation assay as a possible method for the laboratory assessment of FVIII deficiency we established an in-house TGA using a fluorogenic substrate. Changes in fluorescence in plasma were measured in a microtitre plate using an automated reader. The development of fluorescence over a time course was evaluated by studying the rate of increase of fluorescence, which was represented by four derived parameters including the lag time, peak time, peak thrombin generation and area under the curve (AUC). We compared the two different reagents to trigger thrombin production in plasma: the activated partial thromboplastin time (APTT) reagent and the tissue factor (TF), and applied both methods in 18 normal subjects and 42 patients with mild/moderate haemophilia A. Using APTT reagent, the lag time (R = -0.72) and peak time (R = -0.72) demonstrated a significant negative correlation with the one-stage FVIII level. In contrast, using TF activation there was a significant positive correlation of the peak thrombin concentration (R = 0.73) and AUC (R = 0.71) to the FVIII level. For the 42 patients with mild/moderate haemophilia A, there were significant correlations between their FVIII levels (either by one- or two-stage assay) and the TGA results by both triggers.

A clinical study of patients with mild haemophilia A to document the frequency and severity of arthropathy has not been previously published. We conducted a clinical study on ankle arthropathy in 34 patients with mild/moderate haemophilia A. Clinical and radiological evaluation systems for the assessment of haemophilic arthropathy recommended by the World Federation of Haemophilia (WFH) were used, in addition to the visual analogue scale (VAS) pain score. The prevalence of ankle arthropathy in the study group was 52% by the radiology scoring system alone, and 48% by both the physical and radiological systems. In many of the patients pain and disability were major problems. Of 34 patients, nearly half had constant ankle pain and the severity of pain was moderate to severe in nine patients. The most significant physical finding in the 26 patients with a positive physical ankle score was the loss of free range of motion (ROM) of the ankle joint, characterised predominantly by loss of dorsiflexion. The symptom of ankle pain, present in half of the patients, had a high sensitivity (88%) and specificity (94%) for prediction of ankle arthropathy by the radiology system. The presence and severity of ankle arthropathy was most common in patients with a FVIII level of less than 11 IU/dl by the one-

stage assay (6 IU/dl by the two-stage assay). There was a significant relationship between the presence of ankle arthropathy and a history of bleeds into the ankle joint as a child. We conclude that arthropathy of the ankle in these patients is common, is often severe and disabling, and is due to the episodes of bleeding into the ankle joint during childhood.

The current study highlights some important areas of research about haemophilia A, both on the molecular biological level of the basic sciences, and the clinical aspects of diagnostic approach and disease severity. For patients with mild/moderate haemophilia, it is now important to recommend the education to ensure the awareness of the risks of ankle arthropathy, and the consideration by clinicians for early and aggressive treatment of ankle bleeding.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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This thesis is dedicated to the memory of my grandparents.

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ABBREVIATIONS

aa	amino acid
AAOS	American Academy of Orthopaedic Surgeons
ADL	activity of daily living
AHF	antihaemophilic factor
APC	activated protein C
APCC	activated prothrombin complex concentrate
APTT	activated partial thromboplastin time
AR	acidic region
AT	antithrombin
AUC	area under the curve
bp	base pair
BSA	bovine serum albumin
BU	Bethesda unit
°C	Celsius degree
Ca	calcium
cDNA	complementary DNA
CHO	Chinese hamster ovary (cell)
CI	confidence interval
CIP	calf intestinal alkaline phosphatase
COS-7	CV-1 in Origin, SV40 genome - 7 (African green monkey kidney fibroblast cell)
CSL	Commonwealth Serum Laboratories
DDW	double distilled water
df	degree of freedom
dl	decilitre
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
dNTP's	deoxynucleosides
DVT	deep vein thrombosis
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immuno-sorbent assay
ETP	endogenous thrombin potential

FIIa	activated factor II, thrombin
FVII	factor VII
FVIII	factor VIII
FVIII:Ag	factor VIII procoagulant antigen
FVIII:C	factor VIII procoagulant activity
FIX	factor IX
FXa	activated factor X
FXI	factor XI
FCS	foetal calf serum
FFC	fixed flexion contracture
FS	full strength
FU	fluorescent unit
GTE	glucose/tris/EDTA (buffer)
HAT	hypoxanthine-aminopterin-thymidine (medium)
HC	heavy chain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HIV	human immunodeficiency virus
HJHS	Haemophilia Joint Health Score
HLA	human leukocyte antigen
HMWK	high-molecular-weight kininogen
hr	hour
HRP	horse radish peroxidase
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IL	interleukin
IMVS	Institute of Medial and Veterinary Science
ISTH	International Society of Thrombosis and Haemostasis
ITI	immune tolerance induction
IU	international units
kb	kilo base
kDa	kilo Dalton
km	kilometre
LB-Amp	Luria-Bertani – ampicillin (medium)
LC	light chain
Log	logarithmic

mA	milliampere
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mM	millimolar
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
n or N	number
ng	nanogram
NIBSC	National Institute for Biological Standards and Control (UK)
nm	nanometre
nM	nanomolar
OAC	Orthopaedic Advisory Committee (of WFH)
OD	optical density
PAI-1, PAI-2	plasminogen activator inhibitors 1 and 2
PAR-4	protease activated receptor 4
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCC	prothrombin complex concentrate
PCR	polymerase chain reaction
PEG	polyethylene glycol 1500
PF3	platelet factor 3
PK	prekallikrein
PL	phospholipid(s)
PLB	protein loading buffer
PN	pooled normal (plasma)
PN-HI	pooled normal heat-inactivated (plasma)
PNP	platelet neutralisation procedure (product)
pM	picomolar
PPP	platelet poor plasma
PRP	platelet rich plasma
PT	prothrombin time
PVDF	polyvinylidene fluoride (membrane)
R	Spearman correlation coefficient
RAH	Royal Adelaide Hospital

ROC	Receiver-Operator Characteristic (curve)
ROM	range of motion
RPMI	Roswell Park Memorial Institute (medium)
RNA	ribonucleic acid
RNAase	ribonuclease
sec	second
SD	standard deviation
SDS	sodium dodecyl sulphate
Sf-21	Spodoptera frugiperda – 21 (insect cell line)
TAFI	thrombin-activatable fibrinolysis inhibitor
TBE	tris/borate/EDTA (buffer)
TE	tris/EDTA (buffer)
TF	tissue factor
TF/FVIIa	tissue factor-activated factor VII complex
TFPI	tissue factor pathway inhibitor
TGA	thrombin generation assay
TNF	tumour necrosis factor
tPA	tissue-type plasminogen activator
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
UV	ultraviolet-visible
VAS	visual analogue scale
V_{max}	maximum velocity
vWF	von Willebrand factor
WFH	World Federation of Haemophilia
Z-GGR	Z-Gly-Gly-Arg-AMC HCl (fluorogenic substrate)

CHAPTER ONE GENERAL INTRODUCTION

In this review of the literature on some laboratory and clinical aspects of haemophilia A, specific attention has been paid to a number of features, including factor VIII (FVIII) inhibitors and epitope detection, FVIII assay discrepancies and the emerging thrombin generation assay, as well as ankle arthropathy in mild and moderate haemophilia A.

1.1 Coagulation Pathway

Haemostasis is an important biological mechanism that provides a rapid, potent, but tightly localised response to vascular damage. At the site of injury, rupture of vascular endothelium allows exposure of blood to the extravascular tissue. The haemostatic plug, a platelet-rich mass encased in fibrin, forms to occlude the vascular lesion, thereby allowing subsequent cellular ingrowth and tissue repair to take place. The process of normal human haemostasis is comprised of three distinctive stages: primary haemostasis, secondary haemostasis and fibrinolysis (Lasne *et al*, 2006; Lippi *et al*, 2007). Primary haemostasis includes blood vessel contraction, platelet adhesion and aggregation and occurs immediately after tissue injury to arrest blood flow. Secondary haemostasis is the process of blood coagulation. Blood coagulation is initiated by substances in injured tissues and propagated by an interlocking network of zymogen-to-proteinase conversions. Blood coagulation results in the formation of a fibrin clot and is essential to ensure stability of the primary platelet plug (Stassen *et al*, 2004).

1.1.1 Thrombin

It is now commonly acknowledged that thrombin (activated factor II, or FIIa) is the key effector enzyme of the coagulation system (Dahlback, 2000; Mann *et al*, 2003a; Baglin, 2005; Monroe & Hoffman, 2006). Thrombin has the notable biological functions of not only activating platelets and converting fibrinogen to a fibrin network, but also amplifying its own production by activating the cofactors (factors V and VIII) and factor XI (Dahlback, 2000).

Maximum thrombin generation occurs after the formation of the fibrin clot (Mann *et al*, 2003b). This thrombin is important for additional fibrin generation as well as for activation of factor XIII, a transglutaminase that stabilises the clot by covalent cross-linking of fibrin (Davie, 1995), and thrombin-activatable fibrinolysis inhibitor (TAFI) that prevents fibrinolytic attack by attenuation of plasmin-mediated fibrin degeneration (Nesheim *et al*, 1997).

The precise and balanced generation of thrombin at sites of vascular injury is the result of an ordered series of conversions of proenzymes to the respective serine proteases, and other complex interactions between the enzymes. Congenital disease associated with absence or reduced production of thrombin includes important clinical problems such as the haemophilias. Unregulated production of thrombin in an inappropriate location can be the culprit in thrombosis (Mann *et al*, 2006).

1.1.2 Tissue factor pathway

The current model of blood coagulation acknowledges that *in vivo* blood coagulation is primarily initiated by transient exposure of tissue factor (TF) to blood at the surface of extravascular cells, resulting in subnanomolar amounts of thrombin (Brummel *et al*, 2002; Mann *et al*, 2003a; Monroe & Hoffman, 2006; Mackman *et al*, 2007).

TF is a transmembrane glycoprotein constitutively expressed by certain cells within the vessel wall and cells surrounding blood vessels, such as vascular smooth muscle cells, subendothelial pericytes and fibroblasts (Mackman *et al*, 2007). Following tissue injury and laceration of the blood vessels, the TF pathway is activated when TF is exposed to plasma factor VII (FVII), a circulating serine protease proenzyme, with formation of a TF/FVII complex (Figure 1.1).

Upon complexing, FVII is activated to FVIIa, a serine protease, leading to the formation of the TF/FVIIa complex. This complex, in the presence of calcium (Ca) and phospholipid (PL), forms “extrinsic tenase”

"Extrinsic" **"Intrinsic"**

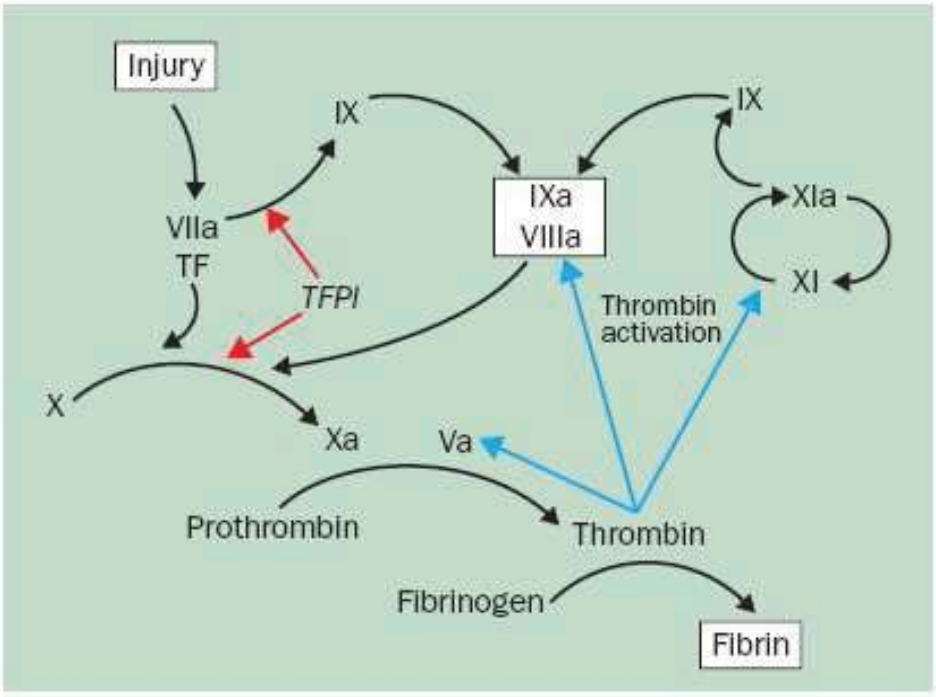


FIGURE 1.1 Schematic Model of Coagulation *in Vivo*

Reprint from Bolton-Maggs & Pasi. Haemophilia A and B. Lancet. 2003; 361: 1801-1809.

Coagulation is initiated when tissue damage exposes tissue factor (TF). Factor VII binds TF and the complex then directly activates factor X to factor Xa and some factor IX to factor IXa.

In the presence of factor Xa, tissue-factor pathway inhibitor (TFPI) inhibits further generation of factors Xa and IXa (arrowed line in red). After this inhibition, the amount of factor Xa produced is insufficient to maintain coagulation.

Further generation of factor Xa, to allow haemostasis to progress to completion, can be achieved only by the factor IX/VIII pathway. Sufficient thrombin has then been generated to activate FVIII (arrowed line in blue), and together with factor IXa (generated by TF/FVIIa) further activation of factor X can proceed.

Augmentation of factor IX activation occurs via thrombin activation of the factor XI pathway (arrowed line in blue).

"Extrinsic", "Intrinsic": Historically the coagulation cascade has been referred to include the two distinct constituents: the classical extrinsic, TF/FVIIa-dependent tissue factor pathway, and the intrinsic, FVIII-dependent contact activation pathway.

"a": The active form of precursor proteins, which are indicated by Roman numbers.

and proteolytically activates factor X to Xa. The TF/FVIIa complex also activates a small amount of factor IX (FIX) to factor IXa (Lawson & Mann, 1991) (Figure 1.1).

Factors IXa and Xa may remain associated with the TF-bearing cells or diffuse into the blood and bind to the surface of the nearby activated platelets that have formed the primary platelet plug (Hoffman *et al*, 1996). Factor Xa then activates factor V (a cofactor) on the surface of the platelets. Thus the "prothrombinase" complex, which is phospholipid-bound and consists of the enzyme (prothrombin), factor Xa and its cofactor Va, converts prothrombin to thrombin. Subsequently, cleavage of fibrinogen to fibrin by the direct action of thrombin and the formation of fibrin network occurs, leading to clot formation. Zymogen factor V is also activated by thrombin (Figure 1.1).

Though rapidly up-regulated, the tissue factor (extrinsic) pathway is short-lived and inhibited by a variety of circulating inhibitors, including tissue-factor pathway inhibitor (TFPI) and antithrombin (AT) (Baugh *et al*, 1998). The small amount of initial thrombin activity is essential and necessary to prime the system for subsequent large-scale thrombin generation by serving as the activator for platelet, factors V, VIII and XI (Brummel *et al*, 2002; Mann *et al*, 2003b).

1.1.3 Contact activation pathway

While the tissue factor pathway is the proposed mechanism by which coagulation is initiated *in vivo* in response to trauma, the contact activation pathway is an alternative mechanism by which coagulation can be initiated (Dahlback, 2000). Activation of this pathway occurs when certain coagulation proteins, including factor XII, factor XI, prekallikrein (PK) and high-molecular-weight kininogen (HMWK), come into contact with a negatively charged surface, usually collagen (Dahlback, 2000; Vine, 2009). These coagulation proteins are found within the blood (hence the term intrinsic) and are known as contact activation factors.

On contact with collagen, contact activation is initiated by the conversion of PK into kallikrein by HMWK. This in turn activates factor XII to XIIa. In the presence of Ca, factor XIIa activates HMWK-bound factor XI into XIa, which then activates factor IX. Subsequently an "intrinsic tenase" is formed to activate factor X (Figure 1.1).

The "intrinsic tenase" complex is formed by activated FIXa, activated (co)factor VIII (FVIIIa) and platelet factor 3 (PF3, a negatively charged phospholipid supplied by platelets) on an activated platelet surface. In the presence of Ca, the tenase complex converts factor X to serine protease Xa (FXa), increasing the maximal velocity (V_{max}) by 200,000-fold, compared to FIXa alone (Mann *et al*, 2006). This is followed by subsequent reactions of the "prothrombinase" complex to convert prothrombin to thrombin, and the resultant fibrin clot.

Activation of factor XI by thrombin is an amplification loop in the contact activation pathway, resulting in the generation of additional FIXa, thus further production of thrombin (Gailani & Broze, 1991; von dem Borne *et al*, 1995) (Figure 1.1). The activation of factor XI by thrombin independent of factor XII is sometimes referred as the "alternative" or "accessory" pathway of coagulation (Mann *et al*, 2003b).

In recent years it has been proposed that while initiation of blood coagulation is ascribed to the TF (extrinsic) pathway, the contact activation (intrinsic) pathway, which is a more slowly up-regulated, but longer-lived response, amplifies the coagulation events in the propagation phase (Mann *et al*, 2003a; Monroe & Hoffman, 2006; Gailani & Renne, 2007; Mackman *et al*, 2007; Tanaka *et al*, 2009). The propagation phase is required for sustained generation of thrombin that influences clot formation and resistance to fibrinolytic degradation (von dem Borne *et al*, 1995; Gailani & Renne, 2007).

When thrombin is initially generated by the TF/FVIIa-driven factor Xa, it stimulates the intrinsic pathway by proteolytically activating several coagulation factors, including factors VIII (Eaton *et al*, 1986) and XI (Naito & Fujikawa, 1991). TF/FVIIa complex also activates factor IX directly (Lawson & Mann, 1991).

Overall, the contact activation (intrinsic) pathway catalyses conversion of factor X into Xa approximately 50-fold more efficiently than the TF/FVIIa-driven factor Xa formation in the tissue factor pathway (Mann *et al*, 2006). The resultant full thrombin explosion in the propagation phase of blood coagulation has multiple actions in addition to clotting fibrinogen. It also stabilises the clot by activating factor XIII (creating covalent links between fibrin monomers) and TAFI (preventing fibrin degradation), and cleaving platelet protease activated receptor (PAR-4) (Monroe & Hoffman, 2006).

The contact activation (intrinsic) pathway is FVIII- and FIX-dependent. In the absence of FVIII or FIX, bleeding will ensue because "the amplification and consolidating generation of factor Xa is insufficient to sustain haemostasis" (Bolton-Maggs, 2003).

1.1.4 Laboratory screening tests for the coagulation pathways

Although there are several interactions between the classical extrinsic and intrinsic coagulation pathways, it is clinically useful to measure these two pathways separately by the prothrombin time and the activated partial thromboplastin time.

The Prothrombin time (PT) measures the extrinsic pathway. Clotting is triggered by adding large amount of TF to plasma to cause TF/FVII complex to activate factor X. The PT is affected by reductions of factors VII, X, V and prothrombin such as occur with vitamin K antagonist therapy or severe liver disease.

The activated partial thromboplastin time (APTT) measures the intrinsic pathway. The APTT is affected by reductions of factors XII, XI, IX, VIII, V, and to a lesser extent, prothrombin. The APTT is used clinically for monitoring of unfractionated heparin, which is a cofactor for AT.

1.1.5 Regulation of blood coagulation

According to the current model of blood coagulation, the TF pathway (extrinsic pathway) is the key mechanism that initiates the system by producing a small amount of thrombin, whereas procoagulant factors of the intrinsic pathway are responsible for the subsequent propagation and amplification of the ''thrombin burst'' (Monroe & Hoffman, 2006; Gailani & Renne, 2007; Lippi *et al*, 2007). For maximum effectiveness, regulation of coagulation is exerted at each level of the pathway, either by enzyme inhibition or by modulation of the activity of the cofactors. The positive feedbacks are all from activated factors such as factor Xa and thrombin that accelerate the coagulant reactions as described above. The negative feedbacks are by a variety of mechanisms.

One mechanism of negative control is the TFPI, which blocks fibrin generation by forming an inactive complex with the TF/FVIIa complex thus switching off the TF pathway soon after its initiation (Broze & Miletich, 1987; Rao & Rapaport, 1987) (Figure 1.1). Consequently elements of the intrinsic pathway, particularly FVIII and FIX, become the dominant regulators of thrombin generation.

Most of the enzymes generated during activation of coagulation are inhibited by the serine-protease inhibitor AT, previously called antithrombin III. The physiological role of AT is to limit the coagulation process to sites of vascular injury and to protect the circulation from liberated enzymes. AT is, in itself, an inefficient serine-protease inhibitor, but heparin and the heparin-like molecules that are present on the surface of endothelial cells stimulate its activity (Lindahl & Kjellen, 1991). This mechanism is the molecular basis for the use of heparin as a therapeutic anticoagulant.

Other circulating plasma inhibitors include activated protein C (APC), which inactivates the cofactors of the procoagulant response (factors Va and VIIIa) through a reaction that is accelerated by protein S. The zymogen of APC, protein C, is activated on the surface of intact endothelial cells by thrombin that has bound to the membrane protein thrombomodulin. Thus thrombin has the capacity to express both procoagulant and anticoagulant functions depending on the context in which it is generated. At sites of

vascular disruption, the procoagulant effects of thrombin are fully expressed. In contrast, in an intact vascular system, thrombin has anticoagulant function since it binds to thrombomodulin and activates protein C (Dahlback, 2000).

1.1.6 Fibrinolysis

The high concentration of thrombin generated by the contact activation pathway (intrinsic pathway) is necessary for the activation of TAFI, which enhances clot resistance to fibrinolysis (Nesheim *et al*, 1997; Bouma & Meijers, 2000).

Fibrinolysis is dissolution and elimination of the blood clot by a highly regulated enzymatic cascade. The fibrinolytic process is initiated by tissue-type plasminogen activator (tPA), which converts fibrin-bound plasminogen to plasmin, and culminates with solubilisation of the fibrin clot. The process is modulated by specific inhibitors, including plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2), α2-antiplasmin, α2-macroglobulin and TAFI (Lippi *et al*, 2007).

1.2 Factor VIII

1.2.1 Role of factor VIII in blood coagulation

The haemophilic factors FVIII and FIX play an essential role in the intrinsic pathway. A very low concentration of FVIII (0.1 μg/ml in plasma) is adequate for procoagulant function in normal persons (Roberts *et al*, 2004); and a substantial reduction of this level (to less than 40% of normal) or absence of FVIII leads to a bleeding disorder, haemophilia A (Bolton-Maggs & Pasi, 2003; Tanaka *et al*, 2009).

When FVIII binds to the phospholipid surfaces of damaged cells and adherent activated platelets, it dramatically increases the V_{max} of the enzyme-substrate reaction of factor IXa with factor X, accelerating it by 200,000-fold (Hoyer, 1981; van Dieijen *et al*, 1981). The effect of the cofactors on activation of factor X by factor IX is as follows (van Dieijen *et al*, 1981):

	V_{max}
IXa	0.0022
IXa + Ca	0.0105
IXa + Ca + PL	0.0025
IXa + Ca + PL + VIIIa	500

1.2.2 Factor VIII molecule and function

The human FVIII molecule is a large plasma glycoprotein comprising a single-chain polypeptide of 2332 amino acids (aa), with a molecular weight of approximately 300 kilo Dalton (kDa). It consists of three homologous A domains, two homologous C domains and a unique B domain, which are arranged in the following order: NH_2-A1(aa 1-372)-A2(373-740)–B(741-1648)-A3(1,690-2,019)-C1(2,020-2,172)-C2(2,173-2,332)-COOH (Toole *et al*, 1984; Lenting *et al*, 1998; Saenko *et al*, 2002c) (Figure 1.2). The three A domains have approximately 40% sequence homology between each other. In addition, they are homologous to the A domains of factor V, as well as to the A domains in the copper binding protein ceruloplasmin.

Prior to its secretion into plasma, FVIII is processed intracellularly into a series of metal ion-linked heterodimers produced by cleavage at the B/A3 junction and by a number of additional cleavages within the B domain (Saenko *et al*, 2002b). These cleavages generate the heavy chain (HC), consisting of the A1, A2 and B domains, and the light chain (LC), composed of the A3, C1 and C2 domains. The carboxyl-terminal portions of the A1 (aa 337–372) and A2 (711–740) domains and the amino-terminal portion of the LC (1649–1689) contain a high number of negatively charged residues and are called acidic regions (AR1, AR2 and AR3, respectively) (Lenting *et al*, 1998; Saenko *et al*, 2002c; Spiegel & Stoddard, 2002) (Figure 1.2).

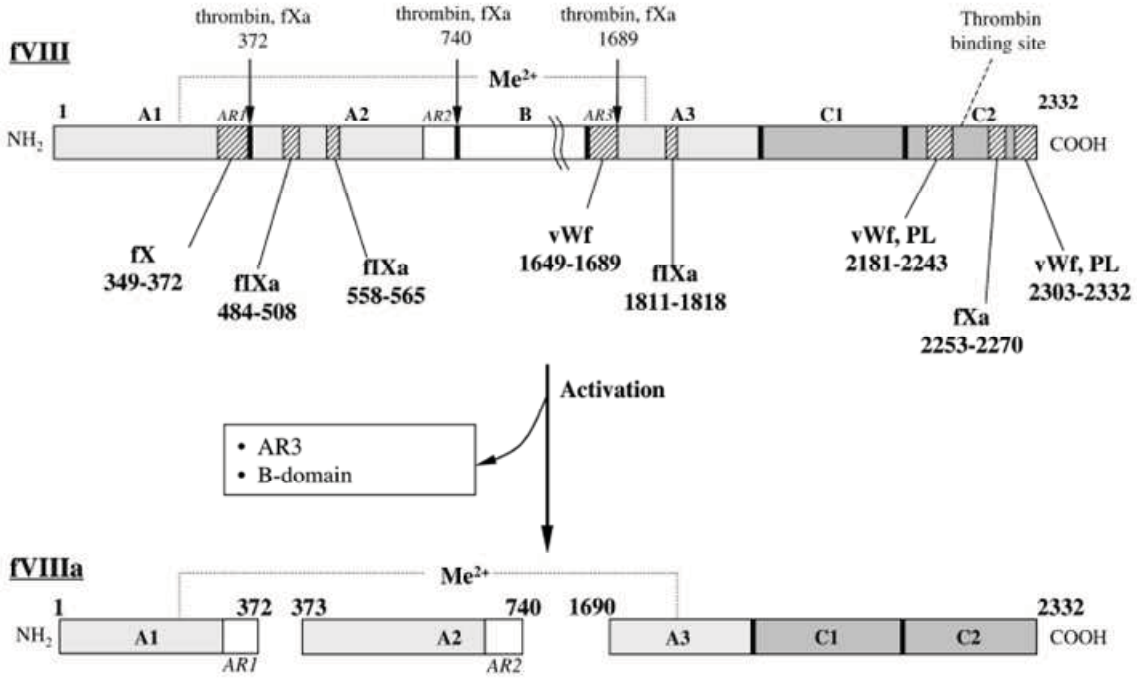


FIGURE 1.2 Factor VIII Structure and Sites Involved in Its Major Interactions

Reprint from Saenko *et al*. Molecular defects in coagulation factor VIII and their impact on factor VIII function. Vox Sanguinis. 2002b; 83: 89-96.

Non-activated FVIII is shown as a multidomain structure, in which A1 and A3 subunits are non-covalently linked via a divalent metal ion bridge (dotted line) and three A domains are flanked by acidic regions (AR1, AR2 and AR3).

The regions of FVIII involved in the binding of vWF, FIXa, FX, FXa and PL are shown as hatched boxes. Activation of FVIII by cleavage after Arg372, Arg740 and Arg1689 (shown by arrows), by thrombin or FXa, leads to release of the B domain and AR3.

Activated FVIII is a heterotrimer consisting of A1, A2 and A3–C1–C2 domains, in which A1 and A3 domains retain the metal ion linkage, and the stable A1/A3–C1–C2 dimer is weakly associated with the A2 subunit through electrostatic interactions.

Immediately after secretion into the circulation, FVIII binds to von Willebrand factor (vWF) with a high affinity and circulates in the plasma as a noncovalent complex with vWF (Saenko & Scandella, 1995; Lenting *et al*, 1998). VWF is a protein 50-fold in molar excess of the amount to FVIII. Complex formation with vWF is required for maintaining the normal FVIII level in plasma. It protects FVIII from proteolysis, and concentrates FVIII at sites of active haemostasis (Bolton-Maggs & Pasi, 2003). The interaction with vWF prevents premature assembly of the tenase complex prior to activation of FVIII and protects FVIII from inactivation by APC, FIXa and FXa (Saenko *et al*, 2002c; Spiegel & Stoddard, 2002). To participate in haemostasis the FVIII must separate from vWF. This requires the cleavage of FVIII by thrombin or FXa.

At the site of a coagulation event, FVIII is activated by the two major physiological activators: thrombin and FXa. Both proteases cleave FVIII after Arg^{372} and Arg^{740} within the HC, and after Arg^{1689} within the LC, producing A1 (approximately 50 kDa), A2 (42 kDa), and A3–C1–C2 (73 kDa) fragments, generating heterotrimeric activated FVIII (Figure 1.2).

It is generally accepted that both A2 and C2 are important functional domains of FVIII, as these two domains include the high-affinity binding sites of the FVIII molecule to its stabiliser (vWF), activators (thrombin and FXa), substrate (FIX) and functionally important molecules such as PL. The vWF binds to FVIII through sites in AR3 and C2 domain. The region in the C2 domain for vWF binding also overlaps with the PL binding site. The thrombin binding site on FVIII is mainly in C2 domain. While the substrate factor IX binds FVIII in its AR1 region, the activated factor IXa interacts with FVIII through both A2 and A3 domains (Saenko *et al*, 2002b; Spiegel & Stoddard, 2002). (Figure 1.2)

More recently, electron crystallography study has illustrated the three-dimensional model of FVIII molecule. It was predicted that the hydrophobic chains in four loops in the C2 domain are the binding sites for PL (Stoilova-McPhie *et al*, 2002) (Figure 1.3).

FIGURE 1.3 Membrane-Bound Factor VIII Model

Reprint from Stoilova-McPhie *et al*. 3-Dimensional structure of membrane-bound coagulation factor VIII: modelling of the factor VIII heterodimer within a three-dimensional density map derived by electron crystallography. Blood. 2002; 99: 1215-1223.

The FVIII model is shown anchored into the top of a phospholipid membrane (coloured white).

A1 domain is in red, A2 domain in yellow and A3 domain in green. C1 (2021 to 2170) and C2 (2171 to 2332) domains are in gold-brown and blue, respectively.

The 4 predicted membrane-binding loops of the C2 domain are shown in green. They include side chains of Met2199/Phe2200, Val2223, Leu2251/Leu2252 and Trp2313/Val2314.

In C2, peptide 2303 to 2332 is shown in magenta, which is reported to accommodate vWF and membrane binding sites. The side chains of residues, Arg2307 (blue sphere) Ile2098, Ser2119, and Arg2150 (golden-brown sphere) are solvent exposed and implicated in vWF binding.

1.2.3 Factor VIII gene

The factor VIII gene was cloned in 1984 (Gitschier *et al*, 1984). It spans 186 kilo bases (kb), approximately 0.1% of the deoxyribonucleic acids (DNA) of the X chromosome, and is located at band Xq28, the distal end of the long arm (Antonarakis, 1998). The FVIII gene comprises 26 exons and 25 introns. The normal factor VIII messenger ribonucleic acids (mRNA) is approximately 9 kb, of which the coding sequence is 7,053 nucleotides. Correspondingly the mRNA predicts a precursor protein of 2351 amino acids, which includes a signal peptide of 19 and a mature protein of 2332 amino acids (Antonarakis, 1998; Lenting *et al*, 1998).

Numerous mutations of the FVIII gene have been described. Mutations causing the absence of the production of the entire protein lead to the severe form of haemophilia A. These mutations typically include gene rearrangements (intron 22 and intron 1 inversions), large deletions, insertions of genetic elements, nonsense mutations (resulting in a stop codon) and splice site mutations (Antonarakis, 1998; Oldenburg & Pavlova, 2006). Missense mutations (single base substitution leading to an amino acid change) are associated with the expression of endogenous but functionally altered protein. They represent the majority mutation type in mild and moderate haemophilia A (Oldenburg & Pavlova, 2006).

In some cases, mutations can result in conformational changes in the structure of the FVIII molecule. The conformational change can lead to loss of FVIII function and a predisposition to inhibitor formation following FVIII replacement therapy (Oldenburg & Pavlova, 2006). An example is the missense mutation Arg2150His in the C1 domain (C1/C2 junction) in mild haemophilia A. Patients with such mutations may develop FVIII inhibitors that neutralised wild type FVIII but not endogenous mutant FVIII (Peerlink *et a*l, 1999). It was hypothesised that the C1/C2 domains are specifically critical for the immunogenicity of FVIII, perhaps because of its role in biding to vWF/PL (Figures 1.2 and 1.3). Any changes in the three-dimensional structure of this critical part of FVIII molecule may affect its immunogenicity (Oldenburg & Pavlova, 2006). Hence in missense mutations, the inhibitor prevalence depends on the location of the mutation, rather than on the severity of the patient's phenotype. This is further supported by the study in

26 mild/moderate haemophilia A with missense mutations clustered in A2 and C2 domain (C1/C2 junction) showing a high occurrence of inhibitors, of up to 40%, similar to that in severe haemophilia (Hay, 1998).

A database of mutations in the factor VIII gene was first set up in 1991 and has been updated through the internet (the Haemophilia A Mutation, Structure, Test and Resource Site: HAMSTeRS, accessed via http://europium.csc.mrc.ac.uk).

1.3 Haemophilia A

1.3.1 Definition and pathogenesis

The word *haemophilia* is defined by The Oxford English Dictionary as "a constitutional (usually hereditary) tendency to bleeding, either spontaneously or from very slight injuries; haemorrhagic diathesis" (Simpson & Weiner, 1989). This disease was known to the ancient world, with the earliest written references in Jewish texts of the second century A.D. (Ingram, 1976). The first modern description of haemophilia is attributed to Dr. John Conrad Otto who clearly appreciated, in his publication in 1803, the three cardinal features of haemophilia: an "inherited" tendency of "males" to "bleed" (Ingram, 1976).

However, the biochemical basis of haemophilia was not elucidated until the early 1950s, when it was demonstrated that some proteins from normal plasma would correct the abnormal clotting of haemophilic blood (Pavlovsky, 1947; Aggeler *et al*, 1952). Most of the patients with haemophilia were found to be missing the protein known as antihaemophilic factor (AHF), and the disease was termed haemophilia A. Some patients with identical clinical and genetic features were found to be lacking a different protein, Christmas factor, and their disorder was termed haemophilia B. Standardised names were assigned to the various coagulation factors by the International Committee on Thrombosis and Haemostasis in 1962: the factor missing in haemophilia A was subsequently termed factor VIII, and the factor missing in haemophilia B was termed factor IX (Wright, 1962).

Haemophilia A is the most common inherited clotting factor abnormality, after von Willebrand disease. It accounts for about 80% of the two main forms of the haemophilias (A and B). Haemophilia A is described as an inherited blood coagulation disorder that is due to defective and/or deficient functional factor VIII, a plasma protein which is essential for blood to clot (Bolton-Maggs & Pasi, 2003). This recessive, X-linked genetic disease affects approximately one out of 10,000 live male births, and is found in all ethnic groups.

1.3.2 Clinical presentation

Haemophilia A is characterised by excessive bleeding. Based on the factor VIII level and severity of clinical manifestation, haemophilia A has been broadly classified as mild, moderate and severe, although there is overlap between the categories. The clinical classification of haemophilia A is summarised as follows (adopted from Bolton-Maggs & Pasi. Haemophilias A and B. Lancet. 2003; 361: 1801-1809):

Concentration of FVIII:C	**Classification**	**Clinical**
< 0.01 IU/ml (< 1% of normal)	Severe	Spontaneous joint and muscle bleeding; Bleeding after injuries, accidents and surgery
0.01–0.05 IU/ml* (1-5% of normal)	Moderate	Bleeding into joints and muscles after minor injuries; Excessive bleeding after surgery and dental extractions
> 0.05–0.40 IU/ml (5–40% of normal)	Mild	Spontaneous bleeding does not occur; Bleeding after surgery, dental extractions and accidents
0.5-1.5 IU/ml (or 50-150 IU/dl) (50-150% of normal)	Normal Range	

*: International Society of Thrombosis and Haemostasis (ISTH) definition for moderate haemophilia A. However, in Australia and UK, a FVIII level of 0.02-0.05 international units (IU)/ml is more often used to identify the moderate cases. We use this latter definition in this thesis.

In severe haemophilia A, patients have spontaneous and recurrent bleeding into soft tissues, muscles, and joints, particularly the knees, ankles, hips, elbows and shoulders. The repetitive presence of blood in joints leads to osteoarthritis and then severe joint deformity, which is known as disabling haemophilic arthropathy. Internal bleeding such as oropharyngeal and central nervous system bleeding accounted for the reduced life expectancy of haemophilia A patients before effective replacement therapy was introduced at the end of the 1960s.

In moderate haemophilia A, haematomas and haemarthroses occurs less often (usually associated with trauma) and are less disabling than in severe patients. With a factor VIII level of 6-30% of normal, the mildly affected patients have infrequent bleeding episodes, and only after significant trauma or surgery. A review of haemophilic arthropathy in mild and moderate haemophilia A is presented in Section 1.7.

The laboratory investigations usually reveal a prolonged APTT, with other tests such as PT, platelet count, bleeding time being normal. Specific bioassays to quantitate FVIII levels are essential to distinguish haemophilia A from haemophilia B.

1.4 Laboratory Evaluation of Haemophilia A

The FVIII protein can be identified in two ways, by functional and immunological methods. Factor VIII procoagulant activity (FVIII:C) refers to its functional property in blood coagulation, while factor VIII procoagulant antigen (FVIII:Ag) refers to its antigenic character as measured by immunoassays (Hoyer, 1981; van Dieijen *et al*, 1981).

1.4.1 Factor VIII assays (one-stage and two-stage assays)

The FVIII:C level (coagulation activity) in plasma is usually measured by a one-stage assay, which is automated and standardised against the International Reference Plasma for FVIII (National Institute for Biological Standards and Control [NIBSC], Hertfordshire, UK). The one-stage assay is based on the

activated partial thromboplastin time. The same proportions of test or standard plasma are mixed with FVIII-deficient plasma, followed by addition of an APTT reagent (with components of PL and Ca), and the clotting time recorded at 37°C. The FVIII level in the test sample is calculated from readings of clotting time against a standard curve.

Another less commonly used assay to detect factor VIII level is the two-stage FVIII assay. This factor Xa-based test is difficult to automate and is considered to be very labour-intensive in the environment of modern diagnostic laboratories. During the first stage of the assay, a combined reagent containing PL, Ca, bovine factor V and human serum (a source of factors IX, X, XI and XII) is mixed with test or standard plasma, in which prothrombin is removed by absorption from aluminium hydroxide [$Al(OH)_3$] (Duncan *et al*, 1994; Keeling *et al*, 1999; Rodgers *et al*, 2007). The mixture is incubated for 10 to 12 min at 37°C. During this period of time, factor Xa is generated without causing a clotting reaction due to the absence of prothrombin. The amount of Xa generation is, however, dependent on the level of FVIII:C in the test or standard sample. During the second stage of the assay, subsequent to adding a source of prothrombin and fibrinogen, the time to clot formation is recorded and this is dependent on the amount of factor Xa produced in the first stage, which is ultimately defined by the FVIII:C in the initial sample. A standard curve for clotting times at different dilutions of FVIII is also used in this assay.

1.4.2. Factor VIII assays discrepancies

It has been recognised for decades that in some haemophilia A patients, there is a discrepancy between their FVIII levels measured by the one-stage or the two-stage assay (Denson & Biggs, 1976; Barrowcliffe, 1984; Parguet-Gernez *et al*, 1988). In patients with inherited mild or moderate haemophilia A, members from certain families showed consistent higher (at least two-fold) FVIII level by the one-stage assay than that of the two-stage assay (Duncan *et al,* 1994; Rudzki *et al*, 1996; Rodgers *et al*, 2007). This phenomenon of assay discrepancy (and usually familial) is not seen in normal population and in severe haemophilia A, where the FVIII results from both assays usually agree.

The discrepancy between the assays has now been well described in a number of haemophilia centres in other countries and is likely to be present in most or all populations (Duncan *et al,* 1994; Keeling *et al*, 1999; Schwaab *et al*, 2000; Pipe *et al*, 2001; Lloyd in Ingerslev *et al*, 2008). It is now well described that these discrepancies are due to underlying mutations of FVIII gene (Lloyd in Ingerslev *et al*, 2008). At least 17 different missense mutations have been described (reviewed by Lloyd in Ingerslev *et al*, 2008). The mutations are in the regions at the interface between A1-A2, A1-A3, and A2-A3. This renders the FVIII less stable so that activated FVIII is inactivated more quickly than normal (Pipe *et al*, 2001; Hakeos *et al*, 2002; Rodgers *et al*, 2007).

On the basis of FVIII:C results by one-stage or two-stage assays, mild and moderate haemophilia A patients can be classified into two subgroups: the *equivalent* subgroup whose one-stage FVIII result agrees with two-stage results, and the *discrepant* subgroup whose one-stage FVIII result usually is equal to or greater than double the two-stage results (Duncan *et al*, 1994; Lloyd in Ingerslev *et al*, 2008). The argument for importance of using the two-stage FVIII assay is that some cases of mild haemophilia may not be diagnosed when only the one-stage assay is used (Keeling *et al,* 1999).

It is estimated that one-third of the mild and moderate patients will fall in the *discrepant* subgroup based on the assay discrepancy (Lloyd in Ingerslev *et al*, 2008). This is an important issue for haemophilia centres because diagnosis of these patients can be difficult and in some cases not possible when only the one-stage assay is available. Studies using a longer incubation time for some commercial FVIII chromogenic assays were shown to be suitable for diagnosis of the *discrepant* patients and thus may offer an answer to address the problem (Rodgers *et al*, 2007; Lloyd in Ingerslev *et al*, 2008).

1.4.3. Thrombin generation assay

1.4.3.1 Development and application

As described above, thrombin is the key effector enzyme in the coagulation cascade, acting directly on fibrinogen, and providing feedbacks to amplify the haemostatic process (Figure 1.1). While the

traditional clotting assays such as APTT and PT use the time to clot as the end point of the test, a thrombin generation assay (TGA) measures the amount of thrombin production over a time course, both before and after clot formation (Hemker *et al*, 2000). Theoretically if performed in whole blood, the use of the TGA could reflect most of the overall effect the influences of the interactions between all the procoagulant and anticoagulant factors. Hence it is proposed that the measurement of thrombin may have the potential to reflect the global function of individual's coagulation system and determine the "coagulation phenotype" (Baglin, 2005).

In haemophilia A, the TGA may be used to study the thrombin generation profile as a result of FVIII deficiency, and provide extra information on the evaluation of individual's FVIII coagulation activity.

The TGA has been used for more than 50 years and was originally developed by Macfarlane and Biggs for use in whole blood and simultaneously by Pitney and Dacie for use in citrated plasma (Macfarlane & Biggs, 1953; Pitney & Dacie, 1953). Both methods were similar in that blood or plasma were placed in glass tubes with normal saline, subsamples were removed into a fibrinogen solution and the time taken for the fibrinogen to clot was measured. Similar subsampling methods were still used in recent years (Butenas *et al*, 1999; McIntosh *et al*, 2003).

Recent modifications have been made by Hemker and colleagues. The use of a chromogenic substrate to replace the fibrinogen solution changed the test end-point from clotting to the cleavage of the chromogenic substance, which could be detected more precisely by optical density (OD) readings (Hemker & Beguin, 1995; Wielders *et al*, 1997). More recent use of a slow-reacting fluorogenic substrate cleaved by thrombin allowed continuous measuring of thrombin formation in a single tube without subsampling (Hemker *et al*, 2000; Hemker *et al*, 2003). The fluorogenic substrate has the additional feature in that the florescence signal is not disturbed by turbidity hence the occurrence of clot or presence of platelet will not interfere with results. In addition, using a thrombin solution of known concentration as a standard, quantification of thrombin production in the test plasma can be obtained

after correction of fluorescence by a parallel curve using the thrombin standard. Hemker's equipment and software for automated and calibrated TGA has been recently made commercially available (Hemker *et al*, 2003).

The TGA can alternatively be performed in defibrinated platelet-poor plasma (PPP) (Kumar *et al*, 1995), non-defibrinated platelet-rich plasma (PRP) (Gerotziafas *et al*, 2005) or whole blood (Horne *et al*, 2006). The TGA provides several analytical parameters such as the lag time, peak thrombin and area under the curve (Section 1.4.3.2, Figure 1.4). Utilisation of these parameters of the TGA can potentially offer additional laboratory assessment of a large spectrum of clotting abnormalities, from inherited bleeding disorders (e.g., coagulant deficiencies, platelet dysfunction, chronic liver disease), to monitoring haemostatic agents (e.g., coagulant replacement therapies or anticoagulant agents heparin and warfarin) (Hemker *et al*, 2004).

1.4.3.2 Thrombin generation curve and its parameters

The general form of a thrombin generation curve is shown in Figure 1.4. After the addition of a trigger, thrombin generation starts with a lag phase, in which only minute amounts of thrombin are formed (Figure 1.4). The lag phase is immediately followed by a sudden burst of thrombin in nanomolar concentrations (Figure 1.4). As has been known for a long time, clotting occurs at the start of the explosive burst, i.e., soon after the end of the lag phase (Macfarlane & Biggs, 1953). The clotting time therefore is a good approximation of the duration of the lag phase (lag time) and vice versa.

As soon as thrombin appears it is scavenged by the plasma antithrombins, even during the lag phase. The velocity of inactivation increases proportionally with the thrombin concentration. At the peak, thrombin generation and decay are equally fast. Hence there is no sharp distinction between the production phase and the inactivation phase. However the area under the curve (AUC) of thrombin generation represents the total amount of thrombin generated over reaction time (Figure 1.4). This is referred as endogenous thrombin potential (ETP) by Hemker (Hemker & Beguin, 1995).

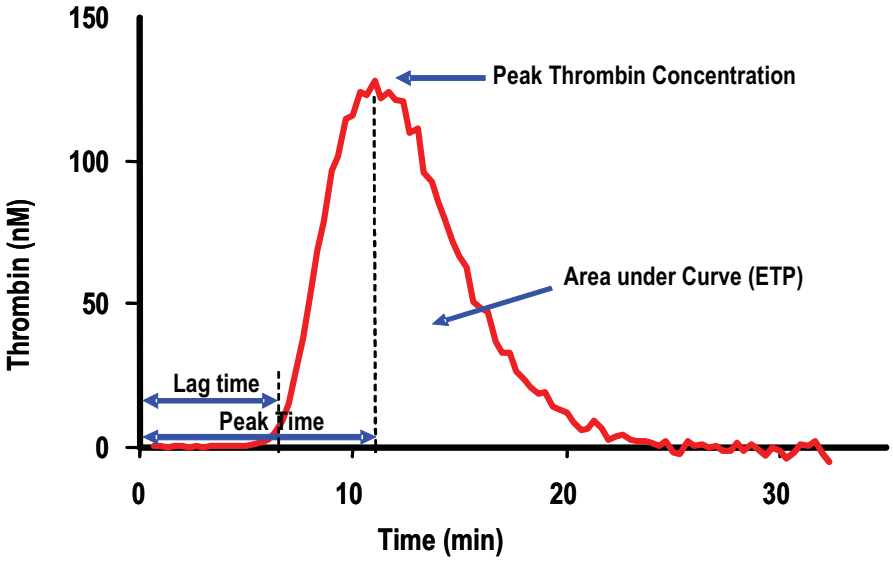


FIGURE 1.4 The Parameters of the Thrombin Generation Curve

The thrombin generation curve comprises a lag phase, a peak production phase and an inactivation phase.

Four parameters are used most often:

A Lag time (min)
B Peak time (min)
C Peak thrombin concentration (nM)
D Area under curve (AUC) = Endogenous thrombin potential (ETP) (nM x min)

1.5 Treatment of Haemophilia A

1.5.1 Multidisciplinary approach

Comprehensive care addresses the treatment and prevention of bleeding, the long-term management of haemophilic arthropathy and other complications of bleeding, the management of significant complications of treatment (the development of inhibitors and transfusion transmitted infections) and the psychosocial support and education required to manage this bleeding disorder (Bolton-Maggs & Pasi, 2003). Comprehensive care, therefore, requires collaboration between several specialists as well as a core team (usually a haematologist, laboratory staff, nurses, physiotherapist and social worker) and access to appropriate and sufficient treatment products

1.5.2 Factor VIII replacement therapy

The mainstay of treatment is to raise the concentration of FVIII sufficiently to arrest spontaneous and traumatic bleeds or to cover surgery. A remarkable evolution in haemophilia A replacement therapy has occurred over the past half century. More than 40 years ago, the treatment for bleeding episodes was by the infusion of whole blood and later frozen plasma or cryoprecipitate, all of which were severely limited therapies. Plasma derived factor VIII concentration became widely available in the 1970s, resulting in very effective treatment of bleeding episodes and the development of self-infusion and home therapy programs. Effective therapy permitted surgery, including major orthopaedic procedures for the restoration of joint function.

Continued manufacturing advances resulted in products of higher purity with fewer contaminants. Viral inactivation steps were introduced in 1986 to eradicated transmission of hepatitis B, hepatitis C and HIV (human immunodeficiency virus). Genetically engineered (recombinant) FVIII concentrates became available from 1992. The third-generation recombinant FVIII products are free of animal and human proteins. Recombinant products are the treatment of choice if available and affordable, because they eliminate the risk of transmission of human and animal infectious agents (Bolton-Maggs & Pasi, 2003).

1.5.3 Prophylaxis

In contrast to treatment by on demand therapy, prophylaxis involves regular infusions of FVIII concentrates to prevent bleeds from occurring. Prophylaxis was pioneered in Sweden (Nilsson *et al*, 1992) and is now the recommended strategy for paediatric patiens with severe haemophilia A beginning in early childhood. Prophylaxis largely prevents spontaneous joint bleeding and the development and/or progression of haemophilic arthropathy. Recently in some countries, prophylaxis has been recommended as the standard of care for patients of all ages with severe haemophilia (Hay, 2007).

1.5.4 Development of inhibitors

Following multiple transfusions, approximately 20-35% of severe haemophilia A patients develop anti-FVIII antibodies (FVIII inhibitors) as an immune response to infused FVIII (Spiegel & Stoddard, 2002; Key, 2004; Berntorp *et al*, 2006; Hay, 2006). FVIII inhibitors neutralise FVIII coagulant activity, rendering the usual doses of replacement therapy ineffective. Patients with inhibitors are usually divided into two basic categories: low responders, whose inhibitor titre usually does not exceed 5 Bethesda units/ml (BU/ml) despite repeated exposure to FVIII; and high responders, who have an anamnestic response to repeated administration of FVIII, and in whom the inhibitor titre rises above 5 BU/ml (Spiegel & Stoddard, 2002; Key, 2004).

In acute situations, patients with inhibitors can sometimes be treated effectively with very high doses of human FVIII or with porcine FVIII. However, in patients with inhibitors, the long-term treatment options and the potential complications, as well as the costs of treatment, all differ from those of the patients with haemophilia uncomplicated by inhibitors. With better protection against joint disease from FVIII prophylaxis and improved biological safety of transfusion products, the development of FVIII inhibitors is now considered to be the most serious complication of haemophilia A and its treatment (Berntorp *et al*, 2006; Oldenburg & Pavlova, 2006).

1.6 Factor VIII Inhibitors

1.6.1 Prevalence

An exact figure for the prevalence of FVIII inhibitors is difficult to provide, as the percentage varies with the population of patients considered, the method used to detect inhibitors, the threshold level and the frequency at which tests detecting of inhibitors are carried out (Gilles *et al*, 1997). However, it is generally estimated that inhibitor antibodies to FVIII develop in up to 35% (20-50%) of patients with severe haemophilia A, and less frequently (5%, 3-13%) in mild/moderate haemophilia (Spiegel & Stoddard, 2002; Bolton-Maggs & Pasi, 2003; Key, 2004; DiMichele, 2006; Berntorp *et al*, 2006; Hay, 2006; Oldenburg & Pavlova, 2006). Factor VIII inhibitors can also develop spontaneously as autoantibodies in one per million general population without hereditary deficiency of FVIII, causing acquired haemophilia (Spiegel & Stoddard, 2002).

1.6.2 Risk factors

1.6.2.1 Environmental influences

The impact of non-genetic factors on inhibitor development is still unclear. Putative environmental risk factors that have been investigated include: age at first FVIII exposure; treatment-related variables such as treatment intensity, type of product used, mode of administration; and immune system challenge (e.g., concomitant infection or vaccination) (Berntorp *et al*, 2006; Hay, 2006). Two studies suggested that inhibitor risk was increased by very early FVIII exposure (Lorenzo *et al*, 2001; Van der Bom *et al*, 2003), but this was disputed by a third study (Goudemand *et al*, 2006). Larger studies addressing the question are ongoing. Studies investigating the relative inhibitor risk of plasma-derived versus recombinant FVIII have been similarly inconclusive (Hay, 2006). Overall, currently there is no definitive data on the evaluation of the non-genetic factors to recommend clinicians to avoid specific treatment approaches that would otherwise constitute adequate and successful clinical practice (Berntorp *et al*, 2006).

1.6.2.2 Genetic risk factors

More importantly, in addition to environmental factors, genetic or hereditary risk factors are recognised to play a decisive role in inhibitor development. Foremost amongst these are haemophilia severity, family history of inhibitors and race (African origin).

Factor VIII genotype analysis, which determines disease severity, offers some predictive value in the assessment of inhibitor risk. In severe haemophilia A, mutations that result in an absent or truncated FVIII protein, such as large deletions, stop mutations and intra-chromosomal recombinations, are more likely to develop inhibitors than those with small deletions and missense mutations (Schwaab *et al*, 1995; Tuddenham & McVey, 1998). Among these defects, the most common is the intron 22 gene inversion, which has been detected in 45% of patients with severe haemophilia A. In this mutation there is intra-chromosomal DNA rearrangement of the tip of the long arm of the X chromosome, with a region within intron 22 of the FVIII gene. This leads to gross disruption of the gene and absence of FVIII protein.

Patients with mild or moderate haemophilia, in which missense mutations represent the main mutation type and measurable levels of endogenous FVIII are detected, are much less likely to develop an inhibitor than those with severe haemophilia (Tuddenham & McVey, 1998). This discrepancy may be explained by a lack of immune tolerance of the FVIII antigen of any kind in patients with the more severe mutations. Nevertheless, certain mutations clustered in the A2 and C2 domains that result in mild or moderate haemophilia A are also associated with an increased risk of inhibitor development (four-fold greater than with mutations outside this region) (Hay, 1998; Oldenburg & Pavlova, 2006).

On the other hand, not all patients with the intron 22 inversion, large deletions, or nonsense mutations form inhibitors, suggesting that factors other than the FVIII mutation contribute, such as inherited immune-response characteristics of individuals. It has been reported that the risk of inhibitor development in the haemophilic sibling of an inhibitor patient is approximately 50%, whereas the risk for

an extended family member is only about 10% (Gill, 1999). This finding supports the existence of other critical genetic factors important in the induction of FVIII inhibitor expression.

From the studies available thus far, a weak association of certain HLA (human leukocyte antigen) MHC (major histocompatibility complex) class I or II haplotypes, with inhibitor formation amongst northern European patiens with the intron 22 inversion has been found (Hay *et al*, 1997; Oldenburg *et al*, 1997). Recently, a significant association between inhibitor formation and polymorphisms in genes coding for cytokines (interleukin-10, IL-10) and other immunoregulatory factors (tumour necrosis factor-alpha, TNF-α) has been shown (Astermark *et al*, 2006). This is likely to be only the first of many such relatively weak, interacting, immunogenetic influence that direct the patient's immune responses to FVIII (Hay, 2006).

1.6.3 Characteristics of FVIII inhibitors

Antibodies to FVIII are polyclonal high affinity immunoglobulins of the IgG class, most commonly the IgG_4 isotype, or a combination of two or more subtypes (Fulcher *et al*, 1987). Occasional inhibitors that are immunoglobulins of class A or M have been reported (Gilles *et al*, 1997; Pruthi & Nichols, 1999). Most FVIII inhibitors arise early in the patient's treatment life, after a median of 9 to 12 days post-exposure (Hay, 2006). The inactivation of FVIII:C by FVIII inhibitors is known to be time and temperature dependent, and is irreversible both *in vivo* and *in vitro* (Hoyer *et al*, 1984). In addition, FVIII inhibitors have some degree of species specificity and usually have a relatively lower cross-reactivity to porcine FVIII.

A standard procedure for the detection of FVIII inhibitors is the Bethesda assay, in which the ability of patient's plasma to inactivate FVIII in normal plasma in a clotting assay is tested. The Nijmegen modification of the Bethesda assay has significantly improved reliability and sensitivity and is recommended in most countries for the quantitation of inhibitors, especially for low levels of inhibitors

(Verbruggen *et al*, 1995). FVIII inhibitors are commonly defined as either "high" or "low" responder type, with high responder inhibitors defined as those with a peak activity of > 5 BU/ml.

When FVIII in the test mixture is inactivated in a dose-dependent linear manner, the inhibitor is usually referred to as a "type I" antibody. Most commonly, inhibitors occurring in patients with severe haemophilia A behave in this manner. In contrast, "type II" inhibitors usually exhibit complex kinetic behaviour and do not fully inactivate FVIII activity in the test mixture, even after prolonged incubation. These inhibitors are more characteristic of acquired haemophilia, and are also frequently seen in patients with mild haemophilia A who develop inhibitors (Hay, 1998).

Previous approaches used to determine the kinetics of FVIII inhibitors were complex and the interpretation of the results was confusing rather than practical for a routine laboratory (Biggs *et al*, 1972 a, b). In my previous work concerning the classification of the *in vitro* kinetics of FVIII inactivation, we showed that the plasma dilution studies could successfully discriminate type I from type II inhibitors (Ling *et al*, 2001). A steep slope with complete FVIII:C inactivation at high antibody concentrations was characteristic for type I inhibitors, whereas a FVIII:C plateau with incomplete inactivation was typical for type II inhibitors. The time-course studies could not discriminate between these groups. We recommended that plasma dilution studies be part of any assessment of inhibitor classification (Ling *et al*, 2001).

Both allo- and autoantibodies against FVIII have been found to be directed mainly towards functional epitopes in the A2 or C2 domains (Scandella *et al*, 1989; Scandella *et al*, 2001). FVIII inhibitors typically bind to several critical binding sites in FVIII molecule (Prescott *et al*, 1997), leading to steric hindrance of the interaction with FIXa, PL and/or VWF. Occasionally, other inhibitory mechanisms have been described: e.g, the binding to the epitopes formed by the complex of FVIII with vWF (thereby preventing release of FVIII from vWF); the inhibition of FVIII activation by thrombin; or the inhibition of FVIII binding to one of its substrates, factor X (Ananyeva *et al*, 2004).

Recently, it was demonstrated that in some patients with inhibitors, FVIII neutralisation might be explained by intrinsic proteolytic activity associated with so-called "catalytic antibodies" to FVIII (Lacroix-Desmazes *et al*, 2002). Another mechanism proposed, but not with strong evidence, is that of binding to non-functional regions and increasing the clearance of FVIII from plasma (Nilsson *et al*, 1990). The prevalence of non-neutralising (non-inhibitory) antibodies in the absence of a detectable inhibitor is, however, disputed. While some groups reported that non-inhibitory anti-FVIII antibodies were quite prevalent (Gilles *et al*, 1993; Dazzi *et al*, 1996), our previous study on 52 patients with haemophilia A revealed a low detection rate of non-inhibitory antibodies against FVIII by enzyme-linked immuno-sorbent assay (ELISA), using three different formulas of recombinant FVIII as coating antigen (Ling *et al*, 2003).

1.6.4 Epitopes of FVIII inhibitors and mechanisms of inactivation

Cleavages by thrombin of the mature FVIII protein yield an active heterotrimer of three subunits: A1, A2 and LC (A3-C1-C2). Although the anti-FVIII immune response is clearly polyclonal and patients may have multiple antibodies against multiple sites on FVIII molecule, inhibitors tend to cluster to a few regions (Scandella, 1996; Gilles *et al*, 1997) (Figure 1.5). Both the A2 and C2 domains contain the epitopes targeted by most (70%) inhibitory antibodies (Scandella, 1996).

The epitope mapping of FVIII inhibitors was initiated by Fulcher, Scandella and colleagues in a series of studies in the mid-1980s (Fulcher *et al*, 1985a; Scandella *et al*, 1988). Fulcher used thrombin cleaved FVIII fragments and immunoblotting to demonstrate that epitopes from 25 inhibitor patients were confined to either a 44-kDa fragment from the HC, or the 72-kDa LC (Fulcher *et al*, 1985a). Scandella performed the systemic epitope mapping by expressing recombinant FVIII fragments with progressive deletions in *Escherichia coli* (*E. coli*) cells followed by testing of the mutant proteins' ability to bind inhibitors in an immunoblot analysis (Scandella *et al*, 1988). In addition, insect cell lines (e.g., Sf-9) to produce recombinant peptide, immunoprecipitation and inhibitor functional neutralisation assays were used (Scandella *et al*, 1989; Scandella, 1996; Scandella *et al*, 2001). The results demonstrated several

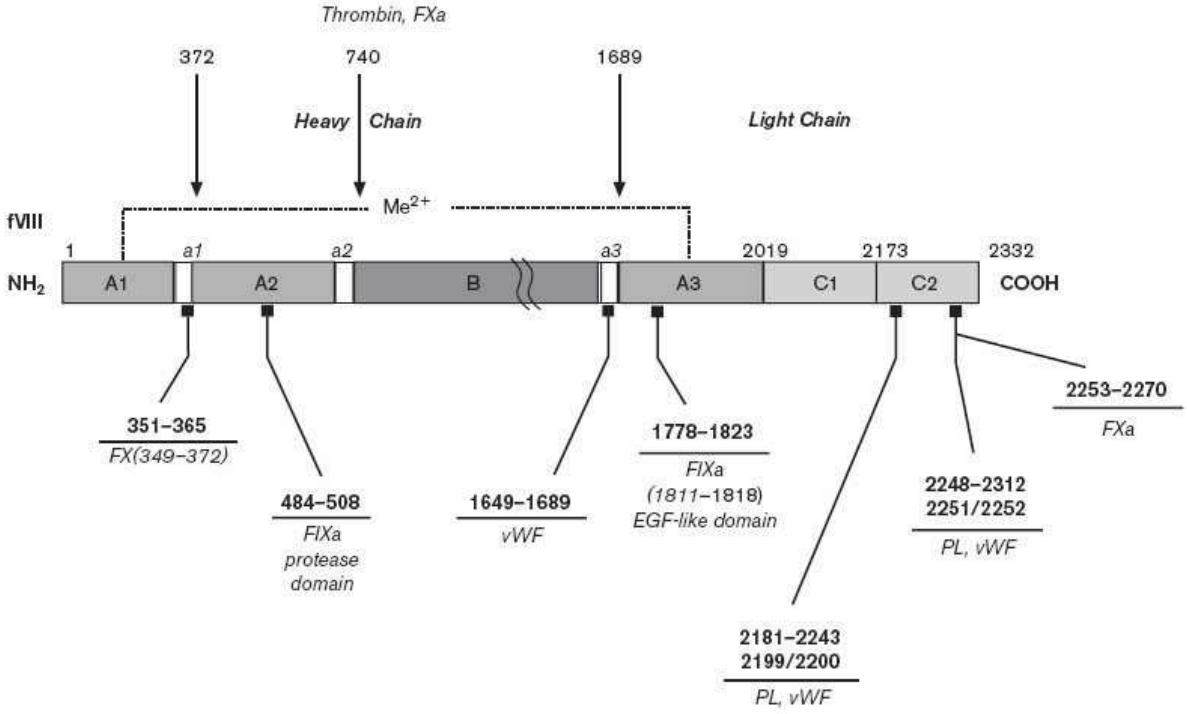


FIGURE 1.5 Factor VIII Structure and Epitopes of Factor VIII Inhibitors

Reprint from Ananyeva *et al*. Inhibitors in haemophilia A: mechanisms of inhibition, management and perspectives. Blood Coagulation and Fibrinolysis. 2004; 15: 109-124.

Non-activated FVIII is a heterodimer, in which the heavy chain consists of the A1, A2 and B domains, and the light chain is composed of the domains A3, C1 and C2. The A domains are flanked by acidic regions a1, a2 and a3, which contain a high number of negatively charged residues.

The A1 and A3 subunits are non-covalently linked via a metal ion-mediated interaction (dotted line). Activation of FVIII (cleavage sites are shown by arrows) leads to release of the B domain and a3.

In activated FVIII heterotrimer, the A1 and A3 domains retain the metal ion-mediated interaction, and the stable A1/A3–C1–C2 dimer is weakly associated with the A2 subunit through electrostatic interactions.

The epitopes of FVIII inhibitors are indicated in bold and overlap with the binding sites (indicated in italic) for vWF, PL, and factors IXa, X and Xa.

dominant epitopes in the A2, A3 and C2 domains, and in the acidic region between the A1 and A2 domains of the FVIII molecule (Scandella *et al*, 2001).

More recently, another approach for epitope mapping based on testing the effects of inhibitory antibodies on fully functional hybrid human/porcine FVIII molecule was introduced (Healey *et al*, 1995). This strategy takes advantage of the observation that human inhibitors have limited cross-reactivity with porcine FVIII (Koshihara *et al*, 1995). When only minimal porcine substitutions are included in the FVIII hybrid, it is less reactive with a given inhibitory antibody to human FVIII. This will identify the residues important in the formation of the epitope targeted by the particular inhibitor. Using this method smaller regions of the epitopes including single amino acid substitutions (e.g., Tyr487 in the A2 domain) were identified as critical for antibody binding (Lubin *et al*, 1997).

Since the completion of the laboratory work for the current project, in more recent years, continuous advances in epitope mapping for FVIII inhibitors have identified smaller regions on FVIII molecule for epitope specificity. A visual illustration of a few well-studied epitopes on FVIII and their inhibitory mechanisms are summarised in Figures 1.5 and 1.6 (Ananyeva *et al*, 2004).

Epitopes in the C2 domain are amongst those that have been extensively described. Anti-C2 inhibitors share a common epitope 2248-2312 at the carboxyl-terminus of C2 and another epitope encompassed by the residues 2181-2243 at the amino-terminal of C2 (Saenko *et al*, 2002a; Spiegel & Stoddard, 2002; Ananyeva *et al*, 2004). Both epitopes include the residues of one of the four hydrophobic “feet” (side chain Leu2251/Leu2252 for epitope 2248-2312, and side chain Met2199/Phe2200 for epitope2181-2243, respectively), which are involved in the PL binding sites as confirmed by direct analysis of 3-dimentional crystal structure of FVIII/PL binding (Figure 1.3) (Stoilova-McPhie *et al*, 2002). This class of human C2 inhibitors frequently targets the two “feet”, interferes with FVIII binding to cell membrane PL and prevents assembly of the “tenase” complex.

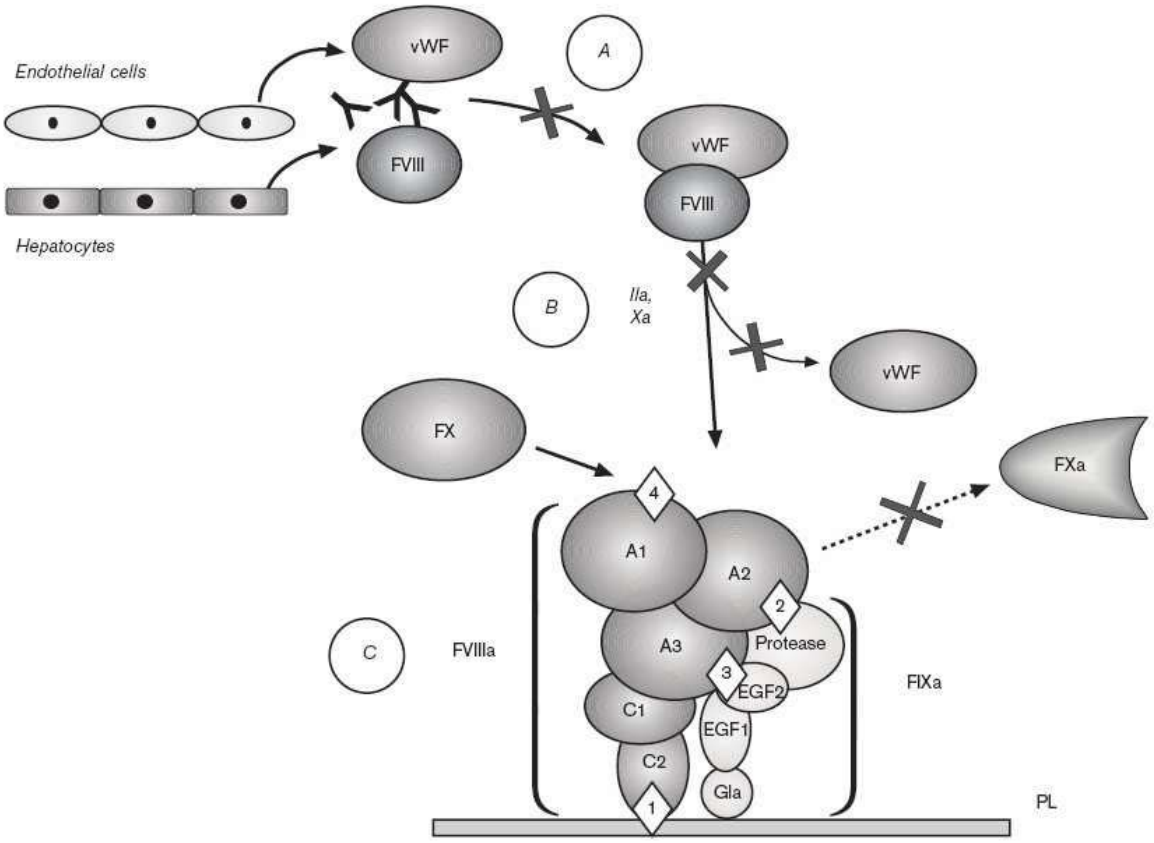


FIGURE 1.6 Inhibitory Mechanisms of Anti-FVIII Antibodies

Reprint from Ananyeva *et al*. Inhibitors in haemophilia A: mechanisms of inhibition, management and perspectives. Blood Coagulation and Fibrinolysis. 2004; 15: 109-124.

A Inhibition of FVIII binding to vWF.
Inhibitors with specificity towards a3 and most anti-C2 antibodies prevent FVIII/vWF interaction, which results in proteolytic degradation of FVIII by FIXa, FXa and APC.

B Inhibition of FVIII activation.
Inhibitors block FVIII interaction with physiological activators, thrombin and FXa, via binding to a thrombin-binding site within C2 or a FXa-binding site and thus prevent FVIIIa release from vWF.

C Inhibition of FVIIIa interactions within Xase.
In a hypothetical configuration of the Xase complex, FVIIIa and FIXa are depicted as multidomain structures; the targets of inhibitors are shown as diamond-shaped indicators 1–4:
(1) inhibitors with C2 epitopes block FVIIIa binding to the PL membrane;
(2, 3) anti-A2 antibodies and anti-A3 antibodies disrupt FVIII interaction with the protease domain and the epidermal growth factor (EGF)-like domain of FIXa, respectively;
(4) anti-A1 inhibitors most probably prevent FVIIIa binding to substrate FX as their epitope overlaps with the FX-binding site 349–372.

It is noteworthy that the residues of the hydrophobic "feet" in the C2 domain involved in binding to PL (in the "tenase" complex) are also critical for binding to vWF (in circulating FVIII/vWF complex) (Gilbert *et al*, 2002). This overlap explains why anti-FVIII antibodies with C2 specificity possess a dual inhibitory mechanism preventing FVIII interaction with both PL and vWF (Saenko *et al*, 2002a). In addition, the acidic region 3 ("AR3" in Figure 1.2 or "a3" in Figure 1.5) of the light chain (aa 1649–1689), representing the major vWF-binding site, was also found to be a target of anti-FVIII inhibitors (Ananyeva *et al*, 2004). As formation of the FVIII/vWF complex plays a protective role, abolition of this interaction by the inhibitors in these epitopes renders FVIII molecule more susceptible to proteolytic inactivation by the APC, factors IXa and Xa.

While the inhibitors against the epitopes in the C2 domain inactivate FVIII:C via interference with PL and vWF binding, another class of FVIII inhibitors targets the A2 or A3 domains and impairs FVIII binding to factor IX (Figure 1.5). Anti-FVIII inhibitors with binding specificity for the A2 domain form a large class and share the major epitope involving the residues 484-508 (Spiegel & Stoddard, 2002; Ananyeva *et al*, 2004). These inhibitors inactivate FVIIIa by a direct blocking of the A2 domain interaction with the protease domain of factor IXa, and hence impair activation of factor X to Xa (Figure 1.6) (Fay & Scandella, 1999). The high-affinity biding of factor IXa to FVIII can also be intercepted by inhibitors that recognise the epitope within the A3 domain (aa 1778-1823) (Ananyeva *et al*, 2004). Occasionally, inhibitors were found to prevent FVIII from binding to its substrate factor X, by acting on an epitope in the acid region 1 (aa 351-365) (Figures 1.5 and 1.6).

In addition, the C1 domain has been identified to contain a novel epitope targeted by an inhibitor that prevented FVIII from binding to vWF (Jacquemin *et al*, 2000). This inhibitor occurred with Arg2150His mutation in the C1 region, which is associated with higher incidence of inhibitor in mild/moderate haemophilia A (Hay, 1998; Oldenburg & Pavlova, 2006). It is proposed that certain missense mutations in mild haemophilia, such as those in the C1/C2 junction including Arg2150His, are especially critical for the immunogenicity of FVIII. These mutations can give rise to conformational changes in the structure

of the FVIII molecule, which leads to attenuated FVIII function (e.g., reduced ability to bind to vWF) and a predisposition to inhibitor formation (Peerlink *et al*, 1999; Jacquemin *et al*, 2000; Oldenburg & Pavlova, 2006). Compared to other regions, the work on epitope detection in the C1 domain is limited.

1.6.5 Treatment of inhibitor patients

The therapeutic options for patients with high-titre inhibitors include the use of materials that bypass the need for FVIII to allow coagulation, such as administration of porcine FVIII, prothrombin complex concentrates (PCC), activated PCCS (APCCs) and more recently, human recombinant activated factor VII (Key, 2004; Berntorp *et al*, 2006).

It is strongly recommended that once an inhibitor is diagnosed, immune tolerance induction (ITI) should be considered as soon as possible (Berntorp *et al*, 2006). ITI regimes using FVIII alone or combined with immunosuppressive drugs are the only proven strategy to achieve antigen-specific tolerance to FVIII in individuals with FVIII inhibitors (Key, 2004).

Various ITI regimens have been developed and many of them start with regular infusion of FVIII and require administration for months or years. While the overall success rate with ITI is 50-70%, the relapse rate is 15% over 15 years and some patients require prophylaxis in order to maintain suppressed production of their inhibitor antibody and avoid frequent joint bleedings. Currently there is an ongoing international ITI study to compare the efficacy, morbidity and cost effectiveness of low versus high dose ITI in good-risk patients (http://www.itistudy.com) (Berntorp *et al*, 2006).

1.7 Haemophilic Arthropathy

1.7.1 Arthropathy in severe haemophilia A

1.7.1.1 History and pathophysiology

Arthritis associated with excessive bleeding was first recognised in 1674 by Philip Hochstetter (Stein & Duthie, 1981). In 1868 Volkmann noted that arthritic joints in people with haemophilia resulted from "bleeding into joints that occurred either spontaneously or following minimal trauma" (Stein & Duthie, 1981). The original classical description of haemophilic arthropathy was however not given until 1892, by an orthopaedic surgeon Konig (Gilbert, 2000). Konig reported that it was bleeding into the joint was the direct cause of the arthritis associated with haemophilia. Since then, the natural history of the unique haemophilic arthropathy has been well studied macroscopically, microscopically, biochemically, pathophysiologically as well as roentgenologically (Stein & Duthie, 1981; Luck & Kasper, 1989).

Since the research in the 1950s on haemophiliac dogs where bleeding was first observed to occur into the synovium, and recurrence of bleeding was observed to cause inflammatory processes in the joint. Since then, there have been many opinions on the mechanisms of joint damage (Swanton, 1959). Some researchers emphasised the importance of the inflammatory changes of chronic synovitis with synovial hypertrophy, as a cause of cartilage destruction (Arnold & Hilgartner, 1977). Others described the importance of iron coupled to haem molecules as a cause of cartilage destruction (Sokoloff, 1975; Stein & Duthie, 1981). The cartilage degeneration and joint destruction resembled the features of both rheumatoid arthritis and osteoarthritis (Arnold & Hilgartner, 1977; Ahlberg & Pettersson, 1979).

Although the exact pathogenesis of the advanced crippling arthritis, termed haemophilic arthropathy, remains incompletely understood, it is recognised to be a multifactorial event (Rodriquez-Merchan, 1997; Lafeber *et al*, 2008). Recent findings from *in vitro* experiments and animal models suggested that the deposit of iron in the joints appeared to exert a central role (Lafeber *et al*, 2008). First, iron may promote the apoptosis of chondrocytes by catalysing the formation of oxygen metabolites: thus the intra-articular blood causes damage to cartilage, independently of synovial changes. Secondly, iron may also

act on the synovial membrane by favouring its proliferation through the induction of proto-oncogenes involved in cellular proliferation and stimulation of inflammatory cytokines as well as abrogation of apoptosis (Lafeber *et al*, 2008). These two processes, one degenerative and cartilage-mediated, the other inflammatory and synovium-mediated could occur in parallel or sequentially (Lafeber *et al*, 2008).

1.7.1.2 Clinical picture

Overall, recurrent episodes of acute bleeding into joints were the hallmark of the clinical presentation in severely affected patients with haemophilia A, before the implementation of prophylactic FVIII therapy in the early 1970s. Haemarthrosis arises either spontaneously (associated with day-to-day use of joints) or following external injuries. Haemarthroses start in early childhood, occur on average about 30 times per year and account for more than three-quarters of total bleeding episodes in severe patients (Rodriquez-Merchan, 1996; Lowe, 1997).

The joints most frequently involved are large joints, such as knees, ankles and elbows (Johnson & Babbitt, 1985; Lofqvist *et al*, 1996; Rodriguez-Merchan, 1996; Lowe, 1997; Bolton-Maggs & Pasi, 2003). In one publication by in 1978, the reported frequency of bleeding episodes in the different joints were found to be 44%, 26% and 15% in the knee, elbow and ankle, respectively (Hoskinson & Duthie, 1978).

Repeated haemorrhages into joints eventually results in progressive synovial hyperplasia, destruction of articular cartilage and other reactive changes in adjacent bone and tissue. Due to the thickened and folded synovium, the affected joint has limitation of motion, and thus has a tendency for subsequent haemorrhages. The affected joint is termed a “target joint”, which is usually characterised by pain, swelling, warmth and restricted motion, when it fails to fully recover between bleeding episodes (Rodriquez-Merchan, 1996; Gilbert, 2000; Bolton-Maggs & Pasi, 2003).

Once a target joint is established, complete resolution is possible, but more commonly there is a slow response to treatment and disabling arthritis will develop due to the vicious cycle of recurrent bleeding

and chronic inflammation (Gilbert, 2000). In affected joints, loss of motion, severe contractures and other deformities are commonplace. Advanced arthropathy, which progresses from joint damage before early adolescence, often causes moderate to severe pain and significant incapacity by the third or fourth decade of life.

A high incidence of joint damage in haemophilic patients was well reported in the literature with an estimated incidence of 80% from the total patient population in the United States prior to the universal availability of the effective treatment with plasma-derived FVIII (Stein & Duthie, 1981). At that time, all patients with severe bleeding disease had some degree of arthropathy by the time of reaching early adolescence (Arnold & Hilgartner, 1977).

1.7.1.3 Management

Successful management of haemophilic joint bleeding aims to prevent the development of chronic arthropathy by reversing the acute bleeding process and restoring normal joint function as early as possible. This requires a multidisciplinary approach led by the haematologist but includes input from orthopaedic surgeons and physiotherapists (Ribbans *et al*, 1997; Gilbert, 2000; Negrier *et al*, 2008).

The treatment for acute haemarthrosis comprises firstly of prompt replacement of the missing FVIII to a level high enough to stop bleeding and for a long enough period of time to prevent recurrence. Secondarily, non-specific conservative measures such as treatment with analgesics, rest, immobilisation of the joint with elastic bandages, splints or slings, and supervised rehabilitation should be in place. In some cases, joint aspiration may be considered (Ribbans *et al*, 1997; Gilbert, 2000).

At the present time, episodic replacement therapy is still the mainstay, with a single transfusion of FVIII achieving a level of 30 to 40% leading a good response in most spontaneous haemorrhages (Gilbert, 2000). Repeated infusion at 12 or 24-hour intervals may be necessary, achieving a level of 50% of normal FVIII levels in cases when clinical evidence of persistent bleeding is present.

For treatment for chronic synovitis or arthritis in haemophilia A however, there are fewer choices and a poor functional prognosis. Ultimately, orthopaedic surgery such as synovectomy (open or arthroscopic, chemical or radioactive) and total joint arthroplasty at a later stage will be considered to offer a solution for relieving pain and improving joint function (Lofqvist *et al*, 1996).

1.7.1.4 Prophylaxis

In early 1990s, a Swedish study in severe haemophilia patients treated with prophylactic replacement of coagulant factors commencing at early age (i.e., 1 to 2 years old) reported on the effectiveness of eliminating haemophiliac arthropathy (Nilsson *et al*, 1992). The benefits of prophylactic therapy was supported by a 5-year longitudinal multicentre study involving United States, Europe and Japan, which demonstrated that long-term prophylaxis improves the orthopaedic outcome by reducing the frequency of haemarthrosis (Aledort *et al*, 1994). When the prophylaxis was intense, there was less deterioration of the joints (Aledort *et al*, 1994).

1.7.2 Arthropathy in mild and moderate haemophilia A

Considerably less data is available on orthopaedic complications in people with the mild or moderate form of haemophilia A, compared to severe haemophilia. This may be partly due to the general impression that bleeding episodes are less frequent in these milder cases.

In one early publication, 12 patients with moderate haemophilia (9 haemophilia A and 3 haemophilia B) had 16 plain X-ray examinations of the knee joints (Pettersson *et al*, 1980). Using the Pettersson scores, which were later universally accepted for radiological evaluation of haemophilic arthropathy, 9 of 32 knee joints scored positively, indicating the presence of arthropathy. The age of the patients when the X-rays with positive results were taken was young: from 9 to 23 years (Pettersson *et al*, 1980).

It was also noted that the radiological changes of the joint once present, would inevitably progress in follow-up examinations. The rate of this progress was independent of patient's age when arthropathy

was first diagnosed on X-ray, or the severity of haemophilia (Pettersson *et al*, 1980). A score of 3 or more suggested a condition resembling progressive and destructive osteoarthrosis, irrespective of further bleeding and treatment of the joint (Ahlberg & Pettersson, 1979; Pettersson *et al*, 1980).

Even though these studies were conducted before the institution of FVIII replacement therapy became available, the results reflect to certain degree the presence and severity of haemophilic arthropathy in patients with mild and moderate haemophilia A.

While prophylaxis was initiated in the severe cases, the orthopaedic complications in the milder form of haemophilia remained significant. Associated with the Swedish study on prophylaxis in haemophilia patients, the authors summarised the orthopaedic surgeries performed in these patients during a 20-year period of time (1970 to 1989) (Lofqvist *et al*, 1996). Among a total of 66 patients (mean age of 13 years, range 6-71) who had a total of 98 orthopaedic surgical procedures, 53 were haemophilia A patients, including 39 severe cases, 7 moderate and 7 mild cases. While the number of patients who underwent surgeries in the severe haemophilia group was reduced from 29 to 10 over the decade since the introduction of prophylaxis, the same number of patients (7 of 14, 50%) from the mild/moderate group required surgery in both 1970-1979 and 1980-1989 decades (Lofqvist *et al*, 1996).

A more recent study on 46 patients with moderate haemophilia A (median age 17 years) with a median follow-up of 8 years also provided some evidence on the prevalence of arthropathy in these “milder” patients (Fischer *et al*, 2000). While approximately 33% of these moderate patients received FVIII prophylaxis, the Pettersson scores were positive in more than half (51%) of the 35 patients who had the radiological evaluation of their knee, elbow and ankle joints (Fischer *et al*, 2000). However, the authors did not provide information on the prevalence of arthropathy as classified by different joints (e.g., knees vs ankles).

1.7.3 Ankle arthropathy

1.73.1 Prevalence and severity

It is well known that in severe haemophilia, large joints are commonly affected with haemorrhages and arthropathy, and the lower extremities are more frequently involved than the upper extremities. Some studies showed that the ankle was the third most frequently targeted joint in haemophilia, following the knee and the elbow (Hoskinson & Duthie, 1978). It is noted that perhaps age, or rather, life activities related to age, has a defining influence on which joint will be likely involved in haemophilic arthropathy.

It was claimed that the ankle joint was the first target joint in childhood (Luck & Kasper, 1989). Another study also stated that the ankle was frequently the target joint before the age of 5, after which the knee joint assumed a more prominent role (Rodriguez-Merchan, 1996). A one-year observation on 75 severe haemophilia patients (73 haemophilia A; mean age 23 years, range 0.5-58) concluded that the knee was the most commonly affected joint during the first and third decades of life while the ankle was the leading joint during the teenage years (Gamble *et al*, 1991).

To examine whether improvements in the medical treatment of haemophilia over the past few decades including effective FVIII replacement therapy had any beneficial effect on ankle arthropathy, Ribbans and colleagues analysed the progression of ankle function in 115 patients with severe haemophilia, by reviewing their serial ankle radiographs, some of which dated back to the 1950s (Ribbans & Phillips, 1996). The results of this cross sectional and longitudinal study suggested that the different generations (grouped by decade) of haemophilia patients demonstrated no significant difference in the pattern of deterioration of ankle arthropathy with age, and that changes in treatment schedules have not yet led to a reduction of the scale of the problem (Ribbans & Phillips, 1996). It was also concluded that progressive ankle arthropathy caused significant disability in many (severe) haemophiliac patients.

A more recent study used clinical, and radiographic scores as well as subjective pain status to assess arthropathy in 79 haemophilia patients (76 haemophilia A; 74 severe and 2 moderate; median age 44

years, range 18-63) (Wallny *et al*, 2002). It revealed that the ankle joint was the most frequently affected joint (83% had arthropathy by radiology assessment), followed by the knee (68%), the elbow (53%) and the hip (12%). Ankle pain was present in 70% of patients. It is important to note that 91% of these patients received FVIII regularly (at least 3000 IU per week) rather than episodically (Wallny *et al*, 2002). This is consistent with the previous study (Ribbans & Phillips, 1996) highlighting that ankle arthropathy remains a significant problem in the care of haemophilia patients today.

1.7.3.2 Ankle arthropathy in mild and moderate haemophilia

In mild and moderate haemophilia, there has only been limited data on arthropathy in general, as summarised in Section 1.7.2. This also includes only scant information on ankle arthropathy in these patients. One early publication, which studied 95 severe, 38 moderate and 24 mild haemophilia patients, found ankle joint changes present in "one half" of patients with severe cases, "one-third" of those with moderate disease and only 1 of 24 with mild haemophilia (Ahlberg, 1965).

In recent years, occasionally available are some data on clinical symptoms such as bleeding and pain associated with haemophilic arthropathy in patients with the milder form of the disease. In a study by questionnaire to analyse levels of health-related quality of life in individuals with haemophilia in UK, results from 66 severe and 100 mild/moderate patients were summarised (Miners *et al*, 1999). All of these individuals were at least 18 years of age, and none of them had received primary prophylaxis with factor VIII. While 57% of the mild/moderate patients had no complaints about bodily pain or discomfort, another 36% experienced pain to a moderate degree and 7% to an extreme degree. In the severe group, the corresponding percentages were 16%, 79% and 5% respectively (Miners *et al*, 1999).

Another publication found that the annual bleeding episodes in Finnish patients with moderate haemophilia (subtotal number 25, 18 haemophilia A), along with severe haemophilia (subtotal number 52, 48 haemophilia A) (mean age 43 years), was still disconcertingly high (Solovieva, 2001). The

annual frequency of bleeding into joints was 28 times per patient for the severe group, and 11-15 times for the moderate group respectively during a 3-year (1994 to 1997) follow-up (Solovieva, 2001).

None of these publications specified which joint the symptom of pain existed or bleeding occurred. Nevertheless such data implied that there might be a reasonable degree of joint disease in mild/moderate haemophilia A.

1.7.4 Evaluation of haemophilic arthropathy

1.7.4.1 Earlier methods

In order to allow comparison between subjects as well as longitudinally in a single subject, arbitrary joint scores have been used to describe various degrees of arthropathy since 1960s. The scores are usually based on both clinical and radiological evaluation of the affected joints.

One Swedish group was amongst the first to develop and deploy a scoring method to study haemophilic arthropathy (Ahlberg, 1965). They used points 1 to 3 to describe the combined effect of clinical joint dysfunction (reduced mobility) and X-ray changes in individual joint. Point 3 meant advanced changes with marked loss of mobility or even ankyloses (Ahlberg, 1965).

Using this method, the same group reported that the mean joint score (sum of points from all affected joints in an individual patient) without prophylaxis was 1.2 for moderate haemophilia A patients, and 9.5 for severe patients , in the 15-19 year old age group (Nilsson *et al*, 1976). The authors also showed that with regular factor VIII replacement, the severe patients could achieve an identical joint score to that of the moderate group (Nilsson *et al*, 1976). This result thus demonstrated the utility of joint scoring.

1.7.4.2 Goniometer to measure (ankle) joint motion

To investigate the range of movement of joints including the ankles, a clinical goniometer can be used. In a publication in 1979, the normal range of motion of joints in 109 healthy male subjects (age from 18

months to 54 years) was measured using an ordinary goniometer (Boone & Azen, 1979). The data were compared with the estimations in the handbook of the American Academy of Orthopaedic Surgeons (AAOS) as reference. The mean degree (+/- standard deviation, SD) for ankle plantar flexion was 56.2° (+/- 6.1°), and for ankle dorsiflexion (extension) was 12.6° (+/- 4.4°) (Boone & Azen, 1979), similar to the AAOS reference ranges (48 and 18 degrees respectively).

Boone and colleagues found that while the range of motion (ROM) of ankle dorsiflexion did not show any statistically significant difference in the subgroups of people greater than 19 years of age and those younger, the ROM of ankle plantar flexion did. There was approximately 4° (mean) decrease of ankle plantar flexion in the adult subgroup (Boone & Azen, 1979). This study is probably the only source on detailed measurements of normal ankle motion according to the subjects' age. The authors also found that in unaffected joints amplitudes of motion of left and right ankle joints were consistently similar therefore the motion of a patient's "healthy" limb can routinely serve as a comparison for the affected side in the presence of disease (Boone & Azen, 1979).

Since Boone's publication in 1979, standard goniometric techniques have been commonly used to measure the range of movement of joints in haemophilic arthropathy. Using a goniometer to study ankle joints in haemophiliacs, Johnson and colleagues accepted a 70° ROM for a normal ankle (20° dorsiflexion and 50° plantar flexion). In the 48 haemophilia patients studied (46 haemophilia A; 32 severe, 8 moderate and 8 mild) the mean total range of motion was reduced: for the right ankle 50.7° (+/-18.9°, 2SD) and the left 54.3° (+/- 15.4°) respectively (Johnson *et al*, 1984). They also noted that loss of dorsiflexion of the ankle, along with loss of flexion of the knee and extension of the elbow, are the earliest signs of arthropathy and suggested that these positions should be managed sensitively clinically (Johnson *et al*, 1984).

In subsequent studies including those recommendations from the World Federation of Haemophilia (WFH), ankle plantar flexion of 50° and dorsiflexion of 20° have been adopted as normal reference range.

1.7.4.3 Pettersson scale to evaluate (ankle) arthropathy radiologically

The biomechanics of the ankle joint including range of motion were also studied using roentgenographic technique, possibly at an earlier time than the use of goniometer, in an attempt to more accurately record the complex kinematic picture of ankle motion. In an early publication, results of the measurements of ankle motion in 50 normal subjects by lateral roentgenogram and clinical examination (methods not detailed) were analysed (Weseley *et al*, 1969). There was a significant variation in the range of ankle motion in individuals but the clinical measurement was mostly higher (range 2-58°) than the X-ray measurement (Weseley *et al*, 1969). A few years later, on examining X-ray films of 26 normal ankles, it was found that there was an average of 44 degree of total range of motion for the ankle joint (Sammarco *et al*, 1973). It was also found that in normal ankles, the degree of plantar flexion tended to decrease with increasing age. In addition, the same study found that in diseased ankles (14 subjects), the range of motion tended to decrease with loss occurring in both dorsiflexion and plantar flexion (Sammarco *et al*, 1973).

Subsequently, a more general approach to evaluation the roentgenographic changes of various joints was developed, in parallel to the development of physical measurements of joints to detect and monitor haemophilic arthropathy. In 1977, Arnold and colleagues categorised haemophilic arthropathy into five stages (Arnold & Hilgartner, 1977). Before long, the Orthopaedic Advisory Committee (OAC) of the WFH adopted Pettersson's radiologic classification as the standard method for assessing haemophilic arthropathy in the early 1980s (Pettersson *et al*, 1980).

The Pettersson scale was developed on the basis of a retrospective review of approximately 1000 radiographs of the large joints from 54 Swedish patients with haemophilia A and B, with clinically severe

and moderate phenotypes. Twenty-four patients had follow-up radiologic examination 2 to 18 years later, before the implementation of any specific coagulant factor treatment (Pettersson *et al*, 1985).

The Pettersson scale includes general radiologic changes that are seen in any damaged joint in haemophilia (Pettersson, 1993). The ideal parameters used to evaluate established arthropathy should not be dependent on a recent bleeding episode but should reflect events occurring over a long period of time. These parameters should be defined and observable irrespective of the technique used and be quantifiable. Radiological evaluation elements "that are associated with acute haemarthrosis, that are dependent on the technique used at the examination and that are considered to be pathologically non-specific" should be omitted (Johnson & Babbitt, 1985; Pettersson, 1993).

The final description of the radiologic parameters that meet the criteria as specified above is recorded in chronological order of the occurrence of the radiological findings. There are 8 parameters: osteopenia, enlargement of the epiphysis, subchondral irregularity, narrowing of the joint space, subchondral cyst formation, erosion, gross incongruence between the bone ends and joint deformity (displacement and angulation) (Table 1.1). The intra-observer and inter-observer variation for individual joint was shown to be very small (Pettersson & Gilbert, 1985). Therefore the method serves as an objective guideline to evaluate joint destruction in haemophilia (Pettersson, 1993).

1.7.4.4 Joint evaluation systems recommended by WFH

Since the early 1980s, the WFH has endorsed both the radiological scoring systems (Table 1.1) and the physical scoring system (Table 1.2), along with data on pain and bleeding episodes, to evaluate haemophilic arthropathy (Pettersson & Gilbert, 1985). Both scoring systems are recommended by the Orthopaedic Advisory Committee of the WFH.

TABLE 1.1 WFH Guidelines for the Physical Examination of Haemophilic Arthropathy (Specified for Ankle Joint)

Physical Signs	Scores	Description
1) Ankle Swelling	0	None
	2	Present
2) Calf Muscle Atrophy	0	None or minimal
	1	Present (difference > 1 cm)
3) Axial Deformity	0	No deformity
	1	Up to 10° valgus or up to 5° varus
	2	> 10° valgus or > 5° varus
4) Crepitus on Motion	0	None
	1	Present
5) Loss of Range of Motion (ROM)	0	Loss of < 10% of total FROM
(full ROM: dorsiflexion 20°, plantar flexion 50°)	1	Loss of 10 - 33% of total FROM
	2	Loss of > 33% of total FROM
6) Flexion Contracture	0	< 15° fixed flexion contracture (FFC)
	2	Equinus at ankle
7) Instability	0	None
	1	Noted on examination but neither interferes with function nor requires bracing
	2	Instability that creates a functional deficit or requires bracing
Total score for each ankle joint	**0 - 12**	(0 for normal ankle)

TABLE 1.2 WFH Guidelines for the Radiological (Plain X-ray) Evaluation of Haemophilic Arthropathy (Pettersson Scale)

Plain X-ray Signs	Scores	Description
1) Osteoporosis	0	Absent
	1	Present
2) Enlarged Epiphysis	0	Absent
	1	Present
3) Irregular Subchondral Surface	0	Absent
	1	Partly involved
	2	Totally involved
4) Narrowing of Joint Space	0	Absent
	1	Joint space > 1 mm
	2	Joint space < 1 mm
5) Subchondral Cyst Formation	0	Absent
	1	1 cyst
	2	> 1 cyst
6) Erosion of Joint Margins	0	Absent
	1	Present
7) Gross Incongruence of Articulating Bone Ends	0	Absent
	1	Slight
	2	Pronounced
8) Joint Deformity (Angulation and/or Displacement)	0	Absent
	1	Slight
	2	Pronounced
Total score for each ankle joint	**0 - 13**	(0 for normal ankle)

The physical scoring system is based on physical examination of joints using Gilbert's method, with a score between 0 to 12 assigned to each joint (Gilbert, 1993). The radiological (plain X-ray) scoring system uses the Pettersson scale, with a score between 0 to 13 graded for each joint (Pettersson *et al,* 1980). Using both the clinical (physical) and radiological scoring systems, the state of the joint may be expressed in short terms, allowing easy comparison of progression of arthritis from one time to another in the same patient, or comparison between patients.

Both scoring systems are suitable for assessment of large joints such as knee, ankle and elbow, excluding the situation of recent haemarthrosis. Both systems are indeed well used by clinicians over the years (Hamel *et al*, 1988; Nilsson *et al*, 1992; Funk *et al*, 1998; Aznar *et al*, 2000; Molho *et al*, 2000; Wallny *et al*, 2002).

However, there seems to be limited data on the utility of the above systems in the normal population to identify the presence of "arthritic" joint or measure joint "changes" in healthy subjects of different age groups. It was also noted that despite being adopted as the gold standard for the evaluation of haemophilic arthropathy for the past two decades, the guideline was never formally validated (Manco-Johnson *et al*, 2000; Hilliard *et al*, 2006).

1.8 Aims of the Thesis

Over the past few decades there has been an increase in knowledge concerning the molecular pathogenesis of haemophilia A and the development of FVIII inhibitors. A better understanding of the mechanisms by which these inhibitors are formed and their modes of action will assist new clinical strategies to treat inhibitor patients more successfully. Epitope detection is a part of this research, and has provided information on the location and amino acid sequence of FVIII segments targeted by the inhibitors and how FVIII:C might be inactivated by inhibitors.

Using either recombinant FVIII techniques or porcine/human FVIII hybrids, the epitope mapping on the FVIII molecule has now narrowed the antigen specificity down to a few small regions mainly located in the A2, A3 and C2 domains (Key, 2004). While the epitopes in the A2 or A3 domain overlap with the binding site of FVIII to FIX, the epitopes in the C2 domain are within or in close proximity to the binding site for PL/vWF. In addition, in mild/moderate haemophilia A, the C1 domain has been found to contain a novel epitope which is important in the inactivation of FVIII:C by preventing its binding to vWF (Jacquemin *et al*, 2000).

It was of interest to obtain information on the epitope specificity in our haemophilia patients with inhibitors, and to compare this to existing knowledge. It was hopeful that the inhibitory effect of the antibodies on FVIII:C could be eliminated by using the recombinant FVIII fragment to block the interaction between the inhibitors and the FVIII molecule. The production of a monoclonal anti-FVIII antibodies would, if directed against the C1 or C2 regions, provide an alternative antibody (inhibitor) for use in these studies.

At the time of this study, considerable interest was developing in exploring the possibility of applying the TGA to the assessment of patients with haemophilia. The use of a TGA in our haemophilia A patients, particularly the mild and moderate cases, would potentially provide additional information to the traditional clotting-based assays thus provide a more complete "coagulation phenotype". The TGA might help to explain the observation that some of the mild/moderate haemophilia A patients have *discrepant* results by one- and two-stage FVIII assays (Lloyd in Ingerslev *et al*, 2008).

In addition, we had noted in our clinic that many patients with mild and moderate haemophilia A had chronic ankle arthropathy, and that this was often severe and disabling. Unlike arthropathy in severe haemophilia, which has been well described, arthropathy in mild haemophilia seemed to affect the ankle joint only. There is little or no published information on the frequency and severity of ankle arthropathy in mild/moderate haemophilia A. We considered that it was important to investigate the prevalence of

ankle arthropathy in our patients, taking advantage of the reasonably large population and well-established family pedigrees of mild and moderate haemophilia A here in South Australia, and the readily applicable WFH scoring systems (physical and radiological) for evaluation of haemophilic arthropathy (Pettersson *et al*, 1980; Gilbert, 1993). Results from this project may alter our care plan for the patients.

Therefore the overall aims of this thesis were:

1) i) To establish a cell expression system to produce the domain-specific recombinant FVIII fragments, C1 and C2.

ii) To produce a murine monoclonal anti-FVIII antibody.

2) To use an in-house TGA to examine the thrombin generation profile in mild and moderate haemophilia A, and its correlation with the FVIII level.

3) To investigate the prevalence and severity of ankle arthropathy in mild and moderate haemophilia A and determine the relationship between arthropathy and the patient's FVIII level.

CHAPTER TWO PRODUCTION OF RECOMBINANT FVIII PEPTIDES AND MURINE MONOCLONAL ANTI-FVIII ANTIBODIES

2.1 Introduction

FVIII inhibitors are a major problem in the management of haemophilia A. At present the main approaches of treating this problem are to try to induce tolerance, and to treat haemorrhage by using bypassing agents. However, studies pioneered by Fulcher and Scandella have led to an increasing understanding of the epitopes on the FVIII molecule that react with the inhibitory antibodies (Fulcher *et al*, 1985a; Scandella *et al*, 1988). It may be possible to exploit this knowledge to improve the management of these patients.

Initial work by Fulcher and colleagues on FVIII epitope mapping used immunoblotting with thrombin-digested FVIII peptides, followed by site-directed mutagenesis of the peptides (Fulcher *et al*, 1985a; Fulcher *et al*, 1987; Ware *et al*, 1992). Scandella and colleagues systemically mapped the FVIII epitope by expressing recombinant FVIII fragments containing progressive deletions in *E. coli* and tested these fragments for its ability to bind to inhibitors in the plasmas in immunoassays (Scandella *et al*, 1988; Scandella *et al*, 1989; Scandella *et al*, 1995).

These studies demonstrated that the A2 and C2 domains are the major targets for FVIII inhibitors. Using immunoprecipitation and inhibitor neutralisation assays, in 28 plasmas from haemophiliacs and spontaneous inhibitor patients, 68% had anti-A2 as well as anti-C2 antibodies, 21% had anti-C2 antibody alone and 11% anti-A2 alone (Scandella *et al*, 1995).

This study intended to understand the antigen specificity of the FVIII inhibitors in South Australian patients with inherited haemophilia A. Currently, no data is available on the epitope specificity in Australian patients with FVIII inhibitors. It would be useful to develop a method to screen the patients in South Australia, and ultimately in other states to identify whether their inhibitor were predominantly against the A domain or C domain of FVIII (in particular the A2 and C2 domains), or both.

In addition, the C1 domain has been identified to contain a novel epitope that prevented FVIII from binding to vWF (Jacquemin *et al*, 2000). It is proposed that certain missense mutations in mild haemophilia, such as those in the C1/C2 junction including Arg2150His, can give rise to conformational changes in the structure of the FVIII molecule, which leads to attenuated FVIII function (e.g., reduced ability to bind to vWF) and a predisposition to inhibitor formation (Peerlink *et al*, 1999; Jacquemin *et al*, 2000; Oldenburg & Pavlova, 2006). Compared to other regions, the work on epitope detection in the C1 domain is limited. On the other hand, the C1 domain could also serve as a negative control for the C2 domain in the detection of epitopes, since the C2 domain is the major target for most inhibitors.

We planned to produce domain-specific peptides (e.g., A2, C1 or C2 domain) initially, using the recombinant techniques for the expression in the mammalian cells. Once the expression system was established, it can be readily applied to produce any FVIII fragment of interest, including bigger or smaller sizes.

We planned to test these peptides for their ability to bind to the inhibitors from patient plasmas. The interaction between the peptides and FVIII antibodies/inhibitors could be examined by a simple binding assay such as ELISA, or the more complex immunoblotting (Western blot) or immunoprecipitation assays. This way we hoped to be able to characterise the epitope specificity of the FVIII inhibitors in our patients. Furthermore, the recombinant peptides could be tested for its ability to restore FVIII:C by preventing the FVIII inhibitor from binding to the FVIII molecule in an *in vitro* "inhibitor neutralisation assay", where the peptide is added to the inhibitory FVIII:C functional assay (Bethesda assay).

This study further hypothesised that identifying the epitope of each subject would in long term lead to specific treatment *in vivo* for individuals with FVIII inhibitors, i.e., patient can be infused with peptide specific for his inhibitor in his circulation. Ideally the peptide could overcome the effect of inhibitors by one of these ways: by competitively blocking the binding of the inhibitor to the FVIII molecule, by exhausting the inhibitor in quantity, or by inducing immune tolerance. These recombinant peptides

would mimic the domain containing the epitope region (e.g., C1 or C2 domain), and ultimately possibly be a small molecule and against only a portion of the domain.

This thesis also aimed to establish an animal (mouse) model to produce monoclonal anti-FVIII antibodies, and test their domain specific antigenicity. These antibodies might be used in the competition experiments with the domain specific peptides (e.g., C1 or C2 peptide). This would be useful particularly in helping to develop an *in vitro* test to apply to the patients. It would also be useful for other research groups who are investigating the structure of the FVIII molecule.

There were only several FVIII monoclonal antibodies that were commercially available at the time of this study, except for those being developed and used by individual research groups. The commercial monoclonal antibodies included ESH5 (anti-HC), ESH8 (anti-LC/C2) (American Diagnostica Inc, CT, USA) and MAS530 (anti-HC), MAS531 (anti-LC) (Harlan Sera-Lab Ltd, Leicestershire, UK).

Results from this study will contribute to the knowledge of the immune mechanism and pathogenesis of inhibitor development. This can facilitate the design of new therapeutic approaches in the treatment of FVIII inhibitors. Ultimately it may be possible to produce small peptides that mimic inhibitor binding sites, and use them to preferentially counteract the effect of the inhibitors *in vivo* in individual patients.

The specific aims of this chapter were:

1) To establish a recombinant protein expression system that is able to produce small fragments of FVIII molecule (e.g., C1 or C2 domain):

   i) To use a baculovirus vector (pACGP67B) and insect cell line as preliminary approach;

   ii) To establish a stable mammalian cell expression system using a mammalian cell vector (pSecTagB), and COS-7 and CHO cell lines, for larger scale and more permanent production of the peptide of interest.

2) To produce a murine monoclonal anti-factor FVIII antibody.

3) To detect the domain-specific antigenic epitopes of the inhibitors from patients and the monoclonal anti-FVIII antibody by immunoassays (e.g., Western blot) where the recombinant FVIII fragments were used as an antigen. If successful, our long-term aim was to explore the effect on reducing FVIII:C inhibition by blocking these inhibitors using the specific peptides identified to be the inhibitory epitopes from individuals.

2.2 Methods

2.2.1 Part I: Production of recombinant FVIII peptides (C1 and C2 domain) using the expression vector pSecTagB in the COS-7 mammalian cell expression system

The methods to produce recombinant C1 peptide are described here. A flow chart for the processes of the production of the C1 peptide is shown in Figure 2.1. The same method principle and production process could be applied to other fragments of FVIII molecule. Attempts were also made to produce the C2, C1-C2 and A3 peptides. This work was performed in conjunction with the Division of Molecular Pathology at the Institute of Medial and Veterinary Science (IMVS). Ms Vicky Apostolidis of Molecular Pathology completed the production of C1-C2 and A3 peptides, the results of which are illustrated in Section 2.3.1.3 (Figure 2.6).

Figure 2.1 is the flow chart for the actual experiment processes carried out to produce the C1 peptide in the current study (Section 2.2.1.1 to 2.2.1.3). It also illustrates the principles of the recombinant techniques to produce FVIII peptides, which mainly involved the following six steps (Figure 2.1).

Step one: design and amplification of the target gene (e.g., the C1 DNA) using a polymerase chain reaction (PCR). The primers for the PCR were designed to incorporate the restriction enzyme sites so that the target gene produced contained a BamHI restriction site at the 5'-end and a NotI site at the

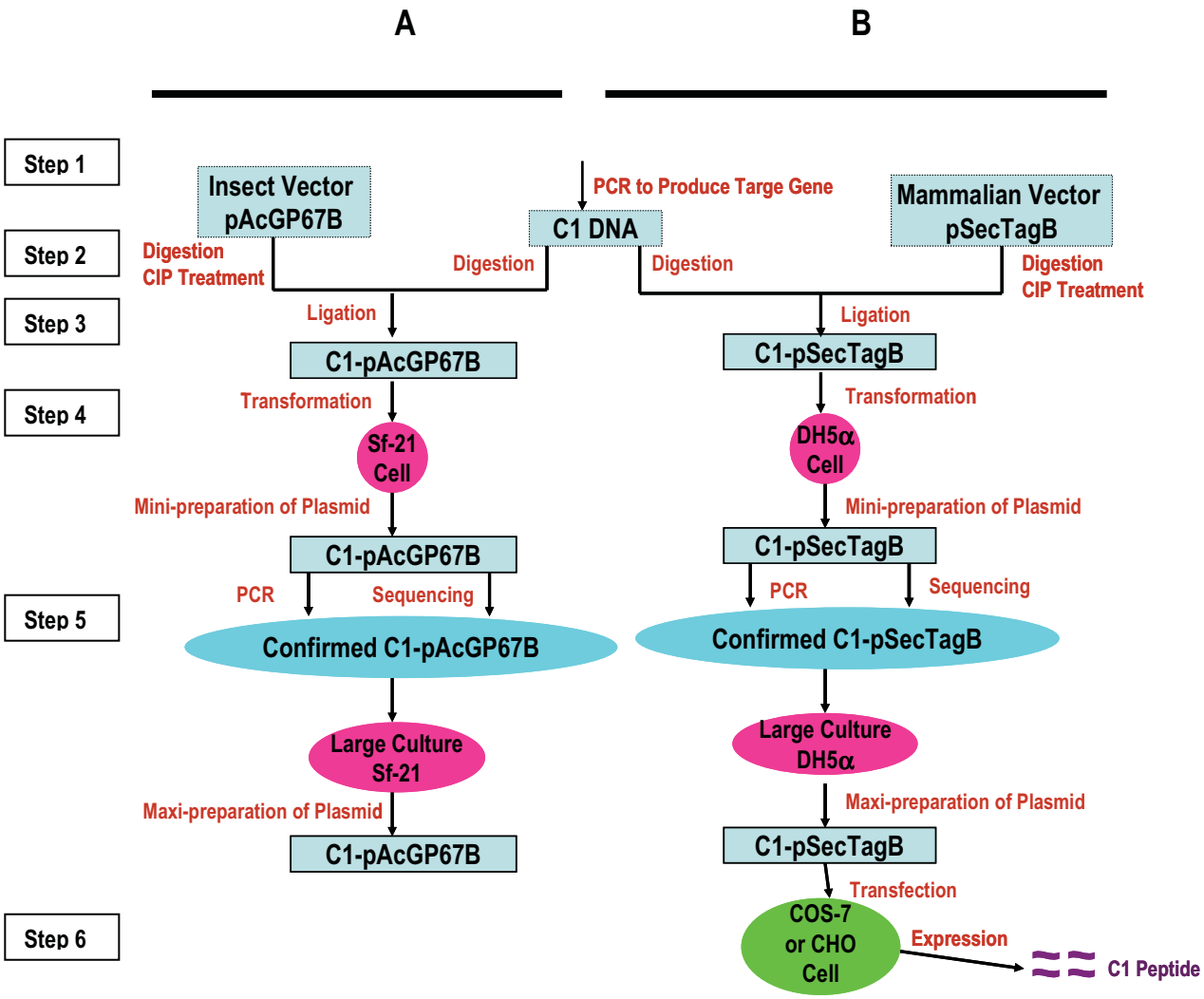


FIGURE 2.1 Flow Chart for the Production of Recombinant C1 Peptide

A. Preliminary production of C1 recombinant plasmid using insect cell vector system (baculovirus vector pAcGP67B and insect cell line Sf-21).

B. Production of C1 peptide using mammalian cell expression system (mammalian cell vector pSegTagB and mammalian cell lines COS-7 and CHO cells).

3'-end. Both sequences were present in the multiple cloning sites of the vector plasmid (e.g., both the baculovirus expression vector pAcGP67B and the mammalian expression vector pSecTagB have BamHI and NotI sites).

Step two: digestion. Both the target gene and the vector were digested by the restriction enzymes (BamHI and NotI). The vector also underwent Calf Intestinal Alkaline Phosphatase (CIP) treatment to prevent self-ligation.

Step three: ligation. In this step, the insert (target gene) and the vector was re-united by a ligase to form a circular DNA again, resulting in a recombinant plasmid with the target gene properly inserted.

Step four: transformation or transfection. The recombinant plasmid DNA (construct), which contained the target gene, was *transformed* into insect cells (e.g., Sf-21) or bacterial cells (e.g., *E coli* DH5α strain), or *transfected* into mammalian cells (e.g., COS-7 or CHO cells). Maxi-preparation of the recombinant plasmid was performed to obtain adequate quantities of the plasmid prior to transfection of the mammalian cells.

Step five: confirmation of the successful construction of the recombinant plasmid. This was the extra step to examine the proper insertion of the target gene by a PCR and direct sequencing of the recombinant plasmid. This was performed after mini-preparation of the plasmid from the cell cultures.

Step six: expression of recombinant peptide. The transfected cell lines were expanded in the selective culture medium containing an appropriate antibiotic that would only sustain the growth of cells with a specific resistance gene. The expressed protein (e.g., C1 peptide) was expected to be secreted into the culture media hence could be harvested in the supernatant. The expressed peptide would carry an extra string of amino acids (e.g., the myc-tag) so that the recombinant protein could be identified when probed by specific antibodies (e.g., the anti-myc antibody).

Figure 2.2 illustrates the structure of mammalian expression vector pSecTagB, which was successfully used to produce the C1 recombinant peptide. The pSecTagB vector was selected as it could be used to express recombinant target proteins in *E.coli,* as well as in a mammalian cell line. The vector has a cytomegalovirus promoter that drives the expression of the target gene, as well as the myc epitope, which is encoded by a myc-tag (green box) that is localised immediately adjacent (3') the multiple cloning site (orange box). Expression of the full length recombinant protein could be detected using anti-myc antibodies against the myc epitope that is concomitantly expressed with the target protein. A Igk leader sequence directs the secretion of a fusion protein. The vector has a Zeocin (bleomycin) resistance gene for selection of stable transfectants in mammalian cell lines. There is also an ampicillin resistance gene that can be used to select for success transformation of the plasmid in *E.coli*.

2.2.1.1 PCR to produce the target gene (C1 DNA)

The complete complementary DNA (cDNA) sequence of human FVIII encoding the 2,351 amino acids (including a signal peptide of 19 aa) of the FVIII molecule was identified by cloning the genomic library of the X chromosome (Wood *et al*, 1984). The C1 target gene starts from H6115 (HC 6115th base pair (bp)) and finishes at H6576 (HC 6576th bp), and the length of the gene is approximately 460 bp. It codes for amino acids from Lys2020 to Asn2172. The cDNA corresponding to the C1 gene was amplified by a PCR, with the two primers designed to insert a BamHI restriction enzyme site at the 5'-end of the C1 PCR fragment, and a NotI restriction enzyme site at the 3'-end of the C1 PCR fragment.

The base pair sequences recognised by BamHI and NotI restriction enzymes are "G↓GA TCC" and "GC↓GGCCGC" separately. Therefore, the primer sequences used in this PCR reaction were designed as follows:

the 5'- (or sense strand) oligonucleotide primer, H6115 BamHI:

5'- CCGTA$_{(\text{flanking nucleotide})}$ **GGA TCC** AAG$_{(\text{Lys2020})}$ TGT CAG ACT CCC CTG GGA ATG -3';

the 3'- (or anti-sense) oligonucleotide primer, H6576 NotI:

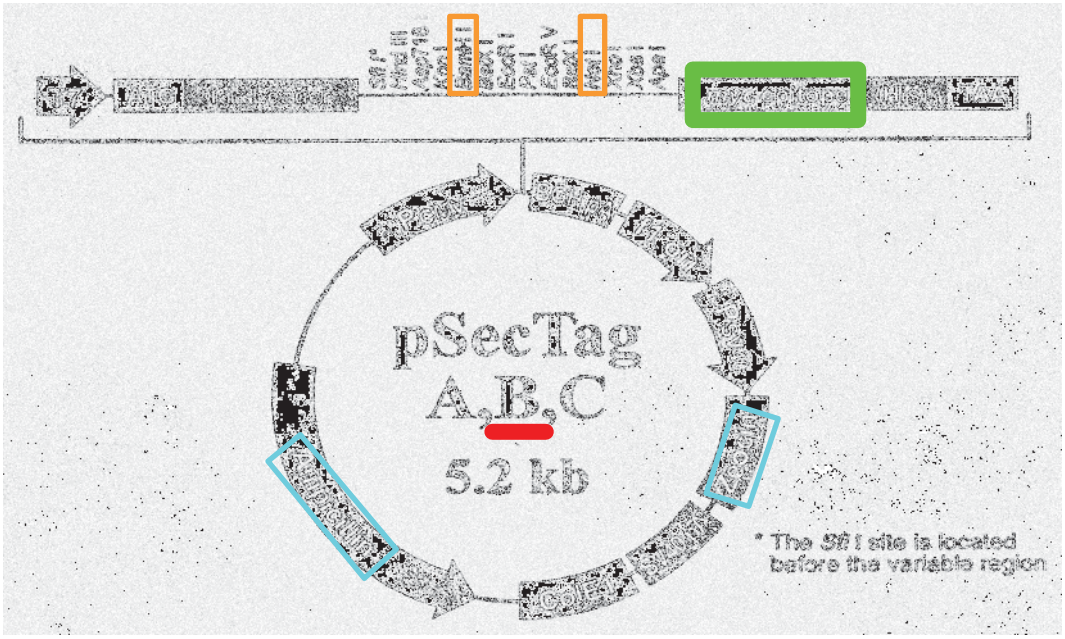


FIGURE 2.2 Structure of the Plasmid Vector pSecTagB

The plasmid vector pSecTagB is of approximately 5.2 kb in size and has multiple restriction recognition sites that can be used to insert target genes.

The restriction sites of interest in this study were the BamHI and NotI sites (highlighted in brown). Correspondingly, the target gene (C1 or C2 DNA) was designed with the restriction sites at its 5'- and 3'-ends respectively. After digested by BamHI and NotI, the linear target gene and the vector re-ligates to form a circular plasmid DNA (i.e., the new construct).

This recombinant plasmid (construct) is then used to *transform* bacterial cells (e.g., *E coli* DH5α cells) or *transfect* mammalian cells (e.g., COS-7 or CHO cells), during which process the plasmid DNA integrates into the chromosome of the host cell. The resistance genes in the vector plasmid ("Ampicillin" or "Zeocin", highlighted in blue) are used to select for successfully transformed/transfected cells in the culture media containing specific antibiotics. This function is more commonly used for the transformation of the bacterial cells (e.g., DH5α) or transfection of the permanent cell lines (e.g., CHO cells) for mass production of the target protein.

When the desired peptide (C1 or C2 peptide) is expressed, it carries the "myc" protein tag, coded by the nucleotide sequence in the "myc epitope" in the vector (highlighted in green). Subsequently an anti-myc antibody can be used to probe for the protein in immunoassays (e.g., Western blot).

3'- CCGTAAAT$_{(flanking\ nucleotide)}$ **G CGG CCG C**TT ATT$_{(Asn2172)}$ TAA ATC ACA GCC CAT CAA CTC CAT$_{(Met2164)}$ -5'.

The primers were purified and diluted to a working concentration of 15 μM in double distilled water (DDW, Greiner Bio-One GmbH, Kremsmueuster, Austria) for the use in the PCR reaction.

FVIII cDNA was amplified using the Pfx High Fidelity PCR System (Invitrogen Corporation, CA, USA). The master mix was in a total volume of 50 μl and composed of:

Pfx buffer (10 x):	5 μl
dNTP's (10 mM):	1.5 μl
$MgSO_4$ (50 mM):	1 μl
Primer H 6115 BamHI (15 μM):	1 μl
Primer H 6576 NotI (15 μM):	1 μl
FVIII cDNA (100 ng/ml):	1 μl
Pfx DNA polymerase:	0.5 μl
DDW:	39 μl

The reaction was performed in a DNA thermal cycler, for 25 cycles with the thermal cycling conditions as follows:

Initial denaturation:	94°C,	5 min
Denaturation:	94°C,	1 min
Annealing:	70°C,	1 min
Elongation:	72°C,	40 sec
Final elongation	72°C,	7 min

The annealing temperature was 4°C below the lower melting temperature for the two primer oligonucleotides (here 74°C for both H6115 BamHI and H6576 NotI). For the elongation step, it was

adapted temperature 72°C for base pair less than 3 kb (here 460 bp for C1), and 45 sec for the size of up to 750 bp.

Following PCR, the products were visualised by electrophoresis on a 2% Agarose gel that were prepared in 1x TBE (Tris/Borate/EDTA) buffer and the pUC19/HpaII DNA molecular weight marker (26-501 bp) (Fermentas International Inc, Ontario, Canada) was loaded alongside to aid in confirming the size of the PCR product. The PCR products were then purified from the unincorporated nucleotides and salts were removed using the UltraClean PCR Clean-Up Kit (Mo Bio Laboratories Inc, CA, USA) according to the manufacturer's instructions. The PCR products were then quantitated using a spectrophotometer by multiplying 50 ng/μl by the OD reading at 260 nm and the dilutional factor.

2.2.1.2 Preliminary production of C1 recombinant plasmid using insect cell vector system (baculovirus vector pAcGP67B and insect cell line Sf-21)

The methods described here details the creation of a recombinant plasmid using an insect cell expression vector pAcGP67B and the methodology used here was similar to the subsequent work (Section 2.2.1.3) involving the cloning of the C1 peptide in the mammalian cell vector system (pSecTagB vector and COS-7 and CHO mammalian cell line). The preliminary production of the C1 recombinant plasmid in the insect cells (Figure 2.1 A) provided the ground work for us to proceed with the mammalian cell expression system (Figure 2.1 B).

2.2.1.2.1 Digestion of target gene and vector by BamHI and NotI restriction enzymes

The recombinant restriction enzymes BamHI (G↓GATCC, 10,000 units/ml) (Promega Corporation, WI, USA) and NotI (GC↓GGCCGC, 10,000 unit/ml) (New England Biolabs, MA, USA) were used to digest both the target gene and the baculovirus vector pAcGP67B (9.765 kb) (BD Farmingen Inc, CA, USA).

In brief, purified C1 PCR product (1 μg) or pAcGP67B vector (2 μg) were incubated first with NotI (10 units) and its buffer (final volume of 100 μl) at 37°C overnight, before heat-inactivation of the enzyme

(65°C for 20 min) followed by extraction of proteins using phenol/chloroform and ethanol wash. The pellet was then suspended in DDW and digested by BamHI (10 units) with its buffer in a final volume of 100 μl at 37°C overnight. Subsequently heat-inactivation of BamHI (80°C for 20 min), extraction of the treated C1 gene or vector were performed and pellet suspended in 10 μl DDW in preparation for ligation.

The NotI/BamHI-digested C1 DNA product was resuspended in a final concentration of 50 ng/μl. The digested vector pAcGP67B underwent further CIP treatment to prevent self ligation of the vectors, by dephosphorylation of the 5'-end of the fragment. This was important in improving the yield of properly ligated product, and reducing the background of improperly self-ligated contaminant. The CIP treatment was carried out by incubating the vector (500 μg in 10 μl DNA mix) with the CIP enzyme (1 unit in 1 μl) at 37°C for 1 hr, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was then resuspended in 10 μl DDW. The final concentration of pAcGP67B after CIP treatment was 20 ng/μl.

2.2.1.2.2 Ligation of vector and insert

Prior to cloning of the C1 insert, the vector was tested for immunity to self-ligation. Poorly dephosphorylated linearised vectors or uncut (circular) vectors that were transformed into bacteria would from colonies on agar supplemented with selective antibiotics. The vectors pAcGP67B used in this study were properly linearised and dephosphorylated as shown by the absence of colony formation on the LB-Amp (Luria-Bertani - ampicillin, 100 μg/ml) agar plate after the enzyme-digested (linear)/CIP-treated vector was incubated with T4 DNA ligase (New England BioLabs, MA, USA) overnight, transformed into *E. coli* XL1-Blue competent cells and plated out onto LB-Amp agar.

Ligation of the enzyme-treated C1 gene and vector pAcGP67B was then performed on ice using 1 μl T4 DNA ligase (stock concentration 20,000 units), 10 ng of vector and 100 ng of C1 DNA in a final volume of 10 μl and incubated overnight at 4°C.

2.2.1.2.3 Transformation of recombinant plasmid into insect cell line Sf-21

One microlitre of the ligation product was added to 100 μl of the Sf-21 (Spodoptera frugiperda) insect cell line, which were maintained and freshly passaged in protein-free GIBCO Sf-900 II SFM medium (Invitrogen), plus 900 μl of LB-Agar broth. The transformed Sf-21 cells were plated out and grown overnight at 37°C. The next day, five colonies were randomly selected, inoculated into 2 ml LB-Agar-Amp broth and grown overnight at 37°C.

2.2.1.2.4 Mini-preparation of recombinant plasmid DNA

After overnight growth, mini-preparation of the plasmid DNA from the 5 cultures were carried out. The bacteria pellet was resuspended in GTE buffer (50 mM Glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8) and lysed by adding NaOH/SDS solution (0.2 M NaOH, 1% SDS). An aliquot of 5 M potassium acetate solution (pH 4.8) was added to neutralise the activity of NaOH and the plasmid DNA precipitated out from the cleared supernatant. The pellet was resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) then treated with RNAase A (ribonuclease) (10 mg/ml stock) to remove RNA contaminants. Proteins was removed from the plasmid DNA preparation by phenol/chloroform/isoamyl alcohol extraction, and precipitated by using ammonium acetate and absolute ethanol. The precipitated plasmid pellet was then washed with 70% ethanol and resuspended in 25 μl TE buffer. The concentration of plasmid DNA (μg/ml) was determined using an ultraviolet-visible (UV) spectrophotometer.

2.2.1.2.5 Confirmation of integrated C1 DNA in pAcGP67B plasmid

The yield of the C1-pAcGP67B plasmid, prepared from 1 ml of the Sf-21 cell culture, of the five colonies were 305, 565, 580, 685 and 3285 μg/ml respectively. Each of the five C1-plasmid were amplified with the same C1 primers and thermal cycling conditions described in section 2.2.1.1 (H6115 BamHI and H6576 NotI). The presence of the PCR product C1 DNA (approximately 460 bp) from all five template samples was confirmed on the electrophoresis (data not shown).

Subsequently the plasmid DNA prepared from one of the five colonies was sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc, CA, USA) according to the manufacturer's instruction. The product submitted for sequencing by an automated sequence analyser (ABI Prism 3700 DNA analyzer, Applied Biosystems, Scoresby, Australia). Proper integration of the C1 DNA in the vector at the BamHI and NotI multiple cloning sites was confirmed (data not shown). This Sf-21 cell line was then grown in a larger culture in plastic flask and a maxi-preparation of the culture yielded of 0.5 ml of the C1- pAcGP67B plasmid at a concentration of 5,735 ng/μl (5.7 mg/ml).

2.2.1.3 Production of C1 peptide using mammalian cell expression system

2.2.1.3.1 Production of recombinant plasmid C1-pSecTagB and transformation into bacterial cells DH5α

The mammalian cell vector pSecTagB (5.2 kb) (Invitrogen) (Figure 2.2) was digested with BamHI and NotI then dephosphorylated using CIP treatment as described in Section 2.2.1.2.1. The final concentration of the linearised and dephosphorylated vector was 10 ng/μl. The vector was also tested for immunity to self-ligate, as described in Section 2.2.1.2.2.

The linearised C1 gene (100ng) was ligated to the pSecTagB vector (10 ng) using 1 μl of T4 DNA ligase in a final volume of 10 μl. The ligation product was transformed into the *E. coli* chemically competent cells, the DH5α strain, and the cells grown on LB-Amp agar plates overnight at 37°C.

Five randomly selected colonies were removed from the agar plates and allowed to grow in a large LB-Amp culture (broth) overnight at 37°C. After mini-preparation of the plasmid DNA, the concentration of the C1-pSecTagB plasmid from the five colonies ranged from 345-810 ng/μl.

Subsequently, PCR amplification of the C1 gene (with the H6115 BamH1 and H6576 Not1 primers) using these plasmids as template was established and the products visualised on agarose gel at the relevant DNA molecular weight. This was followed by direct DNA sequencing of the plasmid from one

of the five colonies to ensure the insert properly integrated into the vector. A large culture (in medium flask) of this colony was then grown overnight and harvested for maxi-preparation of the plasmid DNA. The final concentration of C1-plasmid DNA was 4,665 ng/μl (4.7 mg/ml), ready for transfection to mammalian cells.

2.2.1.3.2 Transfection of recombinant plasmid C1-pSecTagB into mammalian cells COS-7 and CHO cells and expression of C1 peptide

The two mammalian cell lines were used here, the COS-7 and CHO cells, are adherent cell lines. The COS-7 cell line is derived from SV40 virus transformed fibroblasts obtained from African green monkey kidney and the CHO cell line is of epithelial origin from Chinese hamster ovary. Both cell lines were available at the IMVS and maintained at log phase in DMEM (Dulbecco's Modified Eagle's Medium) (for COS-7 cells) and Ham's tissue culture medium (for CHO cells) respectively. Both media were supplemented with 10% foetal calf serum (FCS) and L-glutamine.

During transfection, approximately 5 μg of C1-pSecTagB (1 μl of maxi-preparation product) in 250 μl of DMEM was mixed with 15 μl of Lipofectamine 2000 (Invitrogen), also in 250 μl of DMEM, and incubated at room temperature for 20 min. The mix was then placed in a culture well that was seeded with 4 x 10^5 of freshly passaged COS-7 cells in 2 ml of the growth medium. The culture was allowed for growth at 37°C for 48 hr before harvesting.

During harvest the culture medium in which the transfected COS-7 cells were grown in was removed and reserved for future use. The cells were then washed once with Ham's medium, then digested with trypsin at 37°C for 4 min to separate the adherent cells from the bottom of the tissue culture flask. The cells were transferred to an Eppendorf tube then an equal volume of DMEM medium was added and the sample centrifuged for 30 min. The pellet was dissolved (lysed) in 150 μl of PLB (protein loading buffer, 0.5M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% 2-β-mercaptoethanol, and 0.05% bromophenol blue) and for use in Western Blot. Both the supernatant and the pellet in PLB (or lysate) were kept at 4°C.

When CHO cells were used for transfection, the Lipofectamine 2000 were first incubated with Ham's medium at room temperature for 5 min, before incubation with C1-plasmid. The mix was placed together with CHO cells and for growth at 37°C. After 24 hr, the cells were expanded into a medium flask with 10 ml of Ham's medium supplemented with 10% FCS. A selective medium, Ham's medium plus 10% FCS and Zeocin (phleomycin, at 50 μg/ml), was introduced after over night culture. Zeocin was added to the growth medium each time the cells were passaged. Only successfully transfected CHO cells could survive the selective medium due to the presence of antibiotic-resistant gene contributed by the plasmid DNA (Figure 2.2). The culture supernatant and CHO cell pellet was harvested using the same methods described for the COS-7 cells. The CHO cell line was desirable as a permanently maintained cell line for the purpose of mass production of the C1 peptide.

2.2.2 Part II: Production of murine monoclonal anti-FVIII antibodies

A flow chart of production of monoclonal antibodies is illustrated in Figure 2.3. Briefly, the process involved immunisation of the test mouse with recombinant FVIII, harvesting of post-immunised mouse spleen to perform fusion experiment with myeloma cells, screening for anti-FVIII antibody-secreting hybridomas, cloning of positive cell lines, then harvesting of the purified monoclonal antibodies. In addition, for each anti-FVIII monoclonal antibody, we planned to characterise its binding specificity to the FVIII molecule using the recombinant FVIII fragments (e.g., A2, C1 or C2 domains) produced from the current study.

2.2.2.1 Animal anaesthesia, blood sampling and euthanasia

All the animal experimental procedures in the study were approved by the Animal Ethics Committees of the IMVS (project number 22/01, animal user permit number 201/01) and the University of Adelaide (project number M-56-2001). The animals (mice) were supplied by and housed in the IMVS, where standard mouse food and water were provided *ad libitum*.

FIGURE 2.3 Flow chart for the Preparation of Monoclonal Antibodies

Reprint from Kimball. Monoclonal Antibodies. Online Biology Textbook (http://users.rcn.com/jkimball.ma.ultranet/BiologyPages). 2009.

HGPRT: hypoxanthine-guanine phosphoribosyl transferase, an enzyme involved in the synthesis of nucleotide from hypoxanthine, an amino acid.

After fusion, the cells are cultured in HAT (hypoxanthine-aminopterin-thymidine) medium, where only hybridomas between spleen cells and myeloma cells can survive. This is because the hybrid cells have both the HGPRT gene (contributed by the spleen cells) and the ability to grow continuously (malignant potential contributed by myeloma cells).

To produce monoclonal anti-factor VIII antibody, two groups of four female Balb/c mice were immunised with purified recombinant human FVIII. At the time of fusion, for each immunised mouse, two additional feeder mice (6-week old female Balb/c) were used to provide thymus cells as a source of cytokines and other active biologic factors to optimise the fusion condition. Feeder mice were also used at various stages of the cloning procedures to facilitate the maintenance of healthy hybridoma cell lines.

The mice were anaesthetised by inhalation of Fluothane before performing immunisation (via intraperitoneal injection of purified recombinant FVIII) and blood sampling (via eye-bleeding) on the mice. The mice were sacrificed by cervical dislocation.

2.2.2.2 Immunisation of mice

Each of the eight female Balb/c mice was immunised with three intraperitoneal injections of purified recombinant FVIII over a two-month period. The mice received their first dose of FVIII at the age of six weeks. The initial dose of FVIII injection contained 1 μg of purified unformulated recombinant FVIII (Recombinate, Baxter Healthcare, IL, USA) in PBS buffer diluted 1:1 in Complete Freund's Adjuvant solution (final volume 100 μl). The subsequent injections, of the same dosage of recombinant FVIII (1 μg) but diluted in Incomplete Freund's adjuvant solution, were delivered four and six weeks after the initial immunisation.

Three to four days prior to sacrifice, a booster injection of the same antigen and dosage (FVIII 1 μg in Incomplete Freund's Adjuvant solution) was administered to ensure production of the IgG form of the monoclonal antibodies. For the eight mice used in the study, the average duration between the third injection and the boost injection were approximately two months.

One day before the initial dose of FVIII was administered (pre-immunisation), and one week after the second injection (post-immunisation), the mice were retro-orbitally bled. Approximately 20-30 μl of serum was obtained to test for the presence of antibodies against human FVIII in an ELISA assay.

2.2.2.3 ELISA and Bethesda methods to detect anti-FVIII antibodies

An ELISA was used to screen for polyclonal anti-FVIII antibodies in the post-immunised mouse serum. The method was adapted from our previous work on optimised ELISA method to detect FVIII antibodies in patient plasma (Ling *et al*, 2003). Briefly, each well of microtitre plates (Nunc A/S, Roskilde, Denmark) was coated with 50 μl of the FVIII (Recombinate, Baxter; 5 IU/ml in 0.1M carbonate buffer pH 9.6) and incubated overnight at 4°C. The following day, excess FVIII was washed off with washing buffer (0.05% Tween 20 v/v in PBS pH 8.0) then unbound sites were blocked with 100 μl blocking buffer (0.05% Tween 20 v/v, 1% gelatin w/v in PBS pH 8.0) for 90 min at room temperature.

Fifty microlitres of mouse serum samples (dilutions from 1/100 to 1/256,000 in blocking buffer) were then added to each well and incubated at room temperature for 2 hr. After washing, an aliquot of 50 μl of the secondary antibody comprising horseradish peroxidase (HRP)-linked rabbit polyclonal anti-mouse IgG (DAKO A/S, Glostrup, Denmark), diluted 1/2000 in blocking buffer, was added and incubated for 1 hr. The secondary antibody was then washed off and an aliquot of 50 μl of 1,2-phenylenediamine dihydrochloride (OPD solution, DAKO) was added. Colour was allowed to develop for 10 min then stopped with 50 μl 2.5M H_2SO_4. The OD (at 490 nm wave length) was read after 5 min using a microplate reader MR 7000 (Dynatech Laboratories, VA, USA).

All samples were tested in duplicate. An OD reading of 0.31 was established to be the minimum cut-off reading for a positive ELISA test detecting anti-FVIII antibodies in our previous study (Ling *et al*, 2003). A commercially-available murine monoclonal anti-FVIII antibody ESH8 (American Diagnostica Inc; directed to aa 2248-2285 in the C2 domain of LC, stock inhibitor titre > 5,000 BU/ml; used at 1/10,000 dilution) was used as a positive control in the assay.

In addition to ELISA, Bethesda assay was used to detect the inhibitory anti-FVIII antibodies in mouse serum. The method was adapted from the Nijmegen modification of the Bethesda method (Verbruggen *et al*, 1995). Briefly, test serum (i.e., mouse serum obtained before or after immunisation) or control

serum (FVIII deficient plasma from Dade Behring Diagnostics, Marburg, Germany) was incubated with an equal volume of buffered, pooled normal plasma (pH7.4) for 2 hr at 37°C in a closed plastic test tube. The remaining FVIII:C (IU/ml) was measured by a one-stage assay and the residual FVIII:C (%) expressed as a percentage of the control. The strength of a FVIII inhibitor from the test serum was estimated from a graph, in which the log of residual FVIII:C (%) is plotted against the Bethesda units on a linear scale. An inhibitor with 1 Bethesda unit per millilitre (BU/ml) was defined as the amount of antibody causing 50% reduction in FVIII:C compared to the control incubation.

Both the ELISA and the Bethesda assays were also used to assess the presence of anti-FVIII antibodies/inhibitors in the supernatant of tissue culture medium of the hybridomas/clones after the fusion experiment. The cut-off OD reading for the ELISA using the undiluted supernatant of tissue culture was different to that of the mouse serum described above. The cut-off OD reading of ELISA for culture medium supernatant was 0.05. This was generated by the mean (0.014, 95% CI of mean 0.12-0.16) plus 3-fold of SD (0.012) of the OD from a total of 118 culture media of hybrids (from six fusion experiments), which had a visibly negative ELISA result (no colour reaction by visual inspection).

2.2.2.4 Fusion of mouse immune spleen cells with myeloma cells and formation of hybridoma

For each immunised mouse, the spleen was removed using aseptic techniques within minutes of the sacrifice and a single cell suspension of splenocytes prepared in a sterile culture plate. Briefly, the spleen was repeatedly flushed with serum-free RPMI (Roswell Park Memorial Institute) culture medium using a syringe fitted with a 24 gauge needle. The spleen tissue was then gently teased/spread against the bottom of the plate. This action was repeated as necessary until the spleen capsule was devoid of splenocytes.

The debris-free supernatant was then centrifuged to pellet the cells. The cell pellet was then resuspended in lysis buffer and washed in serum-free PRMI medium to remove red cells. Cell counts were performed and cell suspension adjusted to approximately 1 x 10^8 splenocytes in 25 ml serum-free

medium and then kept at 37°C in preparation for the fusion step. For each spleen harvested, the thymus from two feeder mice (6-week old Balb/c) were also harvested using the same techniques. The washed thymus cells from the feeder mice were resuspended to approximately 2 x 10^8 cells in 4 ml serum-free RPMI and stored at 37°C for the fusion experiment.

The X63 (Balb/c) mouse myeloma cells line, used to create the hybridomas, was maintained in complete medium (RPMI supplemented with 10% FCS). On the day of fusion, the myeloma cells were washed and resuspended in serum-free RPMI medium, at a count of approximately 2 x 10^7 cells in 25 ml of media.

At the time of fusion, the splenocytes and myeloma cells were mixed at a ratio of 4:1 in a 50 ml tube and centrifuged for 10 min at 400 g at room temperature. The supernatant was completely aspirated off, leaving a dry pellet which was subsequently disrupted by flicking the side of the tube. One millilitre of polyethylene glycol 1500 (PEG, Boehringer Mannheim GmbH, Mannheim, Germany) pre-warmed at 37°C, was then added over a period of 1 min while mixing (by gentle stirring) the cells to facilitate fusion of the cells. The PEG-cell mixture was then placed in a 37°C water bath and the tube was gently agitated intermittently for a further minute. The PEG was then diluted out by adding the pre-warmed (37°C) serum-free hybridoma growth medium at a volume of 1 ml, 3 ml, 5 ml and 10 ml, all at an average rate of 1 ml per min, over the next 10 min. The cells were then allowed to recover for 5 min at 37°C before centrifugation for 5 min at 400 g. Following removal of the supernatant the fusion cells were then gently suspended in 10 ml of hypoxanthine-aminopterin-thymidine (HAT) medium (Sigma, MO, USA).

The HAT medium is a selective medium to ensure only hybridomas between the spleen cells and myeloma cells can survive, as it sustains only hypoxanthine-guanine phosphoribosyl transferase (HGPRT)-positive cells. HGPRT is an enzyme involved in the synthesis of nucleotides from hypoxanthine, an amino acid. Myeloma cells are HGPRT-negative while spleen cells (B cells) are

HGPRT-positive. The hybrid cells have both the HGPRT gene (contributed by the spleen cells) and the ability to grow continuously (malignant potential contributed by myeloma cells).

Each 5-ml of the HAT medium with fusion cells (with approximately 0.5 x 10^8 spleen cells) was adjusted to a final volume of 40 ml (5 ml of fusion mix, 2 ml of feeder thymocytes and 33 ml of HAT medium), and plated onto two flat-bottomed 96-well culture plates (200 µl/well). Hence four 96-well plates were usually produced for every fusion experiment. The plates were sealed with micropore tape and cultured at 37°C in a fully-humidified incubator supplemented with 5% CO_2. After twenty-four hours, an aliquot of 100 µl of HAT medium was added to each well. The cultures were fed by removing and adding fresh medium every second day. The plates were examined daily under microscope for colony formation and all HAT-resistant hybridomas were allowed to grow for at least 10-14 days at which time screening was initiated.

2.2.2.5 Selection and cloning of antibody-secreting hybridomas and production of monoclonal antibodies

The established hybrids growing in HAT medium in 96-well plates were screened for the presence of anti-FVIII antibodies using the ELISA method (Section 2.2.2.3). Hybrids found to produce antibodies that reacted with the recombinant FVIII in ELSIA were selected for cloning. Each hybrid was expanded into a 24-well tissue culture plate in the presence of fresh thymus feeder cells, then into a 6-well plate and finally into a small plastic culture flask to ensure healthy growth. ELISA analysis of the supernatants of culture media was repeated at every cell passage to ensure surveillance of ongoing antibody production.

For each selected hybrid line, approximately 100 hybrid cells were counted and taken from the small-flask culture and plated onto a 96-well plate (one cell per well). Any single clone descended from the original hybrid that demonstrated consistently significant positive ELISA results was considered to be a monoclonal cell line. These cell lines were then sent to the IMVS Murine Virus Monitoring Service for

isotyping of immunoglobulin subclass, screen of mycoplasma contamination, expanding of the cell line and purification of the monoclonal antibody.

2.2.2.6 General aspects of tissue culture

2.2.2.6.1. Tissue culture medium

RPMI medium was used in tissue culture for X63 myeloma cells. The medium were supplemented with 10% heat-inactivated FCS as appropriate and correspondingly referred as complete medium. Hybridoma cells were maintained in HAT medium.

2.2.2.6.2 Maintenance of cell culture

All cells were maintained in either tissue culture plates (96, 48, 24, 12 or 6-well plate) or flasks (25, 75 or 150 ml) containing the culture medium supplemented with FCS where necessary. The cells were incubated in a humidified incubator at 37°C with specific 5% CO_2 flow. The tissue culture medium was refreshed every 2-3 days and cells split when the culture reached 80-100% confluence. A laminar flow hood was used to ensure sterility.

The X63 myeloma cell line was an adherent monolayer cell line and required trypsin treatment (4 min 37°C) to release the adherent cells from walls/bottoms of culture flasks/plates when splitting the cells. Fresh medium containing 10% FCS was added to stop trypsin activity. The released cells were washed with RPMI, then an aliquot was stained with 0.4% Trypan Blue (Sigma) to assess cell viability and determine cell count. The cells were then plated out, usually in a 1:4 to 1:6 dilution of the original confluent culture.

2.2.2.6.3 Cryopreservation of cells

The cryopreservation process was started by resuspending 5×10^6 cells with the viability of greater than 90% in 1 ml of “freezing mix” (50% RPMI supplemented with 40% FCS and 10% Dimethyl Sulphoxide (DMSO, BDH Merck Ltd, Poole Dorset, UK)). The freezing mix was added dropwise over 1-2 min then

the cells were transferred into cryotubes (Corning Costar Corporation, MA, USA) that were pre-chilled on ice. The cryotubes of cells were then stored in a cryobox and cooled down either in a -80°C freezer over night, or in a controlled rate freezer, before being transferred to a liquid nitrogen container for long term storage (-196°C).

2.2.2.6.4 Resuscitation of cryopreserved cells

After a cryotube of the cryopreserved cells was removed from the liquid nitrogen container, it was immediately warmed up in a 37°C water bath. As soon as the frozen mixture thawed, the cells were transferred into a 10 ml tube where an equal volume (usually 1 ml) of fresh culture medium with 10% FCS was pre-added. The cells were then washed twice with fresh serum-free medium and plated out to grow in complete medium supplemented with FCS. The average cell viability after thawing was aimed at greater than 80%.

2.2.3 Western blot to detect biding of FVIII peptides and anti-FVIII antibodies

The harvested recombinant C1 (or C2) peptide, the cell pellet (lysate in 150 μl of PLB) or the supernatant, were separated on a 15% SDS-PAGE gel and transferred onto a PVDF (polyvinylidene fluoride) membrane using a semi-dry blotting apparatus. The peptides were identified by blotting with a primary antibody (mouse anti-FVIII antibody), and peroxidase-conjugated rabbit anti-mouse IgG-HRP (DAKO A/S) secondary antibody at 1/10,000 dilution. The probed membrane was reacted with enhanced chemiluminescence (ECL, Amersham Biosciences, Buckinghamshire, UK) for visualisation.

The primary antibodies used included: the commercially available mouse anti-myc monoclonal antibody (Invitrogen) (at 1/1,000 dilution), purified monoclonal mouse anti-FVIII antibody developed from this study (IgM “M3-1F2-A9”, 1.7mg/ml, undiluted), polyclonal mouse serum before and after FVIII immunisation (at 1/2,000 dilution), and the culture media that individual monoclonal cell lines were grown in (undiluted).

In brief, the lysates containing the recombinant proteins were denatured at 95°C in the loading buffer for 5 min then cooled to room temperature. The lysates were then loaded onto a 15% acrylamide gel. The gel was run at 20 mA in SDS-PAGE running buffer for 1 hr. Following electrophoresis, the separating gel, a piece of PVDF membrane, together with 6 pieces of blotting paper were soaked in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3) and transferred at 40 mA for 1 hr. After the transfer, the membrane was blocked with 5% milk prepared in Tris-saline at room temperature for 1 hr.

The membrane was then incubated overnight at 4°C with the primary antibody which was prepared in Tris-saline/0.02% Tween buffer in a sealed bag. The membrane was then washed three times with Tris-saline/0.05% Tween and subsequently incubated with the secondary antibody at room temperature for 1 hr before ECL development.

2.3 Results

2.3.1 Production of recombinant FVIII peptides (C1 and C2)

2.3.1.1 PCR product of target gene

The PCR products of CI and C2 DNA, designed with restriction sites (BamHI and NotI) on both ends, were visualised on a 2% agarose gel after electrophoresis (Figure 2.4). The C1 (upper gel) and C2 (lower gel) DNA is of the size of approximately 460 and 470 bp respectively, as indicated by the relevant DNA molecular weight marker (Figure 2.4). The concentration of the purified PCR product for C1 and C2 DNA was 175 ng/μl and 135 ng/μl respectively.

2.3.1.2 Transformation of recombinant vector plasmid C1- or C2-pSecTagB to DH5α cells

Following digestion and ligation of the target DNA and the mammalian vector pSecTagB, the recombinant vector plasmid (C1-pSecTagB, or C2-pSecTagB) which was inserted with the target gene, was transformed into the chemically competent *E. coli* strain DH5α cells. Five randomly selected colonies were grown and mini-preparation of the plasmid DNA was made. These constructs were then

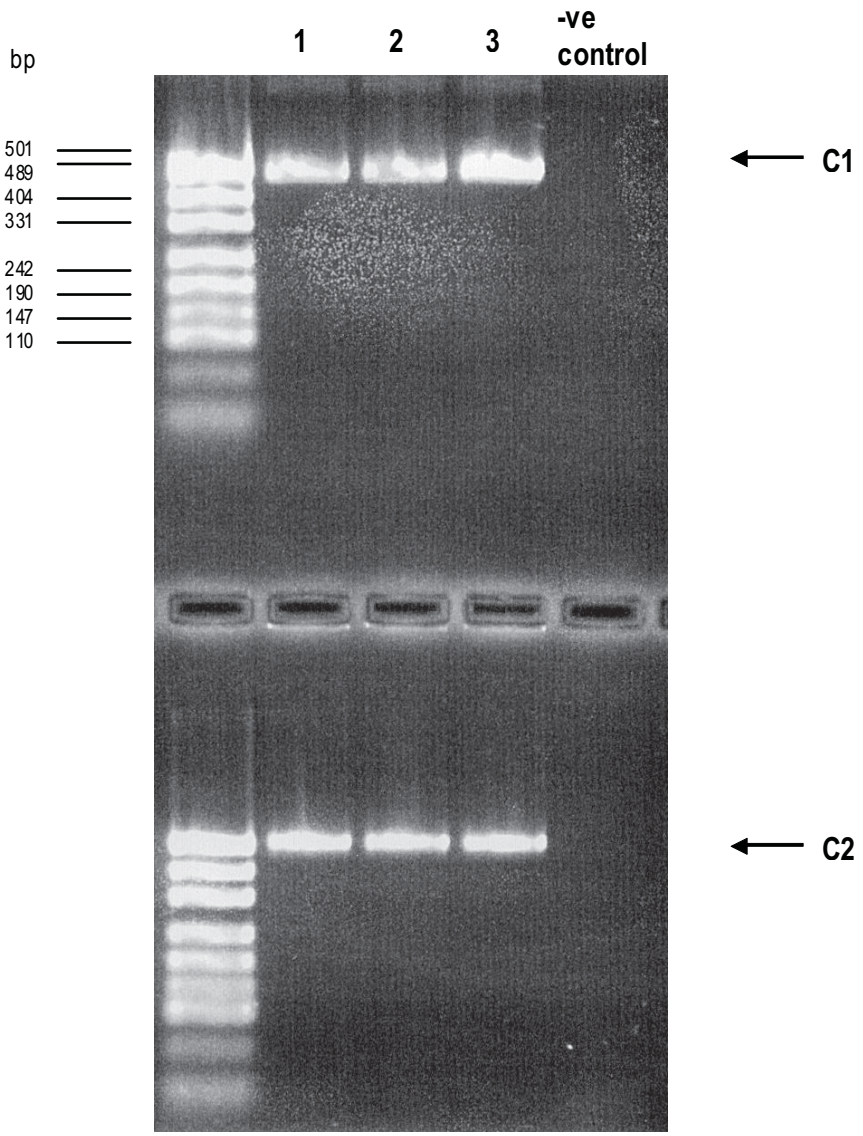


Figure 2.4 PCR Products of C1 and C2 DNA

The PCR product of C1 and C2 DNA was amplified in triplicate using primers H6115 BamHI and H6576 NotI, and visualised on a 2% agarose gel.

The expect size for the C1 and C2 DNA was approximately 460 bp (upper gel) and 470 bp (lower gel) respectively.

The DNA molecular weight marker pUC19DNA/HpaII was on the left side of the gel, with the corresponding base pair size indicated.

Negative control: PCR using DDW as template.

used as the template in the PCR to produce C1 (or C2) DNA (Figure 2.5). Positive control was provided by using the FVIII cDNA. The presence of the PCR product at the corresponding DNA molecular weight confirmed the successful insertion of the target gene in the vector (Figure 2.5 panel A for C1 and panel B for C2).

Subsequently, a direct DNA sequencing of the recombinant plasmid (colony number 2 for C1, and colony number 1 for C2, with reference to Figure 2.5) was performed. This confirmed the correct nucleotide sequence from the vector to the target gene throughout in both cases (data not shown). These two colonies (for C1 and C2 respectively) were grown in larger DH5α cell culture and maxi-preparation of the recombinant plasmid DNA was made, so that there was enough material for transfecting into the mammalian cells.

2.3.1.3 Expression of C1 and C2 peptides in COS-7 cells

All proteins coded by the recombinant vector plasmid pSecTagB will express a myc-tag when induced (Figure 2.2), therefore an anti-myc antibody can be used to probe the expressed desired protein in transfected cells. Figure 2.6 shows the successful expression of the C1 and C2 peptides (both were approximately 17 kDa) as detected by Western blot analysis of the lysate of the cell pellet of the transfected COS-7 cells, using an anti-myc antibody.

In addition, the stability of the expression of the protein (C1 peptide) in COS-7 cells was tested using stored samples of the cell pellet and the supernatant (both at 4°C) two weeks after the initial harvest (Figure 2.7). The results from Western blot reveal that the presence of C1 (probed by anti-myc antibody) was in low dilutions of the cell pellet (Figure 2.7 upper film), but not in any of the diluted or undiluted supernatant samples (Figure 2.7 lower film). This result suggested that the recombinant C1 peptide expressed by the COS-7 cells in the present study was mostly confined in the cell pellet but was inadequately secreted into the culture medium.

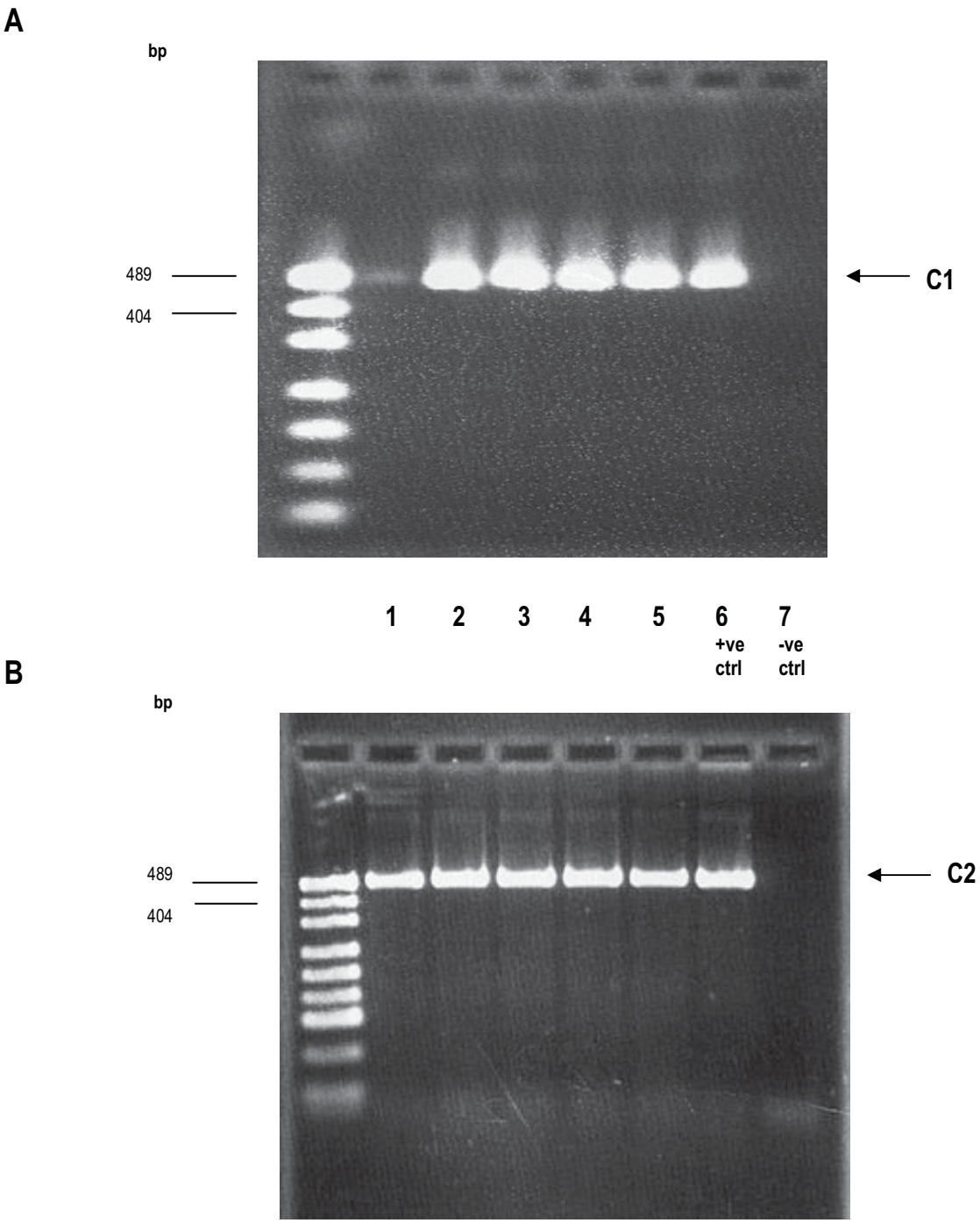


Figure 2.5 PCR of C1 and C2 DNA, Using Recombinant pSecTagB Plasmid DNA Extracted from Transformed DH5α Colonies as Template

Miniprep DNA of C1-pSegTagB and C2-pSecTagB was amplified to confirm the integration of the amplicon into the vector.

Lane 1 to 5: The PCR product of C1 (panel A) and C2 (panel B), using the template of recombinant plasmid DNA (A: C1-pSecTagB; B: C2-pSecTagB), which was extracted from five DH5α cell colonies following transformation. All but colony number 1 for C1 (Lane 1 in panel A) showed a strong result.
Lane 6: Positive control using FVIII cDNA as template.
Lane 7: Negative control using DDW as template.

The DNA molecular weight marker, pUC19DNA/Hpall, was loaded on the left side of the gel.

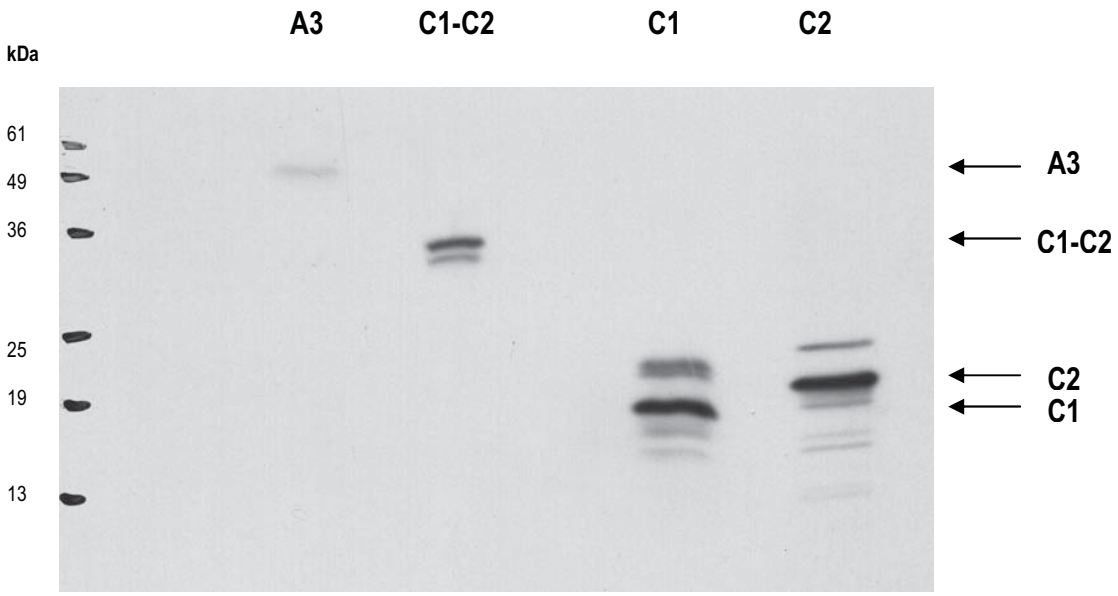


Figure 2.6 Expression of C1 and C2 Peptides in COS-7 Cells

The cell pellet of transfected COS-7 cells was lysed and electrophoresed on a 15% acrylamide gel and probed for “myc” protein using a commercially available anti-myc antibody in a Western blot.

The protein bands shown in the four lanes represented the successful expression of the A3, C1-C2 (both courtesy of Ms Vicky Apostolidis, Division of Molecular Pathology, IMVS), C1 and C2 peptides respectively.

The protein molecular weight marker (in kDa) was indicated on the left. The molecular weight for C1 and C2 peptide is approximately 16.9 kDa (154 aa) and 17.3 kDa (157 aa) respectively.

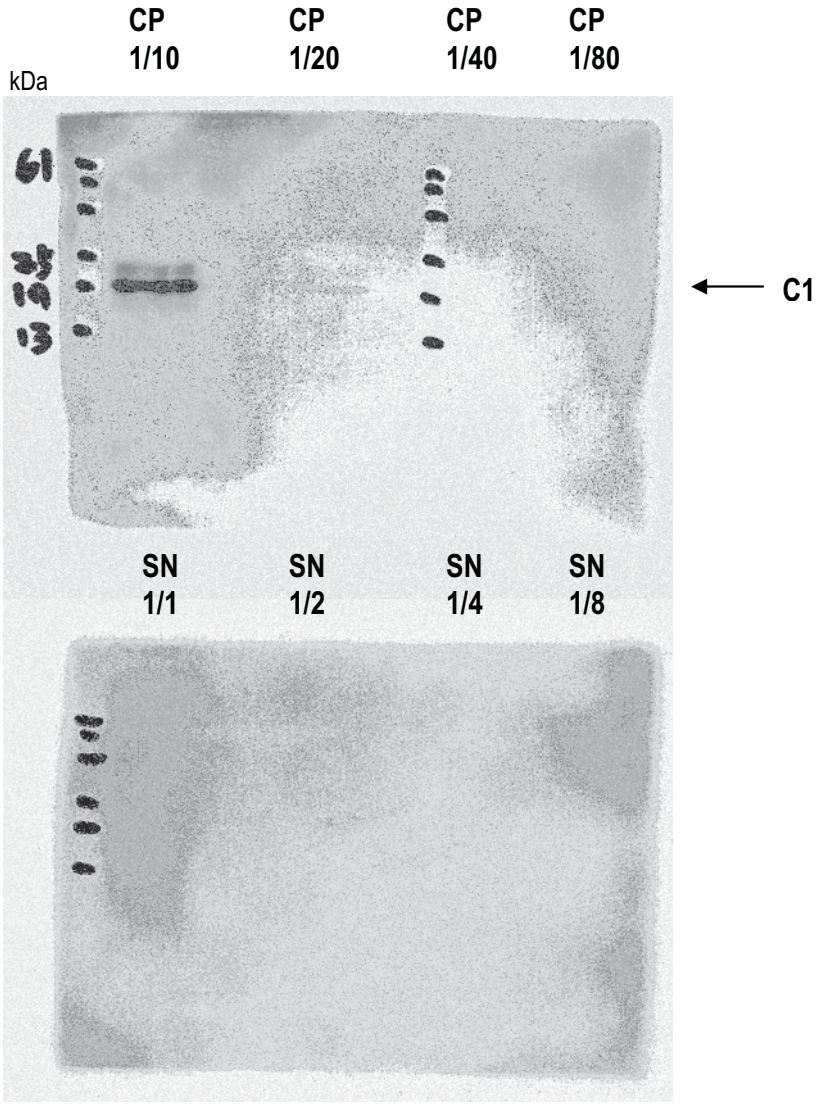


Figure 2.7 Expression of C1 Peptide in COS-7 Cells: Cell Pellet Superior to Supernatant

After the harvest of C1-transfected COS-7 cells, both cell pellet and supernatant were stored at 4°C for two weeks. Expression of the peptides in the crude lysate (prepared from cell pellet) and supernatant were assessed by Western blot analysis. Specifically, different dilutions of the cell pellet (1/10, 1/20, 1/40 and 1/80) and supernatant (undiluted,1/2, 1/4 and 1/8) were loaded onto 15% acrylamide gel, and probing with anti-myc antibody.

The expression of the C1 peptide was confined primarily to the cell pellet, rather than being secreted into the supernatant, as shown by the presence of the C1 peptide band only in two of the eight testing lanes: 1/10 and 1/20 dilution of the cell pellet sample.

The protein molecular weight marker was the same as in Figure 2.6.

CP: Cell pellet.
SN: Supernatant.

In an effort to establish a permanent cell line to produce recombinant peptides in adequate quantity, we used the recombinant plasmid C1- or C2-pSecTagB to transfect the CHO cells. Despite a few attempts, no successful expression of the C1 or C2 peptides was achieved, as Western blot using both the cell pellet or supernatant of the transfected CHO cells failed to reveal any protein bands probed by the anti-myc antibody (data not shown). These results suggest that the CHO cell expression system may not be suitable for expressing the recombinant C1 or C2 peptides.

2.3.2 Production of monoclonal murine anti-FVIII antibody

2.3.2.1 Presence of anti-FVIII antibodies in post-immunisation mice by ELISA and Bethesda assay

All eight mice developed anti-FVIII antibodies after the second injection of recombinant FVIII as indicated by the high OD reading by ELISA in the serum of post-immunisation mice (in serial dilutions from 1/1,000 to 1/256,000) (Figure 2.8). Pre-immunisation serum (in 1/100 and 1/200 dilutions) were also included for comparison (Figure 2.8). Using the cut-off OD value of 0.31 (at 490 nm) (Ling *et al*, 2003), there was a strong positive result in the post-immunisation serum in all eight mice, suggesting that the successful development of polyclonal anti-FVIII antibodies was achieved.

Anti-FVIII antibodies in the mice was not only detectable by ELISA but also confirmed to be functionally inhibitory to FVIII:C by the Bethesda assay. The inhibitory antibody titre in the post-immunisation serum from the eight mice ranged from 52.5 to 500 BU/ml. A summary of the ELISA and Bethesda results in post-immunisation mice is listed in Table 2.1 (left columns).

2.3.2.2 Hybridomas and purified monoclonal antibodies

Seven fusion experiments were successfully carried out, as one of the eight mice died before fusion. Theoretically for each fusion experiment, a maximum of nearly 400 hybridomas (4 x 96-well tissue culture plates seeded) could grow in HAT media. In actual fact, the number of the hybridomas obtained from the seven fusion experiments (M2 to M8) varied between 3 and 205 (Table 2.1 right columns).

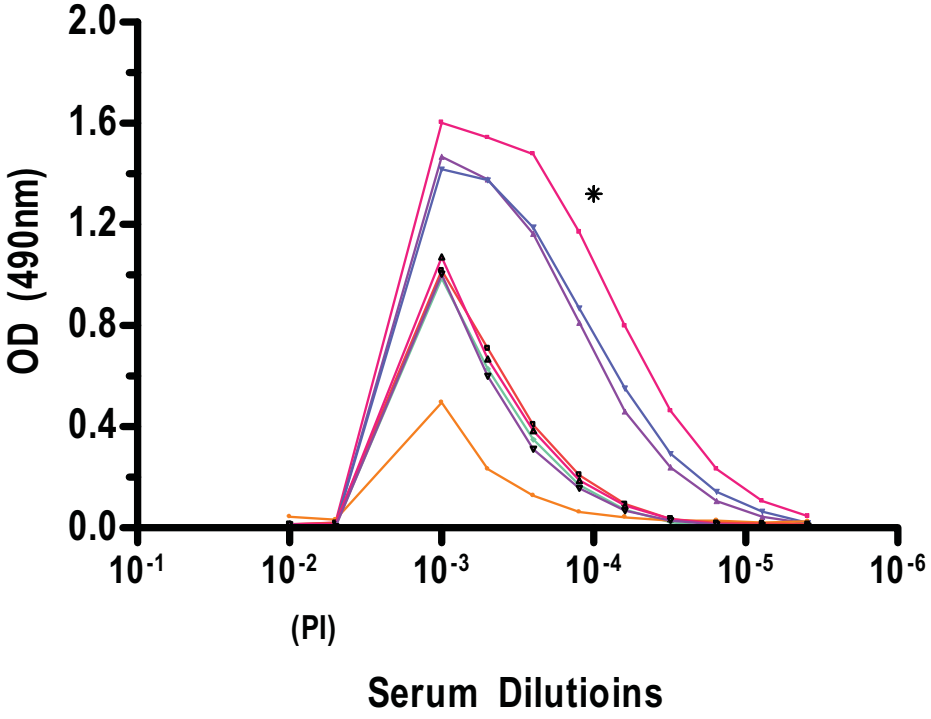


FIGURE 2.8 Screening for FVIII Antibodies in Post-Immunisation Mice by ELISA

Post immunisation serum from eight mice, in a serial dilutions (1/1000, 1/2,000, 1/4,000, 1/8,000, 1/16,000, 1/32,000, 1/64,000, 1/128,000 and 1/256,000), were tested for the presence of anti-FVIII antibodies using the ELISA method. Cut-off OD reading: 0.31.

PI: Pre-immunisation serum (1/100 and 1/200 dilutions) as negative control.
* : ESH8, a commercial murine anti-human FVIII antibody (1/10,000 dilution) as positive control.

TABLE 2.1 Eight Mice Used in the Production of Monoclonal Anti-FVIII Antibodies

Mouse Code	Post-Immunisation Mouse Serum with Polyclonal anti-FVIII Antibodies		Number of Hybrids Post-Fusion		
	ELISA*	FVIII Inhibitor Titre (BU/ml)	Total Hybrids	Hybrids with + ELISA*	Hybrids with + BU†
M1	+	500	(failed to survive before fusion)		
M2	+	420	43	0	--
M3	+	240	9	1	0
M4	+	80	4	0	--
M5	+	52.5	3	0	--
M6	+	52.5	50	0	--
M7	+	60	205	11	0
M8	+	92.5	10	0	--

*** :** The cut-off OD reading for ELISA was 0.31 for mouse serum and 0.05 for the supernatant of culture medium of hybrid, as described in Section 2.2.2.3.

† : Only those hybrids which returned a positive result for ELISA were tested for inhibitory titres by the Bethesda assay.

ELISA screening of the undiluted supernatant of culture medium detected antibody-secreting hybridomas derived from two of the seven mice only: M3 (n = 1 hybridoma) and M7 (n = 11 hybridomas) (Table 2.1 right columns). However none of the antibodies secreted from these hybridomas showed any inhibitory effect on FVIII:C in Bethesda assay (Table 2.1 right columns).

For the 12 hybrids that showed a positive ELISA result, we proceeded with cloning to obtain monoclonal cell lines. Despite the consistent positive ELISA results in the descended single clones from each of the hybridomas, Bethesda assay failed to detect an inhibitory effect in the culture media in any of the monoclonal cell lines (data not shown).

As representatives of the cloning experiments, we obtained three purified monoclonal antibody cell lines: M3-1F2-A9, M7-1C6-E8 and M7-2A9-F2. Isotyping revealed that the monoclonal antibodies produced from these three cell lines were all IgM antibody. None of the cell lines was found to have mycoplasma contamination. The purified IgM from M3-1F2-A9 was also tested in Bethesda assay and Western blot but returned negative results (data not shown).

2.3.3 Detection of anti-C1 and anti-C2 antibodies in polyclonal mouse serum by Western blot

Western blot was performed to demonstrate the biding of the recombinant peptide (C1 and C2) not only to the commercially available anti-myc antibody, but also to the murine anti-FVIII antibody in the serum of post-immunised mouse (Figure 2.9).

C1 and C2 peptides (lysate of cell pellet of transfected COS-7 cells) were loaded in separate lanes in 15% acrylamide gel. After electrophoresis followed by transfer of the protein onto a PVDF membrane, the membrane was first probed by post-immunisation mouse serum (from M3). The result was shown in Figure 2.9 panel A, with the binding of the polyclonal antibodies to a protein band of a similar molecular weight to C1 (or C2).

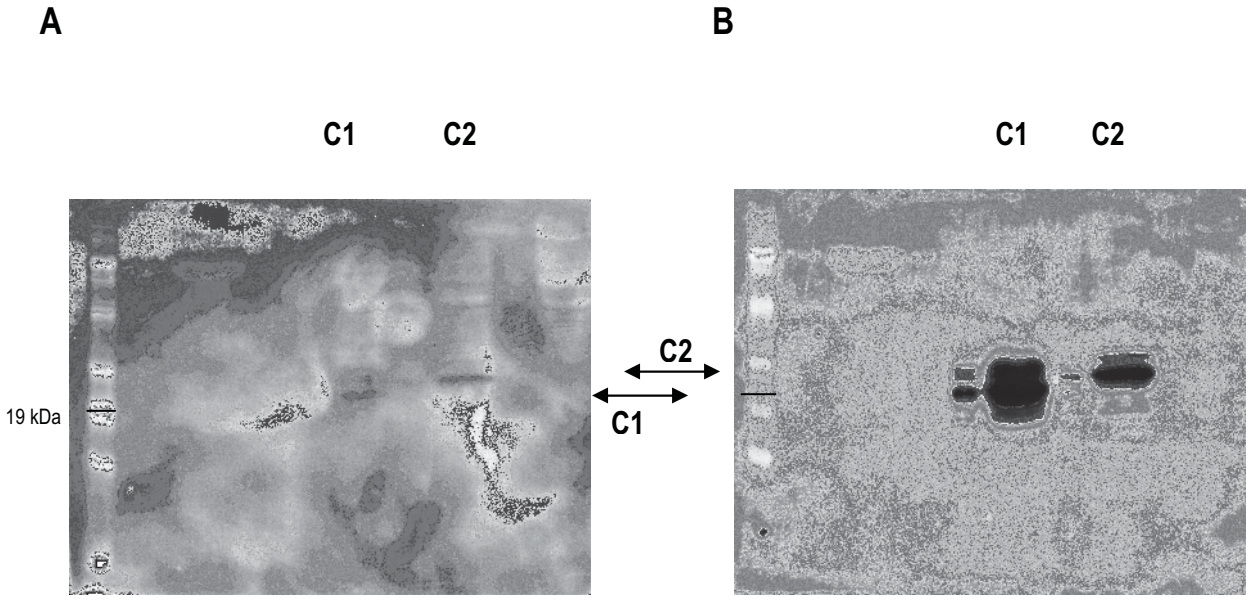


Figure 2.9 Binding of Polyclonal Mouse Anti-FVIII Antibody to Recombinant C1 and C2 Peptide in Western Blot

Both C1 and C2 peptides (cell pellet) were used as the protein antigen in Western blot. The membrane was probed first with post-immunisation mouse serum (panel A), then stripped and re-probed with anti-myc antibody (panel B).

For either C1 or C2, a protein band appeared at identical molecular weight mark when probed with the two different antibodies. This confirmed the presence of anti-C1 and anti-C2 antibodies in the polyclonal mouse serum.

The membrane was then stripped and washed then re-probed with the anti-myc antibody (Figure 2.8 panel B). The relevant protein band in Figure 2.8 panel A was now confirmed to be the recombinant C1 (or C2) peptide that was expressed by the constructs (recombinant plasmid C1- or C2-pSecTagB), because of the presence of the myc-tag as detected by the anti-myc antibody.

Thus, Western blot analysis demonstrated the presence of anti-FVIII antibodies in M3 mouse serum that at least partially bound against the C1 and C2 epitopes of FVIII. The inhibitory effect of these antibodies was previously shown to be strong: 240 BU/ml (Table 2.1 left columns).

In contrast, the purified monoclonal IgM "M3-1F2-A9" produced from the selected hybridomas of this study failed to show any binding against either the C1 or C2 peptide in Western blot (data not shown).

2.4 Discussion

The formation of alloantibodies against FVIII is a severe complication of replacement therapy in patients with haemophilia A, especially when the level of inhibitor is high. Although it is widely accepted that the driving force of inhibitor formation is the presentation of a novel or an immunologically altered FVIII antigen to patient's immune system, the pathogenesis of inhibitor formation is only partly understood. An important advance that suggests new treatment initiatives is the discovery that the inhibitory antibodies are directed against a limited number of epitopes on the FVIII molecules (Scandella *et al*, 1995). In the past two decades, epitope mapping of the anti-FVIII antibodies has added cumulative data to the understanding of the molecular mechanisms of the inactivation of FVIII.

In this project, we were hopeful to determine the domain-specific epitopes in the FVIII molecule. The C2 domain is frequently targeted by FVIII inhibitors. A recent study on the three-dimensional structure of C2 domain revealed putative biding sites (Met2199/Phe2200 and Leu2251/Leu2252) for phospholipids

(Barrow *et al*, 2001). It would be useful to identify any functional epitopes in the C2 domain that are targeted by inhibitors in our haemophilia patients.

A mammalian cell expression system, using the plasmid vector pSecTagB to integrate the target gene, was established to produce the recombinant C1 and C2 peptides. Each step of the recombinant techniques to produce the C1 and C2 peptide was successful, from the design of the target gene and the enzyme treatment of both the gene and the vector, to the insertion of the target gene to the vector and the sequencing of the recombinant plasmid DNA, and finally the expression of the peptides and the detection of expression with specific anti-myc antibody in Western blot. We demonstrated the successful insertion of the C1 and C2 target gene into both the insect cell vector pAcGP67B and the mammalian cell vector pSecTagB, as confirmed by PCR and direct sequencing of the recombinant plasmid DNA. We were successful in expressing a significant amount of the target peptide in a transfected COS-7 cell line. However, the results indicated that the peptide was confined mostly, if not entirely, in the cell pellet.

Our ultimate aim was to use epitope-mimicking peptides to neutralise inhibitors both *in vitro* and *in viv*o. To examine the ability of the recombinant peptides to block the inhibitors and restore FVIII:C activity in a "inhibitor neutralisation assay", and to fulfil the eventual aim of using peptide infusion to treat inhibitor patients, the FVIII fragments are required to be soluble and secreted into the culture supernatant. Expression of the peptides into the culture supernatant would mean the production of the peptides in a relatively pure form and in adequate amounts. Extensive extra work was carried out by others in our laboratory to purify the peptides from the cell pellet, including some quite innovative approaches. It was disappointing that none of these efforts yield any encouraging results. The difficulty in improving the secretion of protein from the cells highlighted the technical obstacles in this area and it was not worthwhile pursuing further within the scope of this study.

As an additional tool for epitope-mapping, we attempted to establish an experimental mouse model for the production of monoclonal anti-FVIII antibodies. The mice successfully produced high titres of polyclonal FVIII inhibitors after intraperitoneal injection with recombinant FVIII, as shown by the positive results of the post-immunisation serum in both ELISA and Bethesda assay.

After the fusion experiment and cloning of antibody-secreting hybrids, monoclonal antibodies of IgM subclass were obtained originating from two of the seven mice. Although most murine monoclonal antibodies against FVIII used by other researchers were of IgG subclass, inhibitory IgM antibodies were produced as well (Shima *et al*, 1988). It was disappointing that none of these monoclonal antibodies had an inhibitory effect on FVIII:C in the Bethesda assay, despite their ability to bind FVIII was repetitively shown by ELISA. These monoclonal antibodies were likely to be directed to the non-functional epitopes on FVIII molecule. Non-inhibitory anti-FVIII antibodies were reported by others in their work to produce monoclonal antibodies to FVIII (Rotblat *et al*, 1983; Fulcher *et al*, 1985b). They can be useful in many studies including those on the structure and immunogenicity of the FVIII molecule. Nevertheless, for our eventual purpose of using the monoclonal antibody to assist the development of new approaches for the treatment of inhibitors, inhibitory antibodies directed against the functional epitopes on FVIII, rather than the non-inhibitory antibodies, were desirable.

The fusion experiment for the other five mice resulted in successful growth of hybridomas, but none secreted antibodies against FVIII. It was possible that the antibodies secreted did not bind to FVIII molecule in the current ELISA method, or was at a very low titre. Further expanding of these hybrids and repeating ELISA analysis of the supernatant of the more mature cultures did not result in positive results either. This suggested that although the standard techniques for the production of monoclonal antibodies are relatively established, the optimisation of the techniques in the production of anti-FVIII antibodies may not be easy.

It was encouraging that as demonstrated by the analysis of Western blot, we had produced murine polyclonal anti-FVIII antibodies from the immunised mice that bound to our recombinant C1 and C2 fragments. These polyclonal antibodies were also shown to have high inhibitory titres by the Bethesda assay. It would be of interest to confirm whether these anti-C1 (or anti-C2) antibodies have a direct inhibitory effect on FVIII:C via their interaction with the C1 (or C2) domain of the FVIII molecule. This could be investigated in the future by pre-incubation of these antibodies with the C1 (or C2) peptides and assessment for the consequential effect on the inhibition of FVIII:C in a Bethesda assay. If the pre-incubation of the polyclonal antibodies with the recombinant peptides could help to restore some of the FVIII:C inhibited by these antibodies alone (in absence of the peptides), this would confirm that the inhibitory effect of the these antibodies was by their direct interaction with the C1 (or C2) domain of the FVIII molecule. In addition, this functional assay would also confirm that our recombinant C1 and C2 peptides not only display antigenicity recognised by the antibodies in denaturation conditions (Western blot), but also have the biological viability to compete with and counteract the inhibitors *in vitro*.

There is no doubt that epitope detection and characterisation is a critical step in understanding the immunochemistry and mechanisms of action of FVIII inhibitors. Our work in the epitope mapping of FVIII highlighted some technical difficulties in the area, including the poor expression of the soluble recombinant peptides in the culture medium of the COS-7 cells and the low yield of inhibitory monoclonal antibodies in a mouse model. While the methodology remains a challenge, the principle and practice of recombinant DNA technology to produce FVIII peptides of any desired size remains to be an appealing way to carry out a thorough study of epitope determination. Future directions may also include experiments studying the inactivation mechanisms by inhibitors with different specificities.

In addition, there has been knowledge gained on the immunobiological aspects of the development of FVIII inhibitors, including the cellular mechanisms leading to the inhibitor synthesis. For instance, a recent study showed that the immune response to FVIII is dependent upon the interaction of different CD4+ T cell subsets (Th1, Th2 and Th3) specific for FVIII (Reding *et al*, 2002). The knowledge of the

immunobiology and the epitope specificity with inhibitory mechanisms of the inhibitors on the physiological and molecular level will lead to one step closer to the understanding of the pathogenesis of not only FVIII inhibitors in haemophilia A, but also other autoantibody diseases.

2.5 Summary

1) Recombinant C1 and C2 fragments were successfully expressed in COS-7 cells using the mammalian cell vector pSegTagB, as demonstrated in Western blot probed with anti-myc antibody.

2) The recombinant C1 and C2 peptides displayed antigenicity recognised by the polyclonal mouse anti-FVIII antibodies in Western blot.

3) Future optimisation of the methods for the production of recombinant FVIII peptides could be directed to improve its secretion from the cell into the culture medium.

4) All eight mice immunised with recombinant FVIII successfully developed polyclonal anti-FVIII antibodies with high inhibitory Bethesda titre.

5) Murine monoclonal anti-FVIII antibodies produced in this study were of IgM subclass and presumably directed against the non-functional epitopes on FVIII.

CHAPTER THREE THROMBIN GENERATION ASSAY

3.1 Introduction

The two basic coagulation tests, APTT and PT, reflect the classical intrinsic and extrinsic pathway respectively and use the end point of fibrin clot formation. These two tests adequately mirror the function of the coagulation system *in vitro* and serve as the essential screen for bleeding disorders, including haemophilia A, where there is a prolonged APTT due to FVIII deficiency in the classical intrinsic pathway.

Greater than 95% of the total amount of thrombin production takes place after initial clot formation (Mann *et al*, 2003b). The thrombin formed after initial clot formation has been proposed to have several important roles including activation of the TAFI to protect the clot against proteolysis, participation in ongoing remodelling of the clot structure and activation of platelets via cleavage of platelet PAR-4 (Monroe & Hoffman, 2006). Thrombin that has bound to the membrane protein thrombomodulin also activates protein C, resulting in the inhibition of the coagulation system by cleaving the phospholipid-membrane-bound cofactors Va and VIIIa thus localising coagulation reactions to the vicinity of an injury (Dahlback, 2004).

The TGA measures the amount of thrombin produced over time, both before and after clot formation. Theoretically if measured in whole blood, the TGA might reflect the influences of all the procoagulant and anti-coagulant factors and provide information for the evaluation of both haemorrhagic and thrombotic risks. Hemker and colleagues initiated the modifications of the TGA method mainly to assess conditions that predispose to venous or arterial thrombosis, and to monitor antithrombotic therapies (Wielders *et al*, 1997). However more recently, some publications have started to explore the usefulness of the TGA in hypo-coagulability states such as during the treatment of haemophilia A (McIntosh *et al*, 2003; Varadi *et al*, 2003; Lewis *et al*, 2007).

The original TGA described in 1953 was a two-stage based assay (Macfarlane & Biggs, 1953; Pitney & Dacie, 1953). The method required subsampling of plasma at regular intervals from the primary reaction tube, where thrombin generation was triggered. The subsampled plasma was then transferred into the secondary indicator tube containing a fibrinogen solution. The clotting time in the fibrinogen solution was then used to estimate thrombin activity. This method was difficult to perform and imprecise.

An important development in the TGA technique was to measure the thrombin production by the cleavage of a chromogenic substrate, instead of by the clotting time of fibrinogen (Hemker *et al*, 1986). The plasma in the primary reaction tube was defibrinated and a chromogenic substrate replaced the fibrinogen solution in the secondary tube. Later on a slow-reacting chromogenic substrate was used so that the continuous registration of thrombin activity in a single test tube became possible without subsampling (Hemker *et al*, 1993). This converted the method to a much simper one-stage assay. However the use of a chromogenic substrate resulted in erroneous measurement of the thrombin-decay process, mainly due to the continued cleavage of the substrate by both free thrombin and the α2-macroglobulin-thrombin complex (Hemker & Beguin, 1995; Wielders *et al*, 1997). Although the α2-macroglobulin-thrombin complex retains its amidolytic activity, it does not act on fibrin (Hemker *et al*, 1993).

Further improvement of the TGA in recent years saw the use of a slow reacting fluorogenic substrate in place of the chromogenic substrate in a single test tube (Hemker *et al*, 2000). The fluorescent signal is not impaired by turbidity that may be caused by the occurrence of a clot or the presence of platelets. Hence there is no requirement for defibrination of plasma. Subsequently, it is also possible to measure thrombin generation in PRP and in the presence of other cellular components.

In haemophilia A, it is a well-known clinical observation that some patients with identical FVIII levels can show large variations in the severity of bleeding tendency even within the same family (Walsh *et al*, 1973; Ghosh *et al*, 2001). This may be because of differences between individuals in the levels of other

clotting factors and their corresponding inhibitors. Because the TGA measures the global effect of all the procoagulant and inhibitory factors, it might provide useful information that is different from that provided by measuring the level of the missing factor (e.g., FVIII).

In addition, it has been well-documented that in approximately one-third of patients with mild haemophilia A, there is a discrepancy between the one- and two-stage FVIII levels (Duncan *et al*, 1994; Rodgers *et al*, 2007; Lloyd in Ingerslev *et al*, 2008; Trossaert *et al*, 2008). It is not known whether the one- or two-stage assay better reflects the clinical phenotype of these patients. The TGA may offer an alternative tool to study this discrepancy.

At the time of this study (2003) there was little published data on the usefulness of the TGA in a cohort of patients with haemophilia A. Diminished thrombin generation was described by Hemker's group in congenital coagulation disorders (n = 88 patients) including severe factor II (thrombin), V, VII, X and XI deficiency, but they provided no data on FVIII or FIX deficiency in this publication (Al Dieri *et al*, 2002). Another study using plasma from 2 patients with severe haemophilia A showed delayed onset and attenuated thrombin generation when triggered by activated factor IX (FIXa) (McIntosh *et al*, 2003). Since the completion of the present study, a few papers have been published on the application of TGA in the evaluation of haemophilia A (Beltran-Miranda *et al*, 2005; Dargaud *et al*, 2005a; Trossaert *et al*, 2008) and these will be discussed in Section 3.4.

The current concept of haemostasis suggests that *in vivo* coagulation is initiated by a low concentration of tissue factor via the TF pathway (Mann *et al*, 2003a; Monroe & Hoffman, 2006; Tanaka *et al*, 2009; Vine, 2009). It has been demonstrated by Hemker's group that in haemophilia A, thrombin generation triggered by TF was reduced compared to normal (Hemker *et al*, 2003). Nevertheless, haemophilia A is a condition with predominant impairment of the classical intrinsic pathway as a result of FVIII deficiency. Hence we considered that it was important to compare thrombin production triggered by APTT reagent

with that triggered by TF in haemophilia A plasma. To date, there has been no published systematic study of TGA in haemophilia A that compare the use of the two different triggers, TF and APTT reagent.

There was a fluorometer facility available at our institute that was capable of detecting the fluorescent signal from a reaction in a microtitre plate. Our haemophilia centre has a stable population of mild and moderate patients. It was therefore of interest to establish a semi-automated thrombin generation assay to study patients with haemophilia A.

We hypothesised that because TGA measures "real time" thrombin generation both before and after clot formation, the TGA results in haemophilia plasma may provide more information than the clotting-based conventional FVIII assay in describing the "coagulation phenotype" in these patients.

We investigated the TGA using two different triggers, the APTT reagent and the TF, in a group of normal subjects and patients with mild/moderate haemophilia A between April and December 2003.

The specific aims of this chapter were:

1) To establish the TGA method using a fluorogenic substrate and in-house equipment and evaluate it for the assessment of coagulation deficiency, using either APTT reagent or TF to initiate production of thrombin.

2) To apply the two TGA methods to a group of normal subjects and a group of patients with mild/moderate haemophilia A:

   i) To assess the correlation of TGA parameters with the FVIII level.

   ii) To determine which method of the TGA, by the APTT activation or TF activation, is better in assessing haemophilia A.

   iii) In the two subgroups of mild/moderate haemophilia patients with either *equivalent* or *discrepant* FVIII levels by the one- and two-stage assays, to examine whether there is any difference in the TGA results.

3.2 Methods

3.2.1 Establishment of the TGA

3.2.1.1 Equipment

The fluorometer LS55 Luminescence Spectrometer (Perkin Elmer, MA, USA) was used to detect fluorescent signal in the reaction mixture, according to the manufacturer's manual. The wave lengths of operation were 390 μm and 460 μm for excitation and emission respectively. A clear, round-bottomed, 96-well ELISA microtitre plate Nunc-Immuno Module MaxiSorp (Nunc A/S) was chosen following experiments demonstrating superior results of minimal background signal, compared to the other two microtitre plates available in the laboratory (data not shown).

3.2.1.2 Reagents

The APTT reagent Actin FSL from Dade Behring (lot number 527360) was used undiluted in the TGA triggered by the APTT reagent.

The recombinant human TF Innovin (Dade Behring; lot number 526906 and 527360) was reconstituted and used at 1 in 500 dilution in normal saline in the TGA via TF activation. When 10 μl of the 1 in 500 dilution was added to the plasma reaction mixture (total volume of 120 μl) it gave a final concentration of TF 1 pM. This low level of TF was used by Hemker *et al* (2000).

The in-house phospholipid products of washed platelets from the "platelet neutralisation procedure" product (PNP) (batch number D160902-L191003) was used undiluted in TGA via TF activation. In brief, the PNP washed platelets were prepared as follows. The aliquots of pooled platelet concentrate from the Australian Red Cross (Adelaide, South Australia) were mixed with Tris-buffered saline, centrifuged as for PRP to remove blood cells. The PRP then was transferred to another tube and centrifuged for 10 min at minimum of 2,200 g and the supernatant discarded. After resuspending the platelet pellet in Tris-buffered saline and it was centrifuged for another 10 min at 2,200 g. This washing procedure were repeated twice

more and washes discarded. Finally the platelet pellet was resuspended in Tris-buffered saline and platelet count adjusted to 200,000-300,000/μl. The aliquots of PNP product (washed platelets) were stored at - 70°C. The preliminary experiments comparing PNP with two other phospholipid agents including a commercial product Diagen platelet substitute (Diagnostic Reagents, Oxon, UK) demonstrated relatively consistent results of TGA with the PNP product (Section 3.3.1.3.3.3).

The fluorogenic substrate Z-Gly-Gly-Arg-AMC HCl (Z-GGR) was obtained from Bachem (Bubendorf, Switzerland, lot number 0557036) and made up to a final concentration of 5 mM in a calcium-containing buffer (pH 7.35), which contained 10% DMSO, 60 mg/ml bovine serum albumin (BSA), both from Sigma, and 20 mM HEPES, 150 mM NaCl and 0.1 M $CaCl_2$.

In addition, pooled normal heat-inactivated plasma (PN-HI), commercially available FVIII- or FIX-deficient plasmas (Dade Behring), and plasmas from patients with FVII- or FXI-deficiency were also used during the establishment of the TGA method using either the APTT reagent or the TF.

3.2.1.3 Subjects studied

In total, the in-house TGA utilising the LS55 fluorometer was carried out in PPP from 18 normal subjects and 42 patients with mild and moderate haemophilia A. The majority of the blood samples from the haemophilia patients (34 of 42) were collected at clinical interviews held in 2003 during the time of this project. The normal subjects were randomly selected from one of the donor batches of February 2002 from the Australian Red Cross (Adelaide).

In the 42 patients with mild/moderate haemophilia A, 28 had equivalent FVIII levels by the one- and two-stage FVIII assays, henceforth referred as the "*equivalent* subgroup" or "*equivalent* patients" where appropriate. The other 14 patients had discrepant results by the two FVIII assays, henceforth referred as the "*discrepant* subgroup" or "*discrepant* patients" where appropriate.

3.2.1.4 Plasma samples

Blood was collected into one tenth volume 109 mM trisodium citrate anticoagulant. Plasma and cells were separated by centrifugation at 3,000 rpm (2,000 g) in a Beckman J-6B centrifuge for 10 min at 15-20°C. Plasma was transferred into a clean tube and re-centrifuged for another 10 min at 3,000 rpm. The supernatant, free of platelet contamination (hence termed PPP), was then removed and frozen in aliquots at -70°C and thawed at 37°C immediately before the start of the experiment.

3.2.1.5 Some general aspects of the establishment of TGA

3.2.1.5.1 Well reading time

The LS55 Luminescence Spectrometer is equipped with a single detection probe and capable of reading either a single tube or a microtitre plate. The manufacturer's manual recommends a reading time of a fluorescent signal for a mircrotitre well of 2 sec per well. We performed a preliminary experiment to compare a serial of different well reading time: 0.1, 1, 2 and 5 sec per well.

We found that either the 2-sec or 5-sec per well reading time resulted in a much more stable and smooth fluorescent signal curve, compared to the reading time of 0.1 and 1 sec per well (data not shown). We chose a reading time of 2 sec, rather than 5 sec so that we could include a reasonable number of samples for one experiment for our TGA method.

Because only a single probe is available with the LS55 fluorometer, there is the requirement of approximately 1 sec for the probe to transfer from one well to the next. In allowing a reading time of 2 sec for each well, the fluorescent signal detection for one well is completed within 3 sec. A 5-sec per well reading time would require a total working time of at lest 6 sec for each well.

In addition, we adopted a time interval of 30 sec for each cycle of readings for our TGA method. A 30-sec interval for each reading in TGA was used by Hemker *et al* (2000). We considered this was reasonable. A total working time of 3 sec per well would allow eight samples to be tested comfortably

within 30 sec, while a 6-sec per well working time (5-sec per well reading time) only completes reading for four samples safely within the same time duration.

Hence using a reading time of 2 sec per well we could study eight wells per plate. One of the wells was reserved for a blank control. Instead of plasma, 80 μl of normal saline was used in the blank well and other reagents added as per the rest of the plate. This blank control provided a background signal readings for the purpose of subtraction in the data analysis for the other seven testing wells. Thus any interference from the background signals was corrected for each experiment.

3.2.1.5.2 Dispensing

The LS55 fluorometer is not equipped with simultaneous reagent dispensers for different wells on a microtitre plate. To minimise the errors from different timing of starting the reaction, we used a multi-channel pipette to synchronise the time for dispensing reagents to the testing wells, including the calcium-containing fluorescence substrate solution as the final step to start reaction.

3.2.1.5.3 Pre-incubation (time and temperature)

There were few details on the pre-incubation period in the published TGA methods at the time of this project. For the TGA using the APTT reagent (activation via the classical intrinsic pathway), a 30-sec pre-incubation of the testing plasma and the APTT reagent (Actin) at 37°C was described once (Wielders *et al*, 1997). Another publication on TGA via the classical intrinsic pathway but with the use of FIXa as a trigger used a 1.5-min incubation at 37°C (McIntosh *et al*, 2003). To our knowledge, for the TGA using TF activation, pre-incubation of plasma sample with TF and phospholipids was not generally required (Hemker *et al*, 2000; Turecek *et al*, 2003).

The LS55 fluorometer is not equipped with a built-in 37°C incubation facility for microtitre plates. We chose to pre-incubate PPP with the APTT reagent Actin for 15 min at room temperature to ensure

contact activation. Experiments comparing different incubation time (1, 2, 5, 10, 15, 20 and 30 min) of a normal plasma and the APTT reagent were carried out and the results shown in Section 3.3.1.1.1.

3.2.1.5.4 Reaction (time and temperature)

The reaction time for the TGA were according to the test system, triggers used and the samples investigated (Hemker *et al*, 2000; Veradi *et al*, 2003; McIntosh *et al*, 2003). Using the same final concentration of TF (1 pM) in the reaction mixture as ours, Hemker and colleagues showed a 30-min reaction time was sufficient for normal PPP; thrombin generation reached a peak and returned to baseline within 10 min (Hemker *et al*, 2000). We chose a 30-min duration for our reaction time for the TGA method with both triggers, the APTT reagent and TF.

Some researchers used 37°C as reaction temperature for TGA to best mimic the physiological condition (Butenas *et al*, 1999; Hemker *et al*, 2003; Varadi *et al*, 2003), while many others did not disclose the reaction temperature of their TGA methods. Our TGA was performed at room temperature since the LS55 fluorometer is not equipped with a 37°C incubation facility for microtitre plates.

3.2.1.5.5 Reproducibility of duplicate samples

Having determined that seven testing wells could be used on a single microtitre plate in our TGA method (Section 3.2.1.5.1), it was essential to then examine the reproducibility of the results for duplicate samples on the same plate. This information would be useful to decide how many individual plasma samples could be tested in a single experiment (one plate) in our TGA method.

To examine the reproducibility of duplicate samples on the same microtitre plate, plasmas from a normal subject and two patients with mild haemophilia A were tested by TGA using the APTT reagent. The plasmas were placed in different order of reading (sequential or non-sequential) and results shown in Section 3.3.1.1.3.

In brief, there was high reproducibility of results with duplicate samples. Therefore a single well was used for individual plasma sample in our TGA method, allowing a maximal seven different plasmas to be tested in a single experiment. Typically the plasmas from normal subjects and haemophilia patients were both included on the same microtitre plate.

3.2.1.6 TGA using APTT reagent as a trigger

The TGA experiments were carried out between April and December 2003 using in-house equipment and methods as described above, following the assay principles published by Hemker *et al* (2000). We used the same reagents (fluorescent substrate and its buffer, TF reagent) and the same final concentrations as described in Hemker's publication. We also used the same volumes of plasma (80 μl), trigger reagent (20 μl) and the calcium-containing fluorescent substrate solution (20 μl) for the reaction mixture.

To perform TGA using the APTT reagent (Actin FSL) as a trigger, the experiment was started when each well of the mircrotitre plate were loaded with 80 μl of PPP (from either normal subjects or patients with mild/moderate haemophilia). Twenty microliter of undiluted APTT reagent was then added. The mixture was incubated at room temperature for 15 min. The coagulation reaction was then started at time zero by adding 20 μl of the calcium-containing fluorescence substrate solution, making the final volume of the reaction mixture 120 μl.

Fluorescence was measured immediately from time zero for the subsequent 30 min, at a 30-sec time interval. Thus each sample well underwent 61 cycles of fluorescence detection; the first cycle was at time zero and the last cycle was at time 1,800 sec (30 min).

3.2.1.7 TGA using TF as a trigger

Similarly, in the TGA using the TF reagent as a trigger, 80 μl of PPP was first loaded on the microtitre plate. Ten microliter of Innovin (at 1 in 500 dilution) and 10 μl of phospholipid (PNP product, undiluted)

were then added, with no incubation time required. The reaction was started at time zero upon the addition of 20 μl calcium-containing fluorescence substrate solution. The final volume of the reaction mixture was 120 μl, as per the TGA using the APTT reagent. Similarly, continuous measurement of the fluorescence signal at a 30-sec interval was recorded for a 30-min duration.

3.2.2 Analysis of data on thrombin generation curve

3.2.2.1 Analysis principles

The data on thrombin generation in plasma as measured by a fluorogenic substrate are usually presented in two curves. The first is the "fluorescent signal curve". When thrombin generation is started, a continuous increase in the fluorescence intensity is observed (Figure 3.1 panel A, (A)). Because the thrombin substrate is present in the assay mixture, this curve represents the accumulated effect of all thrombin that is generated and splits the fluorogenic substrate during the reaction.

A method for further analysis is to calculate the derivatives of the fluorescence signal curve. In other words, the rate of increase in the fluorescence intensity (fluorescence unit/min, FU/min), i.e., the first derivatives (dF/dt) of the fluorescence signal curve (Figure 3.1, panel A, (B)), reflects the actual effective free thrombin concentration, showing time-dependent formation and subsequent inactivation of thrombin (Hemker *et al*, 2000; Varadi *et al*, 2003). Hence the second curve in TGA is the "derivative curve". The maximum rate of the increase of the fluorescence intensity indicated the highest free thrombin concentration in the reaction.

The arbitrary unit for the rate of increase of fluorescent unit (FU/min) could be converted to thrombin equivalent concentrations in nanomolar (nM), with the use of a reference curve prepared by measuring the rate of substrate conversion by a purified thrombin of known concentration (Hemker *et al*, 2000; Turecek *et al*, 2003). It was of note that in general, the term "thrombin generation curve" (or "thrombogram"), refers to the derivative curve rather than the original fluorescent signal curve.

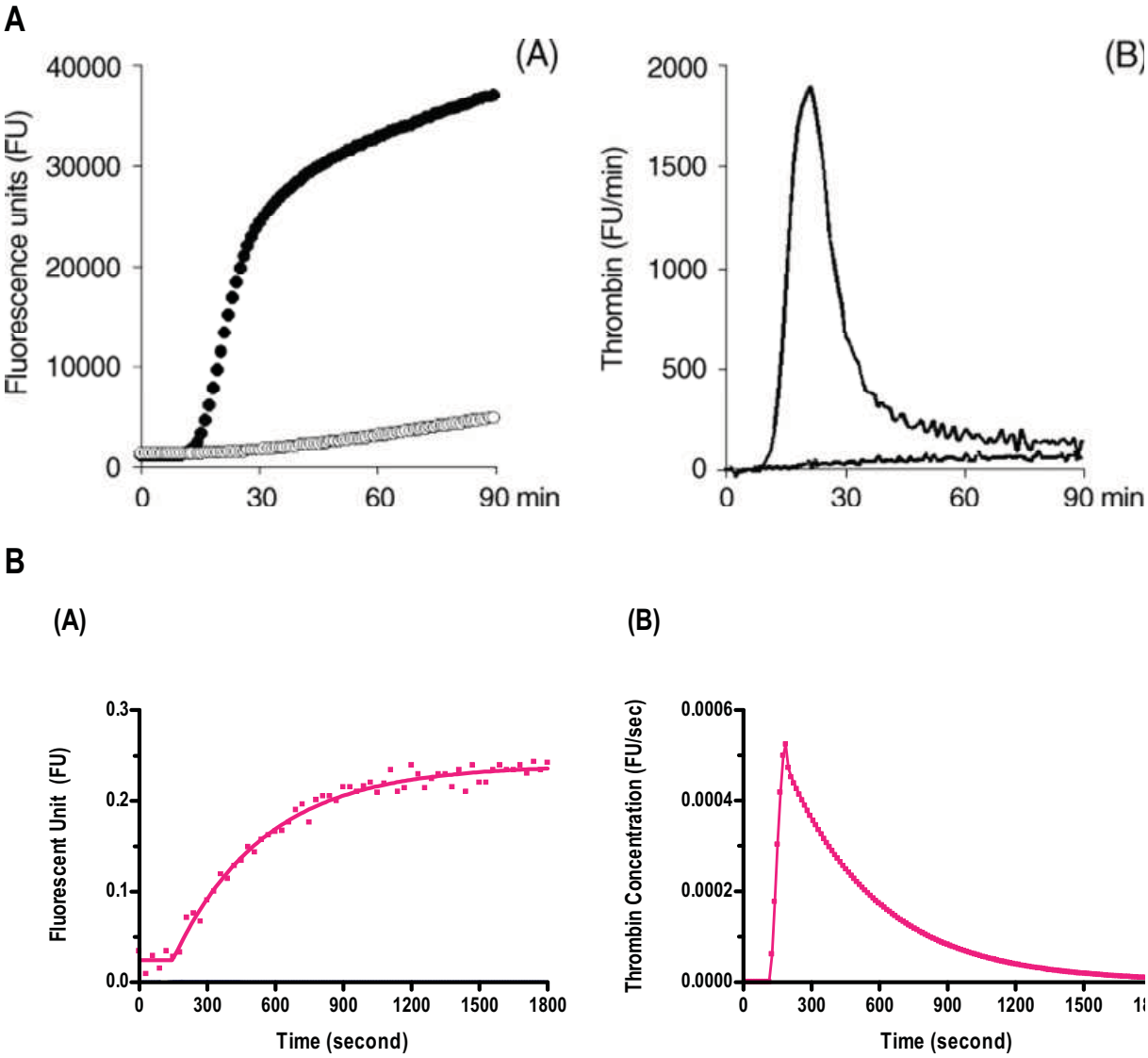


FIGURE 3.1 Thrombin Generation Curve

A. Reprint from Varadi *et al*. Monitoring the bioavailability of FEIBA with a thrombin generation assay. Journal of Thrombosis and Haemostasis. 2003; 1: 2374-2380.

Continuous determination of thrombin generation in a normal and in factor FVIII inhibitor plasma.
(A) Accumulation of the fluorescence signal as detected in normal plasma (●) and in a high-titre FVIII inhibitor plasma (○).
(B) First derivative of the curves from (A).

B. Fluorescent signal curve and first derivative curve from the current study.

(A) Fluorescent signal curve over 30 min in a normal plasma in TGA using the APTT reagent.

(B) The first derivative curve of (A).

The sharp rise of the rate of fluorescent signal increase (FU/sec) from the baseline appeared slightly different to graph (B) in the reprinted publication as shown in panel A. The reading interval of 30-sec (instead of 15-sec, for example) and/or the limitation on sensitivity of signal detection by the LS55 fluorometer may be responsible for our test system's inablity to detect a more gradual and smaller changes of the fluorescent signal.

The derivative curve (thrombin generation curve) can be characterised by four main parameters (Figure 1.4). The first parameter is the lag time, which is the time required for thrombin generation to start (duration from time zero to the onset of significant thrombin generation). In general, the lag time is considered to reflect the clotting time (Hemker *et al*, 2004; Beltran-Miranda *et al*, 2005).

The second and third parameters are the peak time (or time to peak) and the peak thrombin concentration (or peak height) (Figure 1.4). The peak thrombin is the highest Y value on the thrombogram. The peak time is the time required to reach the peak of thrombin generation.

The last parameter is AUC (Figure 1.4). This is the calculated total area under the free thrombin generation curve, representing the fluorescent signal generated during the entire reaction time. The AUC is also referred as ETP since Hemker's publication in 1995 (Hemker & Beguin, 1995).

3.2.2.2 Fluorescent signal curve and curve fitting in current study

We adopted the methodology used by others (Hemker *et al*, 2000; Varadi *et al*, 2003) and analysed the TGA results according to the principles as described above.

The thrombin generation was first presented as the fluorescence signal curve, where the original data of fluorescence signal (FU) obtained from the LS55 fluorometer were plotted over a time course for 1,800 sec (30 min) (Figure 3.1 panel B (A)).

We then used the software Prism 4 (GraphPad software, CA, USA) to perform a non-linear curve fitting for the fluorescent signal curve. In consultation with expertise from the Pharmacology Department (University of Adelaide), it was decided the equation to use was as follows: Plateau then increase to (Plateau + Span): $Y = IF [X < X0, Plateau, Plateau + Span*(1 – exp (-k* (X - X0)))]$; plateau until X = X0, then exponential increase to Plateau.

The curve fitting was successful for each plasma sample tested and consistent throughout the study: the median goodness of fit R^2 was 98% for TGA with activation by the APTT reagent, and 94% for TGA by TF activation in the 60 samples tested.

3.2.2.3 First derivative curve and the parameters in current study

From the fitted fluorescent signal curve, a first derivative curve was obtained using Prism 4 (Figure 3.1 panel B (B)). The X axis of the derivative curve was the time (sec) and the Y axis was the rate of increase of fluorescence signal per time unit (FU/sec), reflecting the actual free thrombin concentration.

We used the four main parameters of the first derivative curve, namely, the lag time (sec), peak time (sec), peak thrombin concentration (FU/sec) and AUC to summarise the results for TGA using either the APTT reagent or TF in this study.

The values of the four parameters for each plasma tested were generated by the Prim 4 software program automatically when graphing the derivative curve. The lag time was represented by the X value (sec) when the first Y value exceeds zero. This was equivalent to the lag time on the fluorescent signal curve (Figure 3.1 panel B (A)). The peak thrombin concentration was the highest Y value (FU/sec), and the peak time was the time to the peak height. The calculated AUC of the derivative curve represented the ETP of the plasma.

3.2.2.4 Specific aspects of analysis of thrombin generation curve in current study

3.2.2.4.1 In relation to the thrombin reference

In TGA, the thrombin concentration in nM for the test sample can be determined from a thrombin reference curve. This is through the comparison of the rate of substrate conversion in the testing plasma to that from the reference thrombin of known concentrations, although the mathematical calculation details was not described (Turecek *et al*, 2003).

We attempted to establish a reference curve for thrombin concentrations in order to convert the fluorescent signals to the equivalent quantifiable thrombin in nM. However our preliminary experiments using a stock purified human thrombin T-6759 (Sigma, lot number 104H9314) at 100 nM repeatedly failed to generate significant fluorescent signal in the current test system (data not shown).

In addition, the Thrombin Calibrator (main component α_2-macroglobulin-thrombin complex) from Hemker's group (Synapse BV, Maastricht, the Netherlands) was trialed as a source for generating the thrombin reference curve. Twenty microliters (576 nM) of the Thrombin Calibrator was added to a parallel non-clotting sample of the testing plasma, in place of the trigger reagent, according to the manufacturer's recommendations. There was only low levels of fluorescent signal detected (data not shown). However, we did not have the technical resource to apply an appropriate algorithm to process this data in order to compare the splitting of the substrate by a known thrombin activity against the thrombin activity of the testing samples, and convert the rate of fluorescent signal increase to the equivalent thrombin concentration in nM.

According to Hemker, using the Thrombin Calibrator in conjunction with their dedicated software program, the systemic errors in TGA caused by the "fluorescent substrate consumption" and the consequent "inner filter effect" of the substrate could be corrected (Hemker *et al*, 2003).

The fluorescent substrate consumption refers to the constant amidolytic activity of α_2-macroglobulin-thrombin complex, which does not represent free thrombin but is a result of continuously degrade substrate. This might not be overcome by adding excessive substrate (Hemker *et al*, 2003). The "inner filter effect" refers to the non-linearity of fluorescence intensity with increasing concentration of fluorescent molecules, partially as a result of the substrate consumption. It was suggested by Hemker that these errors would result as the first derivative of the signal produced did not "directly" reflect the thrombin concentration in nM (Hemker *et al*, 2003).

Therefore, due to the lack of a suitable thrombin reference in this study, the fluorescence data presented were not corrected for the inner filter effect or substrate consumption of the fluorescent substrate and not expressed in equivalent quantifiable thrombin in nM. Instead, the thrombin concentration was express in arbitrary unit of FU/sec as described (Figure 3.1 panel B). Other researchers have used the same approach (Varadi *et al*, 2003).

3.2.2.4.2 In relation to the shape of the derivative curve

Figure 3.1 (panels A and B) illustrates the comparison of the fluorescent signal curve and the derivative curve in TGA in our study and that from a publication (Varadi *et al*, 2003). In both our study and the publication, similar methods for data analysis of the fluorescent signal curve were used, including the use of the arbitrary unit to represent free thrombin concentration in the derivative curve (thrombin generation curve).

The fluorescent signal curve in our study (Figure 3.1 panel B (A)) was similar to that from the publication (Figure3.1 panel A (A)). Both displayed a lag time first, then the exponential increase of the fluorescent signal followed by a plateau.

On the derivative curves, however, the difference were noted for the abrupt rise of the thrombin concentration from baseline in our study (Figure 3.1 B (B)), compared to a more gradual pattern in the publication (Figure 3.1 A (B)). It appeared that our derivative curve did not reflect small changes of the abrupt fluorescent signal increase during the very beginning of the thrombin production phase.

The reasons behind this were unclear. It could be due to the reading interval of 30 sec was too long so that smaller changes of the fluorescent signals in between the time points were not detected and reflected. In other words, a 10- or 15-sec reading interval might reveal more gradual changes of the fluorescent signal and more closely resemble the derivative curve of Varadi *et al* (2003). It could also be due to the systemic limitation on the sensitivity of the signal detection of the LS55 fluorometer.

Consequently, the values for the lag time and peak time for each sample tested in our study seemed close to each other as though they were not overlapping. They were two separate parameters generated from the derivative curve by Prism 4. Both parameters were used to summarise the findings.

3.2.3 Statistics

Curve fitting for fluorescent signal curves and analysis of the derivative curves, including the calculation of the AUC, were performed using the software program Prism 4 (GraphPad software, CA, USA). A two-tailed T test was performed to compare continuous numeric variables from the two different groups. A non-parametric T test was used for non-parametric data or parametric data but without normal distribution. A non-parametric Spearman correlation was used to analyse associations between two groups of parameters. Statistical significance was set at $P < 0.05$.

3.3 Results

3.3.1 Part I: Establishment of the TGA

3.3.1.1 Some general aspects of the establishment of the TGA

3.3.1.1.1 Pre-incubation time for TGA using APTT reagent

Clotting assays based on the activation of the classical intrinsic coagulation pathway (contact activation pathway) such as the APTT test and one-stage FVIII assay require a period of pre-incubation time of the testing plasma with the APTT reagent. Therefore in our TGA using the APTT reagent as a trigger, pre-incubation time was also applied. The experiment comparing different pre-incubation times (1, 2, 5, 10, 15, 20 and 30 min) for a normal plasma and the APTT reagent was carried out.

The results are shown in Figure 3.2. There was a very similar pattern of the fluorescent signal curves for the different pre-incubation time. Subsequently we chose to pre-incubate the plasma with the APTT reagent for 15 min at room temperature to ensure a complete activation through the classical intrinsic pathway.

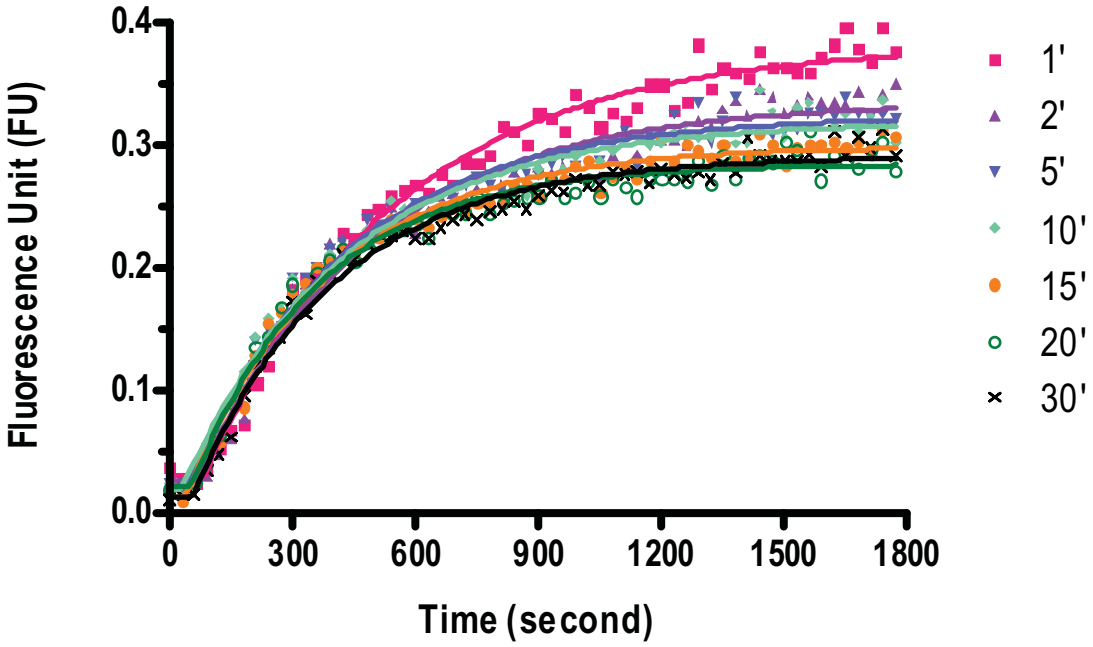


FIGURE 3.2 Effect of Different Pre-Incubation Time in TGA Using APTT Reagent

Seven identical samples of a normal plasma (80 µl) was pre-incubated with the APTT reagent Actin FSL (20 µl) for 1, 2, 5, 10, 15, 20 and 30 min separately before the addition of 20 µl calcium-containing fluorescent substrate at time zero. Continuous measurement of the fluorescent signal at a 30-sec interval was recorded for the next 30 min.

The overlapping fluorescent signal curves indicated that the different pre-incubation time with the APTT reagent did not have significant influence on the thrombin generation in the normal plasma. We adopted a 15-min pre-incubation time for our TGA using the APTT reagent.

3.3.1.1.2 Examination for contact activation in absence of known triggers

To examine whether there might be any contact activation in our test system in absence of any known triggers for blood coagulation, we examined the TGA in plain plasma without the addition of the APTT reagent or TF for a prolonged period of time (60 min). We used plasma from a normal subject, a patient with mild haemophilia A and a commercially available FVIII deficiency plasma. The results are shown in Figure 3.3.

As demonstrated in Figure 3.3, no fluorescent signal was recorded in both plasma of the mild haemophilia A and FVIII deficient concentrate over the 60-min duration. Low level of thrombin generation was detected in the normal plasma at approximately 3,000 sec (50 min). This might reflect a degree of weak contact activation after a prolonged period of time in normal plasma.

It was obvious that in our TGA, for the 30-min reaction time, there was no evidence of contact activation for either the normal or FVIII deficient plasma of mild or severe degree (Figure 3.3).

3.3.1.1.3 Reproducibility of duplicate samples

To examine whether it was necessary to test duplicated wells for each sample on one plate, we examined the reproducibility of the TGA results using the APTT reagent in plasma from one normal subject and two patients with mild haemophilia A.

We examined the results when duplicated samples of the same plasma were placed in adjacent wells (Figure 3.4 A), and when they were placed apart on the same plate. In the latter situation, the three different plasma samples were loaded in sequential order (Figure 3.4 B).

The results showed that regardless of the placement of the duplicated samples, either separate or in adjacent well, the fluorescent signal curves of the duplicates for individual plasma displayed high reproducibility (Figure 3.4).

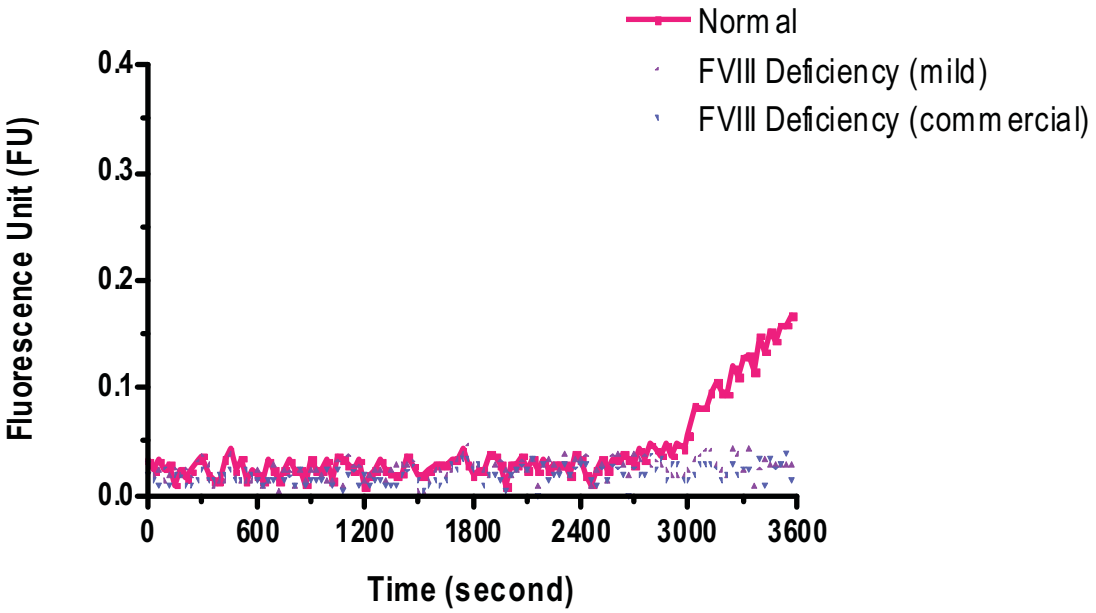


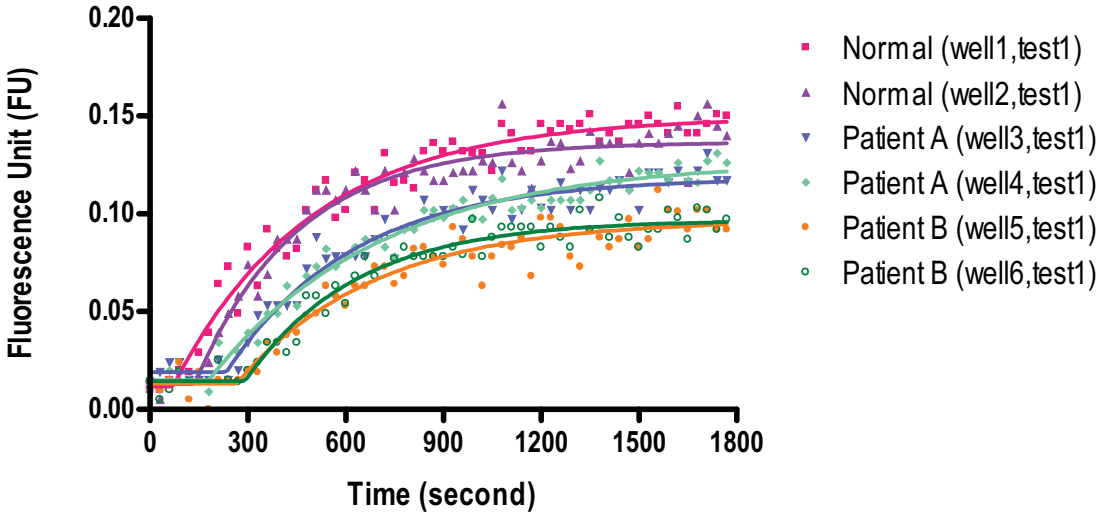
FIGURE 3.3 Examination for Contact Activation in Absence of Triggers

Plasma from a normal subject, a patient with mild haemophilia A and a commercially available FVIII deficient plasma (80 µl) were mixed separately with 20 µl of normal saline, instead of the trigger (APTT reagent or TF). Twenty microliter of the calcium-containing fluorescent substrate was then added. Continuous measurement of the fluorescent signal at a 30-sec interval was recorded for 60 min.

In the absence of any known contact activation or TF activation, there was no thrombin generation for the 60-min course in the two FVIII deficient plasmas of either mild or severe degree. Only the normal plasma displayed a low level of thrombin generation after a remarkable delay of almost 50 min.

Within the 30 min reaction time in our TGA method, there was no evidence of contact activation in either the normal or haemophilic plasma.

A



B

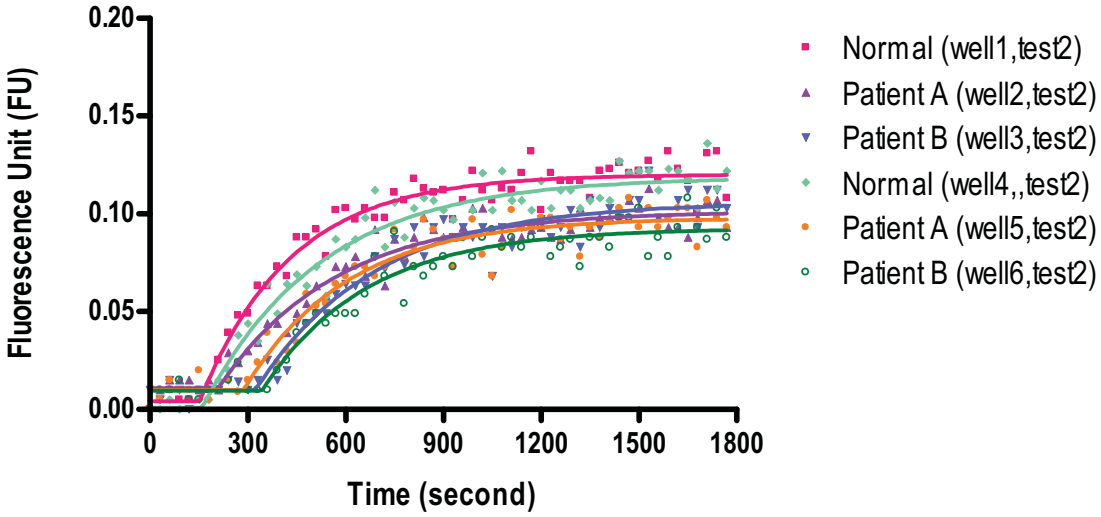


FIGURE 3.4 Reproducibility of Duplicated Samples

TGA of a normal subject and two patients with mild haemophilia A using the APTT reagent as a trigger showed good reproducibility of duplicated samples for the same plasma.

A. Test 1: the duplicated samples of the same plasma were placed in adjacent wells.

B. Test 2: the duplicated samples of the same plasma were placed separately but in a forward sequential order (Normal→Patient A→Patient B→Normal→Patient A→Patient B).

Hence to include duplicated wells for each plasma on one plate was unnecessary given the limitation that only seven testing wells could be used in a single experiment in our TGA method. From a practical point of view, we were confident with the data generated when a single well was used for each plasma in TGA in the current study.

3.3.1.2 TGA using APTT reagent as a trigger

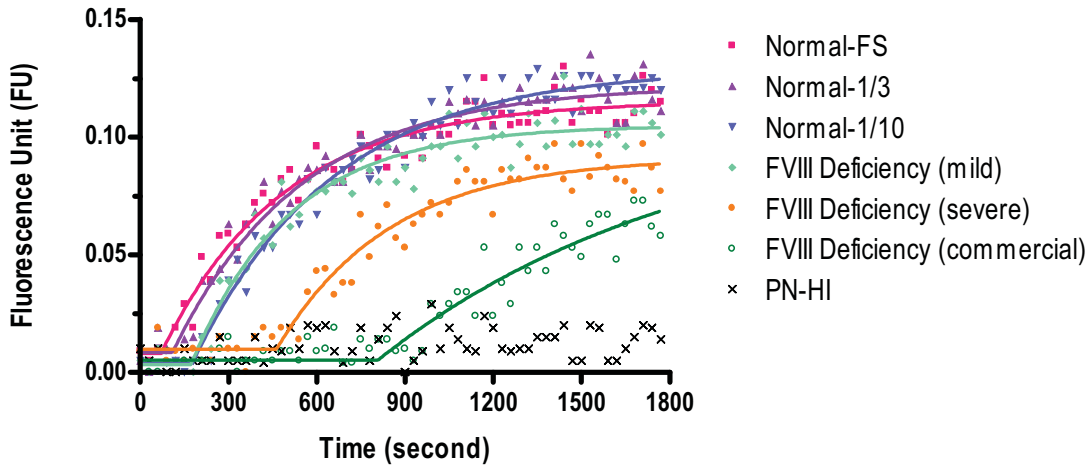
3.3.1.2.1 Effect of FVIII concentration

To investigate the effect of different FVIII levels on thrombin generation in our TGA using the APTT reagent as a trigger, we compared the results from a normal subject, a patient with mild haemophilia A and a patient with severe haemophilia A (Figure 3.5 A and B). The normal plasma was tested at three different dilutions: full strength, 1/3 and 1/10 diluted with commercially available FVIII deficient plasma. A sample of pooled normal heat-inactivated (PN-HI) plasma was included as a negative control (zero level of FVIII).

Figure 3.5 panel A illustrates the fluorescent signal curves: fluorescence unit was plotted on Y axis over a time course of 1,800 sec (30 min) (X axis). In the normal plasma (in full strength), after a short period of lag phase (less than 100 sec) with the baseline level of fluorescent signal close to zero, the fluorescent signal started to rise and reached a plateau by 1,800 sec (30 min).

Overall, for the normal plasma and its 1/3, 1/10 dilutions, and the plasma from the patient with mild haemophilia A, the fluorescent signal curves were not substantially different (Figure 3.5 A). All reached a plateau of similar fluorescence strength by 30 min. However, with the increasing dilutions of normal plasma from full strength to 1/10, the lag phase appeared to be gradually prolonged (Figure 3.5 A). This change in the lag time was subtle but noticeable. This corresponded to the decreasing FVIII levels in these samples.

A



B

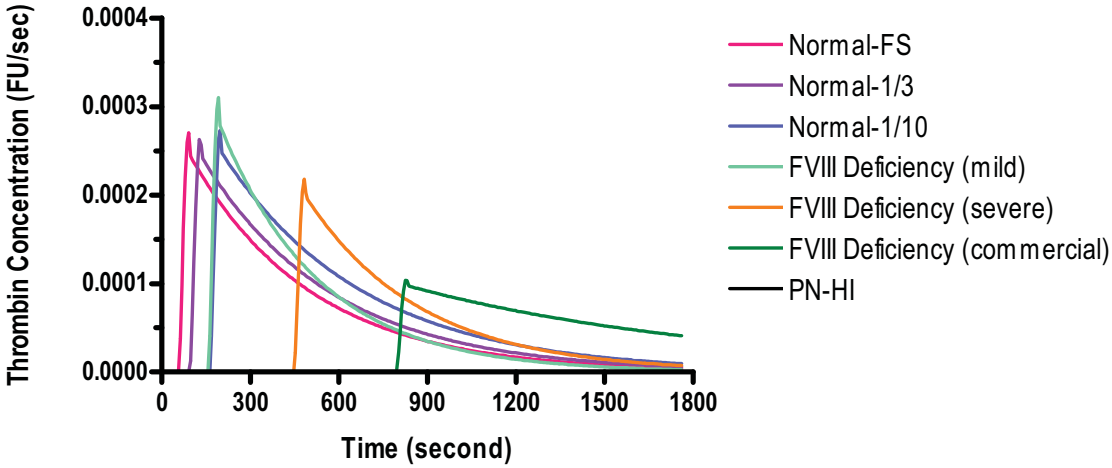


FIGURE 3.5 TGA Using APTT Reagent as Trigger – Effect of FVIII Concentration

A. Fluorescent signal curves of TGA using the APTT reagent in:
a normal plasma (FVIII 137 IU/dl by one-stage assay) in full strength, and its 1/3 and 1/10 dilutions in a commercially available FVIII deficient plasma;
plasma from a patient with mild haemophilia A (FVIII 14 IU/dl);
plasma from a patient with severe haemophilia A (FVIII < I IU/dl);
a commercially available FVIII deficient plasma;
and pooled normal plasma after heat-inactivation of coagulant factors.

B. The corresponding first derivative curves of "A", with the Y axis (rate of fluorescence unit increase) representing free thrombin concentration.

FS: Full strength.
PN-HI: Pooled normal, heat-inactivated.

The fluorescent signal curve of the plasma from the mild haemophilia A (one-stage FVIII 14 IU/dl) also had a longer lag phase (approximately 200 sec) compared to the normal sample (Figure 3.5 A). This lag time happened to overlap with that from the 1/10 dilution of the normal plasma, which represented a similar FVIII level of approximately 13.7 IU/dl.

When the plasma from the patient with severe haemophilia A (FVIII < 1 IU/dl) was tested, the lag phase was definitely prolonged to just under 500 sec, compared to the normal plasma (Figure 3.5 A). In addition, the fluorescent signal curve reached a plateau of a lower fluorescence strength than the normal sample.

It was of note that in the commercially available FVIII deficient plasma, the thrombin generation also showed a prolonged lag phase but did not reach a plateau (Figure 3.5 A). As a negative control, the PN-HI plasma showed no thrombin production (Figure 3.5 A).

Figure 3.5 panel B illustrates the first derivative curves of the fluorescent signal curves from panel A, representing free thrombin generation in the plasma samples. Compared to the normal plasma and its dilutions, the plasma from the patient with severe haemophilia A and the commercial FVIII deficient plasma showed a significant delay of the lag time. The commercial FVIII deficient plasma also displayed an obvious lower peak thrombin concentration than the normal (Figure 3.5 B).

Thus far, the lag time of TGA triggered by the APTT reagent seemed to have the potential to discriminate FVIII deficient plasma from normal plasma. As such, the TGA using the APTT reagent as a trigger was a suitable method for measuring thrombin generation and should be further validated.

3.3.1.2.2 Effect of different coagulant factor deficiencies

As part of the validation of the TGA method using the APTT reagent as a trigger, a series of plasmas with deficiencies of different coagulation factors were tested for thrombin generation. These included

FVII, FVIII, FIX and FXI deficient plasma from patients with hereditary coagulant deficiencies or from a commercial source. Figure 3.6 illustrates the fluorescent signal curves.

Firstly, the FVIII dose-response effect on the lag time of thrombin generation in the plasma from the normal subject, the patient with mild haemophilia A, and the commercially available FVIII deficient plasma were again assessed (Figure 3.6).

Secondly, different factor (FVII, FIX and FXI) deficient plasmas showed different pattern of thrombin generation as triggered by the APTT reagent. These patterns were however, compatible and consistent with the action of the individual factor in normal blood coagulation.

As expected, the patient plasma with severe FVII deficiency showed normal thrombin generation in TGA trigged by the APTT reagent (Figure 3.6). The classical intrinsic pathway of blood coagulation was intact in FVII deficiency. Therefore it was triggered successfully by the APTT reagent to produce thrombin.

Both the commercially available FIX deficiency and the patient plasma from severe FXI deficiency failed to display significant thrombin generation (Figure 3.6). This was consistent with the impaired classical intrinsic pathway, as a result of FIX and FXI deficiency respectively in these two cases.

Based on these results we considered the TGA using the APTT reagent as a trigger was valid and was specific for assessing coagulation insufficiency predominantly within the classical intrinsic pathway. The TGA using APTT reagent was able to detect FVIII deficiency by showing an abnormal pattern of thrombin generation, which was probably represented by a delayed lag phase. More samples with various degree of FVIII deficiency were required to confirm this.

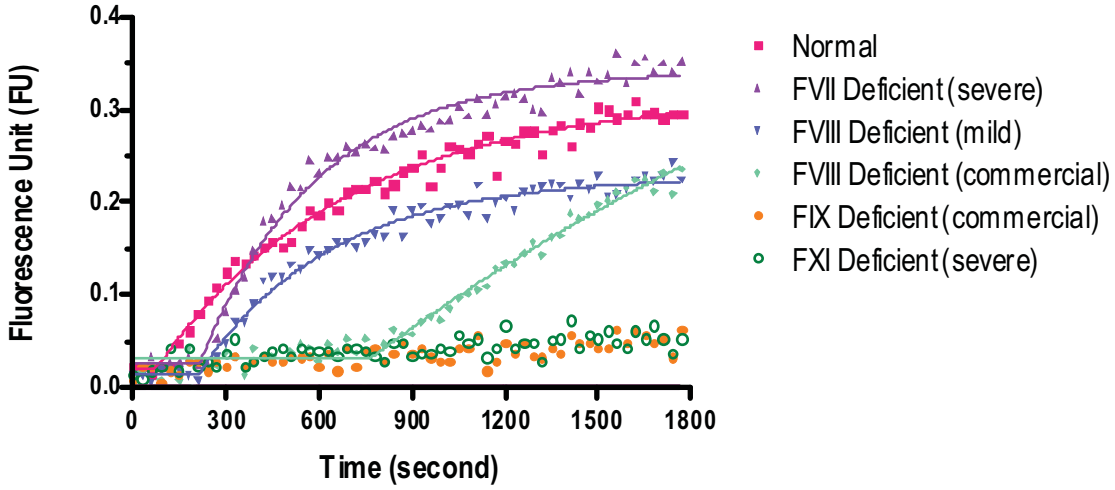


FIGURE 3.6 TGA Using APTT Reagent as Trigger – Effect of Different Coagulant Factor Deficiencies

Fluorescent signal curves of TGA using the APTT reagent in:
plasma from a normal subject;
plasma from a patient with severe FVII deficiency;
plasma from a patient with mild haemophilia A;
a commercially available FVIII deficient plasma;
a commercially available FIX deficient plasma;
and plasma from a patient with severe FXI deficiency.

3.3.1.2.3 Reproducibility

As part of the validation for the TGA method using the APTT reagent, we examined the intra- and inter-experimental reproducibility of the assay in the plasma from two normal subjects (Figure 3.7 A and B).

In panel A, the intra-experimental variations are illustrated when seven samples of a normal plasma were run on the same plate in a single test. In Figure 3.7 panel B, the same plasma was tested repeatedly on four different days and the inter-experimental variations are shown. The inter-experimental variations from another normal plasma were also included (Figure 3.7 B).

Both the intra- and inter-experimental variations for the TGA method using the APTT reagent were acceptable.

3.3.1.3 TGA using TF as a trigger

During the establishment of the TGA method using TF as a trigger, we first examined the appropriate TF concentration. We then examined whether there was any advantage in adding extra phospholipids. We also examined the phospholipids concentration and compared different sources of phospholipids.

3.3.1.3.1 TF concentration

To determine a sensitive concentration of TF for the TGA to adequately discriminate FVIII deficient plasma from normal plasma, different dilutions of TF (1/100, 1/500 and 1/1000) were examined in the absence of additional phospholipids. The results are shown in Figure 3.8 (A and B).

When 1/100 dilution of TF was used, the FVIII deficient plasma (commercially available) demonstrated a thrombin generation close to the normal plasma. With either 1/500 or 1/1000 dilution of TF, appropriate separation of the FVIII deficient plasma from the normal plasma was observed (Figure 3.8 A). Therefore the concentration of TF of 1/100 was not suitable for the detection of FVIII deficient plasma in

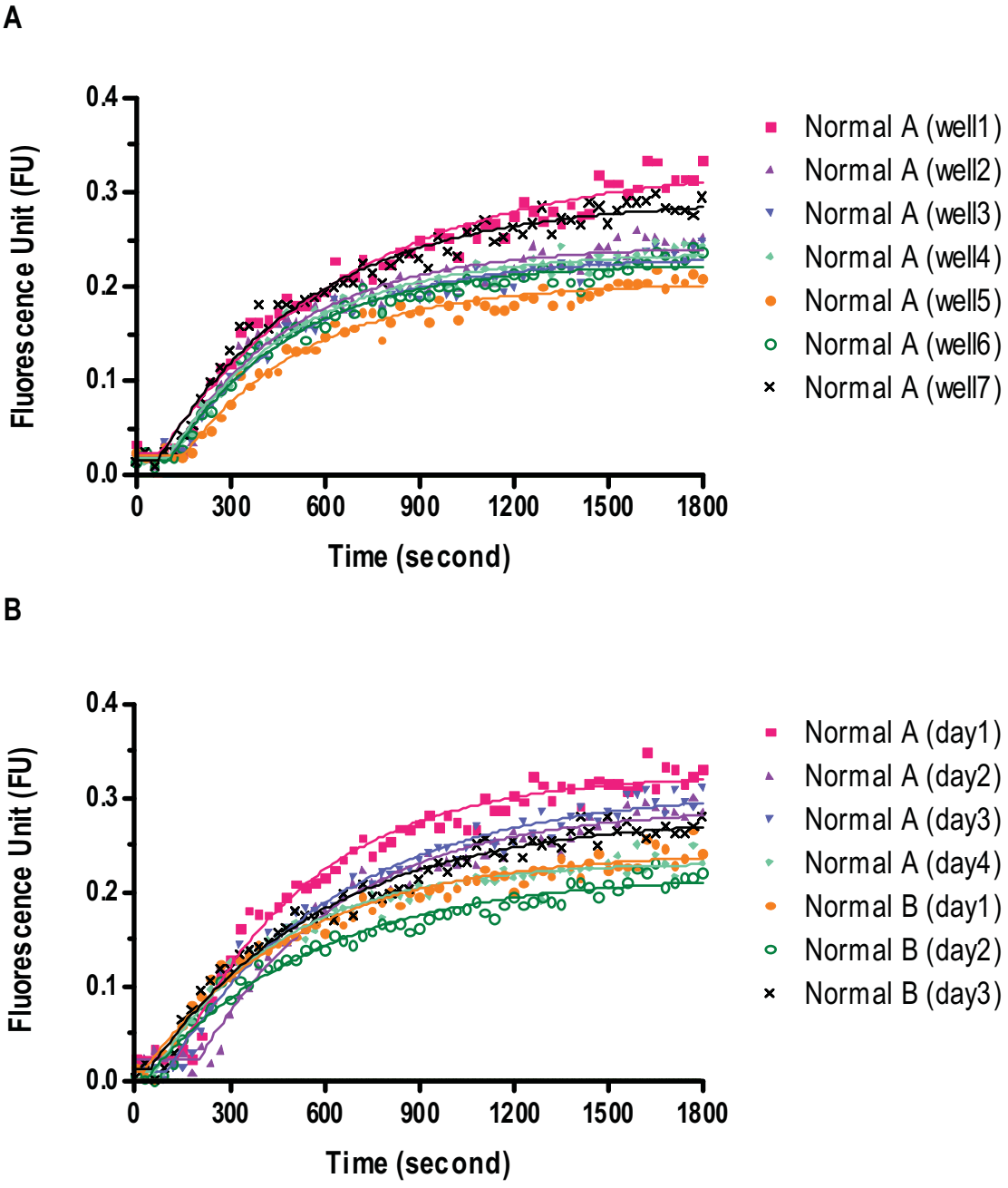


FIGURE 3.7 TGA Using APTT Reagent as Trigger – Reproducibility

A. Intra-experimental variation of the TGA, using APTT reagent as a trigger, in a normal plasma ("Normal A"): identical samples were run in seven parallel wells on a microtitre plate in a single experiment.

B. Inter-experimental variations of the assay in plasma from two normal subjects ("Normal A" and "Normal B"): the samples were assayed randomly on separate days.

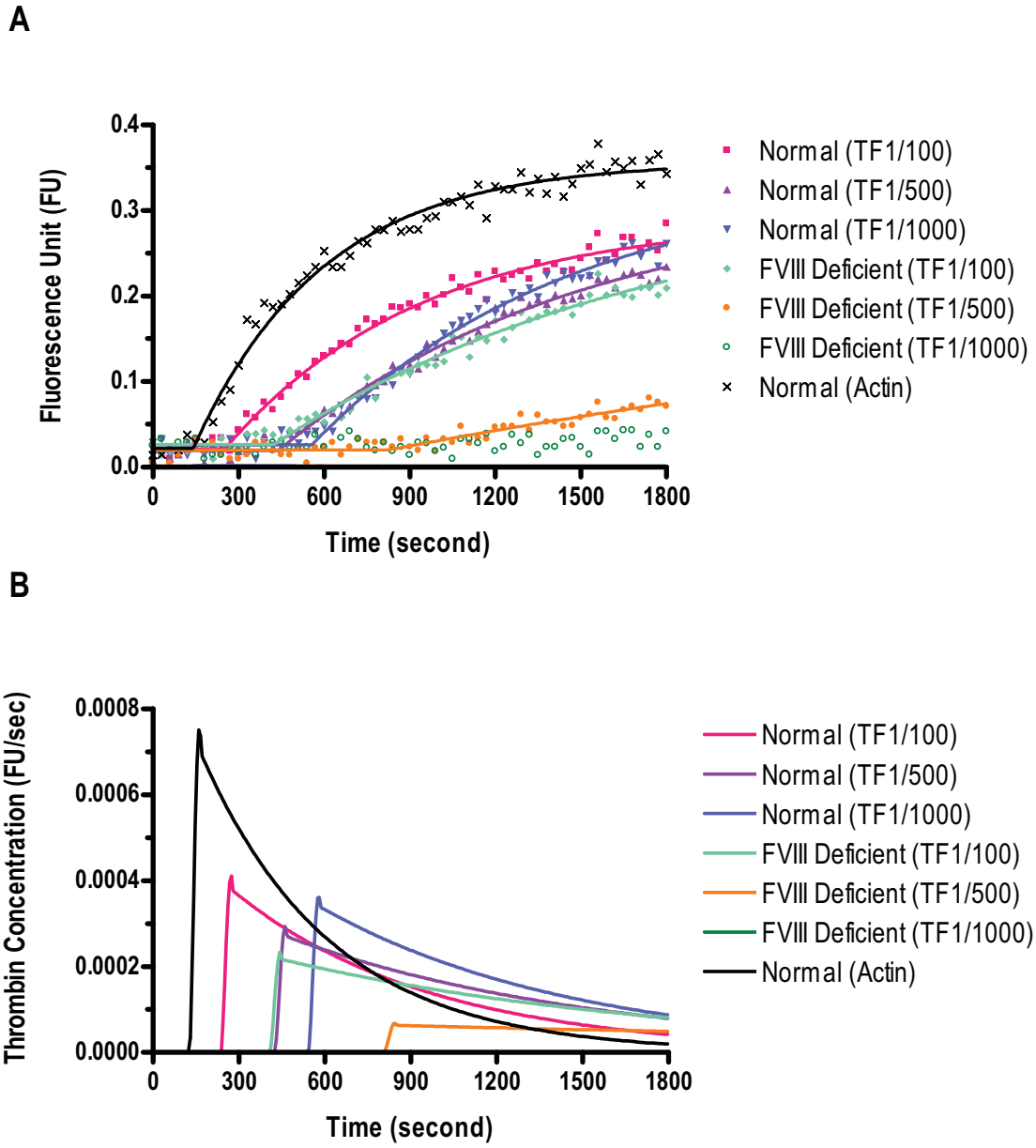


FIGURE 3.8 TGA Using TF as Trigger – TF Concentration in Absence of Additional Phospholipid

A. Fluorescence signal curves of TGA using TF as a trigger in the absence of additional phospholipids in a normal plasma and a commercially available FVIII deficient plasma. Ten microliter of TF plus 10 μl of normal saline were added to plasma, instead of 20 μl APTT reagent.

The different dilutions of TF (1/100, 1/500 and 1/1000) were used to determine a concentration sensitive to FVIII deficient plasma. Both dilutions of 1/500 and 1/1000 of TF appropriately separated FVIII deficient plasma from normal plasma.

B. The corresponding first derivative curves of "A".

Actin: TGA using the APTT reagent as a comparison.

TGA. These results were confirmed in TGA experiments where additional phospholipid was added (data not shown).

We chose TF of 1/500 dilution, instead of 1/1000 dilution, for use in the TGA method, because it gave a final concentration of 1 pM in the reaction mixture. This low tissue factor concentration was suggested by Hemker and Beguin (2000) for TGA in haemophilic plasma and was used frequently by others (Chantarangkul *et al*, 2003; Varadi *et al*, 2003; Beltran-Miranda *et al*, 2005; Dargaud *et al*, 2005a; Lewis *et al*, 2007; Trossaert *et al*, 2008).

It was of note that for the same plasma, compared to the TGA using the APTT reagent, the fluorescent signal curve from the TGA using TF appeared to have a longer lag phase and a lower height of plateau (Figure 3.8 A). The TGA using the APTT reagent resulted in more thrombin production than the TGA using TF in the same plasma.

The differences in the fluorescent signal curves of the normal and FVIII deficient plasma with TF of 1/500 dilutions are also reflected in the corresponding derivative curves (Figure 3.8 B).

3.3.1.3.2 Effect of FVIII concentration

To examine whether variable levels of FVIII will have an effect on the thrombin generation in plasma when triggered by TF, a normal plasma and its 1/2, 1/4, and 1/8 dilutions prepared in a commercially available FVIII deficient plasma, were tested by TGA using TF. The results are shown in Figure 3.9 (A and B).

A lag phase of approximately 600 sec was followed by steadily increasing fluorescence (Figure 3.9 A). There was a good relationship between the FVIII level and the fluorescent signal: lower FVIII concentration (higher dilutions of normal plasma) was associated with reduced fluorescent signal.

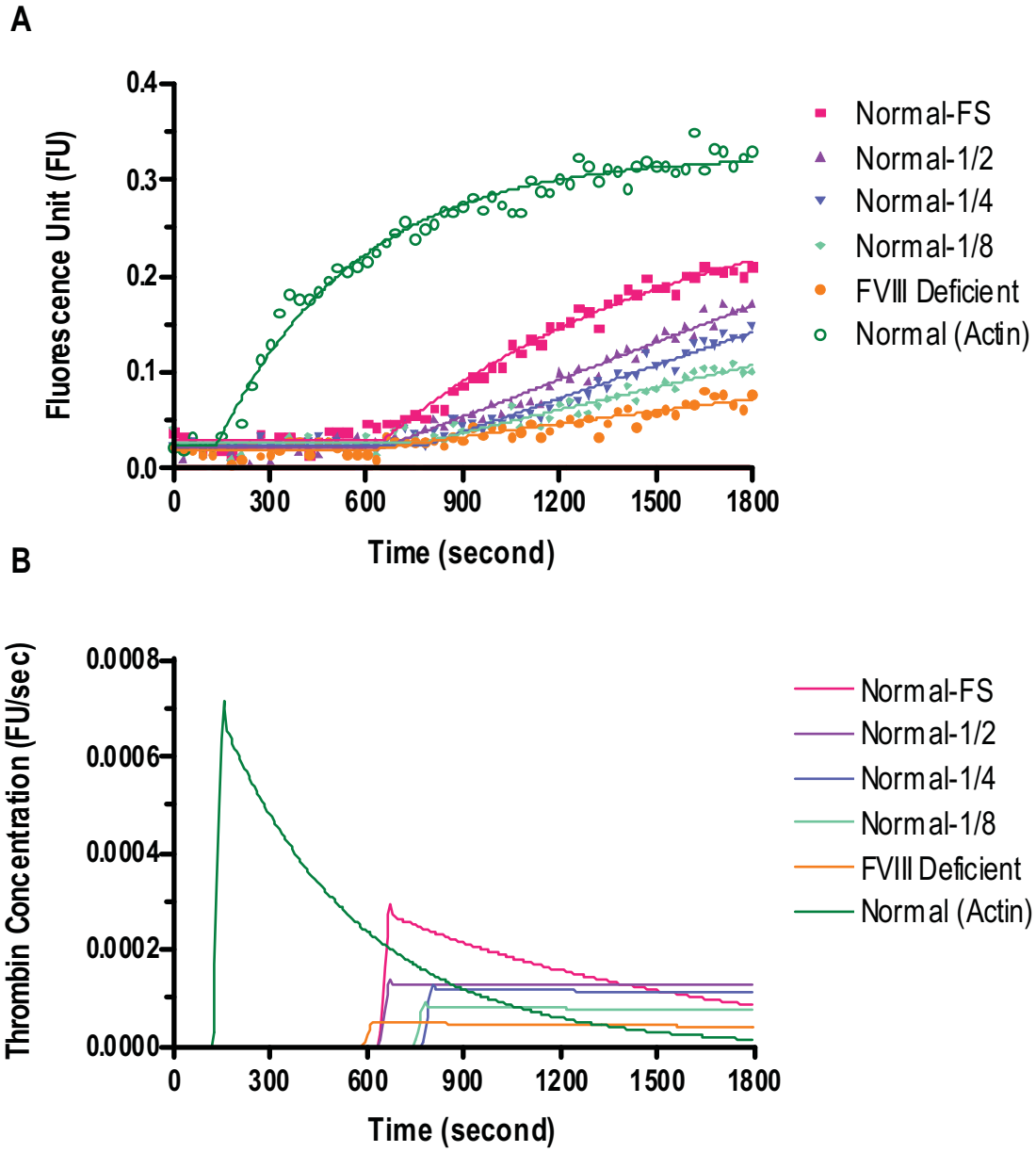


FIGURE 3.9 TGA Using TF as Trigger – Effect of FVIII Concentration

A. Fluorescent signal curves generated using TF 1/500 dilution in the absence of additional phospholipids, in a normal plasma (FVIII 101 IU/dl by one-stage FVIII assay) and its dilutions (1/2, 1/4, and 1/8) in commercial FVIII deficient plasma.

B. The corresponding first derivative curves of "A".

FS: Full strength.
Actin: TGA using the APTT reagent as a comparison.

However, the lag time appeared to be quite similar in the normal and its dilutions, as well as the FVIII deficient concentrate (Figure 3.9 A).

The parallel changes can be seen in the derivative curves (Figure 3.9 B): there appeared to be a relationship between the FVIII level and the peak thrombin concentration or AUC. Both the peak thrombin concentration and AUC showed a tendency to decrease when FVIII level decreases.

3.3.1.3.3 Addition of phospholipids

3.3.1.3.3.1 Effect of additional phospholipids

It has been suggested that when using low TF concentrations in the TGA, it was necessary to add additional phospholipids (especially for PPP) so that the phospholipids in the reaction did not become rate-limiting for the thrombin production (Hemker & Beguin, 2000). The next few experiments investigated the effect of including additional phospholipids in the TGA triggered by TF.

The addition of phospholipids (Diagen, in full strength) shortened the lag time in the normal plasma and enhanced the height of the subsequent increase in fluorescent signal (Figure 3.10 A). With the addition of phospholipids, the plasma from a patient with severe haemophilia A displayed only a very low level of thrombin generation and could be easily distinguished from the normal plasma (Figure 3.10 A).

Therefore we chose to use additional phospholipids in subsequent experiments. This is in accordance with the current practice in the use of TGA to assess FVIII deficiency in PPP (Varadi *et al*, 2003; Chantarangkul *et al*, 2004; Beltran-Miranda *et al*, 2005; Gerotziafas *et al*, 2005; Hemker *et al*, 2003; Collins *et al*, 2006; Matsumoto *et al*, 2006), and sometimes in PRP after one freeze/thawing cycle (Trossaert *et al*, 2008).

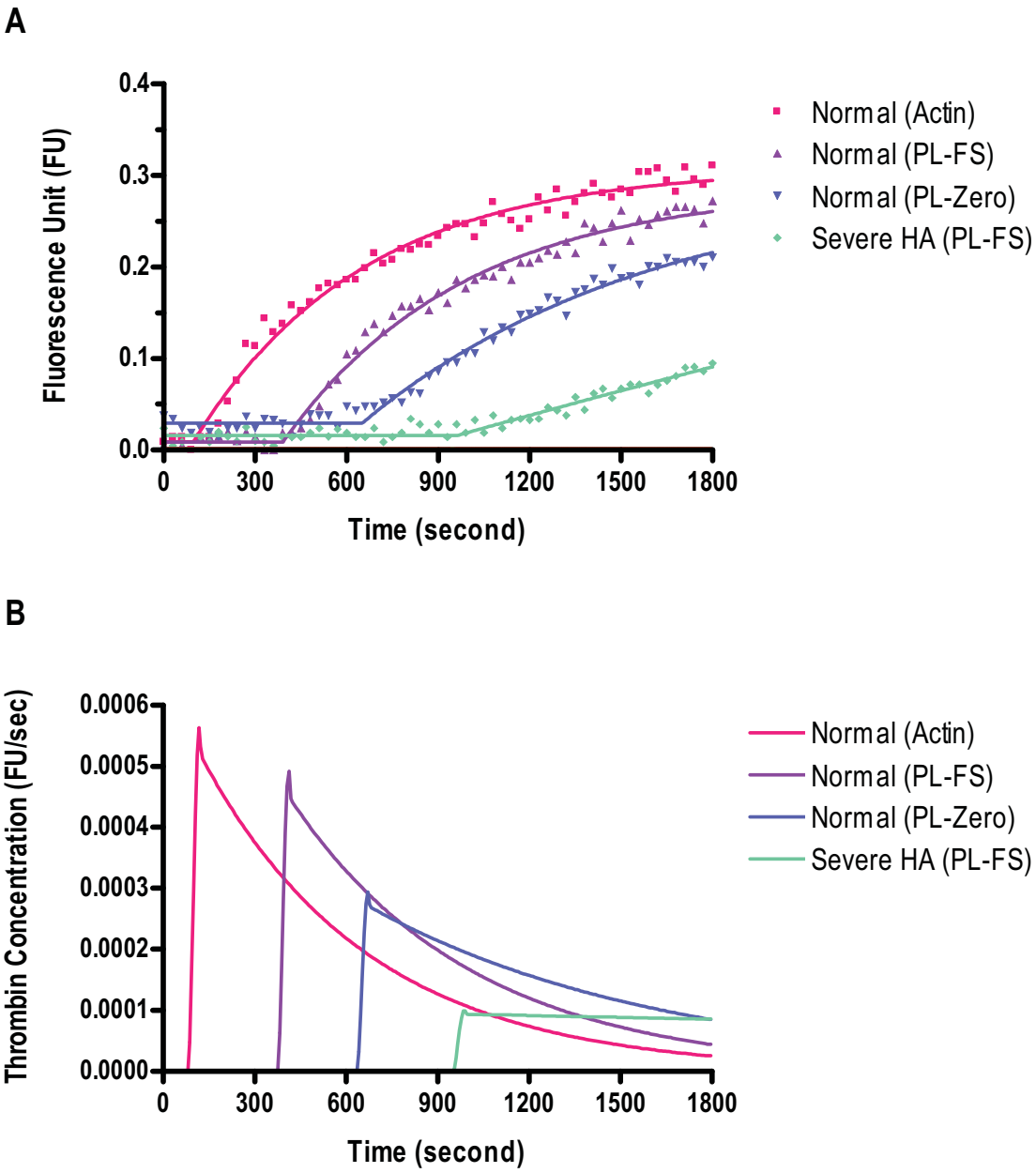


FIGURE 3.10 TGA Using TF as Trigger – Effect of Additional Phospholipid

A. Fluorescent signal curves generated using 10 µl of TF (1/500 dilution) plus 10 µl of phospholipid (full strength) in plasma from a normal subject and a patient with severe haemophilia A.

The additional phospholipid enhanced the thrombin generation in normal plasma but remained discriminatory to the plasma of severe haemophilia A.

B. The corresponding first derivative curves of "A".

Actin: TGA using the APTT reagent as a comparison.
PL: Phospholipid. Results summarised here were using Diagen phospholipid instead of the PNP product, which was used in the main study.
FS: Full strength.
HA: Haemophilia A.

3.3.1.3.3.2 Phospholipid concentration

We next examined the effect of two different concentrations of phospholipid on thrombin generation when TGA was triggered by TF. The experiments were carried out using the Diagen phospholipid reagent. The plasma samples were from a normal subject and from two patients with haemophilia A, one mild and one severe.

The results are shown in Figure 3.11 (A and B). Compared to the 1/10 dilution, phospholipid in full strength resulted in the generation of stronger fluorescent signals in normal plasma. The phospholipid, used undiluted and in a 1/10 dilution, led to a good separation of the fluorescence in the haemophilic plasma from the normal plasma (Figure 3.11).

To take advantage of the higher fluorescence strength we chose to use undiluted phospholipid for subsequent experiments in this study.

3.3.1.3.3.3 Different phospholipid reagents

We also compared two phospholipid reagents that were available in our laboratory for their performance in TGA using TF activation. Results of TGA using the two phospholipid reagents, the PNP product and Diagen platelet substitute, are summarised in Figure 3.12 (panels A, B and C).

In normal plasma, the fluorescent signal generated was the same for both PNP and Diagen (Figure 3.12 A). Similarly, in plasma from severe haemophilia A, the fluorescent signal was greatly reduced with both phospholipid reagents. Therefore there was a good separation of haemophilia from normal plasmas with either phospholipid reagent.

The intra-experimental reproducibility with PNP and Diagen phospholipids in normal plasma is shown in panels B and C of Figure 3.12 respectively. The reproducibility of TGA using PNP was perhaps slightly better than Diagen. Hence PNP was adopted for use in subsequent studies.

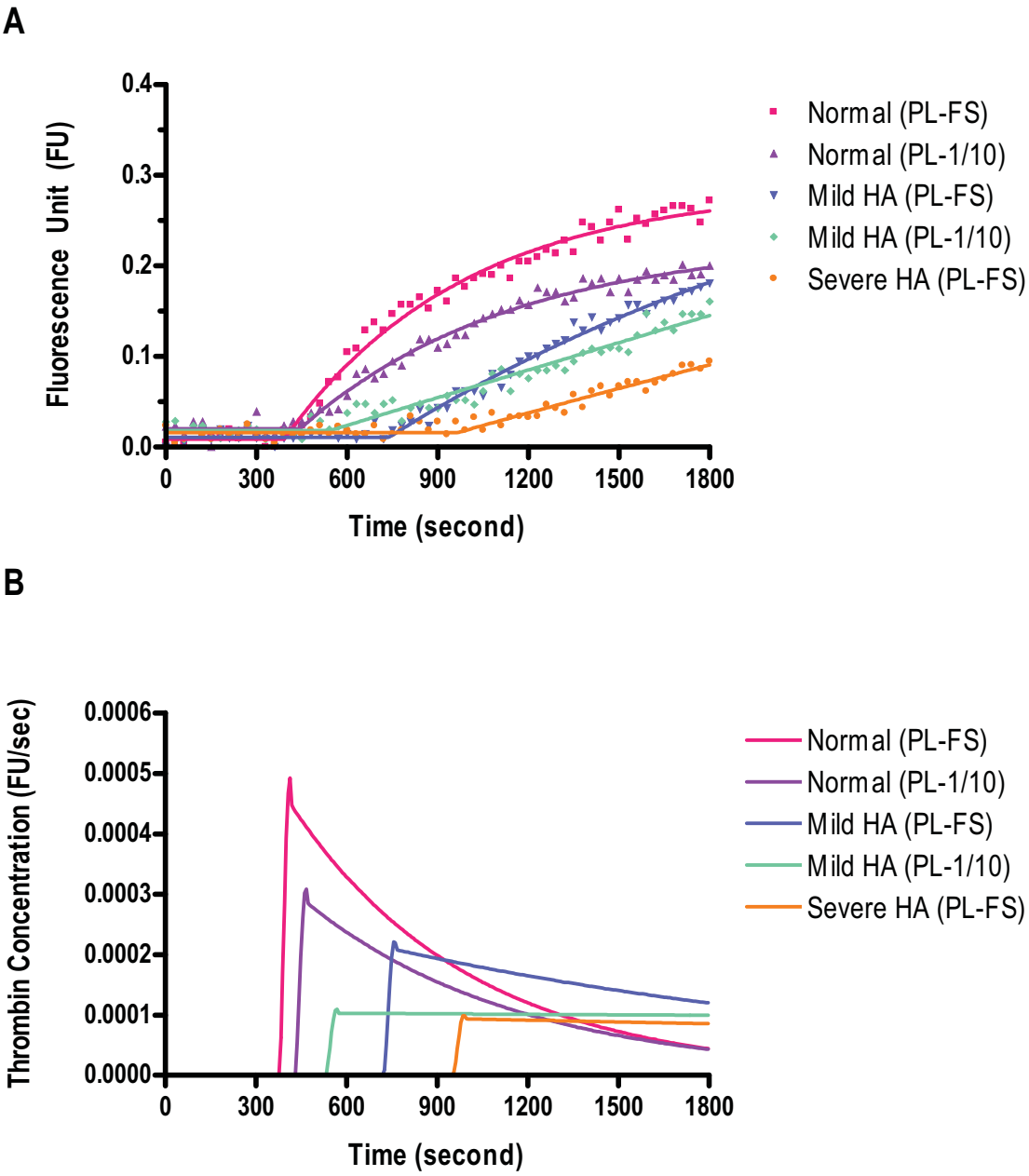


FIGURE 3.11 TGA Using TF as Trigger – Phospholipid Concentration

A. Fluorescent signal curves generated using TF (1/500 dilution), with the addition of phospholipid (FS or 1/10 dilution) in plasma from a normal subject and patients with mild and severe haemophilia A.

Phospholipid in full strength seemed to be suitable in the TGA by TF activation.

B. The corresponding first derivative curves of "A".

PL: Phospholipid. Results summarised here were using Diagen phospholipid.
FS: Full strength.
HA: Haemophilia A.

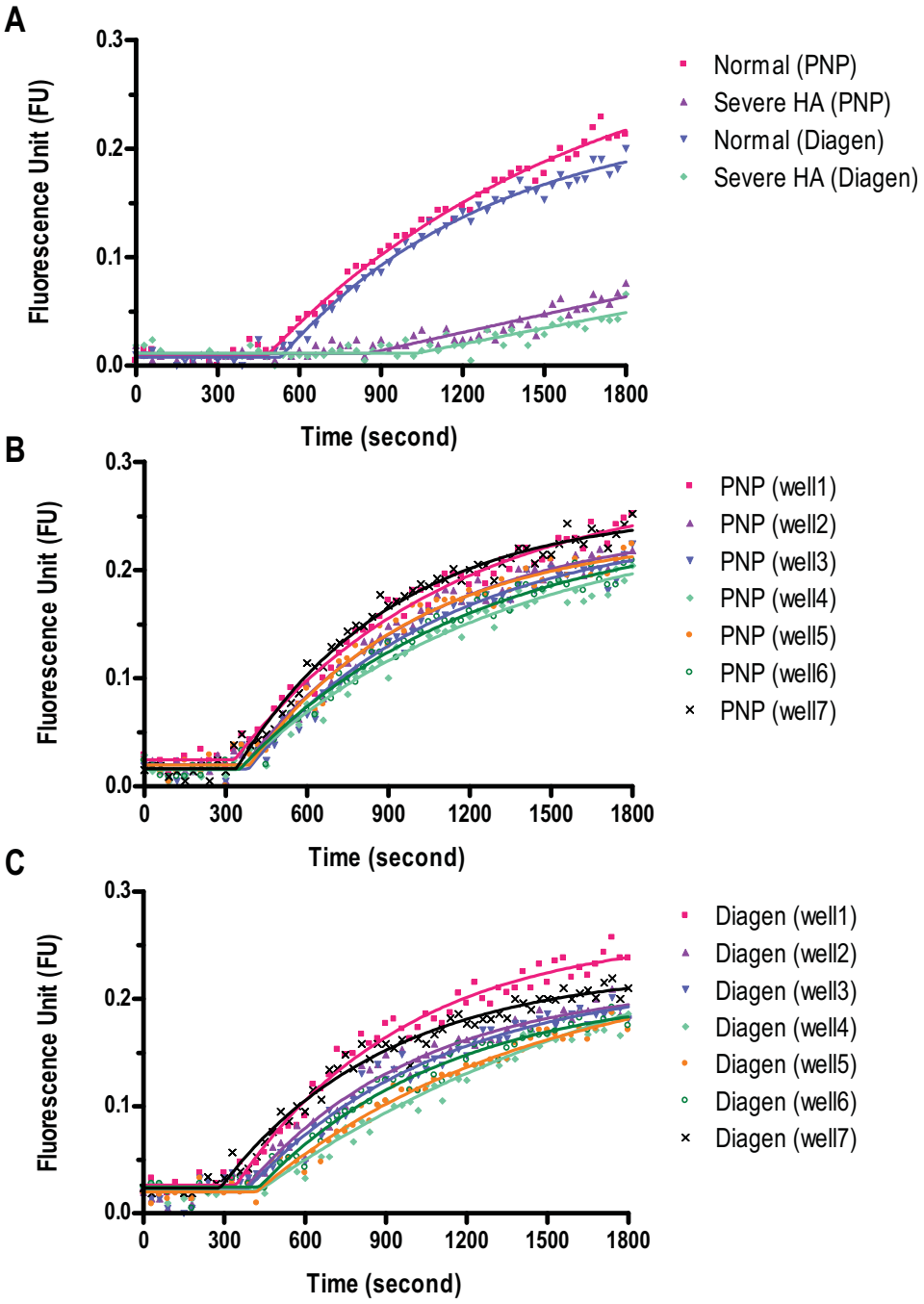


FIGURE 3.12 TGA Using TF as Trigger – Different Phospholipid Reagents

A. Fluorescent signal curves generated using TF (at 1/500 dilution) and two different phospholipid reagents (in full strength) in plasma from a normal subject and a patient with severe haemophilia A.

B. Intra-experimental reproducibility of TGA by TF activation in a normal plasma, using PNP as PL source. Seven identical samples were run in parallel on the same microtitre plate in a single experiment.

C. Intra-experimental reproducibility of TGA by TF activation in a normal plasma, using Diagen PL.

PNP: Products from "platelet neutralisation procedures", adopted for use in the main study.
Diagen: Diagen platelet substitute from Diagen Corporation.
HA: Haemophilia A.

3.3.1.3.4 Effect of different coagulant factor deficiencies

To further valid the method, in addition to the FVIII deficient plasma of haemophilia patients, we also assessed thrombin generation as triggered by TF in plasmas with hereditary FVII and FXI deficiency. The fluorescent signal curves are illustrated in Figure 3.13.

In the plasma from the normal subject, patient with mild haemophilia A and patient with severe haemophilia A, a FVIII dose-dependent pattern of thrombin generation was again observed (Figure 3.13). After the lag phase, while the normal plasma demonstrated a smooth and steady exponential increase of fluorescence, approaching a plateau at the end of experiment, the plasma from the severe haemophilia A showed only minimal level of thrombin activity. The fluorescent signal curve for the plasma from the mild haemophilia A patient fell, as expected, in the middle. Similar to the previous experiment (Figure 3.9), the lag time all seemed to fall in a similar range in these plasmas with different FVIII levels (Figure 3.13).

The plasma from the patient with severe FXI deficiency had a reasonable level of thrombin generation, close to that of the mild haemophilia A. FXIa is responsible for the accessory pathway to enhance FIX activation and the consequent intrinsic tenase (FIXa/FVIIIa catalyst) formation (Naito & Fujikawa, 1991; Butenas *et al*, 2003) (Figure 1.1). Its deficiency will not lead to a defect in thrombin generation unless the reaction is initiated by "extremely" low concentrations of TF (Cawthern *et al*, 1998). The mildly suppressed fluorescence in the plasma with severe FXI deficiency in our study is in agreement with this observation.

The plasma from the patient with severe FVII deficiency offered a negative control in this experiment by showing no thrombin activity. This was simply because FVII is essential to form TF/FVIIa complex to initiate the blood coagulation.

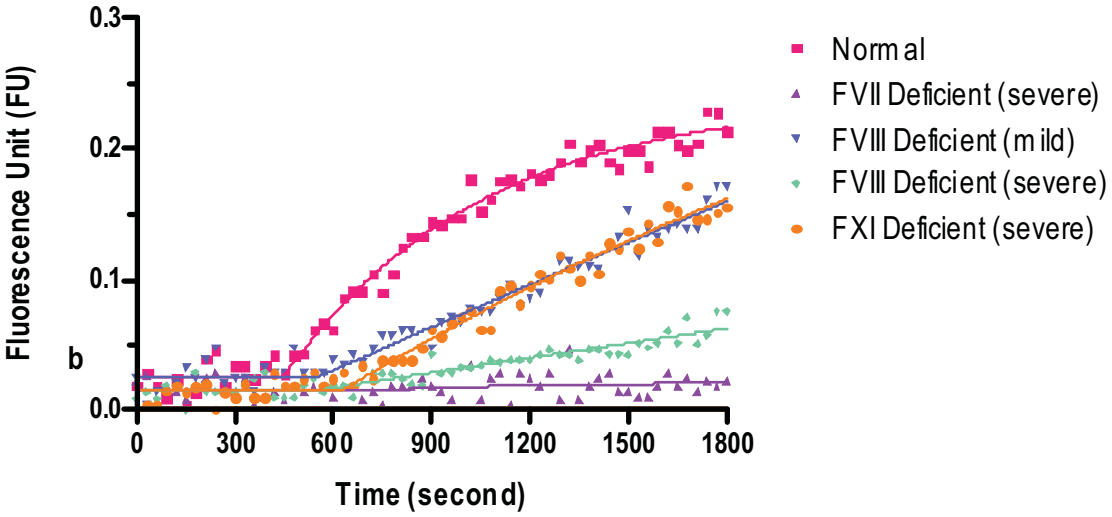


FIGURE 3.13 TGA Using TF as Trigger – Effect of Different Coagulant Factor Deficiencies

The fluorescent signal curves using TF activation system, with additional phospholipid ("PNP") in plasma from:

a normal subject;
a patient with severe FVII deficiency;
a patient with mild haemophilia A;
a patient with severe haemophilia A;
and a patient with severe FXI deficiency.

Thus far we have demonstrated that the TGA method using TF as a trigger was suitable to detect coagulation deficiency in plasmas with FVIII deficiency. The actual pattern of abnormal thrombin generation in the haemophilia plasmas when TF was used remains to be examined. It is anticipations that this may be different to the thrombin generation pattern seen in the TGA using the APTT reagent due to different activation mechanisms.

3.3.1.3.5 Reproducibility

To examine the reproducibility of the TGA method using TF, both intra- and inter-experimental variations of the assay were examined in normal plasma and plasma from a patient with severe haemophilia A.

Figure 3.14 panel A illustrates the inter-experimental variations of the assay on a normal plasma from three tests on separate days. The intra-experimental variations with the same normal sample are previously shown in Figure 3.12 panel B (Section 3.3.1.3.3.3). Both the intra- and inter-experimental variations of the TGA using TF in the normal plasma were acceptable.

For the plasma sample with severe FVIII deficiency (from patient with severe haemophilia A), the intra- and inter-experimental variations are shown in panels B of Figure 3.14. The tests were carried out in two experiments on same day. We considered the variations were acceptable.

In summary, the reproducibility of the TGA method using TF activation was reasonable. This was similar to the results on the reproducibility of the TGA method using the APTT reagent (Figure 3.7).

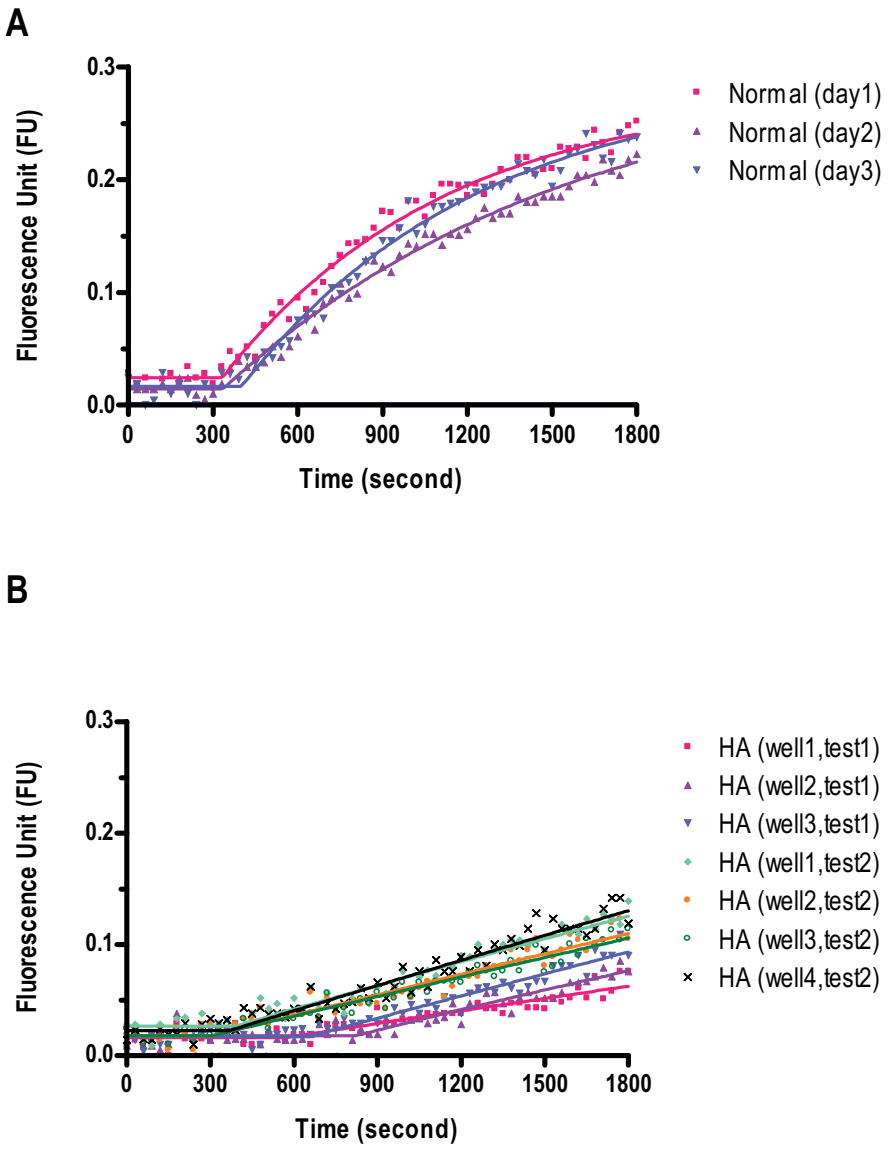


FIGURE 3.14 TGA Using TF as Trigger – Reproducibility

A. The inter-experimental variations of the TGA using TF as a trigger, with added phospholipid ("PNP"), in a normal plasma were tested on three separate days. The intra-experimental variations in the same plasma are previously shown in Figure 3.12 panel b.

B. The intra- and inter-experimental variations of the assay in plasma from a patient with severe haemophilia A; identical samples were run in adjacent wells in two experiments on same day.

HA: Severe haemophilia A.

3.3.2 Part II: Application of TGA using both different triggers in normal subjects and patients with mild/moderate haemophilia A

3.3.2.1 TGA in 18 normal subjects and 42 patients with mild/moderate haemophilia A

To examine the thrombin production in response to different FVIII levels as triggered via either the classical intrinsic pathway or the TF pathway, we applied both TGA methods using the APTT reagent and TF separately to 18 normal subject and 42 patients with mild and moderate haemophilia A. The four parameters from the derivative curve, namely, the lag time, peak time, peak thrombin concentration and AUC, were analysed (Section 3.2.2.1, Figure 1.4).

3.3.2.1.1 TGA using APTT reagent as a trigger

3.3.2.1.1.1 Comparison of normal and haemophilia plasma

The results of the four parameters of TGA using APTT reagent in both normal subjects and mild/moderate haemophilia A are summarised in Table 3.1.

Compared to the normal group, patients with mild/moderate haemophilia A had a markedly prolonged lag time and peak time ($P < 0.0001$). On the other hand, the peak thrombin concentration showed no difference in the two groups ($P = 0.97$) (Table 3.1). The AUC, though by direct comparison was only marginally greater in the normal subjects compared to the haemophilia patients, showed statistical significance of the analysis ($P = 0.01$).

It was of note that the prolonged lag time in TGA was consistent with the prolonged APTT in the haemophilia patients. The APTT was 33 ± 5 sec (mean ± SD) for the 18 normal subjects and 43 ± 7 sec for the 42 patients with mild/moderate haemophilia A, respectively. There was a positive correlation between the APTT and the lag time in these 60 samples (Spearman correlation coefficient $R = 0.76$, data not shown).

TABLE 3.1 Mean and Median Values of Parameters of TGA Using APTT Reagent in Normal Subjects and Patients with Mild/Moderate Haemophilia A

	TGA Using Activation by APTT Reagent		T Test*	
	Normal (N = 18)	Haemophilia A (mild/moderate) (N = 42)	t	P
Lag Time (sec)				
Minimum	6	16		
Maximum	168	443		
Mean ± SD	**71 ± 45**	**179 ± 97**	**4.5**	**< 0.0001**
Median	**67**	**170**		
Peak Time (sec)				
Minimum	54	79		
Maximum	236	514		
Mean ± SD	**135 ± 49**	**245 ± 98**	**4.5**	**< 0.0001**
Median	**133**	**236**		
Peak Thrombin Concentration (FU/sec)				
Minimum	0.00034	0.00028		
Maximum	0.00074	0.00085		
Mean ± SD	**0.00054 ± 0.00011**	**0.00054 ± 0.00016**	**0.04**	**0.97**
Median	**0.00053**	**0.00048**		
AUC				
Minimum	0.112	0.115		
Maximum	0.311	0.321		
Mean ± SD	**0.226 ± 0.04**	**0.190 ± 0.05**	**2.6**	**0.01**
Median	**0.233**	**0.201**		

*: Unpaired t-test of each parameter between the normal and haemophilia groups. Similar results were obtained using non-parametric t-test (data not shown).

3.3.2.1.1.2. Relationship to one-stage FVIII level

It was of interest to compare the results of the TGA using the APTT reagent to that of the conventional one-stage FVIII assay. The lag time, peak time, peak thrombin concentration or AUC (Y axis) is plotted separately against the one-stage FVIII level (X axis) for the normal subjects and patients with mild/moderate FVIII deficiency (Figures 3.15 to 3.17).

The relationship between the lag time and FVIII is illustrated in Figure 3.15 panel A. As shown in the X axis, for the 18 normal subjects and the 42 patients, the ranges of the one-stage FVIII level were 53-248 IU/dl and 4-71 IU/dl respectively.

There was a trend for the lag time to increase when the FVIII level decreases (Figure 3.15 A). This was confirmed by the analysis of Spearman correlation: there was a significant negative correlation between the lag time and the one-stage FVIII level in the 60 samples ($R = -0.72$, $P < 0.0001$).

When transforming the FVIII level to a logarithmic scale, there was a linear relationship to the lag time with a narrow 95% confidence intervals (CI) (Figure 3.15 panel B).

The relationship between the peak time and the FVIII is shown in Figure 3.16 panel A. Similar to the lag time, the peak time also demonstrated a trend to increase when the FVIII level decreases. This was also confirmed by a statistically significant negative correlation ($R = -0.72$, $P < 0.0001$) between the peak time and the one-stage FVIII in the 60 samples. Similar to the lag time, the peak time also had a linear relationship to the one-stage FVIII on a logarithmic scale (Figure 3.16 B).

Subsequently we examined the relationship between the peak thrombin concentration and AUC and the one-stage FVIII level (Figure 3.17 A and B). Unlike the lag time and peak time, the peak thrombin concentration failed to show any correlation with the one-stage FVIII ($P = 0.12$) (Figure 3.17 A). The AUC displayed only a weak positive correlation with FVIII ($R = 0.39$, $P = 0.002$) (Figure 3.17 B).

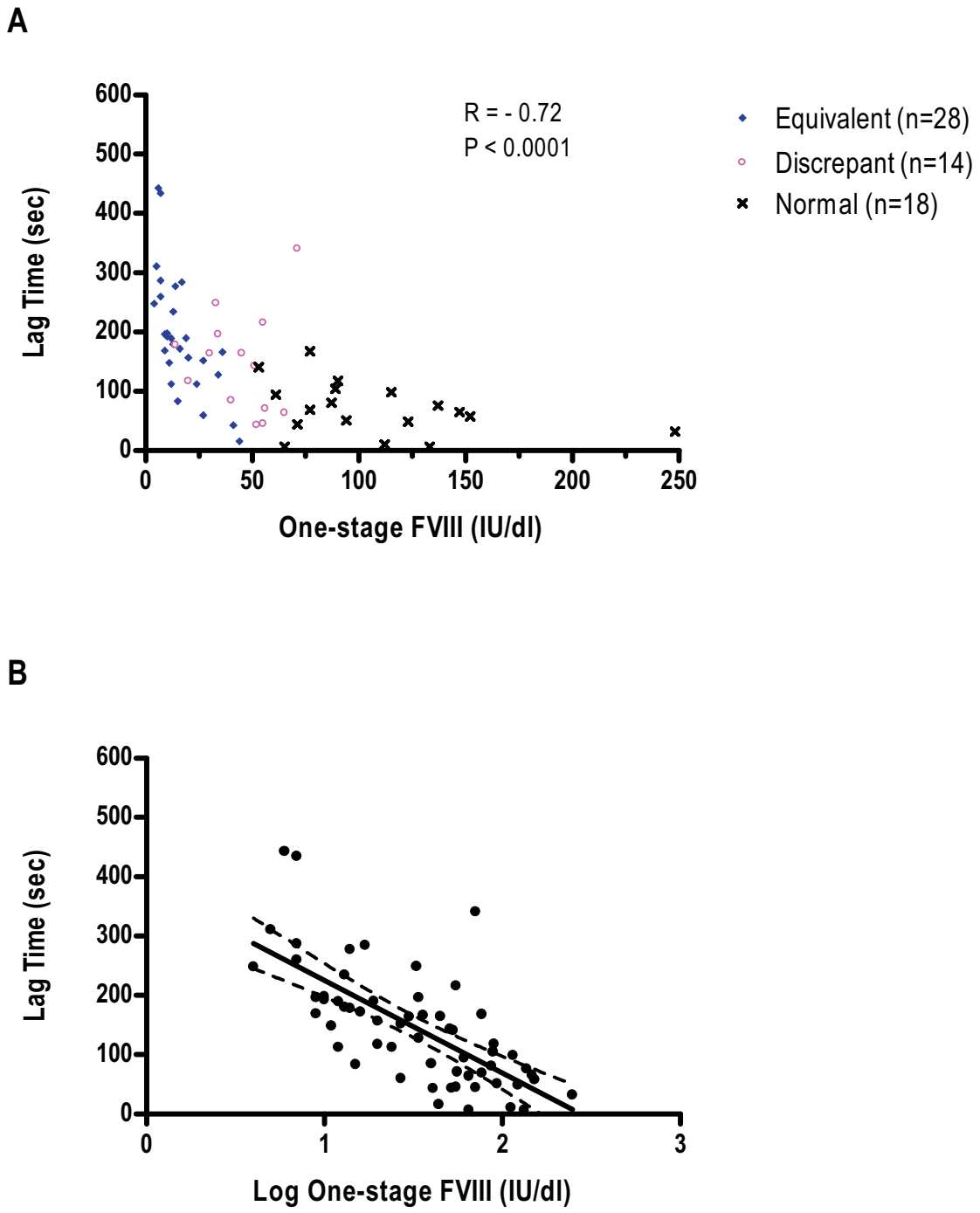


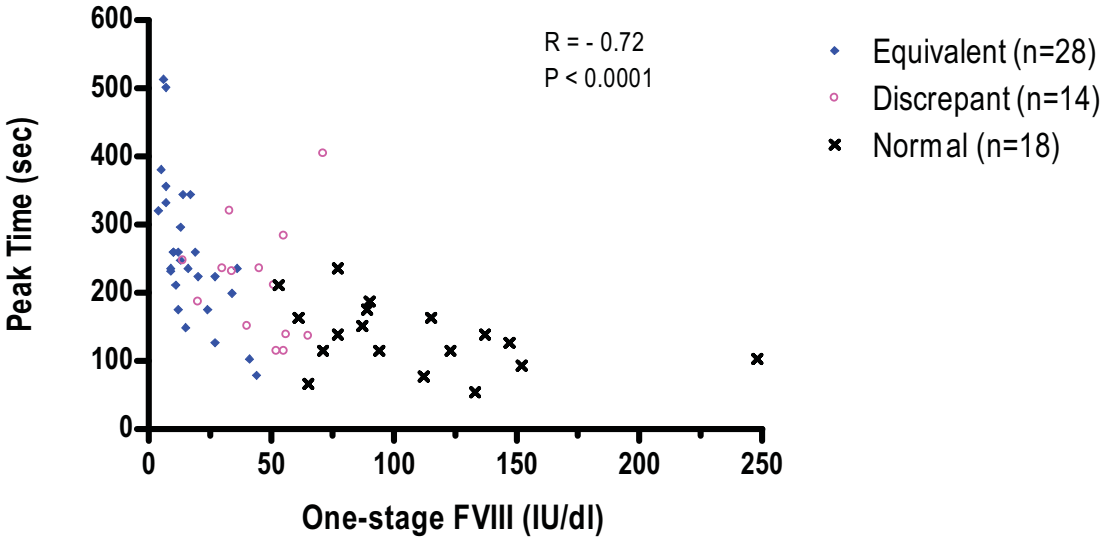
FIGURE 3.15 The Relationship between Lag Time of TGA Using APTT Reagent and One-stage FVIII Level in 60 Subjects

A. One-stage FVIII level in linear scale.

B. One-stage FVIII level in logarithmic scale.

The 60 subjects include 18 normal controls and 42 patients with mild/moderate haemophilia A.
R: Spearman correlation coefficient.
Dashed line: 95% confidence intervals for linear regression.

A



B

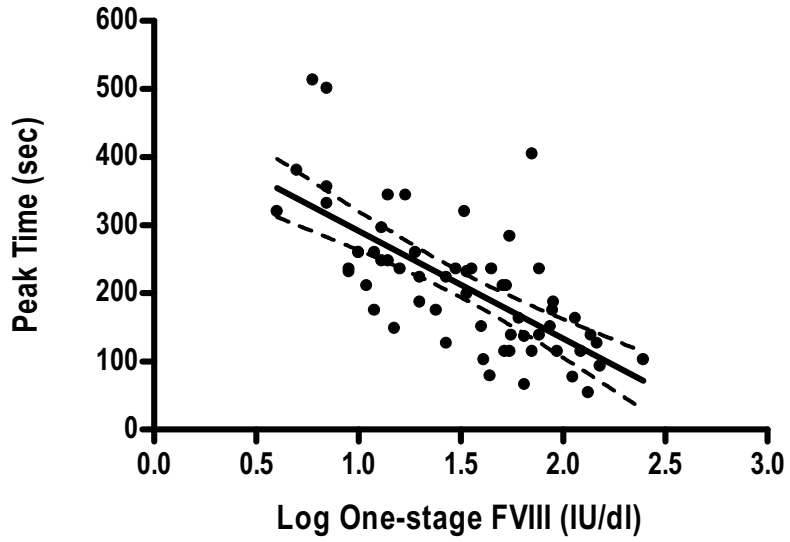


FIGURE 3.16 The Relationship between Peak Time of TGA Using APTT Reagent and One-stage FVIII Level in 60 Subjects

A. One-stage FVIII level in linear scale.

B. One-stage FVIII level in logarithmic scale.

R: Spearman correlation coefficient.
Dashed line: 95% confidence intervals for linear regression.

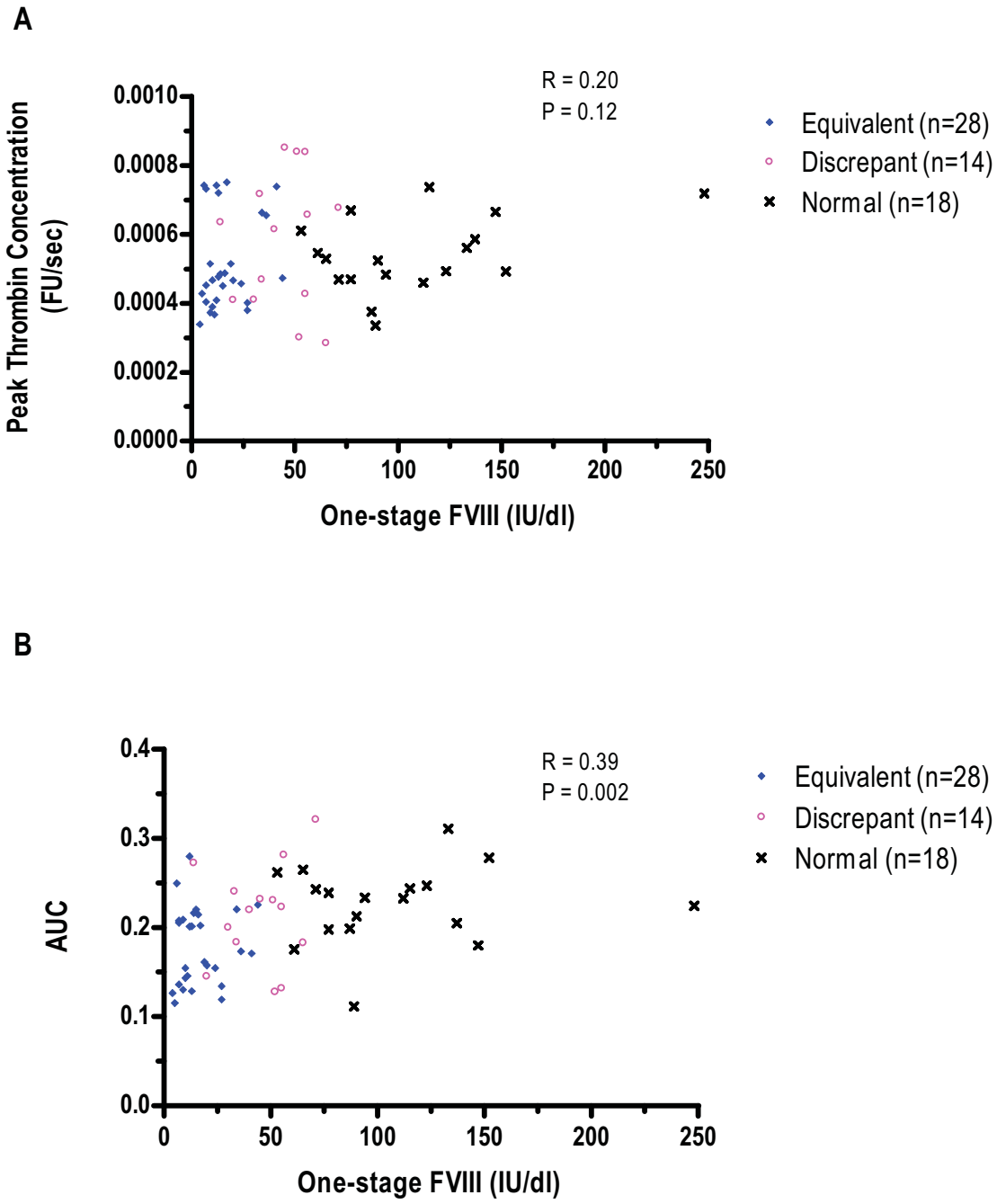


FIGURE 3.17 The Relationship between Peak Thrombin Concentration and AUC of TGA Using APTT Reagent and One-stage FVIII Level in 60 Subjects

A. Peak thrombin concentration and one-stage FVIII level:
Spearman correlation coefficient R = 0.20, P = 0.12.

B. AUC and one-stage FVIII level:
Spearman correlation coefficient R = 0.39, P = 0.002.

3.3.2.1.2 TGA using TF as a trigger

3.3.2.1.2.1 Comparison of normal and haemophilia plasma

In parallel to the TGA triggered by the APTT reagent, we applied the TGA triggered by TF in the 18 normal subjects and 42 patients with mild and moderate haemophilia A. The mean and median values of the TGA parameters are summarised in Table 3.2.

Similarly to the TGA triggered using APTT reagent, in the TGA using TF the lag time and peak time were both prolonged in the patients with mild/moderate haemophilia A compared to the normal subjects (both P = 0.001) (Table 3.2). However, the magnitude of the delay of the lag time, for example, was less substantial in the TF method than in the APTT method. The delay was by a magnitude of 0.3-fold for the TF method compared to 1.4 -fold for the APTT method, respectively.

In contrast to the lag time and peak time, the peak thrombin concentration was markedly lower (by 2.3-fold, P < 0.0001) in the patient group compared to the normal group (Table 3.2). The AUC was also reduced by 1.4-fold in the patient group (P < 0.0001).

3.3.2.1.2.2 Relationship to one-stage FVIII level

Figure 3.18 illustrates the relationship between the lag time (panel A), or the peak time (panel B), and the one-stage FVIII level in 18 normal subjects and 42 patients with mild/moderate haemophilia A. In contrast to the TGA using the APTT reagent, where both the lag time and peak time correlated reasonably well (both R = - 0.72) with the one-stage FVIII level (Figures 3.15 and 3.16), either the lag time or the peak time for in the TF method showed only a weak negative correlation with FVIII (R = - 0.39, P = 0.002) (Figure 3.18 A and B).

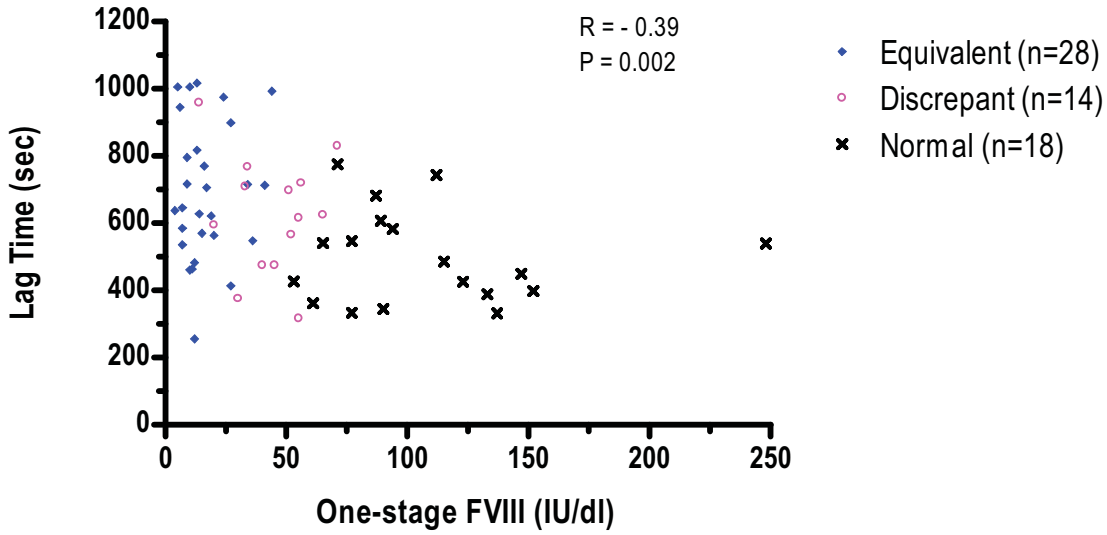
On the other hand, the peak thrombin concentration showed a quite strong positive correlation (R = 0.73, P < 0.0001) with the one-stage FVIII level in the 60 subjects (Figure 3.19 A). Logarithmic transformation

TABLE 3.2 Mean and Median Values of Parameters of TGA Using TF in Normal Subjects and Patients with Mild/Moderate Haemophilia A

	TGA Using Activation by TF		T Test*	
	Normal (N = 18)	**Haemophilia A (mild/moderate)** (N = 42)	**t**	**P**
Lag Time (sec)				
Minimum	331	255		
Maximum	775	1016		
Mean ± SD	**498 ± 139**	**671 ± 197**	**3.4**	**0.001**
Median	**468**	**642**		
Peak Time (sec)				
Minimum	364	292		
Maximum	890	1051		
Mean ± SD	**533 ± 139**	**706 ± 196**	**3.4**	**0.001**
Median	**503**	**677**		
Peak Thrombin Concentration (FU/sec)				
Minimum	0.00013	0.000010		
Maximum	0.00039	0.00015		
Mean ± SD	**0.00026 ± 0.00007**	**0.00008 ± 0.00004**	**13**	**< 0.0001**
Median	**0.00025**	**0.00007**		
AUC				
Minimum	0.119	0.005		
Maximum	0.246	0.145		
Mean ± SD	**0.179 ± 0.03**	**0.075 ± 0.04**	**10**	**< 0.0001**
Median	**0.179**	**0.060**		

*: Unpaired t-test of each parameter between the normal and haemophilia groups. Similar results were obtained using non-parametric t-test (data not shown).

A



B

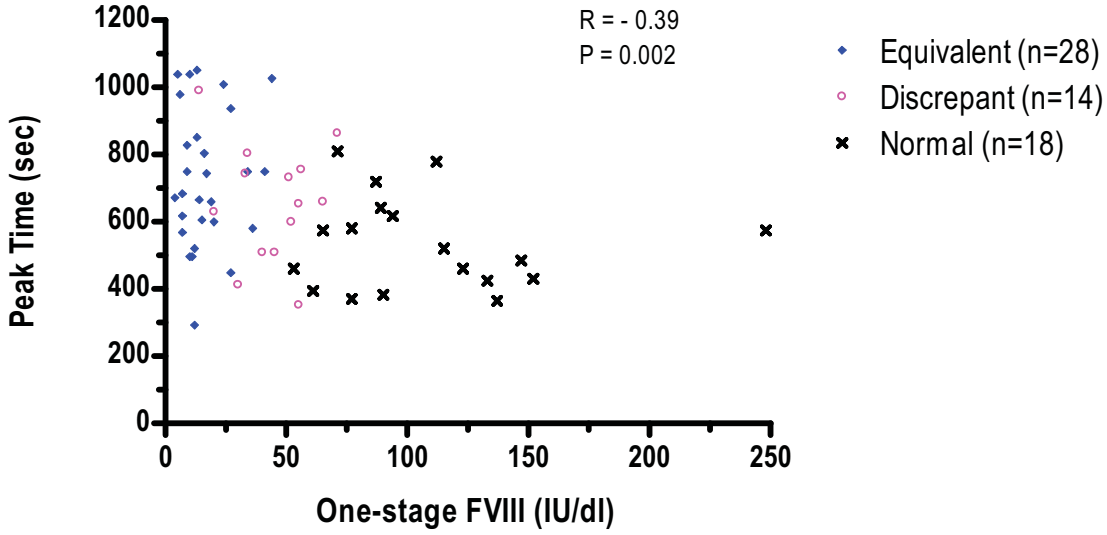


FIGURE 3.18 The Relationship between Lag Time and Peak Time of TGA Using TF and One-stage FVIII Level in 60 Subjects

A. Lag time and one-stage FVIII level:
Spearman correlation coefficient R = - 0.39, P = 0.002.

B. Peak time and one-stage FVIII level:
Spearman correlation coefficient R = - 0.39, P = 0.002.

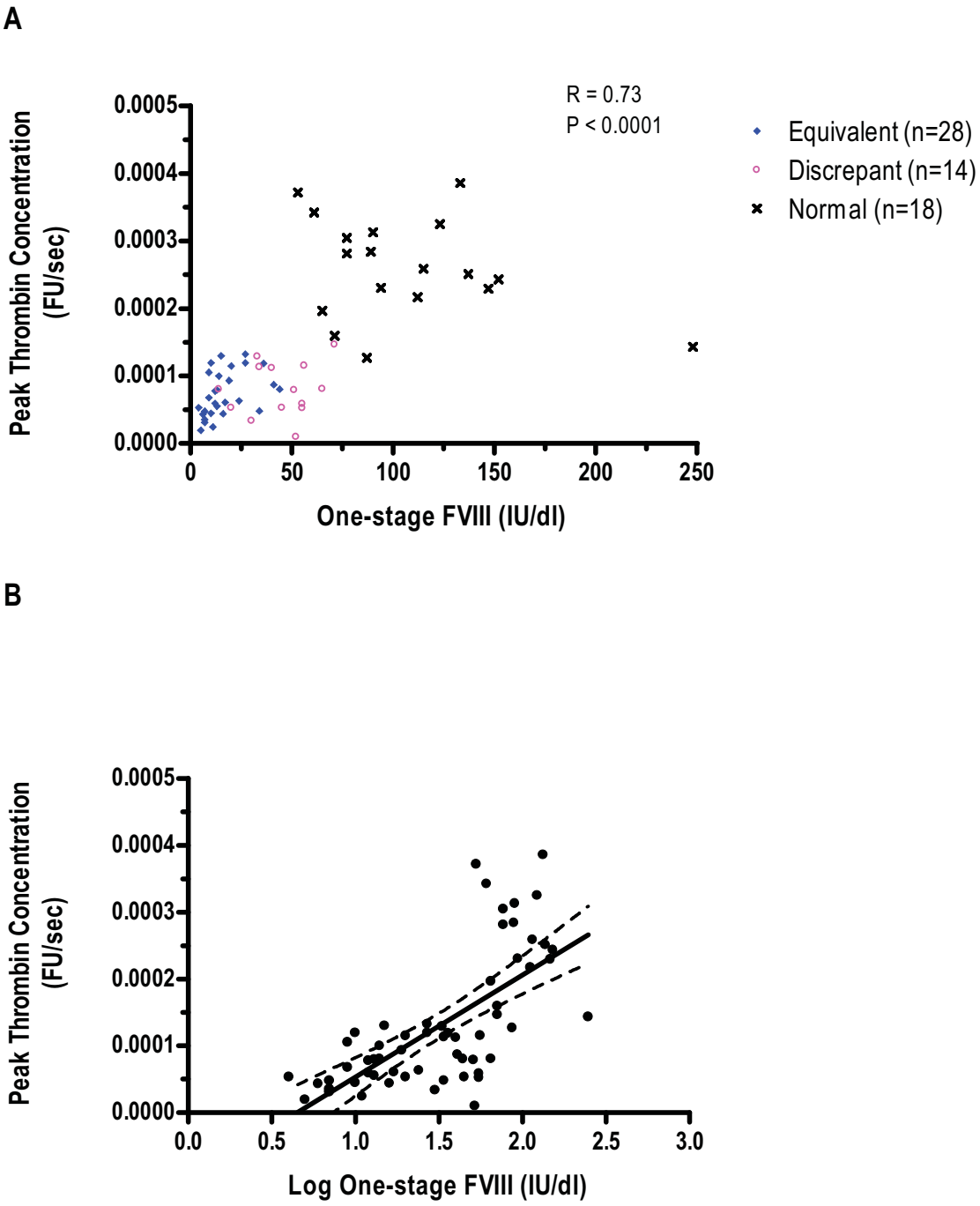


FIGURE 3.19 The Relationship between Peak Thrombin Concentration of TGA Using TF and One-stage FVIII Level in 60 Subjects

A. One-stage FVIII level in linear scale.

B. One-stage FVIII level in logarithmic scale.

R: Spearman correlation coefficient.
Dashed line: 95% confidence intervals for linear regression.

of the FVIII level revealed a linear pattern of FVIII and the peak thrombin concentration, with narrow 95% confidence intervals (Figure 3.19 B).

Similarly, the AUC was positively correlated to the one-stage FVIII level with Spearman correlation coefficient of 0.71 ($P < 0.0001$) (Figure 3.20 A). The logarithmic transformation of the FVIII data also returned a linear correlation with the AUC (Figure 3.20 B).

The analysis of the relationship between the TGA parameters from the two methods (using either the APTT reagent or TF as a trigger) and the one-stage FVIII in the 60 subjects are summarised in Table 3.3.

It is of interest that all parameters showed a correlation with one-stage FVIII level except for the peak thrombin concentration when the APTT reagent was used. The most marked effects of FVIII level were on the lag and peak time of the TGA using the APTT reagent, and the peak thrombin concentration and AUC of the TGA using TF. These were reflected in the relatively strong correlations of these parameters to the one-stage FVIII level (Table 3.3).

3.3.2.2 TGA in 42 patients with mild/moderate haemophilia A: Results compared to FVIII level by one- or two-stage FVIII assay

In this section of the results, the TGA parameters in the 42 patients with mild and moderate haemophilia A alone but not in the normal subjects were examined. The descriptive data on the lag time, peak time, peak thrombin concentration and AUC of the TGA method triggered using either the APTT reagent or TF in the 42 patients have been reviewed in the previous Sections 3.3.2.1.1.1 (APTT reagent, Table 3.1) and 3.3.2.1.2.1 (TF, Table 3.2) respectively. Here, we examined the relationship between the TGA results and the one- and two-stage FVIII levels in these patients.

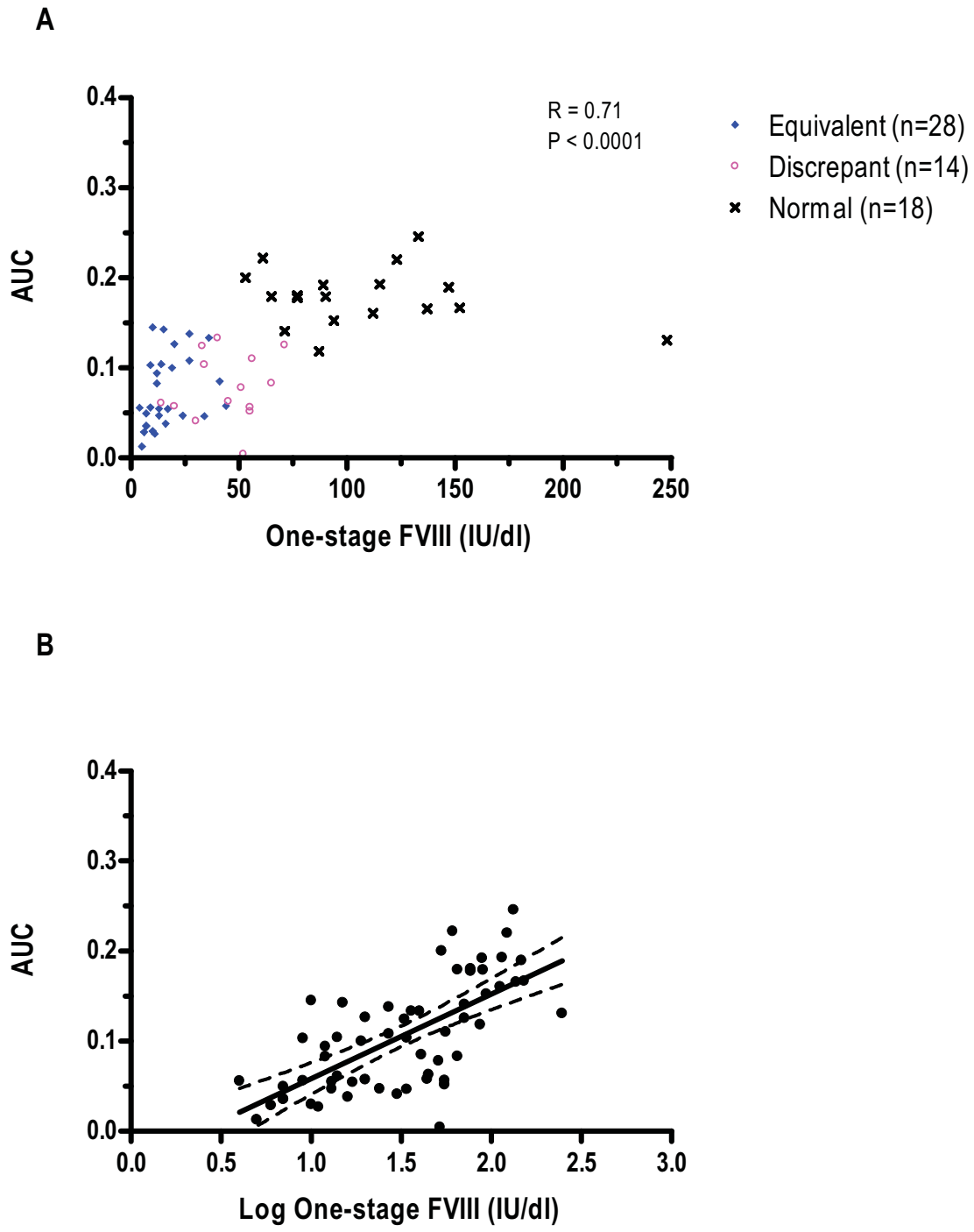


FIGURE 3.20 The Relationship between AUC of TGA Using TF and One-stage FVIII Level in 60 Subjects

A. One-stage FVIII level in linear scale.

B. One-stage FVIII level in logarithmic scale.

R: Spearman correlation coefficient.
Dashed line: 95% confidence intervals for linear regression.

TABLE 3.3 Analysis Using Spearman Correlation for Relationship between TGA Results and One-stage FVIII Levels in 60 Subjects

	TGA Using Activation by APTT Reagent	TGA Using Activation by TF
Lag Time	**R = - 0.72**	R = - 0.39
	P < 0.0001	P = 0.002
Peak Time	**R = - 0.72**	R = - 0.39
	P < 0.0001	P = 0.002
Peak Thrombin Concentration	R = 0.20	**R = 0.73**
	P = 0.12	P < 0.0001
AUC	R= 0.39	**R= 0.71**
	P = 0.002	P < 0.0001

The 60 subjects included 18 normal subjects and 42 patients with mild/moderate haemophilia A.

R: Spearman correlation coefficient.

3.3.2.2.1 TGA using APTT reagent as a trigger

Previously we demonstrated that using the APTT reagent, the lag time and peak time showed the best level of correlation with the one-stage FVIII in the 60 normal and haemophiliac subjects (Figures 3.15 to 3.17, Table 3.3). Here we compared the correlation between the lag time (or peak time) with both the one- and two-stage FVIII levels in the 42 patients (Figure 3.21 panels A, B, C and D). In the 42 patients with mild/moderate haemophilia A, the range of the one-stage FVIII level was 4-71 IU/dl (mean 26 IU/dl and median 20 IU/dl). The range of the two-stage FVIII level was 2-33 IU/dl (mean 9 IU/dl and median 7 IU/dl).

Figure 3.21 panels A and B show that both the lag time (panel A) and peak time (panel B) had a moderate correlation with the one-stage FVIII in the 42 patients. The Spearman correlation coefficient for the lag time and peak time were - 0.57 and - 0.56 respectively.

Figure 3.21 panels C and D demonstrate the results when the two-stage FVIII level, instead of the one-stage FVIII, was plotted on the X axis. Firstly there was clearly a shift of the *discrepant* patients (open pink circle) from the right to the left side of the X axis (compared to panels A and B of Figure 3.21). This was consistent with that in these patients, the two-stage FVIII level was at least more than 2-fold less than the one-stage FVIII level. It was of note that in the *equivalent* patients (blue diamond), there was also some drift of the two-stage FVIII level towards the left side of the X axis.

Secondly the correlation of the two-stage FVIII to the lag time (Figure 3.21 panel C) and peak time (panel D) were moderate: Spearman correlation coefficient – 0.52 and – 0.51 respectively. These results were not significantly different from those of the one-stage FVIII level (Figures 3.21 panels A and B).

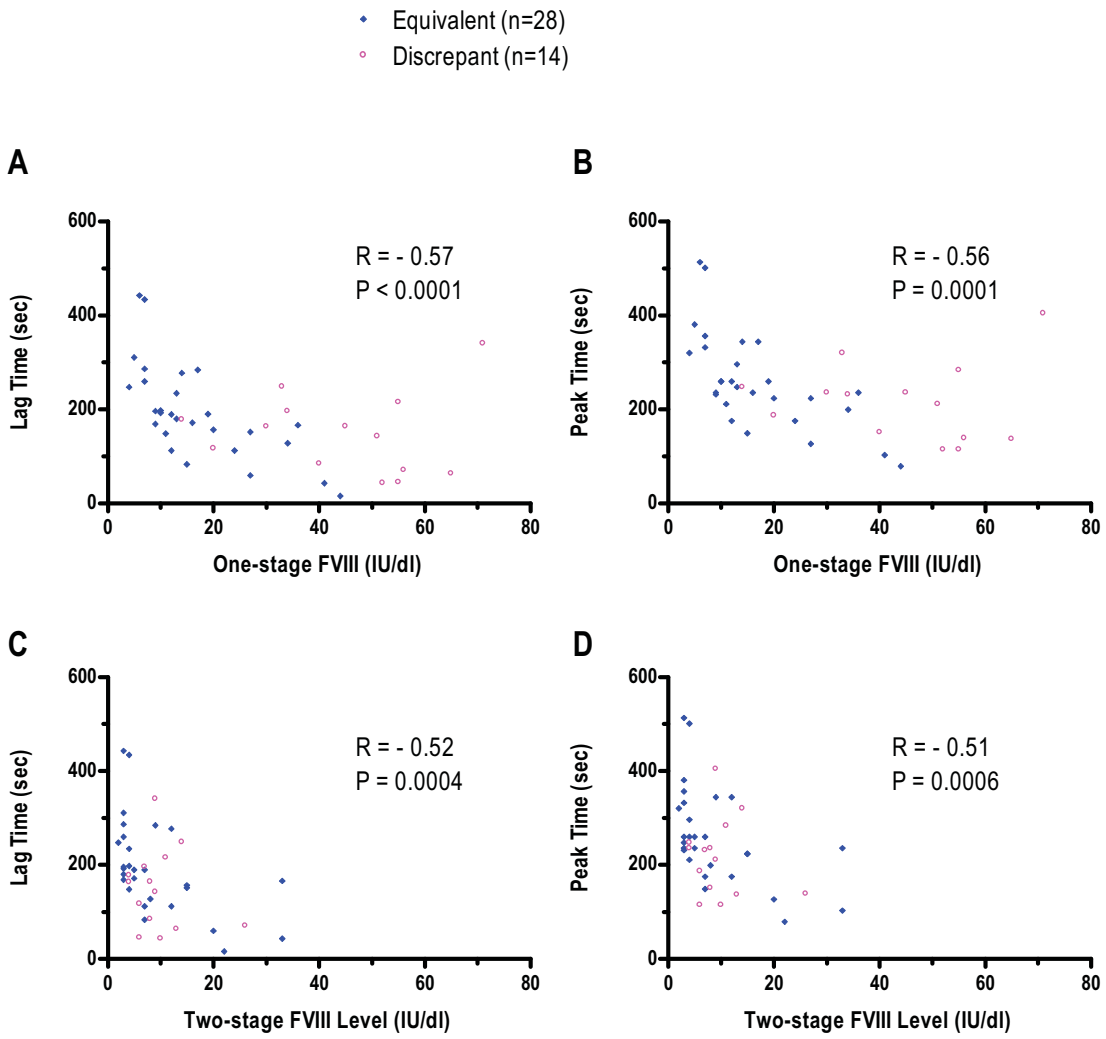


FIGURE 3.21 The Relationship between Lag Time and Peak Time of TGA Using APTT Reagent and FVIII Levels Determined by One- or Two-stage FVIII Assays in 42 Patients with Mild/Moderate Haemophilia A

A: Lag time and one-stage FVIII level in 42 patients, 24 of which had equivalent results by one- and two-stage FVIII assays ("*equivalent*" patients) and 18 had discrepant results ("*discrepant*" patients).

B: Peak time and one-stage FVIII level in the 42 patients.

C: Lag time and two-stage FVIII level.

D: Peak time and two-stage FVIII level.

R: Spearman correlation coefficient.

The results of analysis using Spearman correlation on the relationship between the TGA parameters and the one- or two-stage FVIII levels in the 42 patients are also summarised in Table 3.4. Using the APTT reagent (Table 3.4 left columns), in contrast to the lag and peak time, the peak thrombin concentration and AUC did not show any significant correlation with either the one- or two-stage FVIII levels in the 42 haemophilic patients.

3.3.2.2.2 TGA using TF as a trigger

Using TF activation, the peak thrombin and AUC of the TGA displayed the best level of correlation with the one-stage FVIII level in the 60 samples (Figures 3.18 to 3.20, Table 3.3). In the 42 patients with mild/moderate haemophilia A, the peak thrombin concentration had a significant but weak correlation (R = 0.36) with the one-stage FVIII (Figure 3.22 panel A).

Compared to the one-stage FVIII data, the two-stage FVIII results had a slightly stronger correlation (R = 0.51) with the peak thrombin concentration in 42 patients (Figure 3.22 panel C).

The results of the AUC for their correlation with the FVIII levels in the 42 patients were quite similar to that of the peak thrombin concentration. The AUC had a weak correlation with the one-stage FVIII (R = 0.35) (Figure 3.22 panel B) and the correlation was slightly stronger for the two-stage FVIII (R = 0.48) (Figure 3.22 panel D).

In contrast, the lag time and peak of the TGA using TF did not form any significant correlation to either the one- or two-stage FVIII level in the 42 patients (Table 3.4 right columns).

To sum up, for the TGA using activation by the APTT reagent, the lag time and peak time both had a moderate correlation with either the one- or two-stage FVIII assay. For the TGA activated using TF, the peak thrombin concentration and AUC correlated slightly better with the two-stage FVIII than the one-stage FVIII, although the correlations were only weak to moderate (Table 3.4).

TABLE 3.4 Analysis Using Spearman Correlation for Relationship between TGA Results and One- or Two-stage FVIII Levels in Patients with Mild/Moderate Haemophilia A

	TGA Using Activation by APTT Reagent		TGA Using Activation by TF	
	Correlation to One-stage FVIII	**Correlation to Two-stage FVIII**	**Correlation to One-stage FVIII**	**Correlation to Two-stage FVIII**
Lag Time	**R = - 0.57**	**R = - 0.52**	R = - 0.12	R = - 0.10
	P < 0.0001	P = 0.0004	P = 0.45	P = 0.51
Peak Time	**R = - 0.56**	**R = - 0.51**	R = - 0.12	R = - 0.10
	P = 0.0001	P = 0.0006	P = 0.46	P = 0.53
Peak Thrombin Concentration	R = 0.21	R = 0.20	**R = 0.36**	**R = 0.51**
	P = 0.17	P = 0.22	P = 0.02	P = 0.0006
AUC	R = 0.28	R = 0.22	**R = 0.35**	**R = 0.48**
	P = 0.07	P = 0.16	P = 0.02	P = 0.001

The 42 patients with mild and moderate haemophilia A comprised of 28 with equivalent FVIII results by the one- and two-stage FVIII assays, and 14 with discrepant results by the two FVIII assays.

R: Spearman correlation coefficient.

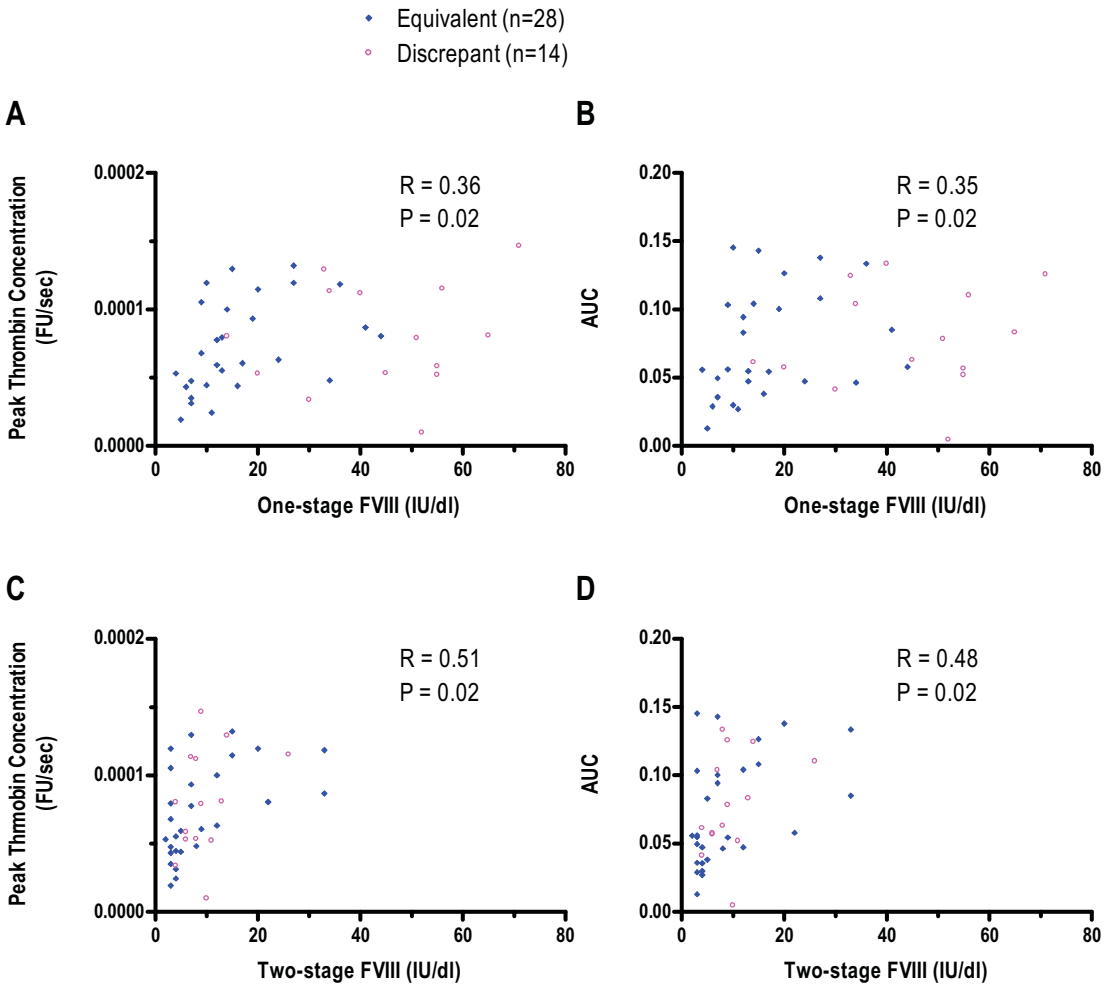


FIGURE 3.22 The Relationship between Peak Thrombin Concentration and AUC of TGA Using TF and FVIII Levels Determined by One- or Two-stage FVIII Assays in 42 Patients with Mild/Moderate Haemophilia A

A: Peak Thrombin concentration and one-stage FVIII level in 42 patients (24 *equivalent* and 18 *discrepant* patients).

B: AUC and one-stage FVIII level in the 42 patients.

C: Peak thrombin concentration and two-stage FVIII level.

D: AUC and two-stage FVIII level.

R: Spearman correlation coefficient.

3.3.2.3 TGA in 42 patients with mild and moderate haemophilia A: Results compared in the *equivalent* and *discrepant* subgroups

To further examine the results of TGA in the mild/moderate haemophilia A, the 42 patients were divided into two subgroups: the *equivalent* subgroup comprising of 24 patients with equivalent one-stage and two-stage FVIII levels, and the *discrepant* subgroup of 18 patients who had discrepant results by the two FVIII assays (one-stage FVIII 2-fold higher than the two-stage FVIII).

We examined the relationship between the TGA results and the one- and two-stage FVIII levels for the *equivalent* subgroup and the *discrepant* subgroup separately and compared them (Table 3.5).

As summarised in Table 3.5 upper rows, in the *equivalent* subgroup, there was a good correlation of the lag time (R = - 0.74) and peak time (R = - 0.70) of TGA using the APTT reagent with the one-stage FVIII level. The correlations were slightly worse if the two-stage FVIII was analysed at R = - 0.64 and - 0.60 for the lag time and peak time respectively.

Using TF as a trigger for TGA, in the *equivalent* subgroup, the peak thrombin concentration and AUC also had a significant correlation with the one- or two-stage FVIII level (R 0.44-0.58) (Table 3.5 upper rows).

In contrast to the *equivalent* subgroup, in the *discrepant* subgroup (Table 3.5 lower rows), there was no significant correlation of the FVIII levels, either by the one- or two-stage assay, and the parameters of the TGA, i.e., the lag time and peak time of TGA using APTT reagent, or the peak thrombin concentration and AUC of TGA using TF.

In the earlier analysis of the 42 patients as a whole (Table 3.4), the correlations between the TGA parameters (lag time and peak time in the APTT reagent method, peak thrombin concentration and AUC in the TF method) and the FVIII were only modest (R 0.35-0.57) (Table 3.4). This probably reflected the

TABLE 3.5 Analysis Using Spearman Correlation for Relationship between Selected Parameters of TGA and One- or Two-stage FVIII Levels in *Equivalent* and *Discrepant* Subgroups

		TGA Using Activation by APTT Reagent		TGA Using Activation by TF	
		Correlation to One-stage FVIII	Correlation to Two-stage FVIII	Correlation to One-stage FVIII	Correlation to Two-stage FVIII
Equivalent **Subgroup (N = 28)**	**Lag Time**	R = - 0.74 P < 0.0001	R = - 0.64 P = 0.0003	---	---
	Peak Time	R = - 0.70 P < 0.0001	R = - 0.60 P = 0.0007	---	---
	Peak Thrombin Concentration	---	---	R = 0.58 P = 0.002	R = 0.51 P = 0.005
	AUC	---	---	R = 0.46 P = 0.002	R = 0.44 P = 0.02
Discrepant **Subgroup (N = 14)**	**Lag Time**	R = - 0.20 **P = 0.48**	R = - 0.02 **P = 0.94**	---	---
	Peak Time	R = - 0.23 **P = 0.42**	R = - 0.04 **P = 0.88**	---	---
	Peak Thrombin Concentration	---	---	R = 0.19 **P = 0.52**	R = 0.36 **P = 0.20**
	AUC	---	---	R = 0.23 **P = 0.42**	R = 0.36 **P = 0.21**

R: Spearman correlation coefficient.

combined effect of the moderate correlation observed in the *equivalent* subgroup and the lack of significant correlation in the *discrepant* subgroup (Table 3.5).

3.4 Discussion

Prior to our study, the TGA method had not been evaluated in a cohort of haemophilia A patients with a range of FVIII levels. We therefore applied this method to a group of patients with mild/moderate haemophilia A. In addition, we compared the use of TF and APTT reagent to trigger thrombin generation in plasma.

We established a semi-automated TGA to assess coagulation deficiency in PPP using an in-house fluorometer and the fluorogenic substrate, assay methods and data analysing principles described by Hemker *et al* (2000). After initiation of thrombin generation, there was a lag phase followed by a gradual increase of fluorescence to a maximum level. A first derivative curve was then used to generate the four main parameters of the TGA, namely, the lag time, peak time, peak thrombin concentration and AUC (ETP). Using these parameters, we were able to characterise the thrombin generation profile. Using either APTT reagent or TF as a trigger, there were abnormalities of the fluorescent signal curve with plasmas from a variety of coagulation deficiencies including haemophilia A.

We applied the two TGA method using the two different triggers to a group of normal subjects (n = 18, one-stage FVIII 53-248 IU/dl) and to patients with mild/moderate haemophilia A (n = 42, one-stage FVIII 4-71 IU/dl). We observed a significant correlation between the results of TGA and the FVIII level in these 60 subjects, regardless of whether APTT reagent or TF was used as the trigger (Table 3.3). However, the actual parameters affected were quite different for the two stimuli. Using APTT reagent as a trigger, the parameters that correlated with FVIII level were the lag time and peak time. By contrast, using TF as a trigger, the parameters affected were the peak thrombin concentration and AUC.

The lag time likely reflects the clotting time for the APTT and PT, while the peak thrombin concentration and AUC presumably reflect more the thrombin produced after initial clotting. Hence the prolonged lag time with APTT reagent is consistent with the prolonged APTT in these patients (Spearman correlation coefficient R = 0.64, data not shown). On the other hand, the normal lag time with TF is consistent with the normal PT in these patients. In the future the main utility of TGA in haemophilia may be for measuring the ETP, as reflected by the AUC, rather than the lag time.

In the TGA using activation by APTT reagent, the lag time and peak time demonstrated a significant negative correlation (both R = - 0.72) with the one-stage FVIII level in the 60 subjects (Figures 3.15 and 3.16, Table 3.3). The AUC had only a weak correlation (R = 0.39), while peak thrombin concentration had no statistically significant correlation with the FVIII level.

On the other hand, using TF as a trigger, the peak thrombin concentration (R = 0.73) and AUC (R = 0.71) of TGA demonstrated a significant positive correlation with the one-stage FVIII level (Figures 3.19 and 3.20, Table 3.3). There was only a weak correlation of the lag time or peak time (both R = - 0.39) to FVIII.

It is now appreciated that under physiological conditions, after the initial TF/FVIIa-driven factor Xa formation, the bulk of thrombin production is dependent on the intrinsic FIXa- and FXIa–driven factor Xa formation (Mann *et a*l, 2003b; Monroe & Hoffman, 2006). The post-lag propagation phase was shown to be nearly independent of the initial TF/FVIIa activator concentration over a 10^3-fold range, but was dependent upon FVIII and factor IX when a low TF/FVIIa concentration (below 100 pM) was used in the *in vitro* reconstituted system (Lawson *et al*, 1994; van 't Veer & Mann, 1997).

In our study, the reduced level of FVIII caused a longer lag time and peak time of thrombin generation triggered via the intrinsic pathway. This is consistent with a delayed thrombin amplification/prolongation phase in the patients with mild/moderate haemophilia A. According to some researchers, the absence

of the amplification/prolongation phase is the principle defect observed in haemophilia A, rather than the prolonged clotting time (Cawthern *et al*, 1998; Butenas *et al*, 2002; Mann *et al*, 2003a).

It was note-worthy that the data in the literature on TGA involving activation via the classical intrinsic pathway was scant compared to that via the TF activation. One report examined TGA using FIXa as a trigger to activate reconstituted FVIII deficient plasma that was spiked with FVIII concentrate. They concluded that there was a dose-response effect of FVIII level (0.5-100 IU/dl) on the TGA parameter "time taken to half of the peak thrombin concentration", but not the peak thrombin concentration or AUC (McIntosh *et al*, 2003). This was in agreement with our findings of the TGA method using the APTT reagent.

The lack of correlation between the FVIII level and the lag time or peak time of TGA triggered by a low concentration of TF has also been observed by others in four independent studies (Chantarangkul *et al*, 2003; Beltran-Miranda *et al*, 2005; Dargaud *et al*, 2005a; Trossaert *et al*, 2008). In these four publications, the peak thrombin concentration and ETP (AUC) was shown to be the best discriminator. A significant correlation between ETP and log transformed FVIII ($r = 0.54$) was described in 39 patients with haemophilia A (FVIII 0.2-30 IU/dl), using a TGA with TF at 1pM (and input phospholipid at 0.5 μM) (Chantarangkul *et al*, 2003). In a study on 34 patients with haemophilia A (19 severe, 8 moderate and 7 mild, FVIII 0-30 IU/dl), there was a significant correlation between FVIII level and the ETP (Spearman correlation $R = 0.53$), peak thrombin concentration ($R = 0.6$) and peak time ($R = - 0.52$), but not the lag time (Dargaud *et al*, 2005a). In another study using a similar TGA method (TF of 1 pM and phospholipid of 3 μM) where 23 haemophilia A patients (12 severe, 6 moderate and 5 mild, one-stage FVIII 0-10 IU/dl) were assessed, it was found that only ETP ($r = 0.85$) and peak thrombin concentration ($r = 0.75$) correlated with FVIII (Beltran-Miranda *et al*, 2005). Using TF at 0.5 pM, Trossaert and colleagues reported that in 61 patients with mild haemophilia A (mean one-stage FVIII 14 ± 9 IU/dl), FVIII:C (by one-stage assay or chromogenic assay) was "correlated statistically" with ETP and thrombin peak, but not with "time to peak" (Trossaert *et al*, 2008). It is of note that the strength of correlation found in these

studies were similar to our findings in the current study of 60 normal subjects and haemophilia A patients (R = 0.73 for peak thrombin concentration and 0.71 for AUC).

Our TGA using two different triggers in normal and FVIII deficient plasmas provides evidence of how thrombin generation might be impaired in haemophilia A. The results showed that both the initiation and amplification/prolongation phases of thrombin generation were abnormal in haemophilia A plasma. On the one hand, FVIII deficiency in the TF pathway (initiation phase) appeared to translate to a reduced amount of thrombin produced by this pathway. On the other hand, the effect of FVIII deficiency on the contact pathway (amplification/prolongation phase) effects a delay in thrombin production. The thrombin produced in the prolongation phase is important in stabilisation of the fibrin clot, through multiple actions in addition to clotting fibrinogen (Monroe & Hoffman, 2006; Gailani & Renne, 2007). It is likely that the coagulation mechanism in haemophilia A is impaired due to the attenuated production of the thrombin required to prime the system and the subsequent delayed consolidation of the clot. The relative importance of the impairment of these two pathways *in vivo* in haemophilia is still to be addressed.

We did not examine plasma from severe haemophilia A, except during the establishment of the TGA method. The FVIII deficient plasma from one patient with severe haemophilia A (FVIII < 1 IU/dl) displayed a low level of thrombin generation in the TGA triggered by TF (Figures 3.10 to 3.13). Some have suggested that the TGA might be more sensitive than the conventional FVIII assay for the assessment of FVIII levels less than 1 IU/dl (McIntosh *et al*, 2003; Matsumoto *et al*, 2006).

The two TGA methods using different triggers had a similar sensitivity to the one-stage FVIII level in the 60 subjects. The Spearman correlation coefficient for the one-stage FVIII and parameters of the TGA method using the APTT reagent (lag time and peak time), or TF (peak thrombin concentration and AUC) were all approximately 0.70 (Table 3.3). Hence both TGA methods were equally sensitive and useful in assessing coagulation deficiency in FVIII deficient plasma.

Despite a reasonable correlation between the TGA parameters and the one-stage FVIII level, there was overlapping of the results for haemophilia patients and normal subjects (Figures 3.15 to 3.20). The relatively large spreading around the general trend was also noted by others (Dargaud *et al*, 2005a). Therefore the current TGA method, triggered either by APTT reagent or TF, was not sensitive enough to diagnose FVIII deficiency quantitatively and independently of the conventional one-stage FVIII assay. However, it is possible that it may eventually prove useful in the determination of clinical phenotype of haemophilia.

On the other hand, the overlapping between the TGA results and FVIII level implies that despite similar FVIII levels, different patients with haemophilia A may have different thrombin generation capacity. While the clotting-based one-stage FVIII assay uses the end-point of clot formation in testing plasma, the TGA theoretically detects the global thrombin production over a time course, both before and after clot formation. The "coagulation phenotype", as reflected by the TGA result, may be determined by not only the FVIII level, but also any underlying contributory pro- and anti-coagulant factors in the plasma.

There are studies suggestive that factors other than FVIII level in haemophilia patients may affect thrombin production, including platelets (Siegemund *et al*, 2003). Due to the poor correlation between the results of TGA in whole blood and FVIII activity after substitution therapy in 40 patients with haemophilia A (mild to severe), it was suggested that the haemostatic effect of FVIII was dependent not only on its activity measured in plasma but also on the interplay between coagulation factors and blood cells, including platelets (Bassus *et al*, 2006). The presence of platelets was demonstrated to enhance thrombin generation triggered by a low level of TF in mild/moderate haemophilia A, but not in the severe cases (Chantarangkul *et al*, 2003). The role of platelets in thrombin generation was also investigated in hyper-coagulability status (deep vein thrombosis, DVTs) using TGA in PPP, PRP and whole blood (Regnault *et al*, 2003; Tappenden *et al*, 2007).

It is of interest to examine the results in the 42 patients with mild/moderate haemophilia A alone, excluding the normal subjects. Using APTT reagent, the parameters of TGA (lag time and peak time) displayed a similarly moderate degree of correlation with both the one- and two-stage FVIII levels (Table 3.4 left columns). Using TF, the parameters of TGA (peak thrombin concentration and AUC) showed that the two-stage FVIII correlated slightly better than the one-stage FVIII (Table 3.4 right columns).

However, the correlations of TGA results with FVIII level in the *equivalent* and *discrepant* subgroups were clearly different (Table 3.5). It can be seen from Table 3.5 that in contrast to the *equivalent* patients, who demonstrated a statistically significant correlation of the TGA results with either one- or two-stage FVIII level, the *discrepant* patients showed no significant correlation between the TGA parameters and their FVIII levels (Table 3.5). Hence it would appear that the correlation between the TGA and FVIII in the 42 patients was mainly due to the significant correlation in the *equivalent* patients. The results suggest that even if the TGA eventually has a role in the assessment of mild haemophilia A, this will not apply to the *discrepant* subgroup.

Hemker's group used a "thrombin calibrator", which is an α_2-macroglobulin-thrombin complex (Hemker *et al*, 2003). Others used a purified thrombin to generate a reference curve in their TGA (details not revealed) (Turecek *et al*, 2003). Ideally we would have preferred to use a thrombin calibrator in the present study. However, our attempts at creating a thrombin generation curve using a purified thrombin and the thrombin calibrator donated to us by Hemker were not successful (data not shown).

Some have used a reaction temperature of 37°C in the TGA to best mimic physiological conditions (Butenas *et al*, 1999; Hemker *et al*, 2003; McIntosh *et al*, 2003; Varadi *et al*, 2003), while many others did not comment on the reaction temperature of their TGA methods (Chantarangkul *et al*, 2003; Beltran-Miranda *et al*, 2005; Dargaud *et al*, 2005a; Trossaert *et al*, 2008). Our TGA was performed at room temperature because the LS55 fluorometer was not equipped with a 37°C incubation facility. It is possible that the use of room temperature in our study had an effect on the results.

The capability of TGA to measure individual "coagulation phenotype" may assist in the study of the heterogeneity of clinical phenotype in haemophilia A, if there is evidence that the TGA parameters can predict the clinical bleeding tendency. In this study, we have demonstrated that some parameters of the TGA reflected the baseline FVIII level in patients with mild/moderate haemophilia A. However there have been no indications from the current study that the measurement of TGA is of clinical use at the present time in untreated patients. Studies of a larger patient cohort are required to determine whether the TGA will provide information to aid in determining the variations in "coagulation phenotype" exist in patients with similar FVIII levels. However, currently, the main use of TGA may prove to be in the monitoring of treatment rather than in the baseline assessment of these patients.

The results of this study raises an important question of whether studies of TGA in the future should include testing with both APTT reagent and TF. In haemophilia A, the main effect of FVIII deficiency on the TF pathway is a decrease in the amount of thrombin produced. In contrast, the main defect of FVIII deficiency on the contact activation pathway is a delay in the production of thrombin, rather than the amount of thrombin produced. It is likely that both effects are clinically and diagnostically important. Therefore it would be reasonable in future studies on haemophilia A to include testing of both pathways.

3.5 Summary

1) We established the TGA in PPP using a fluorogenic substrate and an in-house fluorometer. We used both the APTT reagent and TF as the trigger for TGA. Both TGA methods successfully discriminated well the FVIII deficient plasma from normal plasma on the fluorescent signal curve.

2) Subsequently we evaluated the generation of thrombin in plasmas from 18 normal subjects and 42 patients with mild and moderate haemophilia A using both TGA methods of different triggers.

3) We adopted the methodology for further analysis of TGA by obtaining the derivative curve from the fluorescent signal curve. The four parameters from the derivative curve, i.e., the lag time, peak time,

peak thrombin concentration and AUC, were useful in analysing data for thrombin generation. However, with different triggers, the TGA results were characterised differently by the four parameters.

4) In the TGA using APTT reagent as an activation trigger, the lag time and peak time demonstrated a significant negative correlation (both R = - 0.72) with the one-stage FVIII level in the total 60 subjects. The next useful parameter was the AUC, which had a weak correlation to FVIII (R = 0.39). The peak thrombin concentration had no correlation with the FVIII level.

5) In contradistinction to the APTT reagent, the TGA using TF activation demonstrated a significant positive correlation with the peak thrombin concentration (R = 0.73) and AUC (R = 0.71) to the one-stage FVIII level. There was only a weak correlation of the lag or peak time (both R = - 0.39) to FVIII.

6) For the 42 patients with mild/moderate haemophilia A, the overall correlation between the FVIII levels and TGA results by both triggers was moderate (R 0.35-0.57). There was some overlapping of the FVIII levels and TGA parameters. This may infer that the current TGA methods were not superior to the conventional FVIII assay in the diagnosis of haemophilia A. On the other hand, it may reflect the heterogeneity of the "coagulation phenotype" in individuals with similar FVIII level.

7) For patients with mild/moderate haemophilia A, both the one- and two-stage FVIII levels displayed similar degree of correlation to the TGA results when triggered by APTT reagent (lag and peak time). However, in response to TF activation the TGA parameters (peak thrombin concentration and AUC) appeared to have a slightly stronger correlation with the two-stage FVIII level, compared to the one-stage FVIII.

8) In contrast to the *equivalent* subgroup (n = 24), the *discrepant* subgroup (n = 14) showed no significant correlation between their TGA results and the one- or two-stage FVIII levels. Any role of the TGA in the assessment of mild haemophilia A is unlikely to apply to the discrepant subgroup.

9) In patients with haemophilia A, the combined phenomenon of a delayed lag phase in TGA using APTT reagent and a reduced peak thrombin concentration and AUC/ETP, when triggered by TF, was of clinical interest. The impaired initiation of coagulation by a reduced level of thrombin to prime the

system, plus the subsequent delay in the amplification/propagation phase of thrombin production to sustain the clot, were perhaps both responsible for haemorrhage in haemophilia A.

10) Future studies on the TGA would be directed to the use of a thrombin standard to quantify FVIII deficiency, to include larger number of patients to determine whether there is a relationship between the TGA results (“coagulation phenotype”) and the clinical severity of patients with haemophilia A (“clinical/bleeding phenotype”), and whether the TGA is more useful in monitoring FVIII therapy rather than in predicting the baseline clinical phenotype in these patients.

CHAPTER FOUR ANKLE ARTHROPATHY IN MILD AND MODERATE HAEMOPHILIA A

4.1 Introduction

Arthropathy due to recurrent haemarthrosis has been well studied in severe haemophilia A, and predominantly affects large joints such as knees, ankles, hips, elbows and shoulders. However there is only scant data on the prevalence and severity of chronic arthropathy in mild and moderate forms of the disease. In an early study published prior to the availability of specific FVIII treatment, it was observed that "one-third" of the patients with moderate haemophilia A and only very few with mild haemophilia had ankle arthropathy (Ahlberg, 1965). In more recent study that was based only on a survey questionnaire concerning "bodily pain" in a life-quality survey, almost half (43%) of 100 patients with mild/moderate haemophilia A had chronic musculoskeletal pain (Miners *et al*, 1999). A third study showed that more than half (51%) of the 35 patients with moderate haemophilia A had a positive Pettersson score in the combined radiological evaluation of 3 joints (ankle, knee and elbow), despite a third of them having received prophylaxis (Fischer *et al*, 2000). However, this study did not give details of the degree of arthropathy in the different joints (Fischer *et al*, 2000). These data implied that haemophilic arthropathy may be prevalent in these patients.

It is our experience that many patients with mild/moderate haemophilia have arthritis of one or both ankle joints, but do not have arthritis in the knee joints or the other joints that are often affected in severe haemophilia. A number of these patients suffered constant ankle pain, some to the extent of having to abandon long-term employment. This painful and disabling ankle arthropathy can cause significant medical and socioeconomic impact on the patient as well as the health system.

There is a stable and relatively large population of patients with mild/moderate haemophilia A in South Australia. The adult patients are managed in the Haemophilia Centre fro Adult Patients in South Australia, which is based at the Royal Adelaide Hospital (RAH) and the IMVS, Adelaide. Patient details are available on two databases: the Australian Bleeding Disorder Registry, RAH and the Haemophilia Database, IMVS. In addition, the family trees of these patients are recorded in a genetic registry that

was establishment in 1985 following a survey of all patients with haemophilia A in South Australia. The family trees and two databases have been used for a number of projects including investigations on FVIII level by one-stage and two-stage assays, which led to the classification of the *equivalent* (n = 27) and *discrepant* (n = 12) families in our mild/moderate haemophilia population (Duncan *et al*, 1994; Rudzki *et al*, 1996; Rodgers *et al*, 2007).

The information on these databases has facilitated the identification of patients for this study of the prevalence and severity of ankle arthropathy in mild/moderate haemophilia A, and its association with the baseline FVIII level. We consider that the knowledge obtained in this study would form an initial understanding of the presence and extent of ankle arthropathy in mild haemophilia. This knowledge may be helpful to improve prevention of this complication.

To understand the clinical picture of chronic ankle arthropathy in mild haemophilia, we examined a well studied adult patient population of mild and moderate Haemophilia A for their ankle arthropathy in South Australia. We hypothesise that ankle arthropathy is common in patients with mild/moderate haemophilia A, and that the presence of ankle arthropathy is associated with relatively low FVIII level. We also hypothesise that the WFH physical and radiology scoring systems, which has been well used in severe haemophilia, are sensitive enough to identify ankle arthropathy in patients with mild haemophilia. In addition, we hypothesise that the cause of ankle arthropathy is due to recurrent ankle trauma in childhood that caused bleeding.

The specific aims of this chapter were:

1) To determine the prevalence and severity of ankle arthropathy in mild/moderate haemophilia A.
2) To determine the relative usefulness for the detection of ankle arthropathy of:
    i) The presence or absence of pain.
    ii) Assessment by the WFH physical scoring system.
    iii) Assessment by the WFH radiology scoring system.

3) To determine the relationship to FVIII of:

i) Presence or absence of ankle pain.

ii) Presence and severity of ankle arthropathy as assessed by WFH physical or radiology scoring system.

4) To evaluate whether one-stage or two-stage FVIII level better correlates with the severity of ankle arthropathy in the *discrepant* subgroup of patients.

5) To ascertain whether there is evidence that ankle arthropathy is due to recurrent ankle trauma in childhood associated with bleeding.

4.2 Methods

4.2.1 Patients

This study was approved by the Research Ethics Committee of the RAH (protocol number: 010304 and 010304a) and informed consent obtained at clinical interview from individual participants. At the commencement of the study in January 2003, there were 134 adult patients with inherited haemophilia A living in South Australia and registered on the clinical databases in our Haemophilia Centre: the Australian Bleeding Disorders Registry, RAH. Records of FVIII levels were available on the Haemophilia Database at the IMVS. Also, the families of all patients were identified in a genetic registry (South Australian Genetic Registry of Haemophilia A) at the IMVS.

The patients in the clinical database of the 134 patients with haemophilia A were classified according to their two-stage FVIII levels as severe (≤ 1 IU/dl), moderate (2-5 IU/dl) and mild (> 5 IU/dl). There were 24 patients with severe haemophilia, 46 with moderate haemophilia and 64 with mild haemophilia. Of the 110 patients with the mild and moderate haemophilia A, 92 (52 mild and 40 moderate) were enrolled in the database with a a current contact details (address and/or telephone number). Therefore the study population consisted of a total of 92 patients.

Some members belonging to families of mild haemophilia had FVIII levels that were 2-5 IU/dl consistently and hence were classified as having moderate haemophilia. Thirty-three of the 40 patients with moderate haemophilia in this study population (of 92) were from ten families with mild haemophilia by origin (according to family tree and genetic registry of FVIII mutation) and the existence of mixed population of both mild and moderate clinical picture (or FVIII level).

4.2.2 Initial survey and telephone follow-up

A short survey on the symptom of ankle pain was posted to the 92 patients with mild/moderate haemophilia A in March 2003. This asked two questions, each provided with a positive and a negative answer (Appendix Ia). The first asked whether or not ankle pain was present and the second question asked whether the patient was willing to participate in a clinical interview and assessment of the ankle joint.

For people who acknowledged the presence of ankle pain, an additional question on the severity of the pain was indicated, with the relevant description given to define each of the three possible answers. The severity of pain was referred to as “mild, moderate or severe”, in accordance with the WFH description of pain on haemophilic joint evaluation (Pettersson & Gilbert, 1985). Mild pain does not interfere with occupation nor with activity of daily living (ADL), and may require occasional analgesics. Moderate pain has partial or occasional interference with occupation or ADL, and requires use of analgesics. Severe pain interferes with occupation or ADL, and requires frequent use of analgesics, including narcotics.

A patient information sheet and a consent form regarding this study (Appendix Ib), together with a self-addressed stamped return envelope were provided with each survey letter. Approximately one month was allowed for the recipients to return the survey letter. In the cases where no response received, a telephone follow-up was carried out to obtain answers to the survey question.

4.2.3 Clinical interview and assessment of ankle arthropathy

Of the 92 patients contacted by letter, 65 patients returned their answers. Thirty-four of the 65 patients (19 mild and 15 moderate) attended the clinical interview and assessment for ankle arthropathy at the RAH/IMVS Patient Centre from May to October 2003.

None of the 34 patients had any recent events of acute or subacute joint bleeding or joint surgery, including their ankle joint. All except for one patient were on a "treatment-on-demand" therapy. One patient (age 21 years) had been receiving (secondary) FVII prophylaxis for the past five years (Patient code 9.1, Appendix III). Three patients had inhibitors to FVIII (Appendix III). Twenty-three patients were hepatitis C-positive.

4.2.3.1 Interview concerning clinical history and bleeding tendency

Demographic data and diagnostic details (including age, hospital, family history, history of FVIII inhibitors), as well history of bleeding tendency, were recorded during interview. To profile the history of bleeding, patients were required to recall episodes of major bleeding (musculoskeletal bleeding including ankle haemorrhage, other joint or soft tissue haemorrhage, mouth bleeding, severe epistaxis, haematuria and spontaneous bruising etc.) during both childhood, adolescence and adulthood. Episodes of bleeding related to surgery/procedures performed without factor VIII cover were also specifically noted for. The complete assessment pack used for patient interview is included in Appendix II.

4.2.3.2 Assessment of ankle pain

The evaluation of ankle arthropathy was carried out by recording details of ankle symptoms (mainly the symptoms of pain, including restriction of mobility, necessity for walking aids etc.) and by performing the physical examination of both ankle joints.

At the interview the question on the presence of regular ankle pain and its severity (mild, moderate or severe) was repeated. The patients also indicated the degree of pain for the preceding four weeks for each ankle on a standard visual analogue scale (VAS) of 0 to 10 points (Appendix II). When there was bilateral involvement of ankle pain, the VAS pain score used to summarise the results for an individual patient was the higher score obtained from one of the two ankles.

4.2.3.3 Physical examination of ankle joint using the WFH scoring system

The scoring system was adapted from the WFH guidelines (as recommended by its Orthopaedic Advisory Committee), which was based on Gilbert's method (Pettersson & Gilbert, 1985; Gilbert, 1993). This included the assessment of ankle swelling, calf muscle atrophy, axial deformity, crepitus on motion, loss of ankle ROM, flexion contracture and instability (Table 1.1). For each ankle joint, a score of 0, 1 or 2 was assigned for each of the seven signs/items accordingly after a careful examination. The total score for each ankle was 0 to 12, with 0 being a normal ankle and 12 being most affected. The combined score from both the left and right ankles for each patient was used to summarise the results for the physical scoring system in individuals.

The application of the physical scoring system for ankle arthropathy in this study followed the standard methods practised in the physical examination of the extremities (Hoppenfeld, 1976), which was also the common method used by our physiotherapists specialised in treating haemophilia patients. In particular, the ROM of the ankle joint was recorded by measuring the angles of the dorsiflexion and plantar flexion using a goniometer. Although the normal ankle arc is 70 degree (dorsiflexion of 20° and plantar flexion of 50°), loss of less than 10% of this arc is considered within normal limits and therefore scores zero, according to the WFH guidelines.

4.2.3.4 Radiological evaluation of haemophilic ankle arthropathy using the WFH scoring system

All except one (Patient code 49.1, Appendix III) of the 34 patients had plain X-ray examination of both ankles at the time of interview. We adapted the WFH guidelines for the radiology assessment of

haemophilic arthropathy based on the Pettersson scale (Pettersson & Ahlberg, 1980; Pettersson & Gilbert, 1985). The criteria assessed were osteoporosis, enlarged epiphysis, irregular subchondral surface, narrowing of joint space, subchondral cyst formation, erosion of joint margins, gross incongruence of articulating bone ends and joint deformity (Table 1.2). Each of the eight items was assigned a score from 0 to 2. A score of 13 represented the worst affected joint.

Two radiologists reviewed the films independently and the mean value of the two scores was taken as the final score for each joint. The combined score from both the left and right ankles for each patient was used to summarise the results for individuals.

As a pilot study to investigate whether the magnetic resonance imaging (MRI) is sensitive to identifying any early ankle damage in patients with mild/moderate haemophilia A, a MRI (with Gadolinium as a contrast) on both ankles was also performed on five selected cases (Appendix III).

4.2.4 Investigation of FVIII level

4.2.4.1 Classification of patients with mild/moderate haemophilia A by FVIII assay discrepancy

Using the classification based on the FVIII assay discrepancies, patients with mild/moderate haemophilia A can be divided into to subgroups: the *equivalent* subgroup whose FVIII level is similar by the one-stage and the two-stage assay, and the *discrepant* subgroup whose FVIII level by one-stage assay is more than 2-fold higher than that of the two-stage assay (Section 1.4.2). This characteristic is displayed consistently in the members of the same pedigree family (Lloyd in Ingerslev *et al*, 2008). Hence the 39 families (with 92 members) with mild/moderate haemophilia A in our database were classified arbitrarily into 27 *equivalent* families (59 members) and 12 *discrepant* families (33 members) since our previous publications (Duncan *et al*, 1994; Rudzki *et al*, 1996; Rodgers *et al*, 2007).

The data of FVIII level (both one- and two-stage assays) for analysis were the median value of a series of baseline results in our database for individual patient, including the most recent test on blood collected at

the time of interview for the 34 patients. Blood samples were prepared as described in Section 3.2.1.4. Briefly, blood was collected into one tenth volume 109 mM trisodium citrate anticoagulant. PPP was prepared by centrifugation and stored at - 70°C. Tests for FVIII:C activity were conducted on plasma which had not previously been thawed.

4.2.4.2 One-stage FVIII assay

Routine one-stage FVIII assays were carried out by standard automated methods using an MLA Electra 1000C automatic coagulation analyser (Medical Laboratory Automation Inc, NY, USA), as previously described (Duncan *et al*, 1994; Rodgers *et al*, 2007). FVIII deficient plasma was from Organon Teknika Corporation (NC, USA). The reference standard (quality control) plasma was initially purchased from Dade-Behring and later from Unicalibrator (Stago Diagnostics, Asnieres, France). The APTT reagent Actin FSL was from Dade-Behring. Standards were calibrated against the International Reference Plasma for FVIII from NIBSC (UK).

4.2.4.3 Two-stage FVIII assay

Routine two-stage FVIII assays were measured by a semi-automated clotting method, using a Coag-a-Mate X2 (Organon Teknika) and the Diagen Two-Stage Factor VIII Assay Kit (Diagnostic Reagents, Oxon, UK) as previously described (Duncan *et al*, 1994; Rodgers *et al*, 2007). In preparation for the assay, standards and samples were absorbed with aluminium hydroxide – citrated plasma was mixed with 1/10th volume of 2% $Al(OH)_3$ (Alhydrogel, Superfos Biosector A/S, Frederikssund, Denmark), incubated for 5 min at 37°C, centrifuged and the supernatant taken for assay. The reference standard for the two-stage assay was the CSL reference plasma (Commonwealth Serum Laboratories, Melbourne, Australia), which has been calibrated against the International Reference Plasma for FVIII (NIBSC).

4.2.5 Statistics

A two-tailed T test was performed to compare continuous numeric variables of different groups. A Chi-square (χ^2) test or Fischer's exact test were used to analyse contingency tables with categorical variables. A non-parametric Spearman rank correlation was used to analyse associations between two groups of non-parametric data in most cases except for Table 4.7, where a Pearson correlation was performed. A Kappa test was used occasionally as an additional method to test the agreement between the categories (Table 4.7). A positive Kappa coefficient suggests the agreement exceeds chance. The analysis of Receiver-Operator Characteristic (ROC) curve was performed to calculate a series of sensitivity and specificity by different cut-off values of a test (Figure 4.13), using two softwares independently: Prism 4 (GraphPad software, CA, USA) and SAS 9.1 (SAS Institute Inc, NC, USA). Statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Patient characteristics

4.3.1.1 Age

Of the total study population of 92 patients with mild/moderate haemophilia A, 65 (71%) returned answers to the survey. Thirty-four of the 65 patients participated in the clinical interview concerning their ankle joints. Hence we were able to study in detail over a third (34/92 = 37%) of our target patient population. The data summarised here were based on the results from these 34 patients. One patient (Patient code 49.1, Appendix III) failed to attend the X-ray examination, therefore where the radiology assessment was described, there were 33 patients.

The age distribution of the patients is shown in Table 4.1 and Figure 4.1. Comparing the age distribution between the database population (n = 92) and the responses to survey (n = 65), the median age was similar: 46 and 48 years respectively (P = 0.87) (Table 4.1 columns A and B). There appears to be a difference in the age in those who responded to survey (n = 65) and those who did not (n = 27)

TABLE 4.1 Age Distribution

Age Range (years)	A Database (N = 92)	B Responses to Survey (N = 65)	C Failure to Response (N = 27)	D Interview (N = 34)
19 - 30	19 (21%)	9 (14%)	10	6 (18%)
31 - 40	17 (19%)	11 (17%)	6	8 (24%)
41 - 50	23 (25%)	18 (28%)	5	12 (35%)
51 - 60	12 (13%)	9 (14%)	3	6 (18%)
61 - 70	10 (11%)	7 (11%)	3	2 (6%)
71 - 86	11 (12%)	11 (17%)	0	0
Minimum Age (years)	*19*	*21*	*19*	*21*
Maximum Age	*86*	*86*	*66*	*66*
Average Age	*46*	*50*	*38*	*43*
Median Age	***46***	***48***	***34***	***45***

Chi-square test (with two-tailed P value) to compare age distribution between columns:

A and B: $\chi^2 = 1.84$, df = 5, P = 0.87;

B and C: $\chi^2 = 10.6$, df = 5, P = 0.06;

A and D: $\chi^2 = 6.46$, df = 5, P = 0.26;

B and D: $\chi^2 = 7.70$, df = 5, P = 0.17.

df: Degree of freedom.

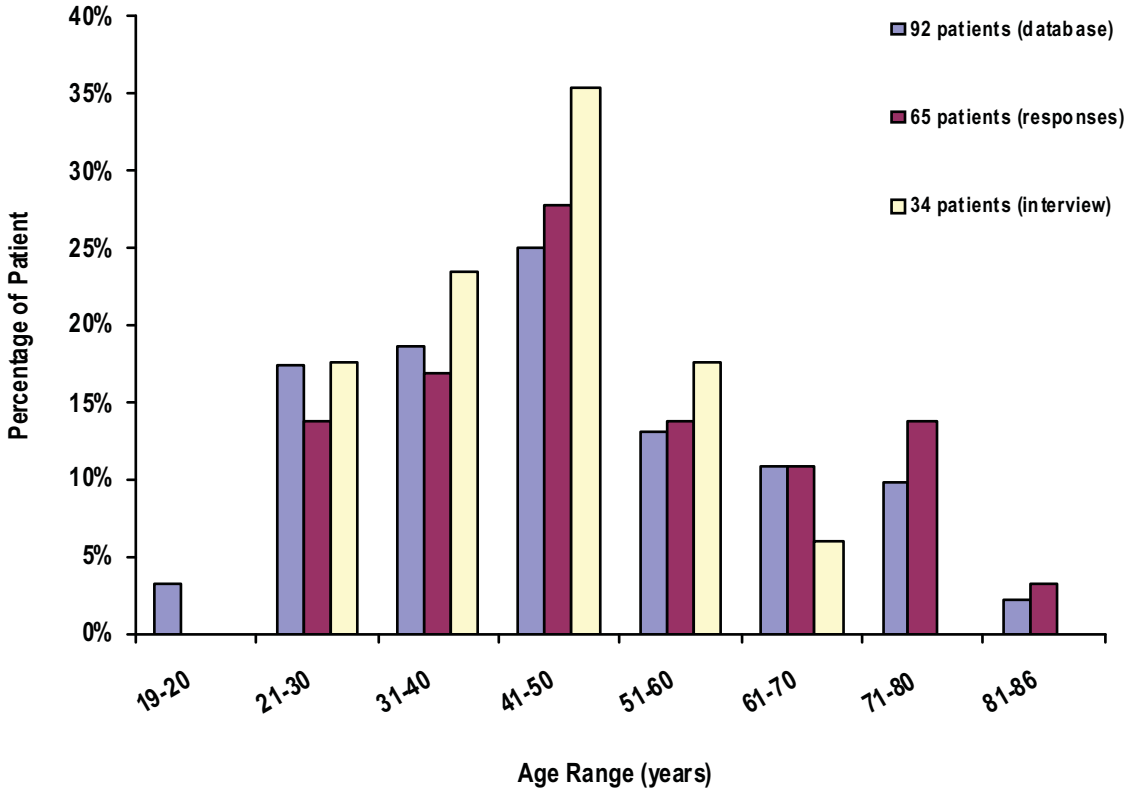


FIGURE 4.1 Age Distribution of Patients

92 patients: Patients with mild and moderate haemophilia A in South Australia enrolled in the RAH/IMVS Haemophilia Centre.

65 patients: Patients from the database who responded to the initial survey about ankle pain.

34 patients: Patients who participated in a clinical interview and assessment for ankle arthropathy.

(Table 4.1 columns B and C), although this did not reach statistic significance (P = 0.06). This could reflect a relatively low response rate from those people at a younger age.

Despite the above, the age distribution in the 34 patients interviewed (Table 4.1 column D) was similar to that of the database population (P = 0.26) and the 65 responses (P = 0.17). Therefore in terms of age, the 34 patients was a reasonable representation of our target study population. This is also illustrated in Figure 4.1.

4.3.1.2 FVIII level

4.3.1.2.1 FVIII level and family tree

The 34 patients in the current study consisted of 19 mild and 15 moderate cases, classified by the two-stage FVIII level (Section 4.2.1). However 12 of the 15 moderate patients were from four pedigree families where there have been mixed members with mild or moderate levels of FVIII. The FVIII levels, whether mild (> 5 IU/dl) or moderate (2-5 IU/dl), have been consistent for individual subjects.

Table 4.2 summarises the 34 patients classified by family of origin. The 34 patients belonged to 19 pedigree families: 16 mild families (including four families with mixed members) and three moderate families (Table 4.2). One large family (Family code 4) with mild haemophilia A had the mutation G6506A Arg2150His. Eight of the nine patients from this family studied for ankle arthropathy had two-stage FVIII levels constantly in the moderate range.

4.3.1.2.2 FVIII level compared in patient populations

A comparison of the frequency of patients in different range of FVIII level between the 34 patients at the interview and the 92 patients in the database is illustrated in Figure 4.2 (A and B). There appeared to be a bimodal pattern for the distribution of patients according to their one-stage FVIII level: while many patients had a FVIII of 1-15 IU/dl, there were also a few patients at the high end of the range of FVIII

TABLE 4.2 34 Patients Classified by Family of Origin (Genetic Registry)

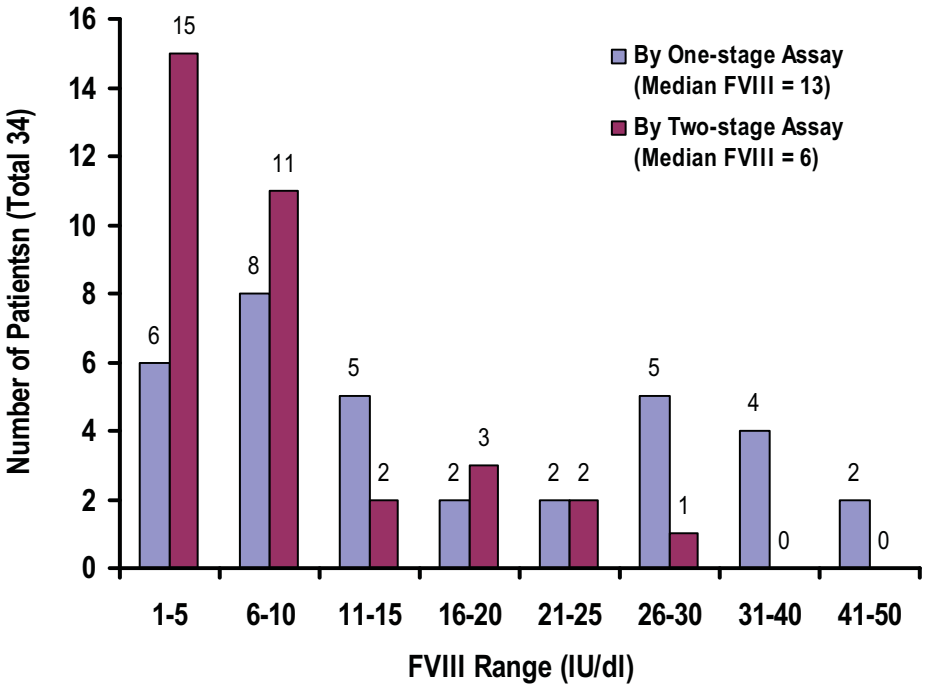
Subgroup		Family Code	Number of Patients Studied in Each Family	Median FVIII (IU/ml)*	
				One-stage	Two-stage
Mild	***Equivalent***	**4****	9	7	5
				2	3
				8	5
				5	5
				8	6
				4	3
				6	3
				11	4
				11	5
		7**	3	15	7
				17	9
				4	4
		16	1	23	10
		24	3	27	24
				40	22
				30	18
		26	1	8	6
		35**	2	6	3
				5	4
		49	2	14	11
				12	9
		101	1	30	27
		121	2	27	20
				24	12
		132	1	16	17
			N = 25		
	Discrepant	**8**	1	34	10
		14	1	37	8
		28	1	41	6
		39	1	44	6
		40**	1	31	5
		46	1	30	6
			N = 6		
Moderate	***Equivalent***	**9**	1	7	3
		97	1	6	4
		138	1	5	4
			N = 3		
Total		**19 Families**	**34 Patients**	**13† IU/ml**	**6† IU/ml**

*: Median baseline FVIII level for individual patient.

**: 4 families (Family 4, 7, 35 and 40) belong to mild haemophilia A by family tree, but have mixed members with either mild or moderate FVIII level (by two-stage assay).

†: Median FVIII level of the 34 patients.

A



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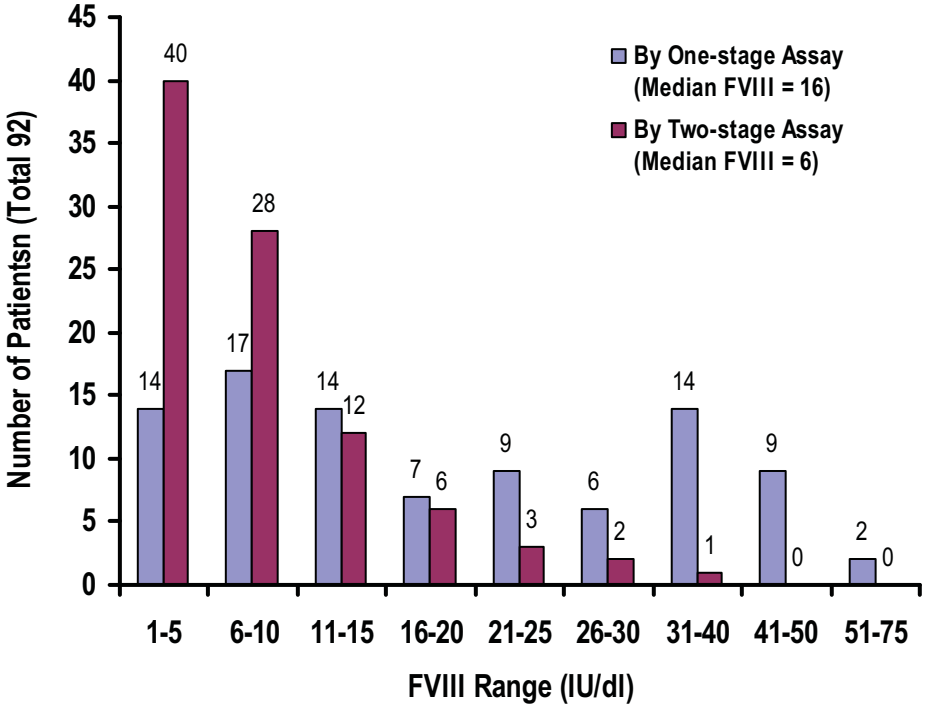


FIGURE 4.2 FVIII Level in the Interview Population and the Database Population

A. Interview population (N = 34).
B. Database population (N = 92).

The comparison of the distribution of patients in different FVIII range in the two populations is not significant: P = 0.72 (χ^2 = 1.35, df = 3) and 0.91 (χ^2 = 0.54, df = 3) for the one- and two-stage results respectively.
df = 3: The contingency table used had four categorised FVIII ranges: 1-5, 6-10, 11-30 and 26-50 IU/dl to ensure adequate numbers in each category.

level (e.g., > 25 IU/dl). This pattern was seen in both the 34 patients at the interview (Figure 4.2 panel A) and the 92 patients in the database (Figure 4.2 panel B).

When the two-stage FVIII results were used, there was a single mode: the majority of the patients had a relatively low FVIII (e.g., < 10 IU/dl) and the patient number tends to decrease with higher FVIII level (Figure 4.2 A and B). This pattern was also reproducible in the two patient populations (interview vs database). There was no difference in the distribution of the patients over the different FVIII levels in the two cohorts: P = 0.72 and 0.91 for one- and two-stage assay results respectively.

The comparison of median FVIII level in the interview cohort and the database cohort is shown in Table 4.3. By the one-stage FVIII assay, the median FVIII level in the 34 patients and the 92 patients were 13 and 16 IU/dl respectively (P = 0.27). By the two-stage FVIII assay, the median FVIII was the same, at 6 IU/dl for both (P = 0.96) (Table 4.3). There was no difference in the median FVIII level by either assay in the two populations. Hence it is reasonable to conclude that the 34 patients studied in detail for ankle arthropathy were a representative of the database population.

4.3.1.2.3 FVIII assay discrepancy in 34 patients

Figure 4.3 displays the results of FVIII level by one- and two-stage assays in the 34 mild/moderate haemophilia A patients. The results are in accordance with our previous publications (Duncan *et al*, 1994; Rodgers *et al*, 2007; Lloyd in Ingerslev *et al*, 2008) in that the *discrepant* patients had a FVIII level by the one-stage assay that is 2-fold greater than that of the two-stage assay. It is noted that there was a tendency in some of the *equivalent* patients to have a higher one-stage FVIII level than previously recorded (Figure 4.3). The reasons for this drift of agreement in one-stage assay over the past few years were not part of the objectives of this thesis, but this is and likely to be multifactorial and is currently under investigation in our laboratory. Possible explanations include a change of international plasma reference standards used, the chronic inflammatory responses in patients with liver disease, or it may simply be a function of the aging process in some patients.

TABLE 4.3 Comparison of Median FVIII in the Interview Population and Database Population

	Interview (N = 34)	Database (N = 92)	Two-tailed T Test		
			P	t	df
Median One-stage FVIII (IU/dl)	13	16	**0.27**	**1.11**	**124**
Median Two-stage VIII (IU/dl)	6	6	**0.96**	**1.98**	**124**

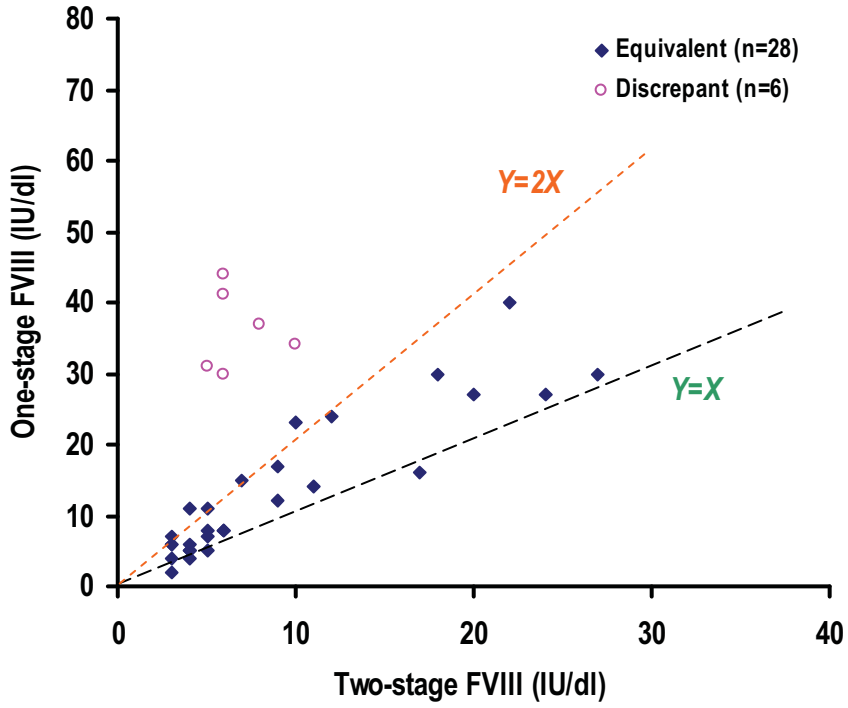


FIGURE 4.3 One-stage and Two-stage FVIII Level in 34 Patients

The median value of all recorded baseline FVIII level for each individual is shown for 28 *equivalent* and 6 *discrepant* patients who participated in the clinical interview: X axis represent the one-stage results and Y axis represent two-stage results.

According to our existing classification on the families with mild/moderate haemophilia A based on FVIII assay discrepancies, the 34 patients participating in the interview for ankle arthropathy were divided into two groups. The *equivalent* subgroup consisted of 28 patients from 13 families (including seven pairs of siblings), and the *discrepant* subgroup consisted of 6 patients from 6 families (Table 4.4). Table 4.4 also includes the number of the *equivalent* and *discrepant* patients in the database population. It was noted that of the 92 patients, the *equivalent* and *discrepant* patients were at a ratio of approximately 2:1 (59:33). However, only 6 *discrepant* patients participated in the interview and hence there were insufficient samples in this study represent this subgroup of patients in the database. It was also noted that the *discrepant* families tend to exist among those with mild (rather than moderate) haemophilia (Table 4.4).

4.3.2 Ankle pain

4.3.2.1 Frequency and age distribution

Of the 65 patients with mild or moderate haemophilia A who responded to our initial survey, 27 (41%) had ankle pain (unilateral or bilateral) of various degrees of severity on a regular basis. The remaining 38 patients denied the presence of ankle pain. There was a similar age distribution in the patients with and without ankle pain in these 65 patients (P = 0.82) (Table 4.5). It is noted that in some cases, ankle pain occurred at a relative early age, i.e., before 40 years, with the youngest being 23.

Of the 34 patients interviewed, almost half (16/34 = 47%) had ankle pain. The reported frequency of ankle pain in mild/moderate haemophilia A obtained from the survey, was therefore consistent with the data obtained for the clinical interview cohort (Table 4.6 A). In the 34 patients interviewed, there was an excellent reproducibility of the answers to the question on ankle pain and its severity on the two occasions (survey and interview) (data not shown). In the 16 patients with the presence of ankle pain, almost two-thirds (10/16 = 63%) had bilateral ankle involvement (Table 4.6 B).

TABLE 4.4 *Equivalent* and *Discrepant* Patients

Subgroup	Interview (N = 34)		Database (N = 92)	
	Number of Families	Number of Patients	Number of Families	Number of Patients
Equivalent				
Mild	10	25	22	52
Moderate	3	3	5	7
Discrepant				
Mild	6	6	12	33
Moderate	0	0	0	0
Subtotal	**19 families**	**34 patients**	**39 families**	**92 patients**

In the database population, approximately 1/3 (33/92) were *discrepant* patients. In the interview cohort, only 6 of 34 were *discrepant* patients.

The classification of mild or moderate families/patients is by family of origin, as in Table 4.2.

TABLE 4.5 Age Distribution and Median Age of Patients with Presence or Absence of Ankle Pain

	Responses (N = 65)		Interview (N = 34)	
Age Range (years)	**Pain Present (N = 27)**	**Pain Absent (N = 38)**	**Pain Present (N = 16)**	**Pain Absent (N = 18)**
19- 30	3	6	1	5
31 - 40	4	7	3	5
41 - 50	7	11	7	5
51 - 86	13	14	5	3
Minimum Age (years)	*23*	*21*	*29*	*21*
Maximum Age	*86*	*79*	*61*	*66*
Average Age	*52*	*49*	*47*	*40*
Median Age	***50***	***47***	***49***	***39***

Chi-square test for age distribution of the two subgroups of patients with presence or absence of ankle pain in the population of:

"Responses (N = 65)": $\chi^2 = 0.91$, df = 3, P = 0.82;

"Interview (N = 34)": $\chi^2 = 3.90$, df = 3, P = 0.27.

TABLE 4.6 Frequency of Ankle Pain

A

Ankle Pain	Responses (N = 65)	Interview (N = 34)
Absent	**38** (59%)	**18** (53%)
Present		
Mild	13 (20%)	8 (23%)
Moderate	6 (9%)	4 (12%)
Severe	8 (12%)	4 (12%)
Subtotal	**27** (41%)	**16** (47%)
Total	**65 patients**	**34 patients**

B

Side of Ankle with Pain	Number of Patients
Bilateral	10 (63%)
Right Only	4 (25%)
Left Only	2 (12%)
Subtotal	**16 patients**

It was noted that although in the 34 patients interviewed the median age of the pain-present subgroup was 10 years older than that of the pain-absent subgroup, statistically there was no difference in the age distribution of these two subgroups (P = 0.27) (Table 4.5).

4.3.2.2 Severity

The frequencies of the patients with different severity of pain (mild, moderate or severe, as defined by WFH guidelines, Section 4.2.2) were very similar among the 65 patients who returned the survey and the 34 patients who were interviewed: i.e., mild degree was reported in approximately 20% of patients, while moderate and severe degree were both reported in approximately 10% of patients (Table 4.6 A).

In addition to the categorisation of "mild, moderate or severe", the degree of ankle pain was expressed in the form of a VAS pain score. The VAS pain score summarised here was the higher score of the two ankles for each individual. We consider that the worse VAS score from a single ankle is representative. Nevertheless, we did a parallel analysis of all relevant results wherever applicable using the sum of VAS scores of both ankles for every patient, and found that there was no difference to the interpretation of the overall results. Occasionally, there were slight changes to the R value in rank correlation of these parameters. The details of the differences were included in the legends of the relevant tables and figures for references.

The results are summarised in Table 4.7. A Similar number of patients were present in the four categories of pain description and the four ranges of VAS score (Table 4.7). The strong agreement between the two measures of pain severity were supported by a positive and high Kappa coefficient (simple and weighted Kappa coefficient: 0.68 and 0.81 respectively) and Pearson correlation coefficient (0.91).

The results of the comparison of VAS pain score versus age are shown in Figure 4.4 panel A. There was no correlation between the age and the pain score (Spearman rank correlation R = 0.29, P = 0.095).

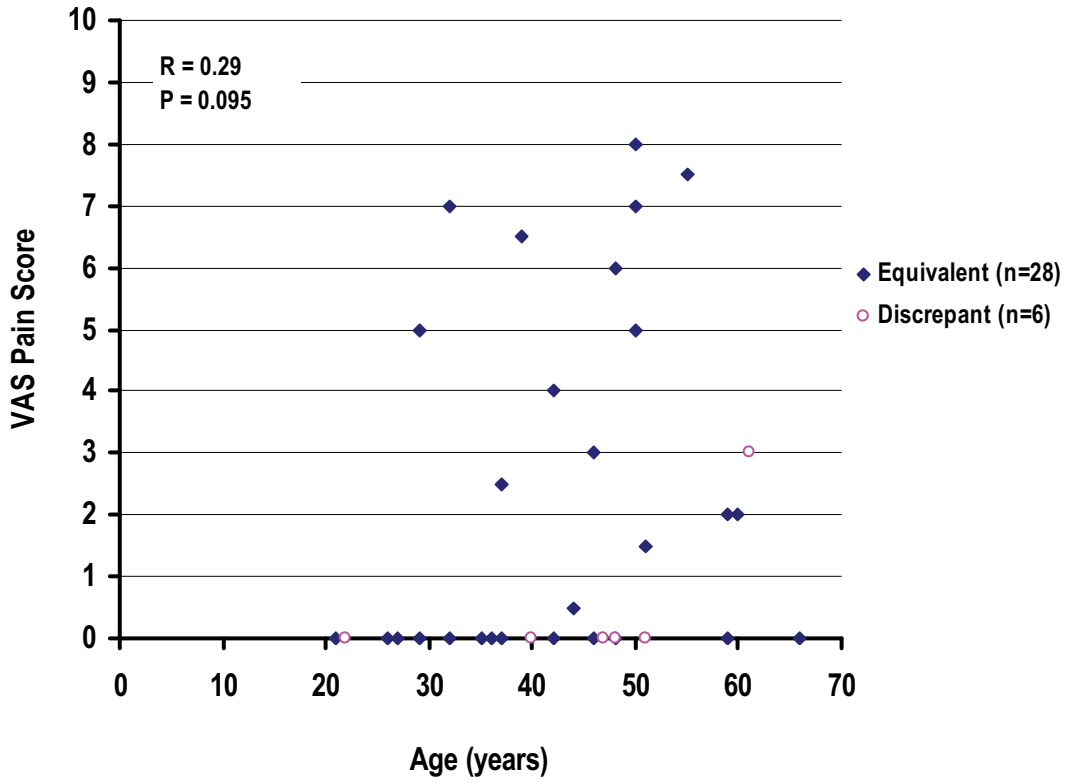
TABLE 4.7 Severity of Ankle Pain

Number of Patients		VAS Pain Score				Subtotal
		0	0.5 - 3	3.5 – 6.5	7 - 10	
Categorisation	No Pain	18	0	0	0	**18**
	Mild Pain	0	6	2	0	**8**
	Moderate Pain	0	1	1	2	**4**
	Severe Pain	0	0	2	2	**4**
Subtotal		**18**	**7**	**5**	**4**	**34patients**
Simple Kappa Coefficient 0.68, P < 0.0001						
Weighted Kappa Coefficient 0.81, P < 0.0001						
Pearson Correlation Coefficient 0.91, P < 0.0001						

We arbitrarily divided the VAS scores into four groups: zero, 0.5 – 3, 3.5 – 6.5 and 7 – 10, in order to correlate the VAS scores to the categorisation description.

In general a Kappa coefficient of 0.41-0.6, 0.61-0.8 and 0.81-1 represents “moderate”, “substantial” and “almost perfect” agreement respectively.

A



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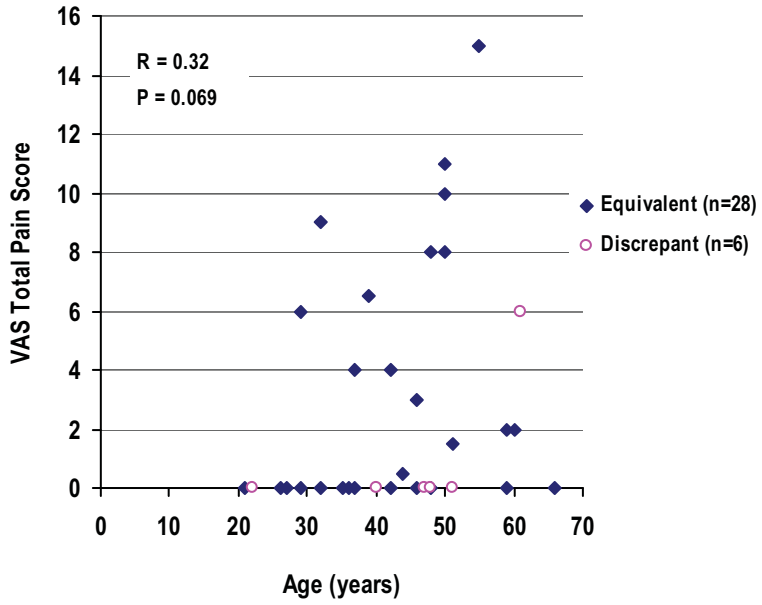


FIGURE 4.4 Age and VAS Pain Score in 34 Patients

A. Age and VAS pain score. Spearman rank correlation coefficient R = 0.29, P = 0.095.

B. Age and VAS "total" pain score (sum of VAS scores of both ankles for individual). There was slight difference to the R and P values compared to "A", with no changes to statistical significance.

All subsequent results in this chapter were summarised using the VAS pain score, with the statistics for the VAS "total" pain score are also given in relevant context.

For instance, two young patients aged only 29 and 32 had a pain score of 5 and 7 respectively, while two patients aged about 60 years had relatively low pain scores of 2. It was also obvious that the ankle pain-free patients (n = 16) were spread among all age ranges. Figure 4.4 panel B gives an example of a similar outcome when the sum of the VAS scores of both ankles (VAS "total" pain score, Appendix II) for each patient was used to summarise the data. As described above, no overall difference of the results was observed.

4.3.2.3 Comparison of FVIII level in patients with presence or absence of ankle pain

For the 34 patients interviewed, it seems that those who had ankle pain tend to have a relatively low FVIII level by the one-stage assay. Figure 4.5 panel A reveals that the median one-stage FVIII was 7 IU/dl in those patients with ankle pain, in contrast to 22 IU/dl for patients who were pain-free. This was statistically significant (P = 0.017). However, the median two-stage FVIII in the two subgroups was similar (P = 0.249), at 4.5 and 7.5 IU/dl for those with the presence and absence of ankle pain respectively (Figure 4.5 B).

The results in Figure 4.5 panels C and D were based on 28 patients; the six *discrepant* patients were excluded from the analysis. The analysis showed that there was no difference in the median FVIII level between the two subgroups presenting with or without ankle pain.

Similar to the results in 34 patients who were interviewed, in the 65 patients who returned the survey, the FVIII levels, as determined by the one-stage assay, in those who had ankle pain (n = 27, median one-stage FVIII 11 IU/dl) was lower than that those who were free of ankle pain (n = 38 patients, median one-stage FVIII 22.8 IU/dl) (P = 0.001, data not shown). In contrast, when the two-stage FVIII results were assessed, the difference in the median FVIII level in the two subgroups was no longer present. The FVIII levels were 4.7 and 6.7 IU/dl for the subgroups with the presence or absence of ankle pain, respectively (P = 0.07). Similar to the clinical interview cohort, in the 65 patients who responded to the survey, after excluding the *discrepant* patients (23 of 65, seven had ankle pain), there was no difference

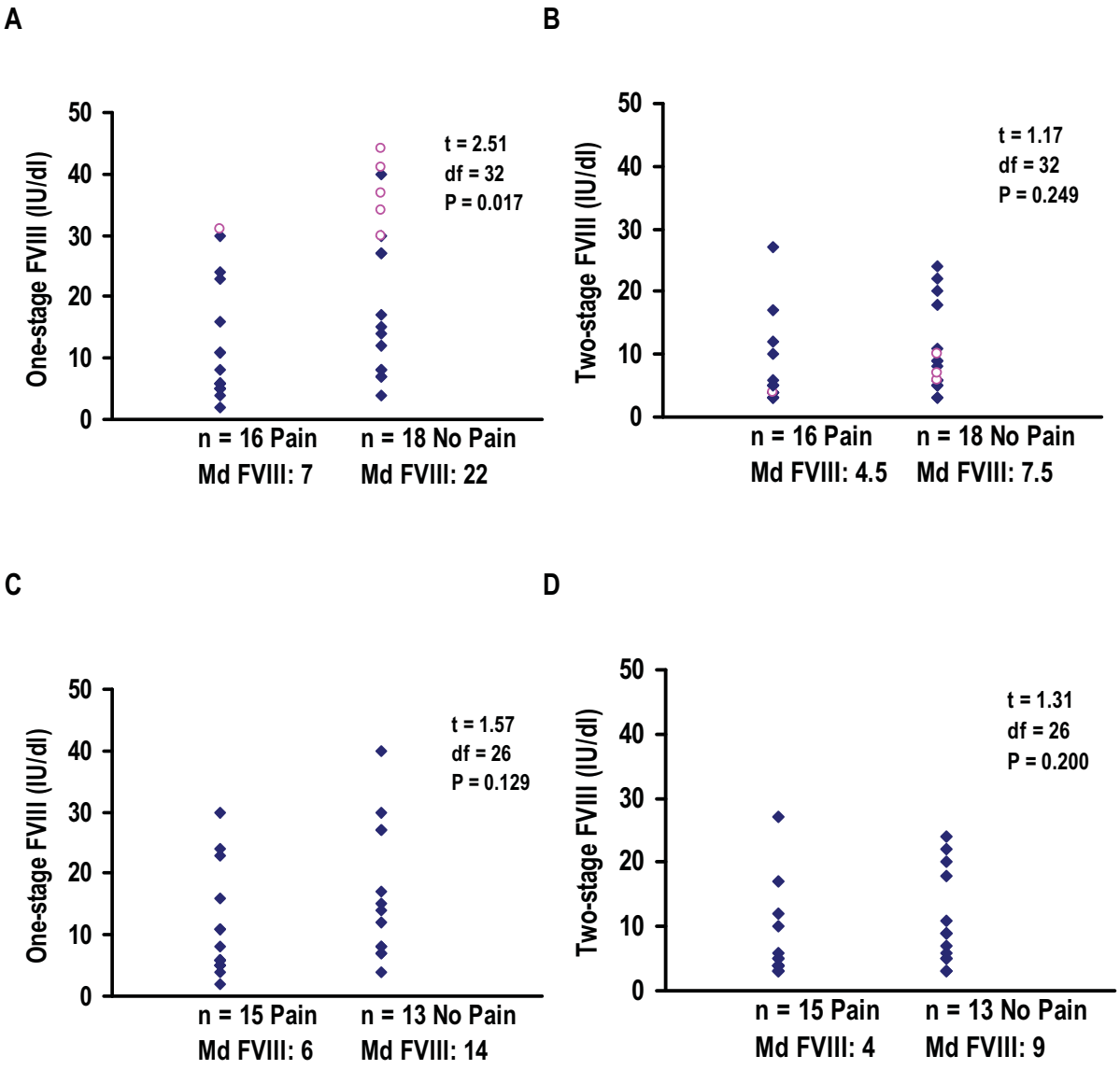


FIGURE 4.5 FVIII Level in 34 Patients with Presence or Absence of Ankle Pain

A. One-stage FVIII in 34 patients (16 had pain), including 6 *discrepant* cases (open circles) (1 had pain);

B. Two-stage FVIII in 34 patients;

C. One-stage FVIII in 28 patients (15 had pain), excluding 6 *discrepant* cases;

D. Two-stage FVIII in 28 patients.

Md FVIII: Median FVIII (IU/dl) of the subgroup with or without pain.

in the median FVIII level in the two subgroups with or without ankle pain by either one-stage (6 vs 10 IU/dl, P = 0.09) or two-stage assay (4 vs 6.5 IU/dl, P = 0.15) (data not shown).

4.3.3 Physical scoring system

Of the 34 patients interviewed, 27 had a positive ankle score by the WFH physical scoring system, where the sum of scores of both ankles greater than zero (range 1-6). Of these 34 patients, the remaining 7 patients had clinically normal ankles. The prevalence of ankle arthropathy by the physical scoring system was therefore 79% (27/34) in this group of 34 patients with mild and moderate haemophilia A.

Among the seven assessing criteria that were included in the WFH guidelines (Table 1.1), the most frequently found sign in these 34 patients was a loss of the range of motion of the ankle joint of greater than 10% of the normal value (ankle arc dorsiflexion plus plantar flexion: 70°) affecting one or both ankles. In other words, the ROM was equal to or less than 63° as measured by a goniometer. This was present in nearly all patients (26 of 27) with a positive physical ankle score (Table 4.8 A). The only patient (Patient code 101.1, Appendix IV) with a positive physical ankle score but preserved ROM instead presented with crepitus on motion in both ankles and swelling in one ankle (scored 4 in total).

Following the loss of ROM, swelling of the ankle joint (measured by circumference over medial and lateral malleoli) (n = 3), calf muscle atrophy (n = 6) and crepitus on motion (n = 2) were also seen in the current study group of 34 patients (Table 4.8 A). There were no other physical signs such as axial deformity, flexion contracture or instability of ankles in these patients.

It was noted in the 27 patients who had a positive physical ankle score, in approximately two-thirds (70%) of cases both ankles were affected (Table 4.8 B). The right side ankle seemed to be more frequently affected compared to the left side.

TABLE 4.8 Physical Scoring System to Assess Ankle Arthropathy in 27 Patients with a Positive Physical Ankle Score

A

Physical Signs	Number of Patients			Total Patients (N = 27)	Total Ankles (N = 46)
	Both Sides Affected	Right Side Only	Left Side Only		
1) Swelling	0	2	1	**3**	**3**
2) Calf Muscle Atrophy	0	4	2	**6**	**6**
3) Axial Deformity	0	0	0	**0**	**0**
4) Crepitus on Motion	2	0	0	**2**	**4**
5) Range of Motion	18	7	1	**26**	**44**
6) Flexion Contracture	0	0	0	**0**	**0**
7) Instability	0	0	0	**0**	**0**

Twenty-seven of 34 patients (79%) had ankle arthropathy by the physical scoring system (physical ankle score > 0). The most frequently observed sign was loss of ankle ROM, which was present in 26 of the 27 patients and in a total of 44 ankles.

B

Side of Ankle Affected	Number of Patients
Bilateral	19 (70%)
Right Only	7 (26%)
Left Only	1 (4%)
Subtotal	**27 patients**

In studying the loss of ankle ROM in these 26 patients, the majority of them (18/26 = 69%) had bilateral ankle involvement (Table 4.9). A total of 44 ankles scored positively. A score of 1 for the affected ankle was most frequently assigned: present alone in 20 patients (13 bilateral and 7 unilateral) (Table 4.9). The remaining six patients had a score of 2 in at least one ankle. In total the majority (35 of 44) of the affected ankles scored 1 for loss of ROM and the remaining nine ankles had a score of 2.

Further analysis of the data on loss of ROM in the 44 affected ankles revealed that the presence of loss of dorsiflexion was more frequently detected, compared to the loss of plantar flexion (Table 4.10). Nearly half (23 of 44 ankles) of the affected ankles displayed loss of dorsiflexion only, while only one ankle had loss of plantar flexion only (Table 4.10). In addition in another half (20 of 44 ankles) of the affected ankles, although both flexions were reduced, the majority (16 of 20 ankles) showed a worse degree of impairment in dorsiflexion compared with plantar flexion (Table 4.10).

Figure 4.6 illustrates the frequency of loss of dorsiflexion, plantar flexion and the total ROM in the 44 ankles. The loss of dorsiflexion was more frequently present and more severe than the loss of plantar flexion (Figure 4.6 panels A and B). Panel C shows that of the total of 44 ankles assessed, 35 had loss of total ROM of between 7° to 25°, which was consistent with a score of 1 (ROM loss of 10-33% from normal, i.e., 7-22°). The remaining nine ankles had more reduced ROM and would attract a score of 2 (Figure 4.6 C). This data was consistent with the individual ankle score for loss of ROM that are listed in Table 4.9. The data detailing the actual measurements of ROM for each ankle joint examined can be found in Appendix V.

Interestingly, a relatively minor loss of dorsiflexion of 5° rarely affected the final score for loss of ROM in that ankle (Appendix V). This was due to the observation in the study that the loss of dorsiflexion of 5° was often an isolated finding in a single ankle hence the total ROM remained in the normal range and scored zero. In other cases, a loss of dorsiflexion of 5° coincided with a loss of plantar flexion of a much more substantial degree. In these cases, the total loss of ROM was determined primarily by the severity of the loss of plantar flexion.

TABLE 4.9 Positive Scores in Loss of ROM of Ankle Joint

Side of Ankle Affected	Score for Loss of ROM* (Right / Left)	Number of Patients	Subtotal
Bilateral	2 / 2	3	
	2 / 1	1	**18** (69%)
	1 / 2	1	
	1 / 1	13	
Right Only	2 / 0	1	**7** (27%)
	1 / 0	6	
Left Only	0 / 2	0	**1** (4%)
	0 / 1	1	
Subtotal Patients			**26 patients**
Subtotal Ankles	with a score of 1:	35 ankles	**44 ankles**
	with a score of 2:	9 ankles	

The majority of the 26 patients (69%) had bilateral involvement.
The majority of the 44 ankles had a score of 1.

*: The score for loss of ROM in each ankle joint is:
- 0: loss of ROM < 10% of normal range (normal ankle arc 70°);
- 1: loss of ROM 10-33% of normal (i.e., 7-22°);
- 2: loss of ROM > 33% of normal.

TABLE 4.10 Loss of Dorsiflexion and Plantar Flexion in 44 Ankles

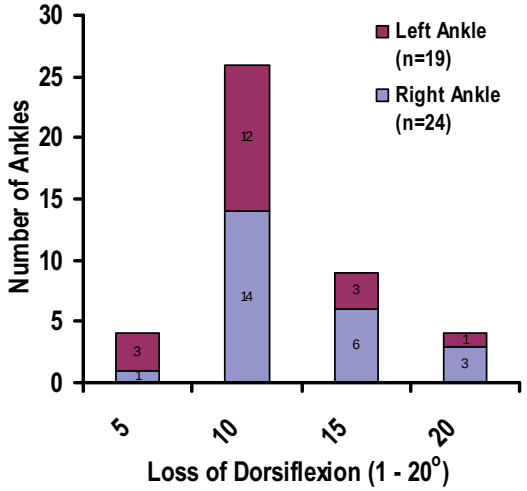
Presence of Loss of Ankle ROM (Dorsiflexion and/or Plantar Flexion)	Number of Ankles
Loss of Both Dorsi- and Plantar Flexion	**20**
loss of dorsiflexion (% of normal range*) > loss of plantar flexion (%)	16
loss of dorsiflexion (%) = loss of plantar flexion (%)	1
loss of dorsiflexion (%) < loss of plantar flexion (%)	3
Loss of Dorsiflexion Only	**23**
Loss of Plantar Flexion Only	**1**
Total Number of Ankles	**44 ankles**

Most ankles (16 of 20) had more severe impairment in the dorsiflexion than plantar flexion if both were present.

Reduced dorsiflexion also served as the sole cause for a positive score (in loss of ankle ROM) in another 23 ankles.

*: Percentage of the corresponding normal range, i.e., 20° for dorsiflexion and 50° for plantar flexion.

A



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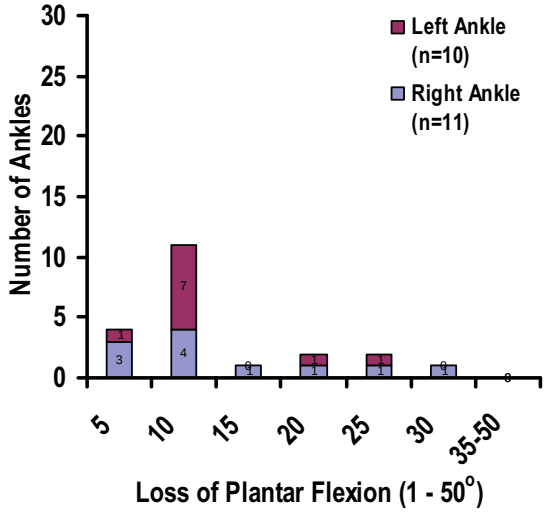

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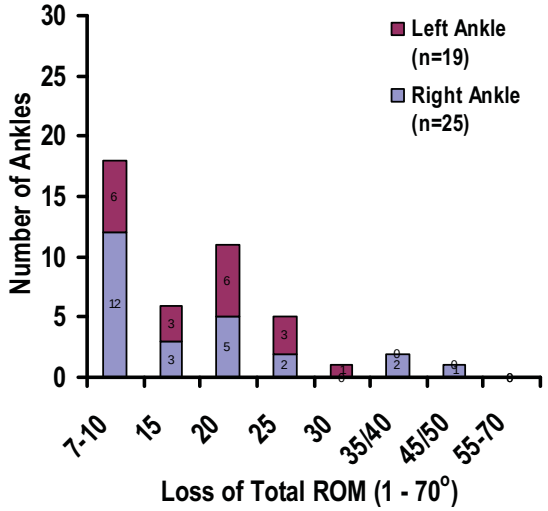


FIGURE 4.6 Loss of Range of Motion in 44 Ankles Assessed

A. Loss of dorsiflexion (total number of ankles affected: 43).
B. Loss of plantar flexion (total number of ankles affected: 21).
C. Loss of total ROM (total number of ankles affected: 44).

The normal ROM of ankle joint is 70° (dorsiflexion 20° plus plantar flexion 50°). Loss of ROM between 10-33% of normal (i.e.,7-22°) attracts a score of 1, while loss of ROM of > 33% attracts a score of 2.

4.3.4 Radiological scoring system

All except one of the 34 patients studied in detail for ankle arthropathy had plain X-ray examination of bilateral ankles at the time of interview. Using the WFH radiology scoring system (Pettersson scale), 17 of 33 were found to have various degrees of haemophilic arthropathy changes on the X-ary. The remaining 16 patients (age 21-66 years) had normal appearing ankles on X-ray. The prevalence of ankle arthropathy by radiology evaluation in this study group was therefore 52% (17/33).

The most frequent sign detected on the ankle X-ray of the 17 patients who had a positive radiology score was irregular subchondral surface (present in 13 patients), followed by narrowing of joint space (11 patients), subchondral cyst formation (10 patients) and enlarged epiphysis (7 patients) (Table 4.11 A). These signs appeared to present in a somewhat clustered fashion in a single ankle. Of the 28 ankles (from 17 patients) with a positive radiology score, 16 had a combination of at least two of the four signs that were most frequently detected (i.e., items 2, 3 4 and 5 of the WFH radiology scoring system), including seven with the combination of three signs and five with all four signs (Table 4.12). The detailed results of the radiology evaluation for ankle arthropathy against the eight assessing items in the 34 patients are included in Appendix VI.

The frequent findings of irregular subchondral surface and narrowing of joint space seemed to coincide with the equally prevalent finding of reduced ROM on physical examination in this group of patients. Osteoporosis and erosion of joint margins were seen in just one and two patients respectively. There was no gross incongruence of articulating bone ends or joint deformity observed in the current study. This was consistent with the clinical findings from the physical examination that no severe alterations such as flexion contracture or instability, were detected in these patients.

About two-thirds of the patients (11/17 = 65%) with a positive radiology score had both ankles affected (Table 4.11 B). This frequency was similar to the findings that, in mild/moderate haemophiliacs with a

TABLE 4.11 Radiology Scoring System to Assess Ankle Arthropathy in 17 Patients with a Positive Radiology Ankle Score

A

Plain X-ray Signs	Number of Patients			Total Patients	Total Ankles
	Both Ankles	Right Only	Left Only	(N = 17)	(N = 28)
1) Osteoporosis	1	0	0	**1**	**2**
2) Enlarged Epiphysis	4	1	2	**7**	**11**
3) Irregular Subchondral Surface	8	3	2	**13**	**21**
4) Narrowing of Joint Space	6	4	1	**11**	**17**
5) Subchondral Cyst Formation	3	6	1	**10**	**13**
6) Erosion of Joint Margins	0	2	0	**2**	**2**
7) Gross Incongruence of Articulating Bone Ends	0	0	0	**0**	**0**
8) Joint Deformity	0	0	0	**0**	**0**

Seventeen of 33 patients (52%) had a positive radiology score, excluding one who did not have X-ray evaluation. The most frequently observed sign in this patient group was item 3 (irregular subchondral surface), followed by items 4 (narrowing of joint space), 5 (subchondral cyst formation) and 2 (enlarged epiphysis).

B

Side of Ankle Affected	Number of Patients
Bilateral	11 (65%)
Right Only	4 (23%)
Left Only	2 (12%)
Subtotal	**17 patients**

TABLE 4.12 Radiology Scoring System: Items 2, 3, 4 and 5 to Assess Ankle Arthropathy in 17 Patients with a Positive Radiology Ankle Score

Positive Radiology Signs: Presence of Items 2, 3, 4 and 5 of Petterson Scale	Number of Ankles Affected
Single Item: 2 or 3 or 4 or 5	**12**
Combination of:	**16**
Two Items	4
Three Items	7
Four Items	5
Subtotal	**28 patients**

The radiology signs appeared in somewhat a clustered fashion in affected ankles: in the four items that were frequently detected in this patient group, a combination of any two or more of these items were commonly found (16 of 28 ankles).

positive VAS pain score or physical ankle score, 63% and 70% of the patients had bilateral ankle involvement, respectively (Tables 4.6 B and 4.8 B).

A pilot study was undertaken to investigate whether MRI is more sensitive in identifying ankle arthropathy at the early stage of joint damage in patients with mild/moderate haemophilia A. MRI was performed on five selected patients (Appendix III), three of them had ankle pain of various degrees and the other two were pain free. The MRI examination was aborted after the pilot study due to insufficient evidence that support early detection of ankle disease by this method in these five patients (data not shown) and partially, due to its relatively expensive costs.

4.3.5 Comparison of physical and radiological scoring systems

4.3.5.1 Correlation

Table 4.13 reveals the comparison of the two scoring systems in the assessment of ankle arthropathy in 33 patients with mild/moderate haemophilia A. By radiological assessment alone, 52% (17 of 33 patients) had ankle arthropathy. Among these 17 patients, 16 were also positive by physical evaluation (Table 4.13). Hence the prevalence of ankle arthropathy in the study group of 33 patients with mild/moderate haemophilia A (excluding one patient without radiology evaluation) were 48% (16/33), by both the physical and radiology scoring systems. On the other hand, 27 of 33 (82%) had positive results by one assessment or the other. Only six patients (age 22-51 years) were free of ankle arthropathy by both systems (Table 4.13).

The case that was excluded from data analysis related to the radiology assessment was a 59 year old patient with mild haemophilia who had no ankle pain but had a physical score of 2 (bilateral reduced ankle ROM) (Patient code 49.1, Appendix III).

Figure 4.7 illustrates both the physical and radiological ankle scores of the 33 patients. The range of positive scores observed for the physical scoring system was 1-6, and that for the radiology evaluation

TABLE4.13 High Prevalence of Ankle Arthropathy in Patients with Mild/Moderate Haemophilia A

Number of Patients		Radiology Scoring System		Subtotal
		+ ve	- ve	
Physical Scoring System	+ ve	**16** (14)*	**10** (1)*	**26** (15)*
	- ve	**1** (1)*	**6** (0)*	**7** (1)*
Subtotal		**17** (15)*	**16** (1)*	**33** (16)*

One patient with a physical score of 2 was excluded from this table due to a lack of radiology assessment.

+ve: Sum of both ankle scores > 0;
-ve: Sum of both ankle scores = 0.

(n)*: Number of patients with ankle pain.

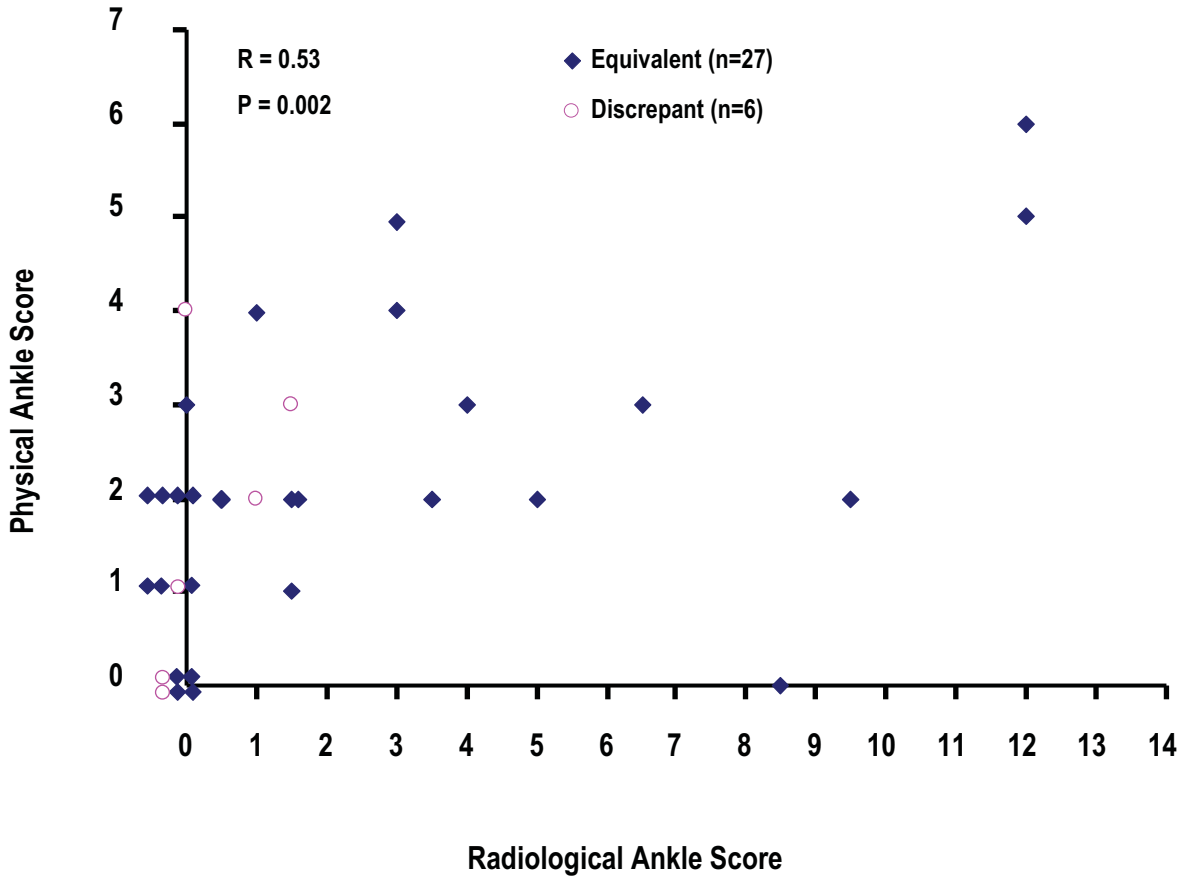


FIGURE 4.7 Comparison of Physical and Radiology Scoring Systems in Assessment of Ankle Arthropathy in 33 Patients

A range of positives scores observed in the study: 1-6 for the physical scoring system and 0.5-12 for radiology system. The radiology score for an individual was based on the average of the two scores determined by two independent radiologists, therefore it may contain decimal numbers.

Ten patients scored 1-4 by the physical evaluation but were normal by the radiology assessment.

One patient scored 8.5 by radiology but was normal by physical examination.

Nonparametric (Spearman) rank correlation: R = 0.53, P = 0.002.
Simple Kappa test for agreement: simple Kappa coefficient 0.3125 (positive).

was 0.5-12 (Figure 4.7). There was a moderate correlation (Spearman rank correlation R = 0.53, P = 0.002) between the two scores. This was confirmed by a simple Kappa test for agreement between the two variables. The estimated simple Kappa coefficient was positive (0.3215), suggesting the observed agreement between the two methods exceeds that expected to occur by chance alone.

There were 10 patients (median age 35.5 years) with normal X-ray evaluation but were found to have a positive physical ankle score (range 1-4) (Figure 4.7 and Table 4.13). The details of these ten patients were listed in Table 4.14. One of them (Patient code 132.1) had a VAS pain score of 5, and the remaining nine patients did not have ankle pain. There was significant reduced ankle dorsiflexion of 10-15° in the majority of the ten patients. In these nine cases, a single ankle was affected in four patients and both ankles were involved in five patients (Table 4.14).

It is of note that amongst the 7 patients who had a normal ankle on physical examination, 6 were also normal by radiology assessment (Figure 4.7 and Table 4.13). The seventh patient (Patient code 35.2, Appendix III, age 48 years) however, had a high radiology score of 8.5 and had moderate degree of ankle pain.

4.3.5.2 Relationship to age

Figure 4.8 reveals a weak association with borderline statistical significance (Spearman rank correlation coefficient R = 0.36, P = 0.04) between the age and the physical ankle score in the 33 patients with mild/moderate haemophilia A (Figure 4.8 panel A). In contrast, age seemed to form a moderate correlation with the radiology ankle score (R = 0.56, P = 0.0008) (Figure 4.8 panel B). Hence age appeared to have a different effect on the radiological scores to that of the physical scores. There was a tendency for the elderly patients to have a higher radiological ankle score than those younger patients.

TABLE 4.14 Ten Patients with a Positive Physical Ankle Score and a Normal Radiology Score

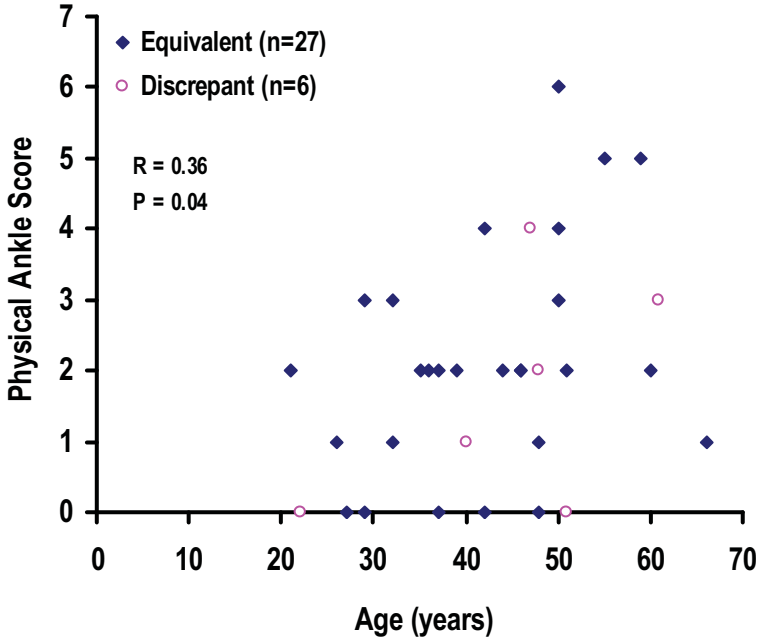
	Patient Code	Age (years)	VAS Pain Score	Loss of Dorsi-flexion (R / L, °)	Loss of Plantar Flexion (R / L, °)	Loss of Total ROM (R / L)	Physical Score (R / L)	Radiology Score (R / L)
1	4.5	46	0	20 / 10	0 / 0	20 / 10	1 / 1	0 / 0
2	4.6	36	0	10 / 5	5 / 0	15 / 5	1 / 0	0 / 0
3	7.2	32	0	5 / 10	0 / 10	5 / 20	0 / 1	0 / 0
4	9.1	21	0	10 / 10	0 / 10	10 / 20	1 / 1	0 / 0
5	24.3	26	0	15 / 5	0 / 0	15 / 5	1 / 0	0 / 0
6	28.1	47	0	15 / 15	10 / 10	25 / 25	2 / 2	0 / 0
7	46.1	40	0	10 / 0	0 / 5	10 / 5	1 / 0	0 / 0
8	49.2	66	0	0 / 0	10 / 5	10 / 5	1 / 0	0 / 0
9	121.1	35	0	15 / 15	5 / 0	20 / 15	1 / 1	0 / 0
10	132.1	29	5	10 / 10	0 / 10	10 / 20	1 / 1	0 / 0

One of these 10 patients had a pain score of 5 (Patient code 132.1), while the other 9 were free of ankle pain. The median age was 35.5 years for the 10 patients.

Reduced ankle ROM with mainly reduced dorsiflexion accounted for the positive ankle scores in these patients.

(R / L, °): Loss of flexion in degree for the right and left ankle.

A



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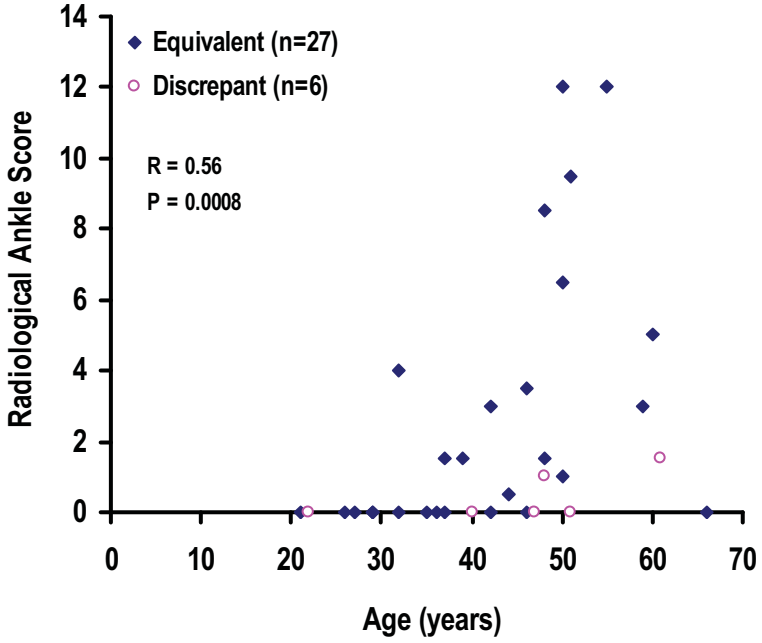


FIGURE 4.8 The Relationship between Age and Physical or Radiology Ankle Score in 33 Patients

Nonparametric (Spearman) rank correlation for:

A. Age and physical ankle score: R = 0.36, P = 0.04;

B. Age and radiology ankle score: R = 0.56, P = 0.0008.

4.3.5.3 Relationship to pain score

Of the 34 patients, 16 had ankle pain, including ten cases of bilateral involvement (Table 4.6 B). It would be useful to know whether the symptom of ankle pain is a reliable indicator for the presence of ankle arthropathy. Table 4.15 panel A reveals that the presence of ankle pain had a high sensitivity (88%) and specificity (94%) in predicting ankle arthropathy as assessed by the radiology scoring system in the 33 patients ($\chi^2 = 22$, $P < 0.0001$). However, pain seemed to have only a moderately sensitive predictor (58%) of ankle arthropathy as detected by the physical evaluation, although the specificity remained good (86%) (Table 4.15 A).

The use of a positive physical score and concurrent presence of pain as a combined parameter to predict ankle arthropathy offers a close association with the radiology system ($\chi^2 = 19$, $P < 0.0001$) (Table 4.15 panel B). This suggests that a combination of the physical scoring system and pain evaluation may provide an alternative to the X-ray exposure in the detection of ankle arthropathy in these patients. The present result indicate that it would be preferable to use the radiology scoring system or the VAS pain scores, rather than the physical scoring system alone, to predict the presence and/or monitor the course of ankle arthropathy in this study.

Figure 4.9 panel A demonstrates a moderate correlation between the VAS pain score and the physical ankle score ($R = 0.63$, $P < 0.0001$). Compared with the physical scores, the radiology scores was more closely associated with the VAS pain score with a higher R (0.79) (Figure 4.9 B). The P value of Fisher's exact test was ($P = 0.0015$ for panel A and $P < 0.0001$ for panel B), confirmed that the presence of a correlation between either the physical or radiology scoring system and the pain score evaluation was statistically significant.

It is noted that two patients (Patient code 4.1 and 39.1, Appendix III) were free of ankle pain but had ankle arthropathy by both scoring systems. The scores were relatively low: at 1 and 2 by physical examination, and 1.5 and 1 by X-ray evaluation respectively (Figure 4.9 A and B).

TABLE 4.15 The Presence of Ankle Pain in Prediction of Ankle Arthropathy

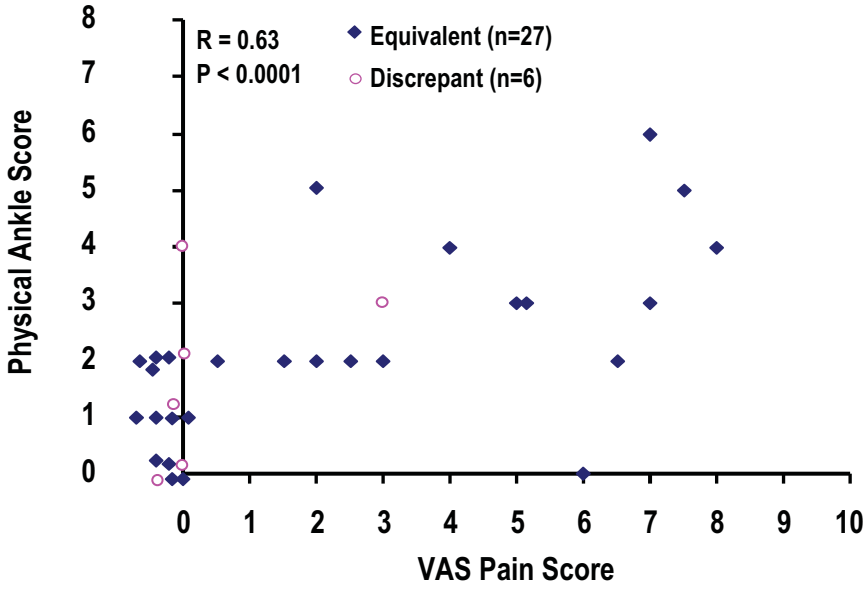
A

Number of Patients		Physical Scoring System		Radiology Scoring System		Subtotal
		+ ve	- ve	+ ve	- ve	
Pain	Present	15	1	15	1	16
	Absent	11	6	2	15	17
Subtotal		26	7	17	16	33
Sensitivity		***58%***		***88%***		
Specificity			***86%***		***94%***	
χ^2		*22.2*		*4.2*		
P		*< 0.0001*		*0.04*		

B

Number of Patients		Radiology Scoring System		Subtotal
		+ ve	- ve	
Combination of Physical and Pain Scoring Systems	+ ve	14	1	15
	- ve	3	15	18
Subtotal		17	16	33
Sensitivity		***82%***		
Specificity			***94%***	
χ^2		*19.3*		
P		*< 0.0001*		

A



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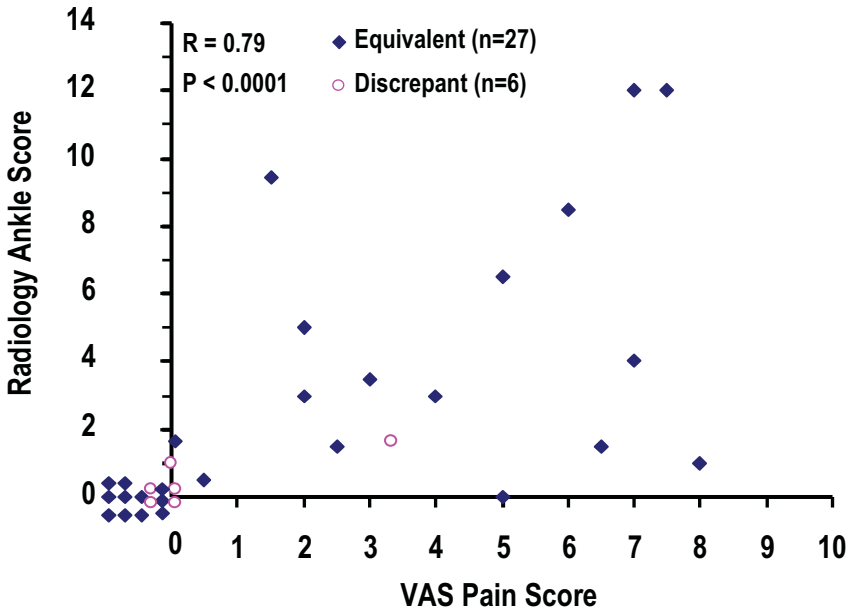


FIGURE 4.9 The Relationship between VAS Pain Score and Physical or Radiology Ankle Score in 33 Patients

Nonparametric (Spearman) rank correlation for:

A. VAS pain score and physical ankle score: R = 0.63, P < 0.0001;

B. VAS pain score and radiology ankle score: R = 0.79, P < 0.0001.

When the VAS "total" pain score was used, R = 0.65 (P < 0.0001) and 0.82 (P < 0.0001) for A and B respectively.

4.3.6 FVIII level and presence and severity of ankle arthropathy

4.3.6.1 FVIII level and pain score

The relationship between the VAS pain score and the FVIII level of the 34 patients was assessed in Figure 4.10 (A and B). There was a weak negative correlation between the FVIII level by one-stage assay and the VAS pain score (R = - 0.37) (Figure 4.10 A). The correlation between the FVIII level by the two-stage assay and the VAS pain score was approaching just marginal significance (P = 0.06, Figure 4.10 B). This result was consistent with what was previously shown in Figure 4.5 (A and B), where a difference in the one-stage FVIII, but not the two-stage FVIII, in the two subgroups with the present or absent ankle pain.

4.3.6.2 FVIII level and physical or radiology ankle scores

4.3.6.2.1 FVIII and physical scores

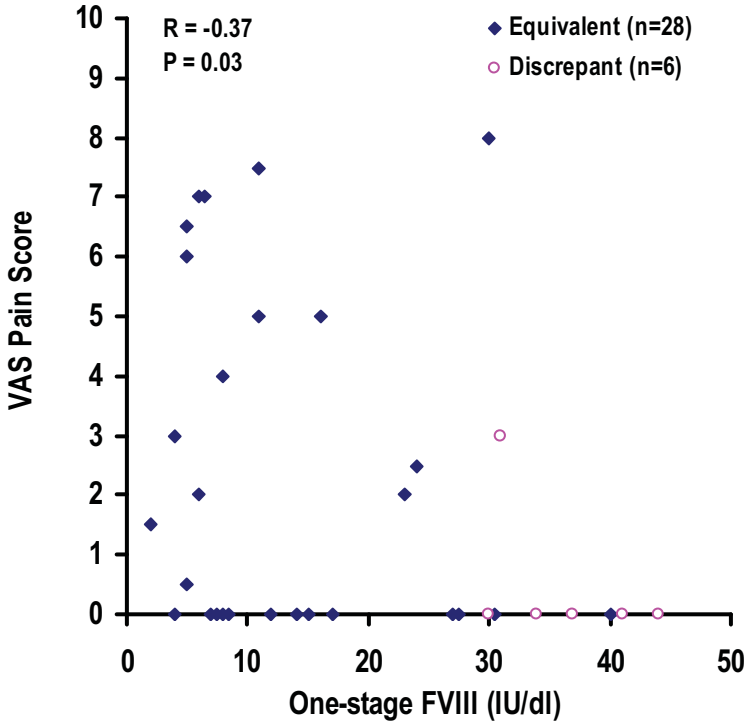
To examine the effect of FVIII level on the presence/severity of ankle arthropathy, as assessed by the two scoring systems in the 33 patients, FVIII level (one-stage and two stage) was plotted against the physical ankle scores (Figure 4.11 A and B) and the radiology ankle scores (Figure 4.12 A and B).

In Figure 4.11, the 26 patients who had ankle arthropathy (score > 0) displayed a wide range of a one-stage FVIII level of 2-44 IU/dl (panel A), and two-stage FVIII of 3-27 IU/dl (panel B). Similarly, in the patients who had normal ankles (n = 7), the FVIII level by the two assays was also spread across a wide range (Figure 4.11 A and B). Hence there was no apparent correlation between the physical ankle score and the FVIII level by either assay. This was confirmed by the analysis of Spearman rank correlation, which failed to show any statistically significant correlation.

4.3.6.2.2 FVIII and radiology scores

In contrast, Spearman rank correlation revealed a significant negative correlation between the radiology ankle score and the one-stage (R = - 0.49) or two-stage FVIII (R = -0.55) in 33 patients (Figure 4.12

A



B

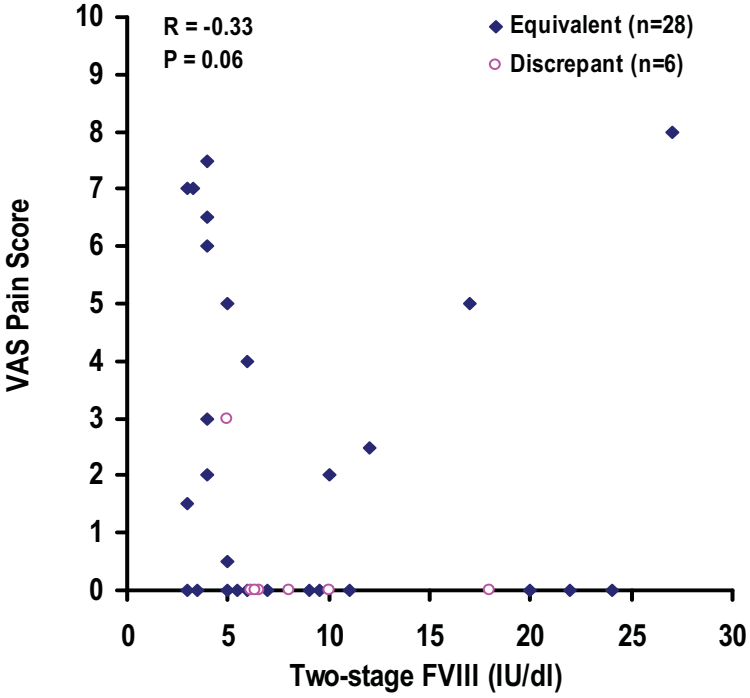


FIGURE 4.10 The Relationship between FVIII Level and VAS Pain Score in 34 Patients

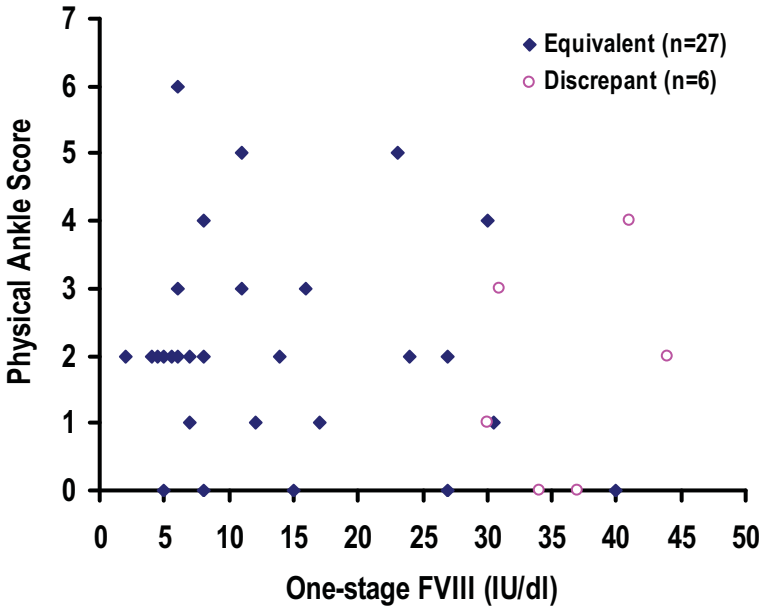
Non-parametric Spearman rank correlation for:

A. One-stage FVIII and VAS pain score: R = - 0.37, P = 0.03;

B. Two-stage FVIII and VAS pain score: R = - 0.33, P = 0.06.

When the VAS "total" pain score was used, R = - 0.37 (P = 0.03) and – 0.36 (P = 0.04) for A and B respectively. Two-stage FVIII correlated with VAS "total" pain score with borderline significance.

A



B

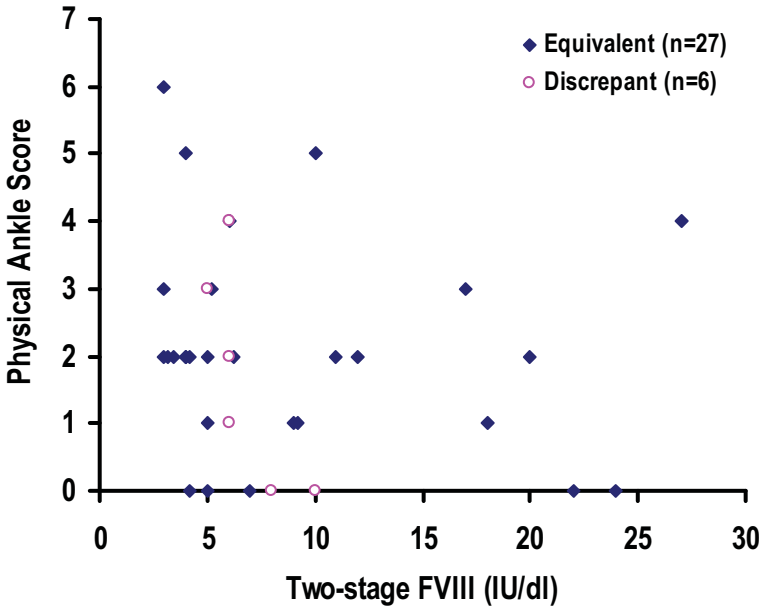


FIGURE 4.11 The Relationship between FVIII Level and Physical Ankle Score in 34 Patients

By visual inspection, there appears to be no apparent threshold effect of either the one-stage or the two-stage FVIII level on the physical ankle scores.

The analysis for correlation (non-parametric Spearman rank correlation) is not significant:

A. One-stage FVIII and physical ankle score: R = - 0.14, P = 0.43;

B. Two-stage FVIII and physical ankle score: R = - 0.25, P = 0.16.

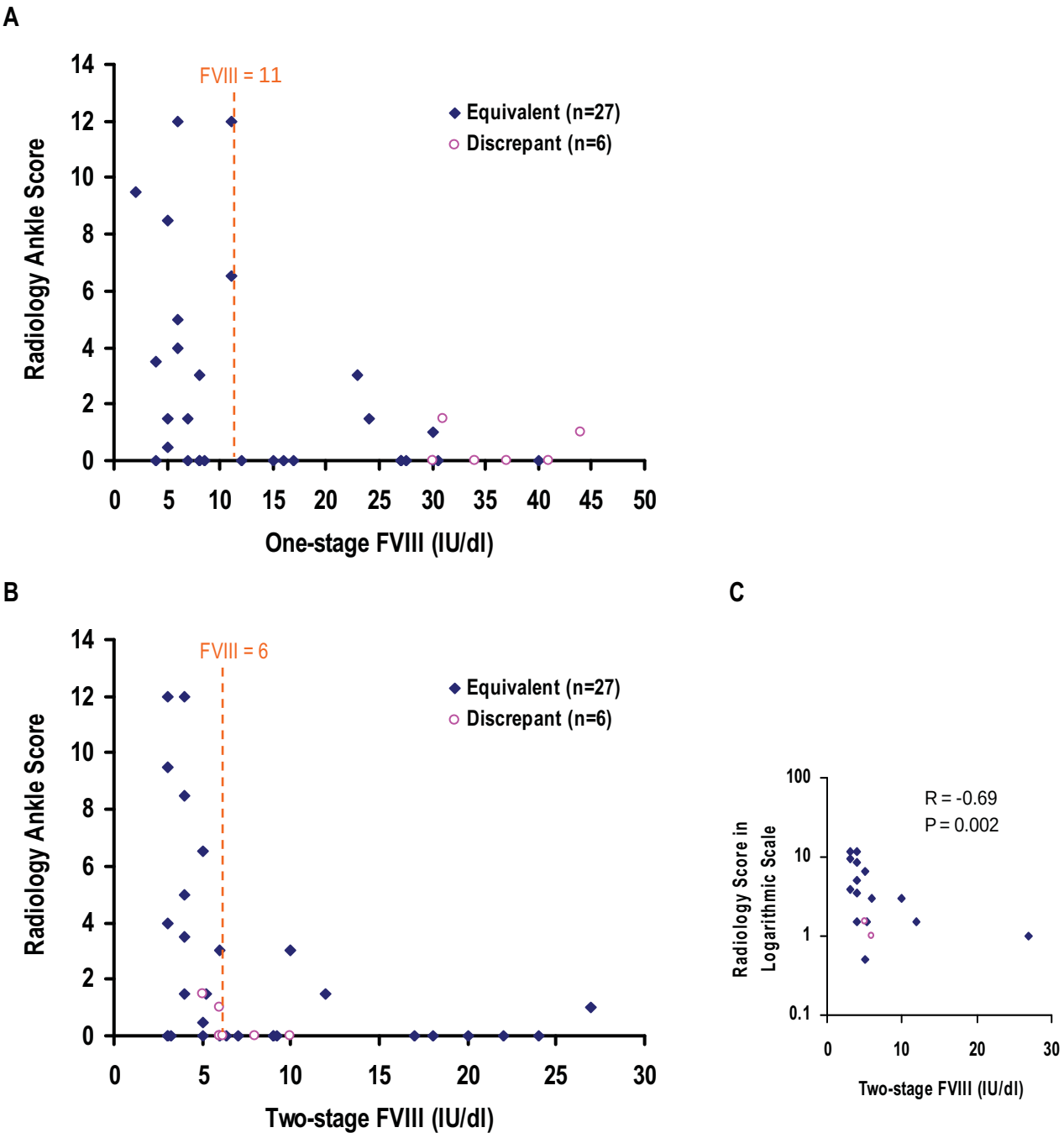


FIGURE 4.12 The Relationship between FVIII Level and Radiology Ankle Score in 33 Patients

By visual inspection, the threshold effect of the FVIII level on the presence of ankle arthropathy by radiology evaluation appears to be: at approximately 11 IU/dl by the one-stage FVIII assay (A), and 6 IU/dl by the two-stage FVIII assay (B).

The analysis for correlation (non-parametric Spearman rank correlation) is significant:

A. One-stage FVIII and radiology ankle score: R = - 0.49, P = 0.004;

B. Two-stage FVIII and radiology ankle score: R = - 0.55, P = 0.001;

C. Examining the 17 patients with a positive radiology score separately (excluding the 16 patients with normal ankles), the correlation between the radiology score (in log scale) and the two-stage FVIII was better (R = -0.69) than that for the total of the 33 patients (R = -0.55), as in panel B.

panels A and B). In the 17 patients with a positive radiology score (excluding the 16 patients with normal ankles), the correlation between the radiology score (in logarithmic scale) and the two-stage FVIII level was good (R = -0.69) (Figure 4.12 panel C). Because the results of the radiology score were not normally distributed, it was important to use the logarithmic transformation of the data for analysis (Figure 4.12 C).

In addition, by visual inspection, there appeared to be a threshold effect of one-stage FVIII on the presence of ankle arthropathy. When the one-stage FVIII was equal to and/or less than 11 IU/dl (n = 16), there was an increase in the occurrence of ankle arthropathy (n = 12) (Figure 4.12 panel A). There was also an increase in the severity of arthropathy (n = 8), as indicated by the presence of the relatively high ankle scores (i.e., those ≥ 3.5), which was absent in the patients with FVIII > 11 IU/dl.

Table 4.16 (A and B) summarises the results of Fisher's exact test used to analyse the threshold effect of one-stage FVIII of 11 IU/dl on the presence (panel A) and severity (panel B) of ankle arthropathy by radiology assessment. The threshold effect was confirmed in both cases (significant P values).

When the two-stage FVIII level was analysed, the threshold effect of FVIII on the presence of ankle arthropathy was again observed (Figure 4.12 panel B). In the patients with a two-stage FVIII levels of ≤ 6 IU/dl (n = 21), 14 had a positive radiology ankle score, eight of whom had a score ≥ 3.5. The Spearman rank correlation was significant for a negative correlation between the two-stage FVIII and the radiology ankle score in the 33 patients (R = - 0.55) (Figure 4.12 B).

Fisher's exact test confirmed the threshold effect of two-stage FVIII levels of 6 IU/dl on the presence of ankle arthropathy (Table 4.17 A). The analysis further confirmed the presence of a more severe arthropathy when FVIII level was equal to or less than the threshold (Table 4.17 B).

TABLE 4.16 Fisher's Exact Test for Association between Radiology Ankle Score and One-stage FVIII Level

A

Number of Patients		One-stage FVIII (IU/dl)		Subtotal
		≤ 11	**> 11**	
Radiology Ankle Score	**> 0**	12	5	17
	= 0	4	12	16
Subtotal		16	17	33
Fisher's Exact Test		***P = 0.01***		

B

Number of Patients		One-stage FVIII (IU/dl)		Subtotal
		≤ 11	**> 11**	
Radiology Ankle Score	**≥ 3.5**	8	0	8
	< 3.5	8	17	25
Subtotal		16	17	33
Fisher's Exact Test		***P = 0.001***		

A. When the one-stage FVIII was ≤ 11 IU/dl, there was in increase in the frequency of patients who had ankle arthropathy by the radiology scoring system.

B. When the one-stage FVIII was < 11 IU/dl, there was also an increase in the frequency of patients who had a relatively high positive radiology scores (≥ 3.5).

Fisher's exact test for the above observations were both significant.

TABLE4.17 Fisher's Exact Test for Association between Radiology Ankle Score and Two-stage FVIII Level

A

Number of Patients		Two-stage FVIII (IU/dl)		Subtotal
		≤ 6	**> 6**	
Radiology Ankle Score	**> 0**	14	3	17
	= 0	6	10	16
Subtotal		20	13	33
Fisher's Exact Test		***P = 0.01***		

B

Number of Patients		Two-stage FVIII (IU/dl)		Subtotal
		≤ 6	**>6**	
Radiology Ankle Score	**≥ 3.5**	8	0	8
	< 3.5	12	13	25
Subtotal		20	13	33
Fisher's Exact Test		***P = 0.01***		

A. When the two-stage FVIII was ≤ 6 IU/dl, there was in increase in the frequency of patients who had ankle arthropathy by the radiology scoring system.

B. When the two-stage FVIII was ≤ 6 IU/dl, there was also an increase in the frequency of patients who had a relatively high positive radiology scores (those ≥ 3.5).

Fisher's exact test for the above observations were both significant.

4.3.6.2.3 Comparison of physical and radiology scores in the relationship to FVIII

Using Spearman rank correlation, the physical scoring system did not form a significant association with the FVIII level, either by the one-stage or the two-stage assay. By visual inspection, it was also difficult to identify any threshold effect of FVIII levels on the presence of ankle arthropathy by this method (Figure 4.11). In addition, the physical ankle scores showed only a moderate association (R = 0.63) with the VAS pain score (Figure 4.9 panel A).

In contrast, the radiology system showed a good correlation with the VAS pain score (R = 0.79) (Figure 4.9 panel B). Using radiology scores, a threshold effect of either one- or two-stage FVIII was also confirmed (Figure 4.12 and Tables 4.16 and 4.17). Therefore the results thus far favour the radiology scoring system over the physical scoring system as the more objective method to evaluate the presence of ankle arthropathy in the current group of patients.

4.3.6.2.4 Mild vs moderate haemophilia and ankle arthropathy

Sixteen of the 33 (48%) patients had ankle arthropathy by both physical and radiology scoring systems. These 16 patients consisted of 11 with moderate range (2-5 IU/dl) of two-stage FVIII, and five with mild range (> 5 IU/dl). Therefore if dividing the 33 patients into two subgroups of moderate (n = 15) and mild (n = 18) haemophilia, the prevalence of ankle arthropathy in each subgroup was 73% (11/15) and 28% (5/18) respectively (Table 4.18). Ankle arthropathy occurred in almost one-third of patients with mild haemophilia A and in three-quarters of moderate cases.

4.3.6.2.5 FVIII assay discrepancy and ankle arthropathy

Table 4.19 summarises a subgroup of 6 *discrepant* patients in the current study. All had a relatively high one-stage FVIII level (30-40 IU/dl), and a much lower two-stage FVIII level (5-10 IU/dl). Despite pain only presenting in one of the six patients, four had a positive physical ankle score, two of whom also had a positive radiology score. Most of the scores were in the low range. It is possible that while the one-stage FVIII results seem to underestimate the clinical severity of the disease in the *discrepant*

TABLE 4.18 Ankle Arthropathy in Mild or Moderate Haemophilia A

Number of Patients		Haemophilia A**		Subtotal
		Mild	Moderate	
Ankle Arthropathy*	+ ve	5 (28%)	11 (73%)	16
	- ve	13 (72%)	4 (27%)	17
Subtotal		18 (100%)	15 (100%)	33 patients†
Fisher's Exact Test		*P= 0.0015*		
Chi-Square Test		$\chi^2 = 6.8$, *df = 1, P = 0.009*		
Median FVIII (IU/dl)	One-stage	27	6	
	Two-stage	9.5	4	

*: Ankle arthropathy by both physical and radiology scoring systems.

**: Classification of mild (> 5 IU/dl) or moderate (2-5 IU/dl) of haemophilia A based on the two-stage FVIII.

†: 33 patients. One patient with mild haemophilia A and absence of radiology assessment was excluded from this analysis.

TABLE 4.19 *Discrepant* Patients

	Patient Code	Age (years)	VAS Pain Score	Physical Score	Radiology Score	One-stage FVIII (IU/dl)	Two-stage FVIII (IU/dl)
1	8.1	51	0	0	0	34	10
2	14.1	22	0	0	0	37	8
3	28.1	47	0	4	0	41	6
4	39.1	48	0	2	1	44	6
5	40.1	61	3	3	1.5	31	5
6	46.1	40	0	1	0	30	6

Four of the 6 *discrepant* patients had a positive physical ankle score, 2 of whom also had a positive radiology score. Most of the scores were at the low range.

Ankle pain was present only in 1 of the 6 patients (Patient code 40.1), with consistently positive physical and radiology scores and a two-stage FVIII of moderate range.

patients, the two-stage FVIII results may have overestimated it. We do not have any statistical evidence to confirm or reject this suggestion due to an insufficient number of the *discrepant* patients in the study.

4.3.6.3 Analysis of FVIII level and ankle scores using Receiver-Operator Characteristic (ROC) curve

In addition to the methods discussed above, which used visual inspection to identify a cut-off (threshold) value of FVIII, a ROC curve analysis was also used to examine the effect of FVIII on predicting arthropathy. A series analysis for sensitivity and specificity of the prediction of arthropathy according to variable cut-off FVIII levels were obtained by using two statistic softwares: the Prism 4 and SAS 9.1. The results from both softwares were similar and the data summarised here was from the Prism program.

Figure 4.13 (A and B) illustrates that using either the one-stage (A) or two-stage (B) FVIII assay, a ROC curve can be successfully generated to predict a normal or arthritic ankle as defined by the radiology scoring system. The calculated sensitivity and "1-specificity" for each cut-off FVIII level were shown in the Y axis and X axis, respectively. The P value was significant: at 0.026 for the one-stage FVIII (Figure 4.13 A) and 0.017 for two-stage FVIII (Figure 4.13 B). The AUC was 0.728 and 0.744 for the one- and two-stage results respectively.

There is inflexion of the ROC curve in both panel A and panel B (Figure 4.13). The corresponding FVIII level of this inflexion likely represented the best cut-off value of FVIII to predict arthropathy in the study. In Figure 4.13 panel A, the best cut-off value of one-stage FVIII was approximately 11.5 IU/dl, as it gave a moderate sensitivity (75%) and specificity (71%). Alternative cut-offs in the curve seemed to result in compromised results, e.g., a cut-off of 7.5 IU/dl would improve the sensitivity to 88% at the expense of a decreased specificity to 53%. Nevertheless, the results from the ROC analysis were similar to that of the visual inspection method (Figure 4.12 A), in which a one-stage FVIII of 11 IU/dl was found to be significantly discriminative in the prediction/identification of ankle arthropathy.

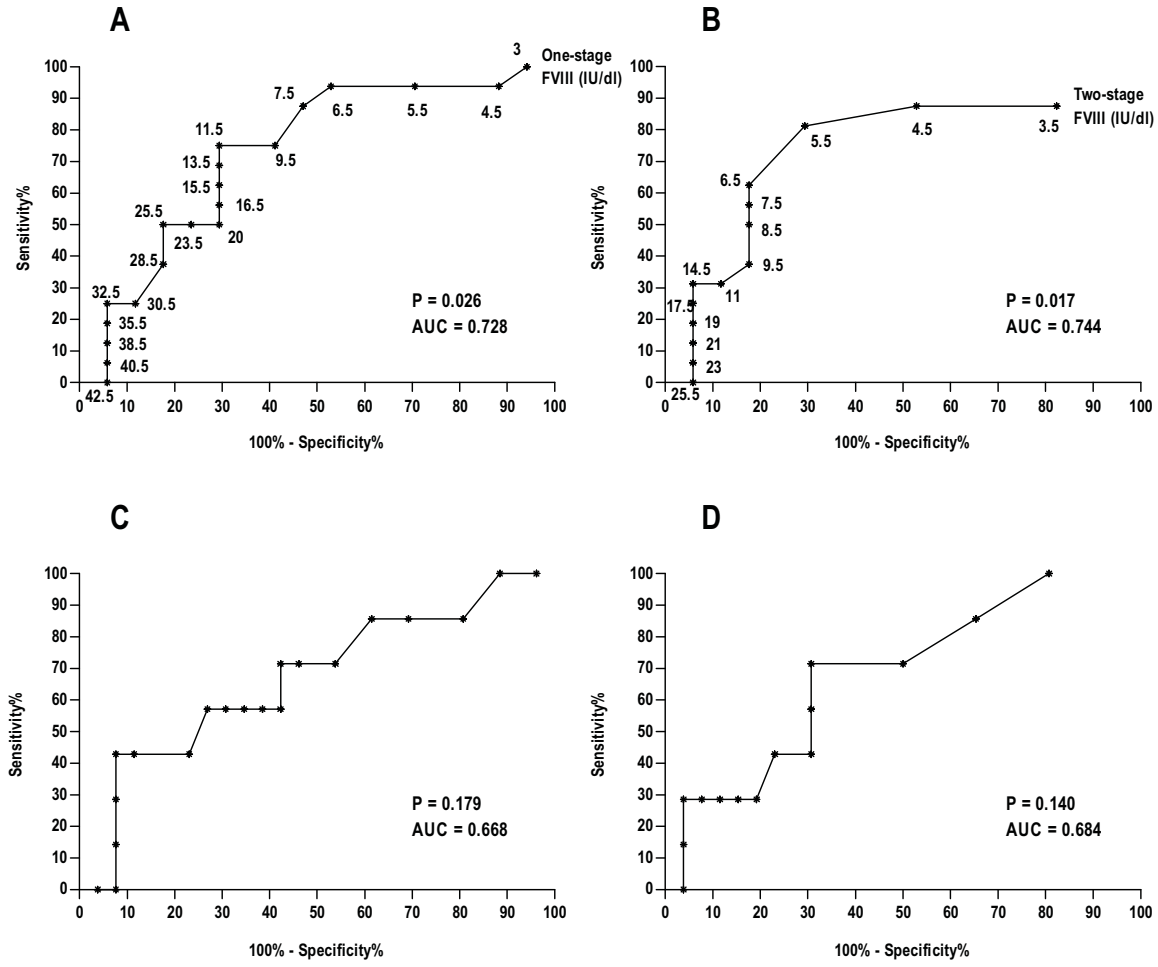


Figure 4.13 ROC Curves for FVIII Level to Predict Ankle Arthropathy

A. One-stage FVIII to predict arthropathy by radiology scoring system.

B. Two-stage FVIII to predict arthropathy by radiology scoring system.

C. One-stage FVIII to predict arthropathy by physical scoring system (non-significant).

D. Two-stage FVIII to predict arthropathy by physical scoring system (non-significant).

AUC: Area under curve.

Using the two-stage FVIII assay in the ROC curve analysis (Figure 4.13 panel B), a cut-off FVIII of 6.5 IU/dl would give a sensitivity of 63% and specificity of 82%. A closer cut-off value to this would be 5.5 IU/dl, with an increased sensitivity of 81% but a reduced specificity of 71%. These results were also consistent with of the visual inspection method for the threshold level, where a two-stage FVIII of 6 IU/dl was considered to be useful in identifying ankle arthropathy by the radiology assessment (Figure 4.12 panel B).

Figure 4.13 panels C and D show the ROC curves using the one-stage (C) and two-stage (D) FVIII in identifying ankle arthropathy by the physical scoring system. In this case the ROC analysis was unsuccessful statistically (insignificant P values for both one- and two-stage FVIII results). This suggested that in this group of patients, it was unable to identify a discriminative FVIII level that could predict ankle arthropathy as defined by the physical evaluation system. This was consistent with the earlier analysis that using visual observation, it was difficult to identify any threshold effect of FVIII on the presence of ankle arthropathy by the physical scoring system (Figure 4.11).

4.3.7 Correlation between ankle arthropathy and TGA results

We further explored whether the clinical severity of mild haemophilia, as maybe indicated by the severity of ankle arthropathy, could correlate with the TGA parameters. Here we choose the radiology assessment as the main evaluator for ankle arthropathy, as it was shown to be a more objective method than the physical or pain scores in the detection of ankle arthropathy in our group of patients. This was confirmed by a moderate correlation between the radiology score and the (two-stage) FVIII level (R = - 0.55, Figure 4.12), compared with a weak correlation between the pain score and FVIII (R = -0.33, Figure 4.10) or a lack of correlation between the physical score and FVIII (Figure 4.11).

The correlation between ankle arthropathy as diagnosed by radiology evaluation and the results of TGA parameters obtained using APTT reagent (Figure 4.14) or TF (Figure 4.15) are shown. The best

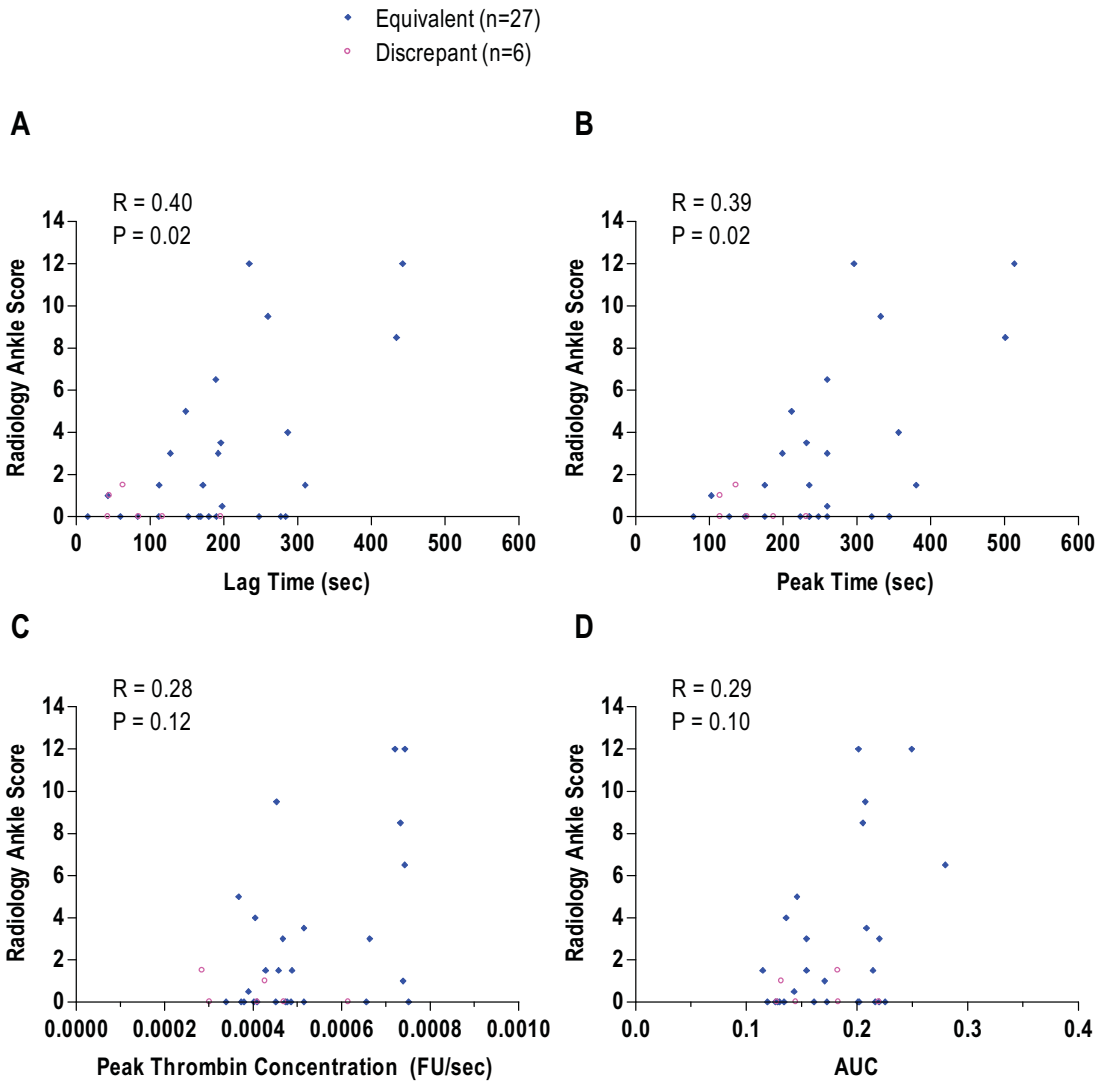


FIGURE 4.14 The Relationship between Ankle Arthropathy and TGA Using APTT Reagent in 33 Patients

A. Radiology ankle score and lag time.

B. Radiology ankle score and peak time.

C. Radiology ankle score and peak thrombin concentration (no significant correlation).

D. Radiology ankle score and AUC (no significant correlation).

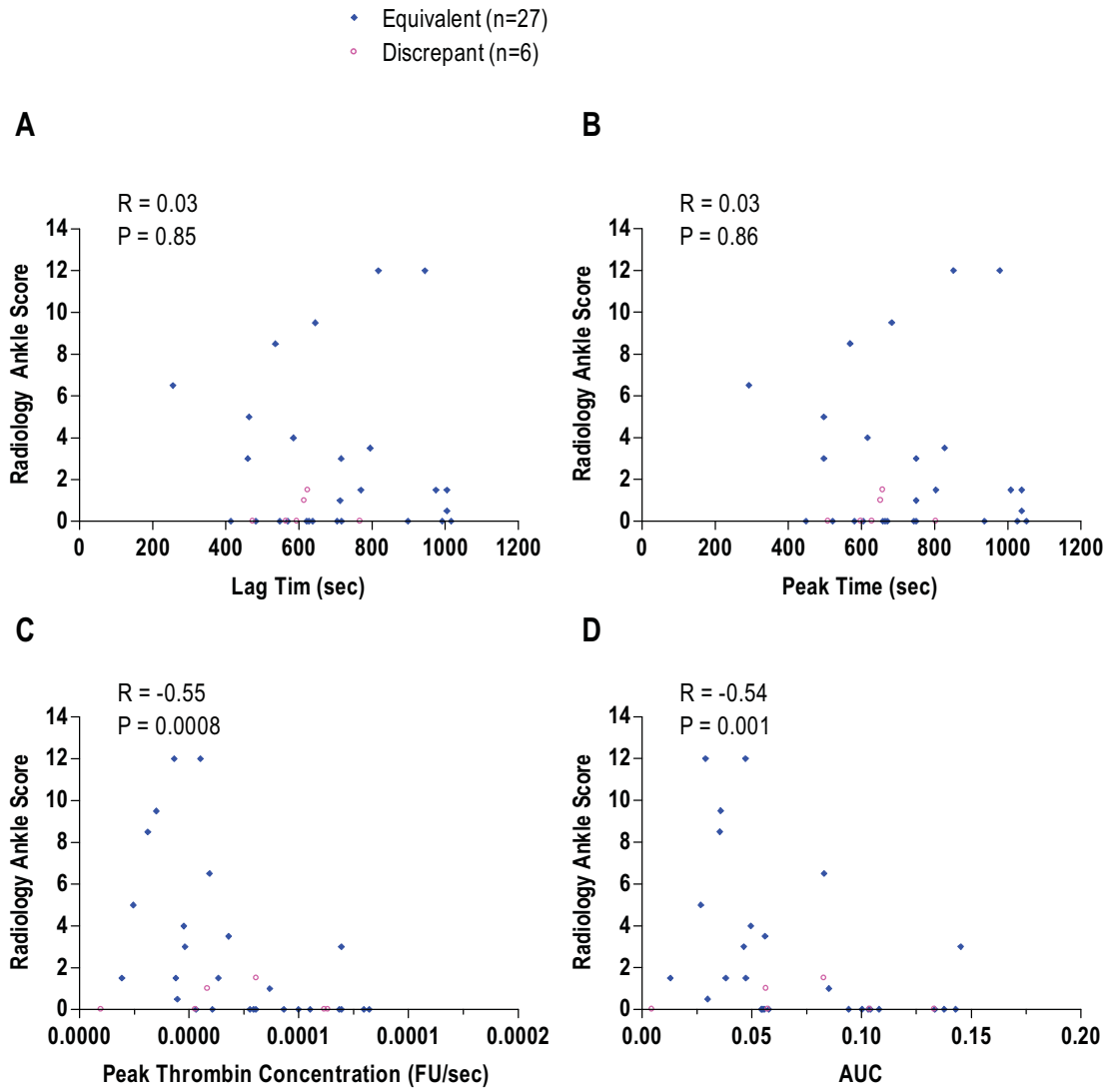


FIGURE 4.15 The Relationship between Ankle Arthropathy and TGA Using TF in 33 Patients

A. Radiology ankle score and lag time (no significant correlation).

E. Radiology ankle score and peak time (no significant correlation).

F. Radiology ankle score and peak thrombin concentration.

G. Radiology ankle score and AUC.

correlations of the radiology scores in the 33 patients were found to be with the peak thrombin concentration (R = -0.55) or AUC (R = -0.54) of TGA using TF (Figure 4.15 panels C and D). This strength of correlation was similar when the radiology score was analysed in conjunction with the one- (R = -0.49) or two-stage (R = -0.55) FVIII levels (Figure 4.12). Hence the TGA in its current form proved just as good as the traditional FVIII assays in providing laboratory evaluation of the disease severity that was phenotypically reflected by ankle arthropathy.

4.3.8 Some clinical aspects of ankle arthropathy and bleeding history

4.3.8.1 Arthritic symptoms and disability

Approximately half of the 34 patients studied (n = 16) suffered from regular ankle pain. The severity of the pain was moderate or severe in at least half of them (Table 4.6 A). In addition to the symptom of pain, ankle arthropathy affected the general mobility in the 34 patients to a significant degree. Among the 16 patients who had ankle pain, 10 had difficulties climbing (up and/or down) stairs and six were able to walk for less than 30 min (four were only able to walk for less than 10 min or 1 km). Five patients attributed lack of regular exercise to their ankle pain. Only three patients had ankle pain but without noticeable limitation on physical activities. Four patients required long-term analgesics including medications with a narcotic component. Although the majority of these patients do not use walking aids regularly, five have to wear modified shoes.

In addition to ankle pain, seven of the 16 patients also had pain in other joints. The joints included knee, hip, shoulder and elbow, though the details of pain in these joints were unknown (Table 4.20 panel A). For the 18 patients who did not have ankle pain, 10 had pain in other joints. The frequency of pain of large joints in the 34 patients is summarised in Table 4.20 A. In our past experience, pain in the knees of patients with mild/moderate haemophilia is not a commonly encountered clinical complaint, in contrast to the pain in the ankles. It may be helpful to include the evaluation of the knee joint in future studies on arthropathy in mild/moderate haemophilia A.

TABLE 4.20 Musculoskeletal Pain

A

Joint Pain	Number of Patients		Subtotal of Patients with Pain of Individual Joint
	In the 16 Patients with Presence of Ankle Pain	In the 18 Patients with Absence of Ankle Pain	
Ankle	16	0	**16**
Knee	6	8	**14**
Hip	2	1	**3**
Elbow	1	3	**4**
Shoulder	2	3	**5**

B

Musculoskeletal Pain		Number of Patients
Pain Present		
Joints		
	Ankle	16
	Other Joints	10
Soft Tissues / Muscles		3
Pain Absent		5
Subtotal		**34 patients**

Table 4.20 panel B summarises the overall presence of musculoskeletal pain in the 34 patients. Besides the 26 patients who had joint pain (involving the ankle or another joins, or both), another three patients instead had pain in soft tissues and muscles, e.g., thigh/calf, neck. In total, there were only five patients (age 7-59 years) who were free of musculoskeletal pain in this study group of 34 patients (Table 4.20 B).

4.3.8.2 Bleeding history and presence of ankle arthropathy

A brief bleeding history was collected during the clinical interview for each of the 34 patients with all due care taken to ensure absolute accuracy of the data. The approximate numbers of bleeding episodes in the 33 patients are summarised in Table 4.21. The result for one patient without radiology assessment was excluded from the analysis.

In the 16 patients who were determined ankle arthropathy by both the physical and radiology examinations, all except for one patient recalled a history of ankle bleeding and/or major ankle trauma (some episodes were treated with FVIII or other blood products) in their childhood and/or adolescence. The eight patients who had a relatively high radiology ankle scores (i.e,. range 3.5-12) inevitably showed multiple episodes (> 8 episodes) of remembered ankle haemorrhages (data not shown).

In the rest of the group (n = 17), where patients either had no ankle arthropathy by both methods (n = 6), or scored positive by only one method (n = 10 for the physical system and n = 1 for radiology system), four had ankle bleeding during early stage of life (childhood/adolescence) and another five had haemorrhage later in life.

Table 4.21 also shows that except for ankle bleeding, episodes of bleedings elsewhere (into other joints, soft tissues, urine etc.) were present in similar numbers of patients as for those with presence or absence of ankle arthropathy. There was no obvious difference in the frequency of the total bleeding episodes in these two subgroups of patients with or without ankle arthropathy either. It is noted that of

TABLE 4.21 Bleeding History

Number of Patients (P) or Episodes (E)	Ankle Bleeding		Other Joint Bleeding		Muscle Bleeding		Haematuria		Severe Epistaxis		Mouth Bleeding		Spontaneous Bruising		Bleeding Post-procedure without Peri-operative FVIII	
	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E
Subgroup with Ankle Arthropathy (N = 16)	15*	m	11	24	11	26	8	22	8	m	2	3	1	N/A	5	10
Subgroup without Ankle Arthropathy (N = 17)	9**	m	11	26	13	35	5	21	8	m	5	6	2	N/A	9	11

The data summarised here are from 33 patients. One was excluded from this analysis due to a lack of radiology assessment.
Sixteen of the 33 patients had ankle arthropathy by both the physical and radiology scoring systems. The remaining 17 patients formed the subgroup "without" ankle arthropathy.

P: Number of patients (who had bleeding events).
E: Number of (bleeding) episodes.
m: Multiple episodes.
N/A: Details not available.

15*: 14 of the 15 patients had ankle bleeds during their childhood/adolescence.
9**: 4 of the 9 patients had ankle bleeds during childhood/adolescence, while the other 5 had ankle bleeding later in life.

the 33 patients, 14 experienced episodes of excessive bleeding during and/or after invasive procedures (i.e., dental extraction, tonsillectomy) without peri-operative FVIII coverage (21 bleeding episodes out of 42 procedures) (Table 4.21).

Table 4.22 panels A and B demonstrates that there are strong statistical evidences supporting the observation that that the presence of ankle arthropathy was associated with the presence of ankle bleeding, multiple episodes of ankle bleeding and ankle bleeding early in life.

4.4 Discussion

The results of this study confirmed our hypothesis that there is a high prevalence of ankle arthropathy in the patients with mild and moderate haemophilia A. Although our clinical study group was not large (15 moderate cases and 18 mild cases by two-stage FVIII), we found almost three-quarters (73%) of the moderate patients and one-third (28%) of the mild cases had ankle arthropathy (Table 4.18). In approximately two-thirds of those who had ankle arthropathy, both ankles were affected.

The only recent published data on arthritis in mild/moderate haemophilia is a study that addressed the target level for prophylaxis in severe haemophilia (Fischer *et al*, 2000). In this study, 51% of the 35 patients with moderate haemophilia A (1/3 were on prophylaxis) were found to have a positive Pettersson score. However the scores were based on the combined assessment of 6 joints (ankles, knees and elbows). The scores for the individual joints were not published (Fischer *et al*, 2000). The median Pettersson score was 1 point (maximum score in the group: of 78 points) at the age of 25 years. Hence the authors considered that the arthritis in these patients was only “mild”. However, it was acknowledged that some patients did experience severe joint pathology: five patients had chronic synovitis and eight patients underwent a total of 11 orthopaedic surgeries, including three arthrodeses of the ankle (Fischer *et al*, 2000).

TABLE 4.22 Ankle Bleeding

A

Number of Patients		Episodes of Ankle Bleeding				Subtotal
		0	1 - 4	5 - 8	Multiple (> 8)	
Ankle Arthropathy	+ ve	1	3	2	10	**16**
	- ve	8	5	1	3	**17**
Subtotal		**9**	**8**	**3**	**13**	**33 patients**

B

Number of Patients		Ankle Bleeding		Multiple Episodes of Ankle Bleeding		Ankle Bleeding Early in Life		Subtota
		+ ve	- ve	+ ve	- ve	+ ve	- ve	
Ankle Arthropathy	+ ve	15	1	10	6	14	2	**16**
	- ve	9	8	3	14	4	13	**17**
Subtotal		**24**	**9**	**13**	**20**	**18**	**15**	**33 patients**
Fisher's Exact Test: P Value		***0.02***		***0.01***		***0.001***		

Sixteen of 33 patients had ankle arthropathy by both scoring systems, while the remaining 17 were ankle arthropathy "- ve".

Of the 16 patients who had ankle arthropathy, the majority had a history of multiple ankle bleeding (> 8 episodes), especially in their childhood and adolescence.

Fisher's exact test confirmed that the presence of ankle arthropathy was associated with early and multiple episodes of ankle bleeding.

The presence of ankle arthropathy in the present study was not only characterised by its high frequency, but also by its disabling consequences. For instance, among the 16 patients who had ankle arthropathy (by both the physical and radiology systems), nine had difficulties climbing stairs; four had a walking distance limited to less than 10 min or 1 km, and five attributed lack of regular exercise to their ankle problems. In addition, ankle arthropathy in this study was found to be highly associated with the symptom of pain. Nearly all of the 16 patients with ankle arthropathy (14/16) had ankle pain on a regular basis, four of them required long-term analgesics including medications with a narcotic component. Bilateral ankle pain was also common (10 of 16).

We consider that the 34 patients interviewed (median age 45 years) were representative of the 92 patients in the target population (median age 46): comparing the age distribution (Table 4.1 and Figure 4.1) and median FVIII level (Table 4.3 and Figure 4.2) there was no difference in the interview cohort and the database population.

Evaluation of Arthropathy

To evaluate ankle arthropathy we used three tools: the VAS pain score, the WFH physical and radiology scores.

Pain

Nearly half of the patients (16/34 = 47%) had ankle pain. This was similar to the proportion of patients with ankle pain (41%) in the 65 patients who returned our initial survey (Table 4.6 A). In both the interview cohort and the 65 respondents, a similar percentage (approximately half) of those with ankle pain described the severity as moderate or severe. This further confirms that the 34 patients from the interview group was a good representation of the database population for the study of ankle arthropathy.

In the 34 patients at the interview, there was an excellent agreement between the categorisation of pain by “mild, moderate and severe” (description of pain severity consistent with WFH definition) and the

VAS pain scores. Eight patients had moderate to severe pain (4-8 on the VAS scale). The pain was disabling in the majority of these eight patients, limiting their general mobility and exercise tolerance.

Surprisingly, the degree of pain did not increase with age (Figure 4.4). This suggests that the scoring of pain is not sensitive indicator of the progression of the arthritis that may have occurred.

Physical Scoring System

Using the WFH physical scoring system to assess ankle arthropathy, 27 of the 34 patients (79%) had a positive ankle score (range 1-6). The most frequently detected sign was reduced ROM of the ankle joint: present in 26 patients (44 ankles affected). The majority (18 of 26) had bilateral ankle involvement.

The reduced ROM accounted for nearly all of the physical score. Other elements of the physical scoring system were not sensitive, although ankle swellings, crepitus on motion and calf muscle atrophy were found in several patients.

It was of interest to study the details of the reduced ankle ROM in the 26 patients. Most of the ankles (35 of 44 ankles) scored 1 (Table 4.9 and Figure 4.6). Loss of dorsiflexion (43 ankles) was more frequently present than loss of plantar flexion (21 ankles), and was more severe (Table 4.10). In most cases, it was the loss of dorsiflexion that contributed to a positive score.

In severe haemophilia, progressive loss of dorsiflexion of the ankle joint often results in fixed plantar flexion (equinus deformity) and greater difficulty in walking. None of our patients at interview were found to have severe permanent damage to their ankle joint such as equinus deformity. Nevertheless, it was noted that in the 34 patients, there were seven patients who either had constant difficulty descending stairs and/or required routine use of modified shoes. Of these seven patients, three had complete loss (20°) of dorsiflexion in one or both ankles, and three had the most severely reduced ROM (35-50°) in a

single ankle in this study. Hence the loss of dorsiflexion and/or total ROM to a severe degree was likely to be associated with clinically functional impairment of the ankle joint in this group of patients.

It is of interest that in the 34 patients, 12 (i.e., 11 of the 33 cohort, Table 4.15 A and Figure 4.9 A) did not present with ankle pain but had a positive score on physical examination. All 12 patients had a positive score (mostly 1 or 2) for reduced ROM; six with bilateral involvement and six with unilateral. Loss of dorsiflexion of 10-15° accounted for the score of 1 in the majority of the ankles affected (Appendix V). We do not know the significance of this small loss of ankle dorsiflexion in absence of pain. It is possible that there is early ankle arthropathy in these patients, as suggested by Johnson & Babbitt (1985). Examining 48 haemophilia patients (32 severe, 18 mild/moderate, mean age 24 years), the loss of dorsiflexion of the ankle (up to 15°), and loss of flexion of knee and extension of elbow, were the earliest likely affected loss of ROM in joints with radiologically defined early haemophilic arthropathy (Johnson & Babbitt, 1985). The authors concluded that early occurrence of ankle dorsiflexion, knee flexion and elbow extension often indicated intraarticular disease on roentgenogram (Johnson & Babbitt, 1985). However, it is also possible that the WFH physical scoring system alone is too sensitive for detecting ankle arthropathy in this group of patients, especially in those with absence of ankle pain.

Radiology

In parallel with the physical scoring system, we used the WFH radiology scoring system (Pettersson scale) to assess ankle arthropathy. By this method, the prevalence of ankle arthropathy was 52% (17/33) (range of score 0.5-12). Two-thirds of the patients (11/17 = 65%) who had a positive radiology score had bilateral arthropathy. This was consistent with the corresponding percentage of patients with bilateral ankle involvement in the evaluation by the pain score (63%) and physical score (70%).

The most frequently detected radiology signs in the 28 ankles from 17 patients with a positive radiology score were (in the order of frequency): irregular subchondral surface (21 of 28 ankles), narrowing of joint space (17 ankles), subchondral cyst formation (13) and enlarged epiphysis (11). These four signs

appeared to present in a somewhat clustered fashion in a single ankle: 16 of the 28 ankles had a combination of at least two of the four signs, including five patients with all four signs (Table 4.12). Other radiological features such as osteoporosis and erosion of joint margins were occasionally observed.

In the current study, the frequent radiology findings of irregular subchondral surface, subchondral cyst formation and narrowing of joint space in the ankles coincided with the equally frequent sign of loss of ankle ROM on physical examination. This observation was consistent with an earlier study, which concluded that the loss of cartilage articular space was the single most important roentgenographic finding related to reduced ROM in haemophilic arthropathy (Johnson & Babbitt, 1985). According to the authors, all the articular changes or those features directly related to the joint articulation and the subchondral bone, especially the cartilage loss, were probably the best reflector of the actual function of joint.

It is necessary to compare both the physical and radiology scoring systems in assessing ankle arthropathy in the current study. There was reasonable agreement between the two systems for identification of ankle arthropathy. Of the 33 patients, 16 were positive by both systems (14 with ankle pain), while 6 were negative by both systems (Table 4.13). The remaining 11 patients had discrepant results; 10 had a positive physical score (mostly 1 or 2) but normal X-ray (9 with absence of ankle pain), while one patient was normal on physical examination but had a high radiology score (8.5). Compared to the radiology system, the physical scoring system alone may be too sensitive for detection of ankle arthropathy in our patient group, especially in those with absence of ankle pain. However, there was a moderate correlation (R = 0.52) between the two scores in the 33 patients (Figure 4.7).

The results of our study on the correlations as well as the discrepancies between the physical and radiology scoring systems agreed with previous studies. A reasonable correlation (Pearson correlation coefficient R = 0.59) between physical (paediatric modification of WFH score) and plain X-ray scores

(Pettersson scale) was reported when it was used to evaluate 165 joints (69 ankles, 53 knees and 43 elbows) from 40 paediatric patients with haemophilia (27 severe haemophilia A, 8 moderate haemophilia A) (Pergantou *et al*, 2006). Inconsistency of the two scores in individuals with haemophilia was also reported previously, e.g, negative X-ray findings in patients with a positive physical score (Lofqvist *et al*, 1997; Hill & Ljung, 2003), or positive radiology findings in normal joint by physical examination (Nilsson *et al*, 1992; Funk *et al*, 1998). In an earlier study using the standard WFH scoring systems in 52 patients with severe haemophilia A (mean age 28 years), the correlation between the physical and radiology scores for the knee joint was "close", but for the ankle and elbow joint was "comparatively poor" (Hamel *et al*, 1988).

There have been reports in recent years that both the WFH physical and radiology scoring systems were not adequately sensitive to detect early changes in joints and reveal precise disease process (Manco-Johnson *et al*, 2004; Pergantou *et al*, 2006). Some publications indicate that compared to the physical score, the radiology system (Pettersson scale) was more closely (R 0.48 vs 0.69) associated with MRI findings of joint changes in the 40 paediatric haemophilia A and B patients (32 severe and 8 moderate, mean age 12 years) (Pergantou *et al*, 2006). Our results suggested that the current WFH physical scoring system is more sensitive than the radiology scores in our group of mild and moderate haemophilia A patients, as it was able to identify ankle arthropathy in the subgroup with absence of ankle pain (nearly half of the study population). The underlying causes were unclear. It is worth noting that we could not exclude the presence of early ankle arthropathy in the patients with mild and moderate haemophilia that we examined, despite the argument about "over diagnosis" using the physical system alone. This is quite different to the study objects of the severe cases with established arthropathy in the work of others.

It is of practical interest to determine the suitability of using ankle pain in predicting arthropathy. Both the physical (R = 0.63) and radiology scores (R = 0.79) displayed a good correlation with the VAS pain scores. The presence of ankle pain was shown to be a strong indicator for predicting ankle arthropathy

by the radiological assessment, with a high sensitivity of 88% and specificity of 94% (Table 4.15 A). Pain seemed to be only a moderately sensitive (58%) predictor of arthropathy by physical examination. Nevertheless, when the physical scoring system is used in conjunction with the pain scores, it seems to achieve a reasonable sensitivity (82%) and good specificity (94%) in diagnosing ankle arthropathy (Table 4.15 B). This would suggest that combining pain score with the physical score can be an alternative to X-ray examination in the clinical setting in the identification of ankle arthropathy.

Correlation with FVIII Level

Using the radiology scoring system, there was a significant correlation between both one- and two-stage FVIII levels and radiology scores thus confirming our hypothesis that ankle arthropathy is related to a lower FVIII level. In addition, there was a threshold effect of FVIII level on the presence of arthropathy. This was demonstrated by visual inspection on examining FVIII level plotted against the radiology scores (Figure 4.12). When the one-stage FVIII level was equal to or less than 11 IU/dl or the two-stage FVIII level was equal to or less than 6 IU/dl, there was an increase in the frequency of positive radiology ankle scores. It is also of interest to note that the patients with FVIII levels less than the threshold level tend to have more severe arthropathy as indicated by a relatively high radiology score than that seen in patients with FVIII levels greater than the threshold. The above results were supported by a significant Fisher's exact test on the difference of the frequencies (Tables 4.16 and 4.17). In addition, in the 17 patients who had a positive radiology score, the correlation between the score and the two-stage FVIII was good (R = - 0.69) (Figure 4.12 C). This suggested that the radiology assessment was useful in reflecting the severity of ankle arthropathy.

Somewhat surprisingly, the FVIII level by either the one- or two-stage assay showed only weak or borderline correlation with the VAS pain scores (Figure 4.10) and did not form any significant correlation with the physical ankle scores (Figure 4.11). Patients with a wide range of FVIII level were found to have ankle arthropathy with a variable degree of pain and positive physical scores. Likewise, in those

patients who were pain free or had normal ankles on physical examination, the level of FVIII was not always relatively high.

As an alternative way to examine the relationship between the FVIII level and the presence of ankle arthropathy, a ROC curve analysis was performed (Figure 4.13). We found there was a convincingly positive clinical implication when using the radiology scoring system in the ROC curve analysis to predict ankle arthropathy. It was demonstrated in the ROC curve generation using different FVIII cut-off levels to identify the patients who had ankle arthropathy, as determined by a positive radiology score or a positive physical score. There was a significant P value and a moderate value of AUC when the radiology scores were used (Figure 4.13 A and B). This method also produced the best cut-off value of FVIII in this study, namely, 11.5 IU/dl by the one-stage and 6.5 IU/dl by the two-stage assay, very similar to the threshold FVIII observed previously through graphic effect. Both of the best cut-off values gave a moderate sensitivity (63-75%) and specificity (71-82%) in identifying ankle arthropathy by the radiology system in this group of patients (Section 4.3.6.3). The difference of the FVIII cut-off values by the two assays here in the prediction of arthropathy likely reflected the general difference of the FVIII results obtained in the current study (Figure 4.3). There was a degree of the drift of the FVIII assay results in our laboratory, the reasons of which unclear (Section 4.3.1.2.3). Other contributory factors for the difference in the cut-off values also include normal assay variations and the relatively small sample size.

Both the WFH physical and radiological scoring systems were evaluated in this study for their usefulness in detection of ankle arthropathy in mild and moderate haemophilia. We consider that the radiology scoring system is a better system than the physical scoring system at reflecting the presence and severity of ankle arthropathy due to its closer association with the VAS pain score and FVIII level. It is also useful for identifying the patients with a low level of FVIII who are at risk of developing ankle arthropathy. Therefore the radiology scoring system is useful to provide an objective measurement of disease progression and to examine treatment/intervention outcomes.

Other factors, such as age, were found to have a weak effect on the presence of ankle arthropathy in the current study. Age demonstrated a weak correlation with both the physical and radiology ankle scores (R = 0.36 and 0.56 respectively) (Figure 4.8). Although the correlation between age and the VAS pain score was not statistically significant (P = 0.095), there was an apparent trend of association (P = 0.095) (Figure 4.4). The best approach for assessing arthropathy was noted to be by radiological assessment, confirming our previous conclusion that radiology is the more objective and robust method of evaluation of ankle arthropathy.

History of Bleeding

It was very important to investigate the association of ankle bleeding with the presence of ankle arthropathy in the patients with mild haemophilia. However, this is limited by difficulties encountered when trying to produce a chronology of an individual's bleeding history, which may extend as far back as 40 years or more (the median age of patients in the current study: 45 years). This limits the accuracy of the retrospective review. Nevertheless, approximately three-quarters of the patients (24/33 = 73%) had a recollection of previous bleeding episodes into the ankle joint, with a small percentage of those affected claiming to be treated with FVIII or other blood products. While some had only very few episodes of ankle bleeding, more than half of the 24 patients (13 patients) had multiple episodes (> 8 episodes) (Table 4.22 B).

It was our hypothesis that the cause of ankle arthropathy in mild haemophilia A is due to recurrent ankle trauma in childhood that causes bleeding. Our results are consistent with this. There is strong statistical evidence supporting the hypothesis that the presence of ankle arthropathy in the current study was not only associated with the presence of previous ankle bleeding (P < 0.02), but also multiple episodes of ankle bleeding (P < 0,001). Furthermore, ankle bleeding early in life (i.e., childhood and/or adolescence), rather than in adulthood, was more likely to be associated with the presence of arthropathy (P < 0.001) (Table 4.22 B). Other bleeding manifestations, such as episodes of bleeding

into other major joints or haematuria, did not show any significant association with the presence of ankle arthropathy in these patients.

In recent years MRI has been used to improve the sensitivity of diagnosing early stages of arthritic disease and unmasking more advanced changes in cartilage or bones compared with plain X-ray examination. In the work of Pergantou *et al* (2006), the MRI in 165 large joints (69 ankles) from 40 paediatric patients with haemophilia (32 severe and 8 moderate) showed that chronic synovitis occurred in 55% and 50% of joints studied, which were diagnosed as normal by clinical (physical) score and Pettersson score respectively. Our pilot study of MRI showed results that were consistent with standard X-ray examination in five patients, including two patients with normal ankle scores by both physical and Pettersson scores. Further study of more patients would be required to fully evaluate the usefulness of MRI in mild haemophilia.

In addition, this study has provided a strategy to improve clinical care that will probably reduce the frequency of ankle arthropathy in patients with mild and moderate haemophilia A. We consider that in the current generation of patients there may have been recurrent haemorrhages (clinical or subclinical) into ankles that were inadequately treated.

It is our clinical impression that on many occasions, patients with mild/moderate haemophilia do not seek medical assessment for possible ankle sprains to determine whether treatment with FVIII concentrate is advisable to prevent or control haemorrhage. It is sensible to consider that more aggressive treatment on ankle haemorrhages in children with mild/moderate haemophilia A would maximise the chance of prevention and early reversal of joint damage, and hence reducing the risks of ankle arthropathy in adulthood. Therefore we should consider evaluation of protocols which include education of patients and their families. From the practical point of view all patients with mild/moderate haemophilia A should be warned of the dangers of untreated ankle bleeding. Given the inherent variability of FVIII assays and the need for a larger study, it has not been possible to decide on a reliable

FVIII level above which a particular patient is unlikely to develop ankle arthropathy. Pending the results of any further studies we recommend that patients with a FVIII level below 11 IU/dl should be warned to take all due care to avoid this complication.

There are however, limitations in the current study. Firstly, the sample size was relatively small. This also included an insufficient number of the *discrepant* patients in the study, which could negate any observation on which of the two FVIII assays could better predict with the severity of ankle arthropathy. However, the current data were able to highlight the discrepancy between the two assays. To overcome the shortcomings of sample size a multi-centre study is necessary.

We did not have an age-matched normal control group in the study. Bias can arise from the relatively old median age (46 years) in the database patient population. More effective treatment with FVIII replacement for bleeding was only readily available in Australia in the last 20 years or so. Bias can also arise from the recruiting of patients to participate in the clinical interview and assessment. Those who suffered symptoms were likely to be more interested in the study. Nevertheless, the 34 patients were a good representative of the 92 patients in the database, as demonstrated by the similar median age and median FVIII level between the two cohorts (Tables 4.1 and 4.3).

A control group was not adopted by the previous studies on haemophilic arthropathy using the physical or radiological scoring systems. This may be because an overwhelming majority of the subjects in these studies were severe haemophiliacs, where a control group was not required. It was also considered that when undertaking the physical examination, a patient's "healthy" limb could routinely serve as a comparison for the diseased side since the range of motion of left and right joints were consistently similar in the normal situation (Boone & Azen, 1979). Nevertheless, ideally a age-matched normal control group would be useful in the clinical studies of patients with mild and moderate haemophilia.

A further shortcoming in the study was that we did not include knee joints as a comparison to the ankle joint for investigation of arthropathy. Somewhat to our surprise, some of our patients with mild/moderate haemophilia A also gave a positive answer to the question about knee pain at the clinical interview (Table 4.20). To our clinical knowledge of these patients, pain due to arthritis in the knees has not been a commonly encountered problem. It would be of interest to include both the ankle and knee joint in the future study on arthropathy in mild/moderate haemophilia to gain further knowledge of this aspect.

From the current study, we not only identified that ankle arthropathy is prevalent and disabling in our group of adult patients with mild and moderate haemophilia A, we also demonstrated that the presence and severity of ankle arthropathy is positively correlated to a lower FVIII level and a history of ankle haemorrhage, especially when it commenced early in life and involved multiple episodes. There is both scientific and clinical significance of these results. This study is the first to allude to the scale of chronic ankle arthropathy in mild and moderate haemophilia A, a seemingly under-studied and infrequently reported complication. The result further indicated that once ankle arthropathy developed, there is a solid negative impact in these patients. Using three different scores (pain, physical and radiology), this study confirmed the utility of standard methodology in assessment of ankle arthropathy in mild and moderate haemophilia A.

4.5 Summary

1) We were the first to investigate the prevalence and severity of ankle arthropathy in adult mild and moderate haemophilia A. In the 34 patients that we were able to study in detail, the median age (45 years) and median FVIII level (13 and 6 IU/dl by one- and two-stage FVIII respectively) was a good representation of the 92 patients in the South Australia haemophilia database.

2) We studied the relative usefulness of the current WFH physical and radiology scoring systems in the detection of ankle arthropathy in the 34 patients, and also used a VAS pain score to determine

the severity of pain. In addition, we examined the effect of FVIII level on the prevalence of ankle arthropathy.

3) The prevalence of ankle arthropathy in our group of 34 patients with mild/moderate haemophilia A was as high as 52% by the radiology scoring system alone, and 48% by both the physical and radiological systems. Ankle pain (63% bilateral) and reduced mobility were the significant manifestations in patients with ankle arthropathy.

4) The symptom of ankle pain, present in half of the 34 patients, had a high sensitivity (88%) and specificity (94%) for predicting ankle arthropathy by the radiology system. The VAS pain score demonstrated a good correlation with the radiology score (R = 0.79).

5) An important finding in the vast majority of patients with a positive physical ankle score (26 of 27) was the loss of free range of motion of the ankle joint, characterised predominantly by loss of the dorsiflexion. This might represent early arthropathy.

6) The most frequent signs found on standard X-ray examination of the 17 patients with a positive radiology score was the loss of cartilage articular space, which was the single most important roentgenographic finding related to the concomitant reduced ROM in haemophilic arthropathy.

7) The physical and radiology scoring systems had a moderate correlation (R = 0.53). The physical system may be too sensitive for detecting arthropathy in this group of patients, although the significance of having a reduce ROM on physical examination but a normal radiological appearance of the ankle is unclear.

8) The presence and severity of ankle arthropathy, as assessed by the radiology system, was determined by a threshold effect of FVIII level of 11 and 6 IU/dl by one- and two-stage assay, respectively. Below these levels, patients have a higher chance of developing ankle arthropathy. The ROC curve analysis also confirmed the possibility of using a cut-off FVIII level of a similar value, it may be possible to identify a group of patients with the likelihood of developing ankle arthropathy.

9) Compared to the physical scoring system, we favour the radiology system in the evaluation of ankle arthropathy in future studies, because of its close correlation with the VAS pain score and FVIII level.

10) Due to insufficient numbers of the *discrepant* patients, it was not possible to determine whether the clinical severity of the disease, as represented by the presence of ankle arthropathy, correlates better with one- or two-stage FVIII.

11) There was a moderate correlation between ankle arthropathy and the TGA parameters in our patients with mild/moderate haemophilia A. This level of correlation was similar to that between ankle arthropathy and the FVIII level in these patients.

12) There was a significant relationship between the presence of ankle arthropathy and a history of bleeds into the ankle joint as a child. The multiple bleeding episodes are the most likely cause of the arthropathy. It seems likely that better treatment of these episodes may prevent or reduce the severity of the arthropathy.

13) We recommend education about the awareness of ankle arthropathy in the patients with mild and moderate haemophilia and their families. Patients should seek early assessment on ankle bleeding and more aggressive treatment with FVIII should be considered.

CHAPTER FIVE GENERAL DISCUSSION

This thesis tackles three different aspects of the laboratory and clinical evaluation of haemophilia A. We first attempted the production of recombinant FVIII peptides in a mammalian cell expression system. We succeeded in producing the C1 and C2 peptides but the peptides were confined to the cell pellet. To carry out further experiments with the eventual aim of neutralising FVIII inhibitors using epitope-mimicking peptides, a considerable amount of pure recombine FVIII fragments were required. From a practical point of view, a recombinant product secreted to the cell culture media would be necessary. The difficulty in obtaining soluble peptides in the supernatant of the culture media highlighted the technical obstacles in this area. Considerable work has been carried out by others in our department to attempt to purify the peptides from the cell pellets. These efforts were however unsuccessful and the decision was made to abandon the project.

Meanwhile we attempted to produce monoclonal anti-FVIII antibodies. Although the immunised mice developed polyclonal anti-FVIII antibodies that demonstrated a high inhibitory titre to FVIII:C, none of the subsequent fusion experiments led to the production of any monoclonal cell lines that secreted inhibitory anti-FVIII:C antibodies. The monoclonal antibodies yielded were likely against non-inhibitory epitopes on the FVIII molecule and therefore not useful in aiding our study for the detection of functional epitopes and neutralisation of FVIII inhibitors.

Future directions for work on the detection of FVIII epitopes could include the examination of the methodology for the recombinant techniques to improve the production of soluble FVIII peptides, and probably more importantly, the exploration of alternative approaches for antigen production, such as synthetic small fragments, rather than the full-length domain-specific peptides. The FVIII epitopes are now being recognised to be much smaller proportions of the major domains (e.g., the C2 or A2 domain) (Ananyeva *et al*, 2004). Therefore the epitope-mimicking peptides with a potential application in the neutralisation of the inhibitors are likely to be the size of less than 50 amino acids, sometimes even as small as a few amino acids long.

We then carried out studies to develop an in-house TGA method and to evaluate its usefulness in the assessment of coagulation deficiency in mild and moderate haemophilia A. We considered that it was important to compare the use of two different triggers (either TF or APTT reagent). We found that the TGA results by either trigger were effective in revealing abnormal production of thrombin in plasmas with mild to moderate degree of FVIII deficiency. The results were interesting in that with APTT reagent, the abnormality was a marked delay in lag time (and peak time) in haemophilia plasma, whereas with TF, it was the reduced amount of thrombin formed. In light of the current model of haemostasis as proposed by Mann *et al* (2003a), our results are consistent with the idea that the coagulation defect in FVIII deficient plasma is probably due to a combination of both a diminished amount of thrombin during the initiation phase to prime the system and a delayed amplification/prolongation phase of thrombin production to consolidate the clot. We suggest that in future studies involving the use of TGA in haemophilia A, consideration should be given to the use of both triggers.

There was a moderate correlation between the TGA parameters and the FVIII levels in our 42 patients with mild/moderate haemophilia A. However, the TGA in its present form has no role in diagnosing haemophilia, because of the considerable overlap of the results between the normal subjects and haemophilia patients. The lack of a strong correlation between TGA parameter and FVIII level may be due to the variations of the thrombin generation capacity in patients even with similar FVIII levels. It is possible that these variations in the "coagulation phenotype" reflect at least in part variations in the "clinical phenotype". However, the proof of this concept would require that the "coagulation phenotype" is shown to be associated with an individual's clinical phenotype/bleeding tendency. Multi-centre studies of investigations involving the TGA with a standardised method and the clinical severity of haemophilia patients would be required to establish this.

The modifications of the TGA methods by Hemker's group in recent years led to the availability of their instrument, which enables the simultaneous measurement of automated calibrated thrombin generation

in multiple plasma samples (Hemker *et al*, 2003). Because of this standardised method, the TGA has now been widely accepted as a research tool.

It is possible that the eventual role of TGA in clinical practice in haemophilia will be in the monitoring of FVIII replacement therapy rather than defining the baseline "coagulation phenotype" in untreated patients. The TGA may also have a role in the monitoring the administration of the bypassing agents such as APCCs and recombinant FVIIa. For instance, the results of a recent study suggest that use of the TGA may make an important contribution to decision making process of procoagulant therapies in high risk patients such as a case with severe haemophilia and high titre inhibitors (Dargaud *et al*, 2005b). The practical translation of the use of the TGA to routine clinical practice requires further study.

In addition, we investigated in mild and moderate haemophilia A the prevalence and severity of ankle arthropathy. There was a high prevalence of ankle arthropathy in our 34 adult patients with mild/moderate haemophilia A. There was also a significant impact of the arthritis on the patients' mobility and pain status. These findings have not been previously reported.

In patients with mild and moderate haemophilia A, the presence of ankle arthropathy correlated with their FVIII level. It is important that patients at risk are properly advised regarding this complication and its potential impact. To identify which patients are at risk (predisposed to ankle arthropathy), it is necessary to decide what level of FVIII represents a high or low probability of the occurrence of arthritis. From this study we considered that patients with one-stage FVIII level of equal to or ess than 11.5 IU/dl or two-stage FVIII equal to or less than 6 IU/dl are at risk of developing ankle arthropathy. Further studies are required to confirm this.

Although using a cut-off FVIII value (e.g., 11.5 IU/dl by the one-stage assay), either by visual inspection or by ROC curve analysis, would include the majority of the patients (e.g., 75%) who will develop arthritis of the ankle joint, all patients are at some risk. Due to the inherent/unavoidable variations in the

results of FVIII assays, it is not possible to determine an accurate cut-off level in a study of a relatively small sample size as in this thesis. From the present data it would appear that we should be especially concerned about patients with a one-stage FVIII level less than 11 IU/dl. Nevertheless, all patients with mild haemophilia A should be warned of the danger of under treatment of ankle joint injuries, including ankle sprains.

In addition, in the present study we have provided some evidence that ankle arthropathy in mild and moderate haemophilia is likely secondary to recurrent ankle sprains/bleedings during childhood. This finding is important. It should lead to the development of protocols involving the education of patients with mild/moderate haemophilia A (especially paediatric patients) and families to prevent this complication.

Incorporating the previous TGA results, we explored whether the clinical severity of mild haemophilia, as maybe indicated by the severity of ankle arthropathy, was correlated with the TGA parameters. The best correlations of the radiology scores in the 33 patients were found to be with the peak thrombin concentration (R = -0.55) or AUC (R = -0.54) of TGA using TF. This strength of correlation was similar to that between the radiology scores and the one- (R = -0.49) or two-stage (R = -0.55) FVIII levels. Hence the TGA in its current form proved just as good as the traditional FVIII assays in providing laboratory evaluation of the severity of the disease that was phenotypically reflected by ankle arthropathy.

Our study showed chronic ankle arthropathy to be an important clinical problem in mild haemophilia. More studies are required to further evaluate this, including the studies aiming at the prevention of this condition. Ideally future studies would include prospective evaluation of paediatric patients to detect the development of ankle arthropathy over, for instance, a 20-year period of time. From the results of our study, the WFH radiology scoring system (Pettersson scale) proves to be a useful objective measure. The WFH physical scoring system is also important in assessing ankle arthropathy. It is simple to

perform and involved little cost. In addition, the recent modification of the current WFH physical score, named the Haemophilia Joint Health Score (HJHS) (Manco-Johnson *et al*, 2004; Hilliard *et al*, 2006), should be considered in future studies to evaluate haemophilic arthropathy.

This thesis makes important contribution to the use of TGA in characterisation of FVIII deficiency in patients with haemophilia A. It also provides important information on the incidence of ankle arthropathy in mild haemophilia, and gives the direction for improving treatment protocols to prevent this common complication.

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APPENDICES

Appendix Ia Survey on Ankle Pain

A SHOTR SURVEY ON MILD AND MODERATE HAEMOPHILIA A

(Investigators: Dr John Lloyd and Dr Min Ling from the Haemophilia Centre, Royal Adelaide Hospital and Haematology Division, IMVS)

PERSONAL DETAILS

1.
(RAH Patient Label)

Please fill in below if the above details are incorrect:
Name:
Address:

2. What is your date of birth:

3. What is your contact telephone number:

QUESTION 1

a) Do you often have pain in your ankle(s)?
Answer: Yes ☐
No ☐

b) If you answer "Yes" to question a), would you describe the pain in your ankle(s) as being:
Mild ☐
Moderate ☐
Severe ☐

Please Note:
Mild pain --Does not interfere with occupation nor with activity of daily living (ADL); May require occasional pain-killers.
Moderate pain --Partial or occasional interference with occupation or ADL; Requires use of pain-killers.
Severe pain --Interferes with occupation or ADL; Requires frequent use of pain-killers.

QUESTION 2

Are you prepared to come to the RAH Haemophilia Centre for an interview concerning your experience with bleeding and for assessment of your ankle joints?
Answer: Yes ☐
No ☐

Please sign:……..… Date:……..

ML/JVL 6 Mar 2003

Appendix Ib Patient Information Sheet and Consent Form for Study of Ankle Arthropathy

INFORMATION SHEET
FOR PATIENTS WITH MILD/MODERATE HAEMOPHILIA A

NAME OF STUDY: SURVEY ON RELATIONSHIP BETWEEN FVIII LEVEL AND BLEEDING TENDENCY IN MILD HAEMOPHILIA

INVESTIGATORS: DR JOHN LLOYD AND DR MIN LING

One of the big concerns in people with mild/moderate haemophilia A is that some develop arthritis in their ankle joints. This is due to damage caused by bleeds into the joint space.

Arthritis in mild and moderate haemophilia has not yet been well documented in the haemophilia research literature. Therefore we are conducting a study to address this issue.

The aim of the study is to investigate the prevalence of arthritis in people with mild and moderate haemophilia A, and to make an initial exploration of the possible correlations between arthritis, the severity of bleeding and the factor VIII level. We will use history of bleeding into and damage to joints, especially the ankle joint as an indicator of the severity of the bleeding tendency. This study will provide information useful for future prevention and treatment of this problem.

In the study, we are approaching people with mild/moderate haemophilia who are over 18 years and on the RAH database. If you agree, we would like to see you at the RAH for an interview and examination of your joints.

In addition, if you agree, we will examine your ankle joints by X-ray and MRI (Magnetic Resonance Imaging). Both procedures are non-invasive and pain-free. With X-ray, but not MRI, there will be a minimal amount of radiation involved.

Your participation in this survey is voluntary and if you do not with to participate, your usual medical care will not be affected in any way.

If you have any questions regarding this study, you may contact the investigators for further information:

Dr Min Ling	(08) 8222 3592 or min.ling@imvs.sa.gov.au
Dr John Lloyd	(08) 8222 5410

If you with to speak to someone not associated with this study, phone the Chairman of the Human Ethics Committee of the Royal Adelaide Hospital (telephone 08 8222 4139).

ROYAL ADELAIDE HOSPITAL
CONSENT FORM

NAME OF STUDY: SURVEY ON RELATIONSHIP BETWEEN FVIII LEVEL AND BLEEDING TENDENCY IN MILD HAEMOPHILIA

INVESTIGATORS: DR JOHN LLOYD, DR MIN LING

1. The nature and purpose of the research project has been explained to me. I understand it, and agree to take part.

2. I understand that I may not be directly benefit from taking part in the study.

3. I understand that, while information gained during the study may be published, I will not be identified and my personal results will remain confidential.

4. I understand that I may refuse to participate in this study, and that this will not affect my medical care, now or in the future.

5. I give consent to the following medical procedures for the purpose of the study, on the ground that the risks and possible immediate and remote side effects of each procedure have been explained to me:

a.	Blood sampling	Yes	☐	No	☐
b.	X-ray of both ankles	Yes	☐	No	☐
c.	MRI of both ankles	Yes	☐	No	☐

Name of Participant:…….
Signature:……Date:……..……..

I certify that I have explained the study to the patient and consider that he understands what is involved.
Signature of Investigator:……Date:……...

Appendix II Clinical Assessment Pack for Ankle Arthropathy

Relationship between FVIII level and bleeding tendency in mild/moderate haemophilia A: Assessment by interview and examination

Date:……../……../20……

1. PERSONAL DETAILS

Name: DOB:
Address:
Telephone Number:
Main Occupation:……Usual Transportation to Work……..

2. DIAGNOSIS

Primary Diagnosis of Haemophilia A:

When: at……years of age or 19…… Which Hospital:……..
Inherited☐ Sporadic☐ Mild☐ Moderate☐ FVIII Level:.....(1-stage);.....(2-stage)
Inhibitor History: yes☐ No☐ Inhibitor Titre:.....BU/ml

Other Major Medical Diagnosis, especially in muscle-skeleton system:

……

3. EVALUATION OF ANKLES

History and Symptoms

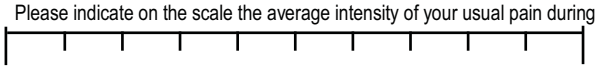
a) Do you often have pain in your ankle joints?
Yes: left ankle ☐, right ankle ☐; No ☐

b) If you have pain in your ankles, how severe is it generally?

- Mild pain; Does not interfere with occupation nor with activity of daily living (ADL); May require occasional non-narcotic analgesic.
- Moderate pain; Partial or occasional interference with occupation or ADL; Use of non-narcotic medications (eg, paracetamol, NSAIDS ibuprofen, voltaren, indomethacin).
- Severe pain; Interferes with occupation or ADL; Requires frequent use of non-narcotic and narcotic medications (eg, codeine, pethidine).

	Left Ankle	***Right Ankle***	***Notes***
Mild	☐	☐	
Moderate	☐	☐	
Severe	☐	☐	

c) Please indicate on the scale the average intensity of your usual pain during the previous 4 weeks:



0 **5** **10**
No pain **Worst possible pain**

d) If you use pain-killers, please describe the medication, dosage, and frequency of use (ie, all the time, most of the time, or occasionally).

e) Do you have pain in other joints in your lower limbs, eg, knees, hips and elbows?

Yes ☐ No ☐
If yes, specify:……

f) Do you have any difficulties climbing up stairs or ramps due to your joint discomforts?
Yes ☐ No ☐

g) Do you use any kind of walking aids?
Yes ☐ No ☐
If yes, specify (eg, Modified Shoes/Walking Sticks/Crutches/Wheelchair):……..

h) What is your comfortable walking distance or walking time:……..
<10m☐ <100m☐ <1km☐ >1km☐; <5 min☐ <30min☐ >30min☐
What are the limiting factors:……

i) Do you have any regular exercise/activity, eg, organised sports/walking/gardening etc?
Yes ☐, I usually do:……
No ☐, it is because of ankle problem, or other reasons:……..

Physical Examination

Shoulder/Elbow/Hip/Knee in general:……..

	L/Ankle	***R/Ankle***
1) Swelling (medial & lateral malleoli): 0 = None 2 = Present (> 1 cm) *(S): added after score if chronic synovitis is present*	…….	…….
2) Calf muscle atrophy: 0 = None or minimal 1 = Present (difference > 1 cm)	…….	…….

	L/Ankle	*R/Ankle*
3) Deformity:		
0 = No deformity		
1 = Up to 10° valgus or up to 5° varus		
2 = > 10° valgus or > 5° varus	.....	.....
4) Crepitus on motion:		
0 = None		
1 = Present	.....	.....
5) Range of motion (full range of motion of ankle: dorsiflexion 20°; plantar flexion 50°)		
0 = Loss of up to 10% of total FROM		
1 = Loss of 10-33% of total FROM		
2 = Loss of >33% of total FROM	.....	.....
6) Flexion contracture:		
0 = < 15° FFC (fixed flexion contracture)		
2 = equinus at ankle	.....	.....
7) Instability:		
0 = None		
1 = Noted on examination but neither interferes with function nor requires bracing		
2 = Instability that creates a functional deficit or requires bracing		
	.....	.....
Total Score (0~12):	**.....**	**.....**

4. HISTORY OF BLEEDING

a) Any joints/muscle/mouth bleeding or severe epistaxis as a child/teenager (<18 yrs)?

Yes ☐ No ☐

If yes, complete the following list:

No.	Age	Site	Sports/Trauma	FVIII Therapy	Hospital Admissions	Duration	Notes
1.							
2.							
3.							
4.							

b) Any joints/muscle/mouth bleeding or severe epistaxis as an adult (≥18 yrs)?

Yes ☐ No ☐

If yes, complete the following list:

No.	Age	Site	Sports/Trauma	FVIII Therapy	Hospital Admissions	Duration	Notes
1.							
2.							
3.							
4.							

c) Any bleeding into urine (haematuria)?

Yes ☐ No ☐

If yes, how many times: ≤2 ☐; 2-5 ☐; >5 ☐;

Extra notes:

d) Any recurrent spontaneous multiple bruising?

Yes ☐ No ☐

If yes, was it as a child (ie, if your mother worried) ☐, or as an adult ☐, or both ☐.

Extra notes:

e) Any sports (eg. football, soccer, hockey, tennis, swimming etc) ever played?

Yes ☐ No ☐

If yes, complete the following list:

No.	What Sport	Duration (from to yrs of age)	Severe bleeds at joints/muscles	Notes
1.				
2.				
3.				
4.				

f) Any surgeries (eg circumcision, tonsillectomy, dental extraction [particular teeth], appendectomy etc) performed <u>without FVIII cover</u> and associated with bleeding?

Yes ☐ No ☐

If yes, complete the following list:

No.	Age	Surgery	Bleeding During/After	Blood Transfusion	Hospital Admissions & Duration	Notes
1.						
2.						
3.						
4.						

Any history of being taken back to theatre after the bleeding following the above procedures?

Yes ☐ No ☐

If yes, specify:.....

Appendix III List of 34 Patients with Mild and Moderate Haemophilia A in the Study of Ankle Arthropathy

	Patient Code by Family	Age	Severity	Equivalent/ Discrepant	VAS Pain Score	VAS "Total" Pain Score	Physical Ankle Score	Radiology Ankle Score	One-stage FVIII (IU/ml)	Two-stage FVIII (IU/ml)	Ankle MRI
1	4.1*	48	Moderate	E	0	0	1	1.5	7	5	
2	4.2	51	Moderate	E	1.5	1.5	2	9.5	2	3	Yes
3	4.3	42	Moderate	E	0	0	0	0	8	5	Yes
4	4.4	44	Moderate	E	0.5	0.5	2	0.5	5	5	
5	4.5	46	Mild	E	0	0	2	0	8	6	
6	4.6	36	Moderate	E	0	0	2	0	4	3	
7	4.7	50	Moderate	E	7	11	6	12	6	3	
8	4.8*	55	Moderate	E	7.5	15	5	12	11	4	
9	4.9	50	Moderate	E	5	10	3	6.5	11	5	
10	7.1	29	Mild	E	0	0	0	0	15	7	
11	7.2	32	Mild	E	0	0	1	0	17	9	
12	7.3	46	Moderate	E	3	3	2	3.5	4	4	
13	8.1	51	Mild	D	0	0	0	0	34	10	Yes
14	9.1**	21	Moderate	E	0	0	2	0	7	3	
15	14.1	22	Mild	D	0	0	0	0	37	8	
16	16.1	59	Mild	E	2	2	5	3	23	10	
17	24.1	27	Mild	E	0	0	0	0	27	24	
18	24.2	37	Mild	E	0	0	0	0	40	22	
19	24.3	26	Mild	E	0	0	1	0	30	18	
20	26.1	42	Mild	E	4	4	4	3	8	6	
21	28.1	47	Mild	D	0	0	4	0	41	6	
22	35.1	32	Moderate	E	7	9	3	3	6	3	Yes
23	35.2*	48	Moderate	E	6	8	0	8.5	5	4	
24	39.1	48	Mild	D	0	0	2	1	44	6	
25	40.1	61	Moderate	D	3	6	3	1.5	31	5	
26	46.1	40	Mild	D	0	0	1	0	30	6	
27	49.1	59	Mild	E	0	0	2	N/A	14	11	
28	49.2	66	Mild	E	0	0	1	0	12	9	
29	97.1	60	Moderate	E	2	2	2	5	6	4	
30	101.1	50	Mild	E	8	8	4	1	30	27	
31	121.1	35	Mild	E	0	0	2	0	27	20	
32	121.2	37	Mild	E	2.5	4	2	1.5	24	12	
33	132.1	29	Mild	E	5	6	3	0	16	17	
34	138.1	39	Moderate	E	6.5	6.5	2	1.5	5	4	Yes

*: Three patients (code 4.1, 4.8 and 35.2) had inhibitors to FVIII. **: Patient 9.1 was the only patient in this study who was on prophylactic FVIII treatment (past five years).

Appendix IV Physical Ankle Scores in 34 Patients

	Patient Code	Physical Score (Right / Left)		1. Ankle Swelling	2. Calf Atrophy	3. Axial Deformity	4. Crepitus	5. ROM	6. Flexion Contracture	7. Instability
1	4.1	1	(1 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 0	0 / 0	0 / 0
2	4.2	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
3	4.3	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
4	4.4	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
5	4.5	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
6	4.6	2	(2 / 0)	0 / 0	1 / 0	0 / 0	0 / 0	1 / 0	0 / 0	0 / 0
7	4.7	6	(3 / 3)	0 / 0	0 / 0	0 / 0	1 / 1	2 / 2	0 / 0	0 / 0
8	4.8	5	(2 / 3)	0 / 0	0 / 1	0 / 0	0 / 0	2 / 2	0 / 0	0 / 0
9	4.9	3	(2 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	2 / 1	0 / 0	0 / 0
10	7.1	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
11	7.2	1	(0 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 1	0 / 0	0 / 0
12	7.3	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
13	8.1	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
14	9.1	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
15	14.1	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
16	16.1	5	(2 / 3)	0 / 2	1 / 0	0 / 0	0 / 0	1 /1	0 / 0	0 / 0
17	24.1	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
18	24.2	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
19	24.3	1	(1 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 0	0 / 0	0 / 0
20	26.1	4	(4 / 0)	2 / 0	1 / 0	0 / 0	0 / 0	1 / 0	0 / 0	0 / 0
21	28.1	4	(2 / 2)	0 / 0	0 / 0	0 / 0	0 / 0	2 / 2	0 / 0	0 / 0
22	35.1	3	(3 / 0)	0 / 0	1 / 0	0 / 0	0 / 0	2 / 0	0 / 0	0 / 0
23	35.2	0	(0 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
24	39.1	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
25	40.1	3	(1 / 2)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 2	0 / 0	0 / 0
26	46.1	1	(1 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 0	0 / 0	0 / 0
27	49.1	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
28	49.2	1	(1 / 0)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 0	0 / 0	0 / 0
29	97.1	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
30	101.1	4	(3 / 1)	2 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0	0 / 0
31	121.1	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
32	121.2	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
33	132.1	3	(1 / 2)	0 / 0	0 / 1	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0
34	138.1	2	(1 / 1)	0 / 0	0 / 0	0 / 0	0 / 0	1 / 1	0 / 0	0 / 0

Appendix V Loss of Ankle Range of Motion (Dorsi- and Plantar Flexions) in 34 Patients

	Patient Code	Loss of Dorsiflexion (R / L, degree)	Loss of Plantar Flexion (R / L, degree)	Loss of ROM (R / L, degree)	Score for Loss of ROM (R / L)
1	4.1	15 / 5	0 / 0	15 / 5 §	1 / 0
2	4.2	10 / 15	10 / 0	20 / 15	1 / 1 #
3	4.3	0 / 0	0 / 0	0 / 0	0 / 0
4	4.4	15 / 10	5 / 10	20 / 20	1 / 1 #
5	4.5	20 / 10	0 / 0	20 / 10	1 / 1
6	4.6	10 / 5	5 / 0	15 / 5 §	1 / 0
7	4.7	20 / 5	30 / 25	50 / 30	2 / 2 #
8	4.8	10 / 5	25 / 20	35 / 25	2 / 2 #
9	4.9	20 / 10	20 / 5	40 / 15	2 / 1 #
10	7.1	0 / 0	0 / 0	0 / 0	0 / 0
11	7.2	5 / 10	0 / 10	5 / 20 §	0 / 1
12	7.3	10 / 10	0 / 0	10 / 10	1 / 1 #
13	8.1	0 / 0	0 / 0	0 / 0	0 / 0
14	9.1	10 / 10	0 / 10	10 / 20	1 / 1
15	14.1	0 / 0	0 / 0	0 / 0	0 / 0
16	16.1	10 / 20	0 / 0	10 / 20	1 / 1 #
17	24.1	0 / 0	0 / 0	0 / 0	0 / 0
18	24.2	0 / 0	0 / 0	0 / 0	0 / 0
19	24.3	15 / 5	0 / 0	15 / 5 §	1 / 0
20	26.1	10 / 0	0 / 0	10 / 0	1 / 0 #
21	28.1	15 / 15	10 / 10	25 / 25	2 / 2
22	35.1	15 / 5	10 / 0	25 / 5 §	2 / 0 #
23	35.2	0 / 0	0 / 0	0 / 0	0 / 0 #
24	39.1	10 / 10	0 / 0	10 / 10	1/ 1
25	40.1	5 / 15	15 / 10	20 / 25	1 / 2 #
26	46.1	10 / 0	0 / 5	10 / 5 §	1 / 0
27	49.1	10 / 10	0 / 0	10 / 10	1 / 1
28	49.2*	0 / 0	10 / 5	10 / 5 §	1 / 0
29	97.1	10 / 10	0 / 10	10 / 20	1 / 1 #
30	101.1**	0 / 0	0 / 0	0 / 0	0 / 0 #
31	121.1	15 / 15	5 / 0	20 / 15	1 / 1
32	121.2	10 / 10	0 / 0	10 / 10	1 / 1 #
33	132.1	10 / 10	0 / 10	10 / 20	1 / 1 #
34	138.1	10 / 10	0 / 0	10 / 10	1 / 1 #
SUBTOTAL **+ ve Patients†**	(of total of 34 patients)	**25:** Both ankles affected –23 R only – 2 L only – 0	**16:** Both – 7 R only – 4 L only – 5	**26:** Both – 25 R only – 1 L only – 0	**26:** Both – 18 R only – 7 L only – 1
+ ve Ankles‡	(of total of 68 ankles)	**48:** R – 25 L – 23	**23:** R – 11 L – 12	**51:** R – 26 L – 25	**44:** R – 25 L – 19

The measurements of both dorsi- and plantar flexion for each ankle joint were listed here. Loss of total ROM of at least 10% of normal value (70°, dorsiflexion 20° and plantar flexion 50°) attracts a positive score: score 1 for loss ROM of 10-33% (i.e., 7-22°), score 2 for loss > 33%. Twenty-six patients with 44 affected ankles had a positive score for loss of ROM.

*: Patient 49.2 was the only patient who had a score of 1 for loss of ROM but no loss of dorsiflexion in the affected ankle.
**: Patient 101.1 had a positive physical score of 3, with preserved full ROM in both ankles.
†: Number of patients with a +ve result, i.e., a positive score for loss of ROM.
‡: Number of ankles with a +ve result.
§: 7 Patients had mild loss (5°) of dorsi- or plantar flexion in single ankle but maintained a total ROM > 63° (90% of normal) therefore scored zero in the affected side.
#: Patients who had ankle pain (n = 16).

Appendix VI Radiology Ankle Scores in 34 Patients

	Patient Code	Age	Radiology Score (Right / Left)		1. Osteoporosis	2. Enlarged Epiphysis	3. Irregular Subchondral Surface	4. Narrowing of Joint Space	5. Subchondral Cyst Formation	6. Erosion of Joint Margins	7. Gross Incongruence Of Articulating Bone Ends	8. Joint Deformity (Angulation &/or Displacement)
1	4.1	48	1.5	1 / 0.5	- / -	- / -	+ / +	- / -	- / -	- / -	- / -	- / -
2	4.2	51	9.5	5.5 / 4	+ / +	+ / +	+ / +	+ / +	+ / -	- / -	- / -	- / -
3	4.3	42	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
4	4.4	44	0.5	0.5 / 0	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -
5	4.5	46	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
6	4.6	36	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
7	4.7	50	12	7 / 5	- / -	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
8	4.8	55	12	7 / 5	- / -	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
9	4.9	50	6.5	6.5 / 0	- / -	+ / -	+ / -	- / -	+ / -	+ / -	- / -	- / -
10	7.1	29	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
11	7.2	32	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
12	7.3	46	3.5	3 / 0.5	- / -	- / -	+ / +	+ / -	+ / -	- / -	- / -	- / -
13	8.1	51	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
14	9.1	21	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
15	14.1	22	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
16	16.1	59	3	0 / 3	- / -	- / +	- / +	- / +	- / -	- / -	- / -	- / -
17	24.1	27	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
18	24.2	37	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
19	24.3	26	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
20	26.1	42	3	0 / 3	- / -	- / -	- / +	- / -	- / +	- / -	- / -	- / -
21	28.1	47	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
22	35.1	32	4	4 / 0	- / -	- / -	+ / -	+ / -	+ / -	- / -	- / -	- / -
23	35.2	48	8.5	5 / 3.5	- / -	- / +	- / -	+ / +	+ / +	+ / -	- / -	- / -
24	39.1	48	1	0.5 / 0.5	- / -	- / -	+ / +	- / -	- / -	- / -	- / -	- / -
25	40.1	61	1.5	1 / 0.5	- / -	- / -	+ / +	+ / -	- / -	- / -	- / -	- / -
26	46.1	40	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
27	49.1	59	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
28	49.2	66	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
29	97.1	60	5	3.5 / 1.5	- / -	- / -	+ / +	+ / +	+ / -	- / -	- / -	- / -
30	101.1	50	1	0.5 / 0.5	- / -	+ / +	- / -	- / -	- / -	- / -	- / -	- / -
31	121.1	35	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
32	121.2	37	1.5	0.5 / 1	- / -	- / -	- / -	+ / +	- / -	- / -	- / -	- / -
33	132.1	29	0	0 / 0	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
34	138.1	39	1.5	1.5 / 0	- / -	- / -	- / -	+ / -	+ / -	- / -	- / -	- / -

N/A: Not available.