Genetic, Phenotypic and Clinical Evaluation of Haemophilia A in Pakistan

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By
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Declaration

The studies described in this thesis were undertaken in Cardiff University School of Medicine, within the Institute of Infection and Immunity and the Institute of Molecular and Experimental Medicine. Except where otherwise stated, the work is solely my own and no part of it has been, or will be, submitted in candidature for any degree elsewhere.

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Fatima Khanum (candidate) Date

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Professor Peter Collins Date

(Joint primary supervisor)

Dr Derrick Bowen Date

(Joint primary supervisor)

I hereby give consent for my thesis, if accepted, and its title and summary to be available for open access according to the policies and regulations of Cardiff University.

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Fatima Khanum Date

Dedication

I would like to dedicate my work to my family, specially my husband, whose continuous efforts and help made it possible.

Acknowledgement

I would like to thank my supervisors Professor Peter William Collins and Dr Derrick John Bowen for their valuable guidance and unmatched support throughout the whole period of my work at Cardiff University. I felt immensely proud to be a part of the teams of Infection and Immunity and Institute of Molecular and Experimental Medicine as they both joined to make this the perfect place to complete my MD. I would like to thank Rebecca Louise Harris and Dr Briedgeen Catriona Kerr for their kindness, time and help during this course. I would like to thank to Steve Lees and Nicky McCartney for their support over the years. I am also grateful to the following centres in Pakistan for sample collection and storage: Fatimid Foundation, Pakistan Institue of Medical Sciences, Pakistan Haemophilia Welfare Society and Armed Forces Institute of Pathology.

Summary

Hereditary haemophilia A (HA), an X-linked bleeding disorder, is caused by mutations in the coagulation factor VIII gene (FVIII abbreviates protein, gene symbol *F8*). The aim of this study was (1) to characterise *F8* mutations in HA cohort from Pakistan, (2) to investigate whether *in vitro* thrombin generation (TG) differs according to mutation type, and (3) to compare haemophilia joint health score (HJHS) and Gilbert score with severity, age, TG and underlying mutation in HA cohort which had minimal access to haemostatic replacement therapy. Methods: One hundred HA individuals and 100 healthy controls were recruited; clinical details were recorded. Results: Phenotypic measurements were re-evaulated in Cardiff; the essential regions of *F8* were screened. Ninty two individuals were diagnosed with HA, 7 with haemophilia B and 1 was normal. The *F8* defects were characterised and comprised point mutations, inversions and frameshifts. Thirty novel variants were identified. No significant difference was observed *in vitro* TG between severes with null mutation and those with a missense change. HJHS was strongly correlated with Gilbert score (r =0.98), both were siginificantly higher in severe compared with nonsevere before the age of 12 years ($P \leq 0.01$) but not thereafter. According to developmental age (<12 years, 12-16 years and > 16 years), both scores were significantly lower in the youngest group ($P \leq 0.001$). In severes there was no correlation between *in vitro* TG and joint score, no significant difference was observed for either joint score according to the underlying mutation type. Whereas in nonseveres, negative correlation between *in vitro* TG and joint score was observed. Conclusions: *F8* defects in Pakistan is heterogenous; in severe HA *in vitro* TG are not influenced by underlying mutation. Haemophilic arthropathy

is correlated with severity and age; among severes, joint health scores did not relate to either the underlying mutation or *in vitro* TG.

Abbreviations

5'-UTR	5-Untranslated Region
AA	Arachidonic Acid
ADP	Adenosine Diphosphate
APC	Activated Protein C
aPTT	Activated Partial Thromboplastin Time
AT	Antithrombin
BDT	Big dye Terminator
Bp	Base Pairs
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CAT	Calibrated Automated Thrombography
CP	Cryoprecipitate
CTI	Corn Trypsin Inhibitor
DIC	Disseminated Intravascular Coagulation
dNTP	Deoxynucleoside Triphosphate
ELISA	Enzyme-Linked Immunosorbent Assay
ETP	Endogenous Thrombin Potential
FDP	Fibrin Degradation Product
FFP	Fresh Frozen Plasma
FII	Prothrombin
FIX:C	Coagulation Factor IX Activity

FV	Coagulation V
FV:C	Coagulation Factor V Activity
FVII	Factor VII
FVIII	Coagulation Factor VIII
FVIII:C	Coagulation Factor VIII Activity
FXI	Coagulation Factor IX
GP	Glycoprotein
HAMSTeRS	Haemophilia A Mutation, Structure, Test and Resource Site
HCh	Heavy Chain
HEPES	n-2-Hydroxyethylpiperazine-n′-2-Ethanesulfonic Acid
HJHS	Haemophilia Joint Health Score
HMWK	High Molecular Weight Kininogen
HSPGs	Cell-Surface Heparan Sulphate Proteoglycans
IL	Interleukin
Ins-1, 4, 5- P_3	Inositol-1, 4, 5-Triphosphate
Inv1	Intron 1 inversion
Inv22	Intron 22 inversion
Kb	Kilobases
LCh	Light Chain
LDL	Low-Density Lipoprotein
LIA	Automated Latex Immunoassay
LRP	Lipoprotein Receptor-Related Protein
$MgCl_2$	Magnesium Chloride
MRI	Magnetic Resonance Imagining
OPG	Osteoprotegrin

ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PG	Prostaglandin
PI	Phosphatidylinositol
PKC	Protein Kinase C
PKK	Prekallikrein
PLA_2	Phospholipase A_2
PL	Phospholipid
PLC	Phospholipase C
PLG	Plasminogen
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PT	Prothrombin Time
ROIs	Reactive Oxygen Intermediates
SNP	Single Nucleotide Polymorphism
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGT	Thrombin Generation Test
t-PA	Tissue Plasminogen Activator
TT	Thrombin time
Ttpeak	Time to peak
TxA_2	Thromboxane a_2
USA	United States of America
VEGF	Vascular Endothelial Growth Factor

VWF	von Willebrand Factor
VWF:Ag	VWF Antigen
Xg	multiples of g - gravitational constant

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Publications

Khanum F, Collins PW, Harris RL, Bowen DJ. Characterization of F8 defects in haemophilia A in Pakistan: investigation of correlation between mutation type and the in vitro thrombin generation assay. Haemophilia epub 14th Oct 2013.

F. Khanum, D. J. Bowen, B. C. Kerr, P. W. Collins. Joint health scores in a haemophilia A cohort from Pakistan with minimal or no access to factor VIII concentrate: correlation with thrombin generation and underlying mutation. Haemophilia epub 20th Dec 2013

Khanum F, Collins P, Bowen DJ. Diagnosis of haemophilia in Pakistan: current picture. J Coll Physicians Surg Pak. 2013 Jun;23(6):450-1.

Abstracts and Conference Presentations

- Xth International Haemophilia Forum Dubai, UAE (October 2013)
- Poster Presentation: Haemophilic Arthropathy. Haemophilia Academy, Edinburgh, UK (November 2012)
- Poster Presentation: Effect of disease severity and patient age on haemophilic arthropathy. Cardiff Institute of Infection and Immunity Annual Meeting (September 2012)

Chapter 1

Introduction

Haemostasis is the process by which blood loss is prevented. Three major stages of haemostasis comprise primary haemostasis (formation of the platelet rich soft clot), secondary haemostasis (fibrin formation) and tertiary haemostasis (fibrinolysis, breakdown of the clot). In primary haemostasis platelets adhere, activate and aggregate, the activation brought about by various agonists. Secondary haemostasis involves a series of reactions that ultimately generate thrombin which converts soluble fibrinogen into insoluble fibrin, the structural component of a blood clot. Tertiary haemostasis is the formation of plasmin (clot lysis enzyme), which is responsible for fibrinolysis (1).

1.1. Primary Haemostasis

1.1.1. Platelet Adhesion

When a blood vessel is damaged, the sub-endothelial structures are exposed and platelets adhere to the vessel wall. Adhesion molecules are present in the vessel wall which causes the binding of platelets to the sub-endothelium. These include fibronectin, von Willebrand factor (VWF), laminin and nidogen. Several platelet glycoprotein (GP) receptors are involved in binding to these adhesion molecules. Platelet GP receptors include GPIb-IX-V, GPIIb/IIIa ($\alpha_{IIb}\beta_3$), GPIa/GPIIa ($\alpha_2\beta_1$) and GPVI (2).

One of the most important interactions for platelet adhesion to the vessel wall is that involving VWF, a multimeric protein synthesized by endothelial cells and megakaryocytes. It is stored in the Weibel-Palade bodies of endothelial cells and α-granules of platelets. The latter bind to VWF via the GPIb-IX-V complex which consists of four subunits, GPIbα, GPIbβ, GPIX and GPV at a ratio of 2:2:2:1. VWF interacts with the GPIbα of the complex and this leads to platelet activation, resulting in cytoskeleton rearrangement, tyrosine phosphorylation and calcium mobilisation (3-5).

The interaction between platelets and VWF initiates signal transduction events that lead to activation of platelet GPIIb/IIIa, which becomes competent to bind VWF or fibrinogen to mediate platelet aggregation. Like other platelet GP receptors, GPIIb/IIIa is a heterodimer with an alpha ($\alpha_{IIb)}$ and a beta (β_3) subunit; it binds to fibrinogen, VWF, fibronectin and vitronectin through the beta subunit.

Platelet adhesion and aggregation are dependent upon shear, a measure of gradient of flow velocity relative to the distance from the vessel wall. There are two important mechanisms that give rise to adhesion and aggregation at the low and very high shear rates found respectively in veins and in stenosed arteries. At shear rates of 500/s, which are found in venules and larger veins, platelets are able to adhere directly to exposed sub-endothelial matrix proteins and maintain stable adhesion independent of VWF and GPIb-IX-V. At shear rates of 100,000/s and above, which are found in stenosed vessels, adhesion and aggregation are facilitated entirely by the interaction of soluble and immobilized VWF with GPIb-IX-V and GPIIb/IIIa respectively (6, 7).

Collagen is an important thrombogenic component of the sub-endothelial matrix. Nine forms of collagen have been described within the vessel wall (types I and III are more predominant in the deeper layer and type VI in the more superficial region of the vessel wall). GPIa/GPIIa and GPVI are the primary receptors for collagen; both play a prominent role in platelet adhesion. Platelet adhesion promoted by GPIa/GPIIa is a further interaction leading to the activation of GPIIb/IIIa. When platelets are activated, the surface expression of GPVI is increased. GPVI mainly binds to collagen type III and has an essential role in platelet adhesion to the vessel wall.

Application of high-resolution imaging technology has revealed that platelets translocate in a stop-start manner through sliding rather than rolling and this assists to minimize the drag forces on adhesive bonds. This stop-start phenomenon involves many of the interactions discussed above. Once platelets adhere to the vessel wall, they activate and spread (8).

1.1.2. Platelet spreading

When platelets come into contact with a surface, they undergo a characteristic set of morphological changes known as spreading. The initial shape change of platelets involves the generation of finger-like projections known as filopodia. Filopodia grow from the periphery of the platelets and then there is subsequent formation of lamellipodia which fill the areas between adjacent filopodia. Actin-myosin stress fibres then serve to strengthen the spread of platelets. As these events progress, granules and organelles are squeezed into

the centre of the platelet, resulting in a characteristic “fried egg” appearance. In combination, the formation of actin-myosin stress fibres and platelet spreading reinforce and secure the growing thrombus against flow and shear forces of the vascular system (9).

1.1.3. Granule secretion, thromboxane A_2 formation and platelet aggregation

Adhesion of platelets to collagen is followed by aggregation. Platelet aggregation can be initiated by a variety of biological agonists *in vivo*, including adenosine diphosphate (ADP), thrombin, collagen and thromboxane A_2 (TxA_2).

There are two pathways for platelet aggregation, one involving TxA_2 and the other an increase in the concentration of free calcium in the cytoplasm. An important enzyme in the later pathway is phospholipase C (PLC), an enzyme that hydrolyses fatty acid off phospholipids. PLC is further classified into PLCβ, γ and δ. PLCγ hydrolyses phosphatidylinositol (PI) yielding water soluble inositol-1, 4, 5-triphosphate (Ins-1, 4, 5- P_3) and diacylglycerol. Ins-1, 4, 5-P_3 binds to intracellular calcium channels, as the result of this binding the channels open and allow an influx of calcium into the cytoplasm. This leads to an increase in the level of free cytoplasmic calcium. The elevated calcium level allows different processes to accelerate, including calmodulin-dependent activation kinase, activation of protein kinase C (PKC) and activation of phospholipase A_2 (PLA_2).

When PLA_2 is activated, arachidonic acid (AA) is released, and within the platelet this acts as a substrate for cyclooxygenase-1 and also lipooxygenases 12 and 15. Cyclooxygenase-1 transforms AA into the prostaglandin (PG) G_2 and PGH_2, which is converted to the active TxA_2 within the platelet. TxA_2 is released and acts as a powerful platelet aggregator and vasoconstrictor.

Platelets contain several types of granules in which bioactive constituents are sequestered. These include dense bodies (containing serotonin, adenosine triphosphate, ADP, and calcium), α-granules (containing fibrinogen, α_1-antitrypsin, VWF, FV, high molecular weight kininogen (HMWK), fibronectin, platelet factor 4, thromboglobin, protein S, tissue factor pathway inhibitor (TFPI) and platelet derived growth factor), lysosomes and peroxisome, although the significance of the latter two is unclear. Fusion of α-granules with the platelet plasma membrane has the potential to increase the expression of GPIIb/IIIa as well as P-selectin on its surface.

Additional changes in platelet phospholipids bring about a reorientation of the lipid bilayer, a mechanism called flip-flop. This translocates procoagulant phospholipids in the intact platelet from inside to outside. As the result of this translocation, platelets become procoagulant. All of the above mentioned mechanisms ultimately trigger the cross linking of platelets through bivalent and multivalent ligands and produce platelet aggregation.

The processes of platelet adhesion to sub-endothelium, discharge of granular contents, and platelet aggregation all overlap and can rapidly produce a platelet plug and cessation of bleeding. However, fibrin is necessary for the

longer term prevention of bleeding and its formation occurs in parallel with platelet plug development (10-13).

1.2. Secondary Haemostasis

The coagulation system is composed of a series of serine proteases and their cofactors which interact on the phospholipid surface of activated platelets to form a stable fibrin clot. Historically, coagulation was divided into extrinsic and intrinsic pathways respectively involving tissue factor (TF)-factor VII (FVII) and surface-contact factors. The penultimate step of both pathways is the conversion of prothrombin (FII) to thrombin (FIIa) which subsequently converts fibrinogen to fibrin.

1.2.1. Extrinsic Pathway

The coagulation cascade is activated when blood comes into contact with TF that is either constitutively presents in tissues surrounding blood vessels or that is exposed on damaged or stimulated cells such as monocytes. TF is a lipoprotein present in almost all tissues in different concentrations, particularly high in lung, brain, bone marrow, kidney and placenta.

When there is a disruption of vascular integrity, blood is immediately exposed to cells expressing TF, leading to initiation of coagulation. Circulating zymogen FVII is exposed to membrane bound TF, becomes activated to the active serine protease (FVIIa) by an unknown mechanism, leading to the formation of a

high affinity TF-FVIIa complex. This complex then converts coagulation factors IX (FIX) and X (FX) to their activated forms (FIXa and FXa respectively). FXa, in combination with its cofactor, activated coagulation factor V (FVa), converts prothrombin to thrombin (14).

1.2.2. Intrinsic Pathway

The intrinsic pathway is initiated by activation of coagulation factor XII (FXII) to FXIIa by the HMWK-kallikrein complex. FXIIa then activates coagulation factor XI (FXI) to FXIa and this converts factor IX (FIX) to FIXa. The latter forms an activation complex (tenase complex) with its cofactor, activated factor VIII (FVIIIa), that binds with FX at the phospholipid surface of activated platelets. The tenase complex converts FX to its activated form FXa, which itself takes part in another activation complex, the prothrombinase complex. The latter comprises FXa and FVa, and converts prothrombin to thrombin. FV and FVIII circulate as inactive procofactors, and are activated to functional cofactors by the proteolytic action of thrombin.

Thrombin cleaves fibrinogen in the N terminal regions of its alpha and beta chains, releasing fibrinogen peptides A and B and producing fibrin monomers. These monomers polymerise to form long strands of fibrin and become cross-linked by activated coagulation factor XIII (FXIIIa), to give a stable blood clot. This crosslinking increases the strength of the clot and helps to prevent its removal by flowing blood. FXIII itself is activated by thrombin
(15, 16).

1.2.3. The Cell Based Model of Haemostasis

The cell based model proposes that secondary haemostasis occurs in three overlapping steps – initiation, amplification, and propagation – and takes place on different cell surfaces, leading to the formation of thrombin.

1.2.3.1. Initiation Phase

During initiation, the TF pathway remains constantly active in a basal state and generates low levels of activated coagulation factors. The generation of a small amount of thrombin continues to take place outside the vasculature even in a healthy individual. In effect, the initial step of coagulation is happening all of the time, but clot formation does not proceed until injury or inflammation occurs to the blood vessel. The TF pathway is regulated by tissue factor pathway inhibitor (TFPI) which binds to any factor Xa and the TFPI/FXa complex inhibits TF/FVIIa.

1.2.3.2. Amplification Phase

Following vessel damage, important components of the haemostatic system (platelets, FVIII and VWF) come into contact with the limited amount of thrombin that is generated on TF-bearing cells in the initiation phase. Platelets attach to the site of injury, make a plug and become fully activated by thrombin and also by contact with VWF and collagen. The thrombin produced in the initiation phase also plays an important role in activating other coagulation factors. It activates FV and is also responsible for both the activation of FVIII and its release from VWF. Thrombin also activates FXI which binds to the surface of activated platelets. At the

end of the amplification phase, activated platelets are coated with active coagulation factors, and the process moves to the propagation phase.

1.2.3.3. Propagation Phase

On the surface of activated platelets, FIXa combines with its cofactor FVIIIa to give the tenase complex that activates FX. FXa immediately combines with its cofactor FVa, forming the prothrombinase complex that converts prothrombin to thrombin. These reactions are extremely efficient on the surface of activated platelets and lead to a rapid production of a large amount of thrombin (the thrombin burst). Thrombin causes the cleavage of fibrinogen to fibrin monomers, which polymerise to reinforce the initial platelet plug and give a stable clot (17-19).

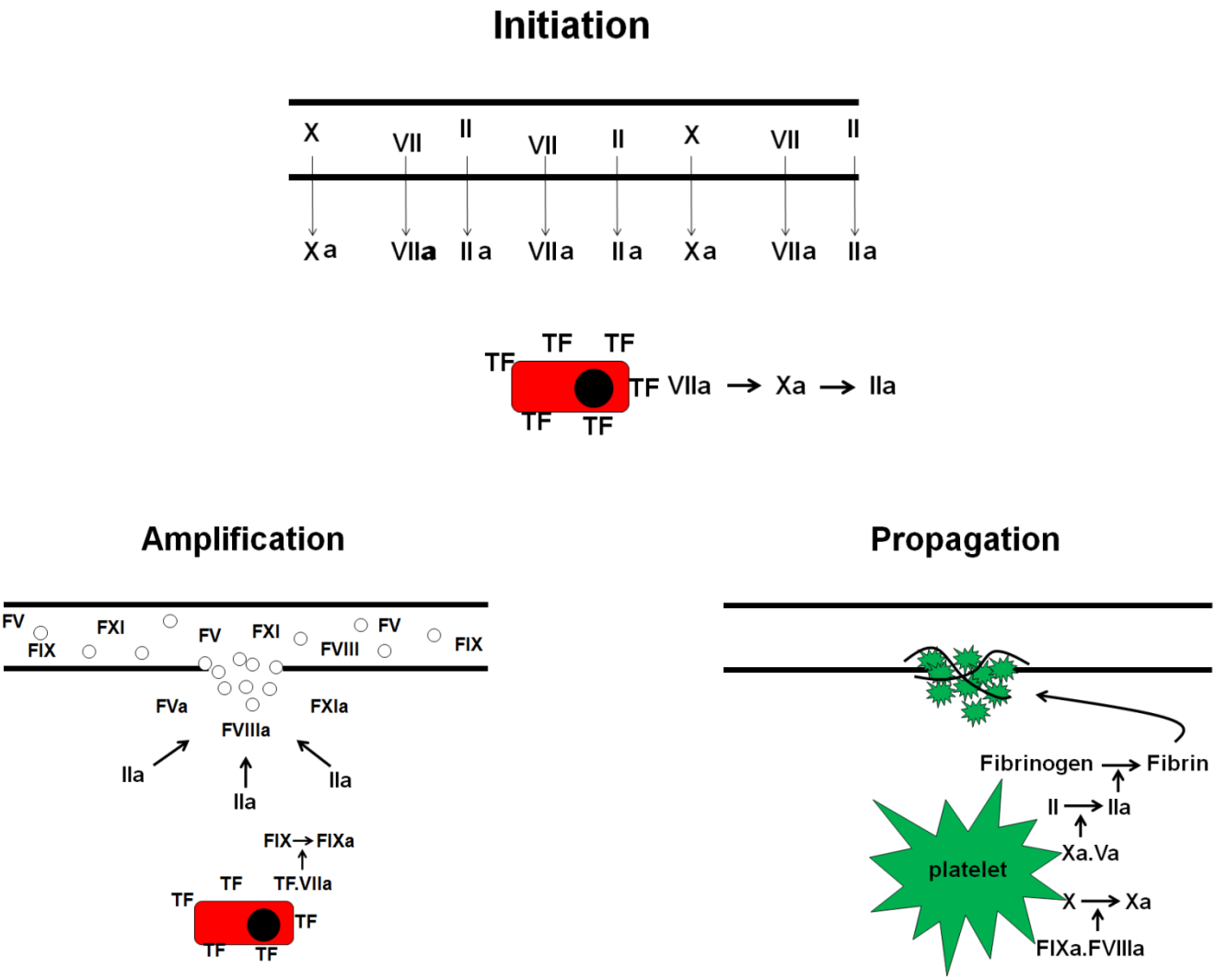


Figure 1.1: A cell-based model of coagulation. The three phases of coagulation (initiation, amplification and propagation) occur on different cell surfaces. Initiation phase occurs on the tissue factor bearing cell, amplification phase on the platelet as it becomes activated and propagation phase occur on the activated platelet surface.

1.3. Fibrinolysis

The principal function of the fibrinolytic system is to ensure that excess fibrin deposition does not occur. Fibrinolysis is a vital, localized, surface-bound phenomenon in which fibrin choreographs its own destruction. The fibrinolytic system is composed of plasminogen (PLG), its activated form plasmin, several endogenous PLG activators (tissue and plasma derived) and a number of inhibitors.

PLG activation is a two-step process, in which tissue plasminogen activator (t-PA) converts it into Glu-plasmin. Glu-plasmin is functionally ineffective and it converts auto- catalytically to Lys-plasmin, the latter interacts with fibrin more efficiently. The major physiological targets of plasmin are fibrin and fibrinogen, which give a series of fragments of gradually decreasing molecular size, fibrin degradation product (FDP) (20, 21).

1.4. Anticoagulation

Physiological anticoagulants are divided into two main groups; one inhibits the serine proteases of coagulation and the other targets the destruction of FVa and FVIIIa. Physiological inhibitors include TFPI, α2-macroglobulin, antithrombin (AT), heparin cofactor II, α1-antitrypsin, nexin 2, C1-esterase inhibitor, α_2-antitrypsin. Protein C and protein S are engaged in the destruction of FVa and FVIIIa (22).

1.5. Haemophilia A and B

Haemophilia A and B are bleeding disorders caused by a deficiency of FVIII and FIX respectively, and are classified into inherited and acquired types. Inherited haemophilia A and B are X-linked, respectively with a frequency of 1 in 10,000 and 1 in 50,000 *per capita* in the general population (23). Haemophilia A is characterised as mild (>0.05 IU/mL), moderate (0.01-0.05 IU/mL) and severe (<0.01 IU/mL) according to the residual amount of FVIII coagulant activity (FVIII: C) present in the blood of the affected individual. Acquired haemophilia A and B are characterised by the production of autoantibodies against the relevant coagulation factor. The annual incidence of acquired haemophilia A is 1.5 per million and unknown in haemophilia B (23).

1.5.1. Structure of the Gene Encoding FVIII

The human gene encoding FVIII (official gene symbol *F8*) is approximately 186 kilobases (kb) long, located towards the telomere of the long arm of the X chromosome (Xq28). The gene contains 26 exons, 24 of which vary in length from 69 to 262 base pairs (bp), the remaining two larger exons (14 and 26) contain 3,106 and 1,958 bp respectively. The *F8* mRNA is approximately 9 kb in length, comprises a short 5-untranslated region (5'-UTR), an open reading frame (ORF) plus stop codon, and a long 3'-UTR (150, 7056, 1806 bp respectively) (24, 25).

1.5.2. Structure and Function of FVIII

Following translation, the signal peptide is proteolytically removed to give a mature FVIII molecule of 2332 amino acids (molecular weight 264,763 Daltons). Mature FVIII has a domain structure arising from internal homologies and unique regions within the protein: NH_2-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH (25). Activated FVIII comprises two chains, heavy chain (HCh) and light chain (LCh). The HCh consists of the A1, A2 and B domains (amino acids 1-336, 373-719 and 741-1648 respectively). The LCh contains the A3, C1 and C2 domains (residues 1690-2019, 2020-2172 and 2173-2332 respectively). The C-terminal region of the A1 and A2 domains and the N-terminal portion of the A3 domain contain a high proportion of negatively charged residues and are called acidic region a1, a2 and a3 respectively (26, 27). Non-activated FVIII is a multidomain structure in which A1 and A3 domains are non-covalently linked via a metal ion-mediated interaction. The C2 domain of FVIII is responsible for its attachment with activated platelet surface, thrombin, FXa. The a3 and A3 domains are respectively involved with the binding VWF and FIXa. The B domain is not directly involved in FVIII procoagulant activity but it has an important role in intracellar processing and trafficking (28, 29).

Immediately after release into the circulation, FVIII as an inactive precursor forms a tight noncovlant complex with VWF. This interaction is mediated by the LCh with contributions from the a3 region (the major binding site) and parts of the C2 and C1 domains. The HCh does not directly bind to VWF, however in its presence, the interaction of FVIII with VWF is increased 10-fold. This interaction results in significant stabilization and survival of FVIII in the circulation (30).

Activation of FVIII is required for its cofactor function in the tenase complex; it has no detectable cofactor activity in the inactivated form. In the tenase

complex, FVIIIa acts as a cofactor for FIXa in the conversion of FX to FXa. In the absence of cofactor, FIXa has a very low catalytic activity. Thrombin and FX are two major physiological activators of FVIII, both proteases cleave the cofactor within the HCh at Arg372 (between the A1 and A2 domains), at Arg740 (between the A2 and B domains) and also within the LCh at Arg1689. This results in a heterotrimer (A1/A2/A3-C1-C2) which is the active form of the cofactor (31). Cleavage at Arg1689 results in the removal of a3, which is vital for the release of FVIIIa from VWF. After this dissociation, FVIIIa effectively binds to the phospholipid (PL) surface and to FIXa during assembly of the tenase complex.

The cofactor activity of FVIIIa in the tenase complex requires three important interactions: binding to the PL membrane, FIXa and FX. There are high and low-affinity interactions between FVIIIa and FIXa which are conferred respectively by the A3 domain in the LCh and the A2 domain in the HCh. These interactions stabilize FVIIIa within the tenase complex.

The activated heterotrimeric FVIII is highly unstable, there is a tendency for spontaneous dissociation of the A2 subunit. There is a rapid loss of the A2 subunit because it is weakly associated with the A1/A3-C1-C2 dimer through low affinity electrostatic interaction and loss of this domain is concomitant with the loss of FVIII activity. In addition FVIIIa is susceptible to proteolytic inactivation by activated protein C (APC) (32, 33).

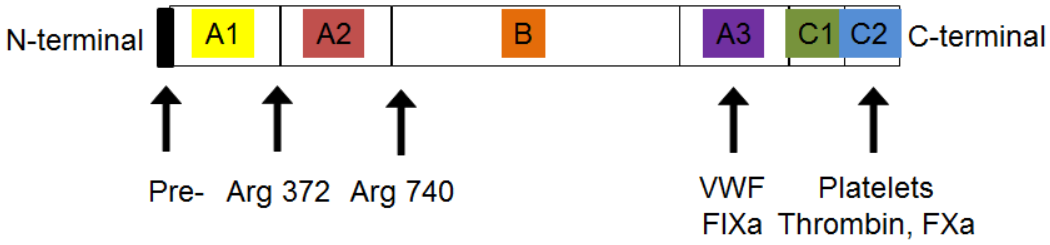


Figure 1.2: Structure of coagulation FVIII. Mature FVIII molecule consists of 2332 amino acids. Activated FVIII constitute of two chains, heavy chain (A1, A2 and B domains) and light chain (A3, C1 and C2 domains). The C2 domain of FVIII is involved for its attachment with FXa, activated platelet surface and thrombin, the a3 and A3 domains are respectively involved with the binding VWF and FIXa. The B domain of FVIII is not directly involved in its procoagulant activity. Two major physiological activators of FVIII are thrombin and FX, both proteases cleave the cofactor within the heavy chain at Arg372, at Arg740 and also within the light chain.

1.5.3. Clearance of FVIII

Removal of FVIII from the circulation involves a receptor mediated catabolic pathway. FVIII is bound and catabolized by low-density lipoprotein receptor-related protein (LRP). LRP is a multiligand hepatic receptor that belongs to the low-density lipoprotein (LDL) receptor superfamily (34). These are endocytic receptors involved in removal and clearance of diverse molecules. LRP-mediated clearance of FVIII is facilitated by cell-surface heparan sulphate proteoglycans (HSPGs), which are one of the major glycoprotein components of the extracellular matrix. The A2 domain of the HCh, and the C2 and A3 domains of the LCh are involved in the interaction of FVIII with LRP (35).

1.5.4. Pathophysiology

F8 defects associated with haemophilia A are divided into several categories: gross gene rearrangements, deletions or insertions of genetic sequence of a size varying from one base pair to entire gene, single DNA base substitutions (point mutations) resulting in either an amino acid replacement (missense) or a premature termination codon (non-sense) and RNA splicing defects.

1.5.4.1. Intron 22 Inversion

About 50 % of severe haemophilia A individuals have an inversion involving intron 22 of *F8* (Inv22). Intron 22 is extremely large (approximately 32

kb), contains a GC-rich region and two transcribed genes (*F8A* and *F8B*). A 9.5 kb sequence (*int22h-1*) within the intron 22 GC-rich region is replicated at two positions approximately 300 kb (proximal, *int22h-2*) and 400 kb (distal, *int22h-3*) telomeric to *F8*. Inv22 involves homologous recombination between the intragenic *int22h-1* and one of the two extragenic homologs (*int22h-2* or *int22h-3*). This recombination occurs during the meiosis of spermatogenesis, resulting in a large inversion in which exons 1-22 are translocated away from exons 23-26, thereby disrupting *F8*. According to which extragenic homolog is involved, the inversion is either proximal or distal. The latter is involved in the majority of cases (15, 36-38).

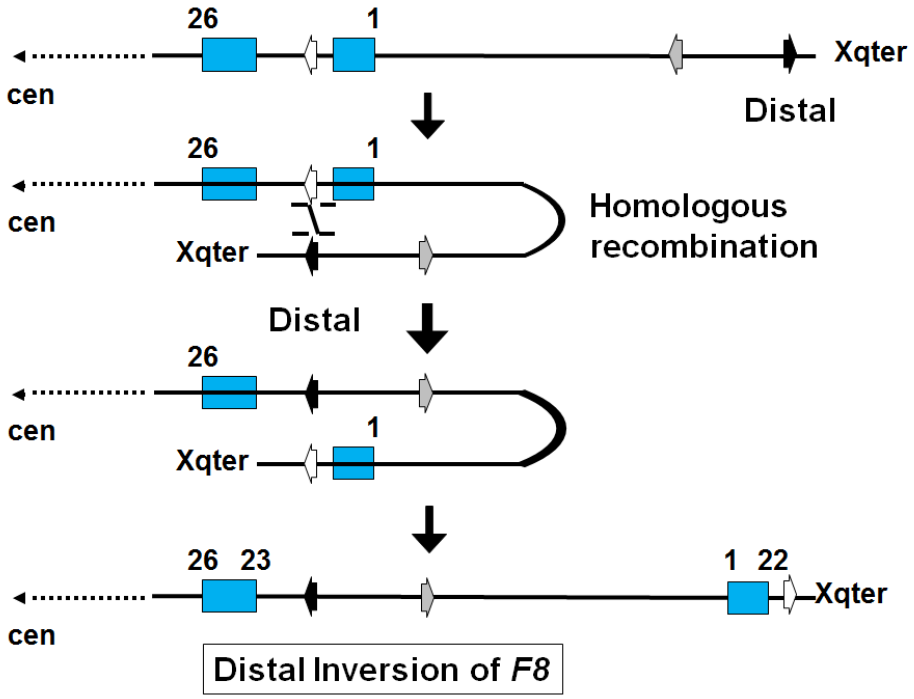


Figure 1.3: *F8* inversion and disruption mediated by the *F8*A gene in intron 22. The genomic organization of the *F8* consists of the three copies of the *F8*A genes (two upstream of extragenic *F8* and one intragenic). The direction of transcription of *F8* is indicated by arrow. This recombination results in an inversion and disruption of the *F8*. Intron 22 inversion is associated with severe haemophilia A.

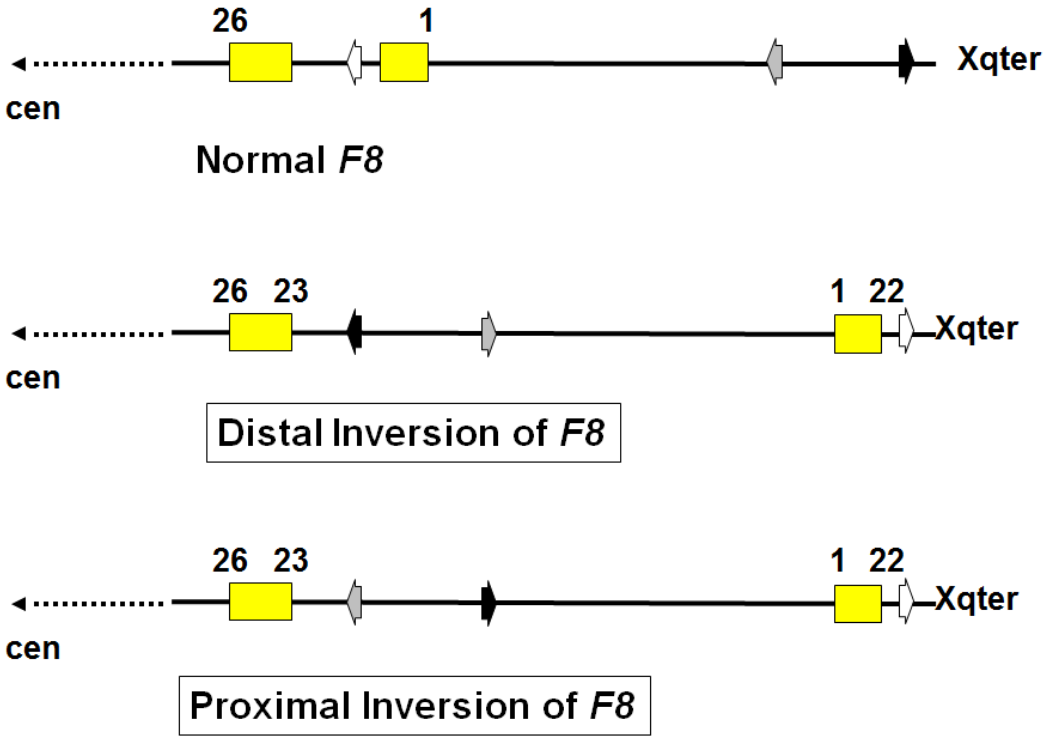


Figure 1.4: Intron 22 proximal and distal inversion of *F8*.

1.5.4.2. Intron 1 Inversion

There is another recombination called the Intron 1 inversion (Inv1) which occurs in about 2-5 % of individuals with severe haemophilia A. This recombination involves a 1.0 kb sequence in intron 1 (*int1h-1*,) and a homologous sequence approximately 140 kb 5' to *F8* (*int1h-2*). As a result of this recombination, there is the separation of the promoter and exon 1 of *F8* from the reminder of the gene (39).

1.5.4.3. Single-base Substitutions in *F8*

Single nucleotide substitutions (point mutations) in *F8* can result either in missense or nonsense changes. The online database for haemophilia A mutations (URL http://hadb.org.uk/) currently lists over 1800 individual reports of *F8* variants from all over the world (time of writing 20/03/13). Missense mutations can be associated with mild or moderate disorder, less so with severe haemophilia A. In contrast, nonsense mutations are associated with severe haemophilia A. In addition to missense and nonsense mutations, single base substitutions can occur at conserved mRNA splice sites (splice site mutation) which interfere with RNA processing. Splice site mutations are found in association with mild, moderate and severe disorder (40-42).

Some mutations are recurrent however in more than 70 % of cases, they are unique to individuals. Interestingly, the clinical phenotype may differ for a given recurrent missense mutation in different individuals (43, 44).

1.5.4.4. Insertions and deletions

The international haemophilia A mutation database catalogues both *F8* insertions and deletions. *F8* deletions are divided into large (>50 bp) and small (<50 bp). A deletion has a high probability of destroying genetic function and there is a very high probability that the functional protein is not formed. Large deletions typically give rise to a clinically severe disorder with no measurable FVIII activity. Large and small deletions may cause frame-shift mutations which are generally

associated with severe haemophilia A, but in a few instances a moderate or mild disorder results (45-47).

Insertions are among the rarer *F8* defects. The majority cause frame-shifts and are associated with severe haemophilia A (48, 49).

When a gene defect abolishes *F8* expression, individuals are at risk of developing inhibitors in response to FVIII replacement therapy. It is therefore not surprising that inhibitor development is higher among individuals with a deletion or an insertion.

1.5.5. Clinical Presentation

1.5.5.1. Severity and Bleeding

The severity and frequency of bleeding in haemophilia A is inversely correlated with residual FVIII level. Individuals with haemophilia A present with bleeding into joints, muscles or other soft tissue, either spontaneously or after trauma or surgery. Bruising and spontaneous bleeding, especially gastrointestinal and central nervous system are also typical of the features of haemophilia.

The main clinical presentation of severe haemophilia A is haemarthrosis (joint bleed), >90 % of all the bleeding occur in the joints and 80 % of haemarthrosis occur in the ankles, knees and elbows, however any joint can be the site of bleeding (50). Acute haemarthrosis usually start with discomfort and mild limitation of joint motion followed by pain, swelling and cutaneous warmth. Repeated episodes of haemarthrosis cause irreversible damage to joints which leads

to haemophilic arthropathy (a polyarticular disease characterized by joint pain, stiffness and severely limited range of motion).

Muscle bleeds can occur at any anatomical site, but in most cases, large load bearing groups of muscles (thigh, calf, posterior abdominal wall and buttocks) are involved. Bleeding into the forearm, calf or perineal muscles can lead to ischemic necrosis and contracture (51).

Haematuria is less common than joint and muscular bleeding in individuals with haemophilia A, but severely affected individuals may have one or two episodes per decade. These symptoms may be painless and resolve spontaneously, but in the case of heavy bleeding, may produce clot colic.

Central nervous system bleeding is uncommon, but can occur after a slight head injury and is an important cause of death in individuals with haemophilia A, especially those without access to factor VIII or IX. Gastrointestinal tract bleeding may present with haematemesis and melena or rarely as an obstruction due to intramural haemorrhage (52).

Oropharyngeal bleeding is often seen in young children but is less common in adults; clinically it may be dangerous because it may extend through the soft tissue of the floor of the mouth and may lead to respiratory obstruction. Tongue bleeding after laceration may be persistent due to the fibrinolytic substances in the saliva.

In the untreated individual with haemophilia A, surgery and open trauma invariably lead to dangerous haemorrhage. If clots are formed, they are febrile, bulky and break off with renewed haemorrhage occurring irregularly over days and weeks.

An individual with haemophilia A may present with bruising, but it remains superficial and self-limiting, and does not usually require treatment. On the other hand, large extending ecchymosis may occasionally require treatment (53, 54).

1.5.5.2. Pathophysiology of Haemophilic Arthropathy

Mechanical, chemical and genetic factors are involved in the development of haemophilic arthropathy, which results in the fibrosis of synovial lining and disintegration of hyaline cartilage. Haemophilic arthropathy shows the characteristics of both inflammatory and degenerative disease in which the extravasation of blood into the joint cavities leads to chronic synovitis and cartilage destruction. Acute inflammation with the infiltration of polymorphonuclear cells and lymphocytes is followed by the extravasation of blood into the joint capsule. In about one week, this acute episode of haemarthrosis is resolved and blood is removed from the joint space by synovial lining cells and invading macrophages. The blood removal capacity is exceeded by repeated episodes of intra-articular bleeding and blood stays longer in the joint space. This leads to the deposition of iron containing red blood cells in the synovial membrane and progressive accumulation of iron as hemosiderin. This deposited iron is involved in both synovial and vascular cells proliferation in the sub-synovial layer.

Analysis of two biomarkers of bone turn over (cartilage oligomeric matrix protein and type II collagen telopeptides) confirmed that the articular cartilage is an early target for the development of this arthropathy (55-58) and changes in the composition of articular cartilage matrix are a prominent feature of this condition. Articular cartilage is composed of water, electrolytes and solid matrix – collagens

and proteoglycans – and its visco-elastic properties are mainly because of water flow through the solid matrix (59). The composition of proteoglycans across the articular surface varies according to location, patient age and compressive load. The continued presence of blood in a joint increases cartilage compliance, which may cause the loss of proteoglycans and the production of degenerative enzymes from iron-laden synovial and sub-synovial macrophages (60). *In vitro*, iron plays several roles: it stimulates synovial cell proliferation, may increase the *c-myc* proto-oncogene expression and promotes DNA synthesis. Iron has an additional effect on cytokine activity, it also induces the expression of *Mdm2* (as an ubiquitin ligase) that binds to p53 (tumour suppressor binding protein) and prevents its function. Other potential genetic modifiers include those that impact the innate immune system (61, 62) – osteopontin (63), interleukin 6 (IL-6) (64), interleukin 8 (IL-8) , interleukin 17 (IL-17) (65, 66) and the chemokine CXCR1/2 (67, 68) – these control neutrophil infiltration into the joint space as well as regulate monocyte/macrophage function. In haemarthrosis, angiogenesis and vascular cell proliferation take place which are influenced by the aberrant expression of vascular endothelial growth factor (VEGF-R) and platelet-derived growth factor (PDGF). In addition to angiogenesis, VEGF is also involved in chondrocyte metabolism (69-75). The exact role of VEGF and PDGF is not known, however it has been suggested that, in the development and progression of haemophilic arthropathy, there is an imbalance between cytokine related cartilage degradation and maintenance of proliferative and synthetic cell responses. In individuals with haemophilia A, a genetic variation between novel receptor activators of nuclear factor kappa-B (RANK) and RANK ligand (RANKL) and osteoprotegrin (OPG) occur which also play an important role in osteoclast activity. It has been assumed that the increased expression RANK and RANKL by both normal and inflammatory neutrophils, together with decrease in

OPG, favour osteoclast differentiation and bone resorption. Haemophilic arthropathy may directly influence the presence of cellular components of the blood: moderate amounts of protein bound heme are very important for various biological functions, however if it is present in large quantity, as occur in haemarthrosis, it has toxic effects mediated by oxidative stress and inflammation. Within the joint microenvironment, chronic oxidative stress leads to the generation of reactive oxygen intermediates (ROIs) that further contribute to the destruction of cartilage and bone (76-84). In addition to this, ROIs may alter the expression of the osteoblast transcription factor, RUNX2 which leads to decrease of osteoblast activity (85, 86). As blood induced joint disease progresses, bone remodelling occurs as well as in the presence of inflammation, osteoclastic activity is also increased, and bone mineralization is impaired. As the result of this there is the net loss of bone and failure of bone repair.

Normally the synovial membrane is thin and mostly avascular but, as a result of proliferation of synovium, the neovascularization of the sub-synovial layer occurs. As the result of neovascularization, an inflamed, villous, friable and highly vascular synovial tissue replaces the avascular synovium which is more susceptible to further haemorrhage even with a minimal stress. Due to the presence of blood in the joint capsule the mechanical distension and pressure in the joint space further increase which additionally induces the apoptosis of chondrocytes and an inhibition of proteoglycan synthesis. As a result, involved cartilage is thereby unable to restore the synthesis of its matrix which leads to long-lasting joint damage. With time, a crippling arthritis is developed, and the final result is a fibrotic and destroyed joint (87, 88).

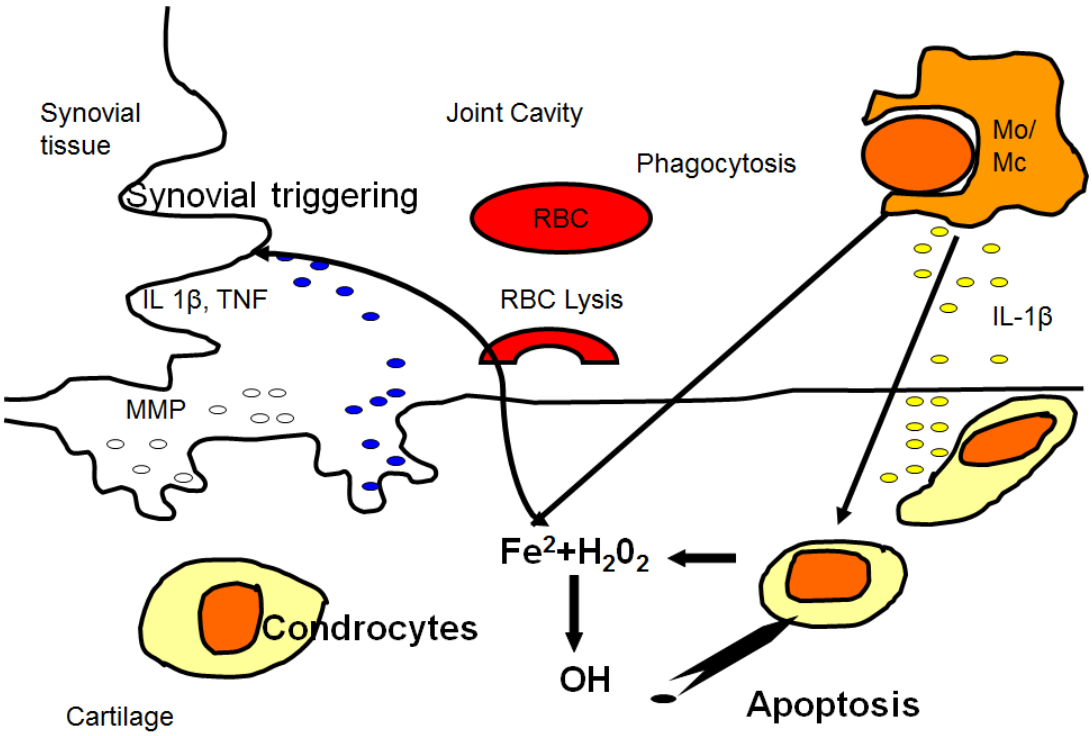


Figure 1.5: Blood induced joint damage. The blood induced joint damage is the result of the formation of hydroxyl radicals in the vicinity of the articular chondrocytes which leads to the apoptosis of these chondrocytes. Hydroxyl radicals are formed when hydrogen peroxide is synthesized by chondrocytes upon stimulation by IL-1 which originates from activated monocytes/macrophages. Chondrocytes apoptosis leads to disturbance and impairment of the cartilage matrix.

Described as above, in the destruction of articular cartilage several mechanisms – enzymatic digestion as well as direct cellular destruction, direct toxic influence by the presence of blood and certain mechanical factors such as local pressure, abrasion , osteopenia and abnormal loading secondary to contractures – are involved. In haemophilic arthropathy, several theories explained the formation of subchondral bone cyst: osteolysis with coalescence of space, post haematoma, infiltration of articular or pararticular surfaces with fibrovascular tissue, ischemic necrosis of bone with resorption and collapse of subcondral bone (89). Pressure may play an important role in tissue necrosis and it is suggested that hydrostatic pressure on epiphyseal vessels by haemoarthrosis may produce subchondral ischemia which leads to cyst formation (90). The centrifugal progression of haemophilic arthropathy from the load-bearing areas of the condyles, intercondylar notch and patella mention a mechanical stress or injury related factor in its pathogenesis (90, 91). As the result of subchondral haemorrhage, the worst event is the microfracture of subchondral bone plate, this fracture may occur with minimal stress in the osteopenic bone of individuals with chronic haemophilic synovitis. Healing of these subchondral microfactures provides a stiffer base for the overlying articular cartilage, ultimately the subchondral bone disappears, the support for the overlying articular cartilage is lost and all is replaced by sclerotic lamellae recessed (92).

1.5.5.3. The Joint Health Assessment

It is not easy to detect a joint bleed with radiography but to visualize the gross arthritic alteration, plain radiography film is usually used (50). In children

with haemophilia who had no clinical sign of haemophilic arthropathy, magnetic resonance imagining (MRI) is very helpful to detect early changes. In haemophilic arthropathy, MRI has several advantages over plain radiography: it can be used to detect the early changes in the soft tissue – synovium and cartilage – and it is anticipated that joint damage starts even after very few bleeding episodes (93). For haemophilic arthropathy, two MRI scoring systems are employed: for severe joint changes a progressive system is used, for osteochondral and soft tissue-related changes an additive system is used. However, MRI has some limitations including its ability to distinguish synovial hypertrophy and hemosiderin deposition (94). To overcome these deficiencies and to access the joint damage, ultrasound can also be used. Ultrasound may detect synovititis as well as bone and cartilage alternation and in haemophilic arthropathy it is a reliable tool to evaluate the joint modification (95).

Measurement of the joint health is critically important when assessing an individual with haemophilia A. Joint scores such as the haemophilia joint health score (HJHS) and Gilbert score (also known World Federation of Haemophilia Physical Examination Score) are a standardised way of assessing the progression of joint disease and can be observed over time.

The HJHS and Gilbert score measure in the domain of body structure and function, the joints most commonly affected by bleeding in individuals with haemophilia. HJHS is an 11-items scoring tool (swelling, duration of swelling, axial alignment, muscle atrophy, crepitus, flexion loss, extension loss, joint pain, instability, gait and strength) for assessing joint impairment of six index joints (elbows, knees and ankles). Data from each assessed item are scored on an ordinal categorical scale and each of six joints is assessed individually. Knees and ankles

are scored using all 11- items, with a total joint score range from 0 to 26. On the other hand, elbows are scored using 9 of the 11 items (giant and axial alignment are excluded). A global gait score is assessed from 0 (indicating all skills are within normal range) to 4 (indicating that no skills are within normal range). All joint and global gait scores are then combined to provide a total score ranging from 0 to 148.

Gilbert score is based on 8 main components: swelling, duration of swelling, muscle atrophy, crepitus on motion, flexion loss, extension loss, joint pain and strength (96, 97). All of the components of Gilbert score are combined together to give a total score ranging from (0-100), excluding the pain and bleeding subscales (0-18).

HJHS is primarily designed for children aged between 4-18 years with mild joint impairment (for example, individuals on prophylaxis). On the other hand the Gilbert score is mainly designed for adults and older children with established arthropathy. It is probably best used for severely affected population such as individuals with uncontrolled inhibitors or little assess to factor replacement, however it can be used as an outcome measurement of physiotherapy intervention (98-101).

1.5.6. Treatment of Haemophilia A

The WFH strongly recommends the FVIII concentrate (plasma derived or recombinant) in preference to the cryoprecipitate (CP) or fresh frozen plasma (FFP) for the treatment of haemophilia A (102, 103). In developed countries, widespread availability of factor concentrate fundamentally improved haemophilia care, resulting in early control of haemorrhages and musculoskeletal damage. However,

in developing countries like Pakistan, supportive therapies (FFP and CP) are usually used to control the bleed. CP and FFP are not a good option of treatment because they are not subjected to viral inactivation procedures – heat or solvent/detergent treatment – which lead to an increased risk of viral pathogen (102). The other disadvantage is that a large volume of FFP (1 mL of FFP contains 1 unit of factor activity) is used to get a required *in vivo* FVIII level and generally, it is difficult to achieve FVIII > 30 IU/dL with FFP alone (104). If factor concentrate is not available, CP is preferable to FFP.

For the prevention of bleeding episodes, the administration of FVIII concentrate, either on prophylaxis or on-demand treatment, is indicated. Prophylaxis may be primary or secondary: primary prophylaxis is defined as the regular, continuous long term treatment of FVIII concentrate which is usually started before the age of 2 years and /or after no more than one joint bleed, whereas secondary prophylaxis is defined as all long-term regular continuous treatment not fulfilling above mentioned criteria (105-107). Several international randomized and observational studies indicated that prophylaxis initiated in early years of life protects from joint damage and decrease the incidence of haemarthrosis and other haemorrhages (53, 108).

The most challenging complication of FVIII replacement therapy for individuals with haemophilia A is the occurrence of inhibitory alloantibodies; approximately 25-30 % of severe individuals within the first 50 days of treatment face this complication. In haemophilia, the inhibitor formation is a multifactorial process in which a number of genetic – ethnicity, *F8* gene mutation and polymorphisms of immune response genes – and acquired – number of FVIII exposure days, age at first exposure and type of FVIII concentrate administered – risk factors are involved (109, 110).

1.6. Coagulation Tests

Coagulation tests are the first line investigation of a suspected inherited bleeding disorder. These tests reflect the history of coagulation investigation and give useful laboratory measures, however these are significantly removed from normal physiology. The most commonly used tests to assess the defect in coagulation are the prothrombin time (PT) and activated partial thromboplastin time (aPTT), these tests require the activity of all of the procoagulant factors except FXIII. Coagulation factors assays are used to identify specific deficiencies but do not always correlate with the severity of bleeding. PT and aPTT measure the time necessary for clot formation. Neither test assesses the coagulation system as a whole, although both are sensitive to deficiencies within the common pathway. They are performed using platelet poor plasma (PPP), and clot formation occurs only when 5 % of total thrombin has formed.

1.6.1. Prothrombin Time

PT measures the extrinsic and common pathways of the classical coagulation cascade. In this test, coagulation is triggered by adding thromboplastin (TF plus PL). PT is sensitive to clotting factors V, VII, X and fibrinogen and less so to prothrombin. PT is prolonged if the concentration of any of these factors is about 10 % or more below the normal range. Vitamin K deficiency, liver disease or disseminated intravascular coagulation (DIC) may prolong the PT, however in haemophilia, which is an intrinsic pathway defect, a normal result is typically

obtained. The sensitivity of this test is influenced by the reagents and techniques used, therefore it is important to establish a reference range locally. The normal range is laboratory specific and is typically between 11-15 seconds in healthy individuals.

1.6.2. Activated Partial Thromboplastin Time

aPTT is a test for the intrinsic pathway; It is the time required for plasma to clot following surface contact activation and optimal PL and calcium concentration are provided. In the presence of a normal PT, aPTT is very sensitive to deficiency of prekallikrein (PKK), HMWK, FXII, FXI, FIX and FVIII. It is therefore abnormal in haemophilia A and B. Additionally, aPTT can be prolonged in any deficiency involving the common pathway (FV, FX, prothrombin and to lesser extent fibrinogen). aPTT is prolonged in the presence of some therapeutic inhibitors of coagulation such as heparin and naturally occurring inhibitors such as a lupus anticoagulant. In the aPTT, PPP is triggered to clot using a negatively charged surface (kaolin or silica) to initiate contact activation; PL and calcium are added to the PPP for the further progression. The normal range for the aPTT is laboratory dependent and is usually between 29-35 seconds.

1.6.3. Mixing Studies

A plasma sample that has a prolonged PT or APTT is further investigated to define the abnormality. This is done using mixing or correction tests. It is very

important to demonstrate whether a defect in patient plasma is corrected with normal plasma in order to exclude the presence of an inhibitor.

1.6.4. Thrombin Time

The thrombin time (TT) is a measure of the conversion of fibrinogen to fibrin by thrombin. It is prolonged (normal range 13-15 seconds) when fibrinogen level is very low (< 1.0 g/L) or in the presence of heparin and heparin-like substances, FDP or dysfibrinogenemia. TT is performed by adding a dilute preparation of thrombin to citrated PPP and measuring the clotting time. PL and recalcification are not required.

1.6.5. One-Stage Clotting Assay for FVIII Activity

This assay is based upon the ability of a test sample to correct the aPTT in standard plasma that is totally deficient in FVIII but containing all of the other factors required for normal clotting. FVIII activity (FVIII:C) is measured according to the degree of correction of the standard plasma.

1.6.6. Quantitative Measurement of FVIII Inhibitor

FVIII inhibitors resulting from replacement therapy in haemophilia A are typically demonstrated by a mixing test in which the normal and test plasma (with prolonged aPTT) are mixed in equal proportions. Failure to correct the aPTT indicates the presence of an inhibitor (correction indicates a coagulation factor

deficiency as discussed above). If FVIII is added to plasma containing an inhibitor and the mixture incubated for some time, the FVIII will be progressively neutralized. If the amount of FVIII and the incubation time are standardized, the strength of the inhibitor is then defined according to the amount of neutralization.

1.6.7. Bethesda Assay

A Bethesda unit is defined as the amount of inhibitor that will neutralize 50 % of one unit of added FVIII in 2 h at 37 ^{o}C. Test plasma dilutions are mixed with normal plasma and the dilutions that gives a residual FVIII:C nearest to 50 % (but within the normal range 30-60 %) is chosen for calculation of the inhibitor. Alternatively, the result can be calculated from each dilution and the average taken.

1.6.8. von Willebrand Factor Antigen

The concentration of VWF antigen (VWF:Ag) in plasma can be measured using immunological assays. Enzyme-linked immunosorbent assay (ELISA) and automated latex immunoassay (LIA) are two commonly used methods.

In ELISA, a primary antibody targeting VWF is coated onto the wells of a suitable plate. Test plasma is added in a series of dilutions and the constituent VWF is bound. The bound VWF is detected using a conjugated anti-VWF secondary antibody. The latter provides a colour reaction from which measurement of VWF is obtained by comparison to reference plasma.

LIA uses latex micro-particles coated with an antibody to VWF. The latex particles agglutinate in proportion to the concentration of VWF:Ag in the plasma sample.

The interpretation of a VWF:Ag value is made in conjunction with other assays (FVIII and VWF activity), the blood group and bleeding history. The reference range for VWF:Ag is 50-150 IU/dL.

1.6.9. Thrombin Generation Assay

Thrombin is an essential part of the coagulation enzymatic cascade. The thrombin generation test (TGT) measures the amount of active thrombin produced in plasma or in whole blood after recalcification and triggering. Thrombin generation (TG) curve (thromogram) is a gold standard tool in coagulation research (111-114) it indicates the overall function of blood coagulation system.

In PPP, TG is very sensitive for pro- and anticoagulation, it expresses all the clotting factor deficiencies except FXIII (115, 116). TG is also very sensitive to the action of oral anticoagulant, direct thrombin inhibitor and all types of heparins (117). In PRP, thrombogram is also very sensitive by platelets, the effect of von Willebrand factor, hypofibrinogenemia and different platelets functions disorders (118). In PRP, TG exhibits the major part of physiological clotting system.

Thrombin generation (TG) can be initiated (triggered) by the addition of exogenous activators such as thromboplastin, or a combination of purified TF and PL, the latter typically of specific composition (119). The clinical utility of this test is extensively reported in haemophilia A (120) in which it is used to tailor individual prophylaxis regimens and, in individuals who have high-titer inhibitors, to predict

optimal dosing of bypassing agents (121). Despite the fact of TG test and its utility in clinical diagnosis and drug monitoring, it is found not be applicable universally because of the technical back ground.

1.6.9.1. Sub-Sampling Method

In this method, TG is triggered by a small amount of thromboplastin. Samples of the clotting blood or plasma are taken at timed intervals and diluted with buffer that contains a chromogenic substrate. TG is measured by a change in optical density at the specific wavelength of the product (122). A major advantage of this method is that TG can be measured in platelet-poor or -rich plasma (PPP, PRP), or in whole blood. Important disadvantages are that the method is very time consuming (at least an hour is required to measure TG in just 1 to 4 samples) and is not automated.

1.6.9.2. Chromogenic Technique

This technique permits continuous measurement of the generation of thrombin. Plasma is triggered by TF in the presence of PL and the amount of thrombin generated is measured by continuous optical density recording of the conversion of a chromogenic substrate. The substrate is cleaved by both free thrombin and also thrombin bound to α_2 macroglobulin, the proportion of the former is then computationally derived. A disadvantage of this technique is the requirement for an optically clear sample; platelet-free or defibrinated plasma must therefore be used (123).

1.6.9.3. Calibrated Automated Thrombography

With calibrated automated thrombography (CAT), a fluorogenic substrate is used to measure TG in plasma that has been triggered with TF and PL. As TG proceeds the substrate is consumed and fluorescence increases. Thrombin concentration is calculated from the conversion of velocity of slow reacting fluorogenic substrate. With the fluorogenic substrate, constant thrombin activity does not correlate a constant increase in the signal output. The relationship between the intensity of fluorescence and amount of thrombin generated is not linear and is influenced by the colour of the plasma (inner filter effect) and ageing of the light source. The method therefore uses a calibrator with a known thrombin activity; this is added to the test plasma and fluoresce measured without triggering. In parallel, thrombin generation is measured in the test sample that has been triggered with TF and PL. In PPP, a standard amount of PL is added as a procoagulant, whereas in PRP, for TG, platelets provide the PL procoagulant.

Main advantages of this technique include the fact that it is not influenced by turbidity and measurements can be done in the presence of platelets or without defibrination of plasma. There is a dependency of this fluorogenic method on fibrinogen, higher concentrations of which lead to an increase in thrombin generation (124). Methods that are not using a calibrator can under- or over-estimate the amount of thrombin generated. *In vivo,* thrombin is neutralized by anti-thrombin and complex formation with α_2 macroglobulin. However, the thrombin-α_2

macroglobulin complex is still able to cleave the fluorogenic substrate and the signal generated by this needs to be subtracted from the total fluoresce.

Software is used to calculate the amount of thrombin generated over time for a sample. The main parameters in this method are: lag time (period required for the generation of 10 nmol of thrombin, it is roughly equivalent to the clotting time), endogenous thrombin potential (ETP, the area under the curve), peak (the maximum concentration of thrombin, also called peak height or thrombin peak), time to peak (ttpeak, the time it takes to reach the peak) and velocity index (peak divided by the ttpeak minus the lag time).

The thrombogram depends upon certain factors, trigger used for the assay is one of them. As excess of TF cause the prothrombinase formation under the activation of extrinsic pathway (coagulation factor VII, V and X) and inhibition of TF pathway. Decrease the TF concentration, TG depends upon FVIII and FIX concentration under the activation of intrinsic pathway. The concentration of PL is also very important, even in the absence of TF; excess amount of PL causes the activation of intrinsic pathway and bust of thrombin formation.

The thrombin generation assay is only sensitive to FVIII deficiency when low concentrations of TF are used to trigger the reactions (125). This is because higher concentrations of TF overwhelm TFPI and bypass the propagation phase of coagulation (126). The amount of TF included in assays is usually achieved by diluting Innovin. Final concentrations of TF of 1 pmol/L are often used to investigate FVIII deficient samples although more information may be derived from using a variety of TF concentrations.

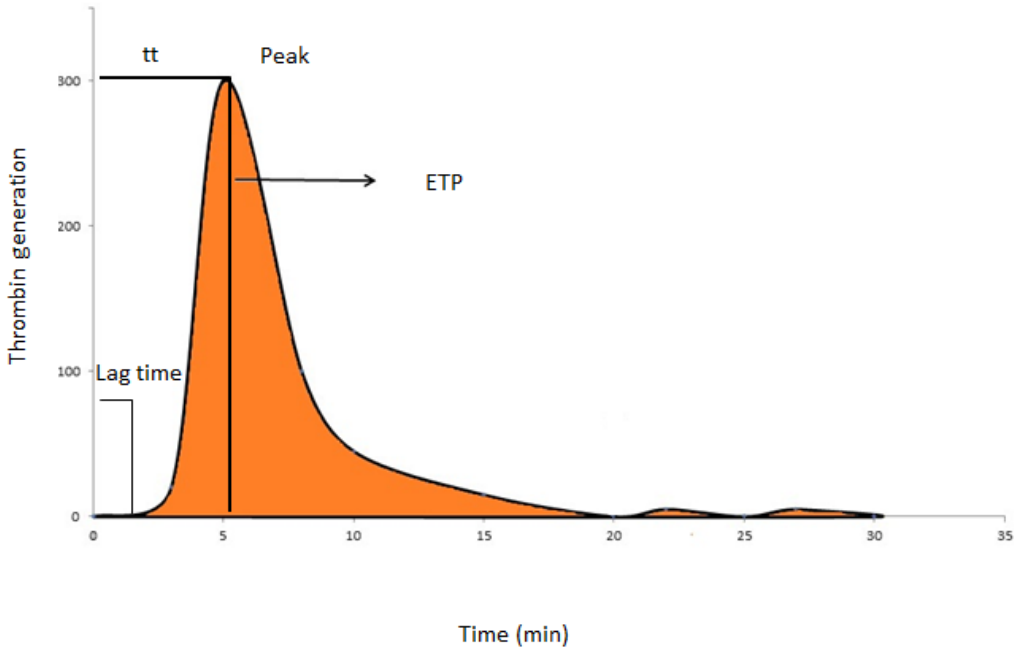


Figure 1.6: The key measures of thrombin generation - lag time (the moment when 10 nmol/L thrombin has been generated, it is equivalent to the clotting time), peak height of thrombin generation (maximum velocity of thrombin generation), time to peak (tt) and endogenous thrombin potential (ETP) represents the total amount of thrombin generated over time and is calculated by the area under the curve) are indicated. Velocity index (VI) (rate of thrombin generation per minute) VI = peak thrombin/peak time-lag time.

1.7. Hypothesis

The severity of haemophilic arthropathy, in individuals with the same baseline FVIII level who are receiving minimal FVIII replacement, is affected by their global haemostatic potential despite the mutation underlying haemophilia A. This cannot be easily studied in patients treated in the UK because their clinical phenotype is modified by prophylactic treatment, but could be studied in haemophilia individuals from Pakistan where prophylaxis is uncommon and the effect of baseline characteristics could be investigated.

1.8. Aims

The aim of this study was to:

a. Undertake genetic, phenotypic and clinical investigation of individuals with hereditary haemophilia A who have minimimal access to treatment (FVIII concentrate).
b. Explore the spectrum of mutations in this geographical group for comparison with other nationalities
c. Investigate predisposing factors that affect joint damage.

The phenotypic studies included global haemostasis (TG) for individuals with (i) identical causative mutations, (ii) similar FVIII:C but different causative mutations, (iii) similar clinical severity but different FVIII:C.

A unique feature of this study was that, unlike many other countries, Pakistan has only very limited access to FVIII concentrate, therefore the joint status of each patient was not modified by treatment.

Chapter 2

Material and Method

2.1. Cohort Recruitment

Two cohorts, each of 100 randomly selected individuals, were recruited in Pakistan. One cohort comprised unrelated individuals previously diagnosed with haemophilia A, and included mild, moderate and severely affected cases. The other cohort comprised healthy controls and was recruited to test whether novel genetic variants found in the haemophilia cohort were neutral polymorphisms. Ethical review was undertaken by the National Bioethical Committee of Pakistan and by Cardiff University (respective reference numbers 4-87/10/NBC-52/RDC/2589; SMREC number 11). Informed consent was taken according to local regulations in Pakistan.

The joint health status of each individual was assessed using both HJHS (version 2.1) (95) and Gilbert score (98). Researcher recorded clinical details and did the assessment of joint score for all individuals. Researcher had training for the assessment of joint score from a qualified haemophilia specialist physiotherapist in Cardiff prior to cohort recruitment.

2.2. Chemicals and Reagents

All general laboratory reagents were supplied by VWF International Ltd (Leighton Buzzard, UK). Sodium citrate anticoagulant tubes were obtained from Greinerbio-one LTD (Gloucestershire, UK). Sodium citrate/corn trypsin inhibitor (CTI) blood collection tubes were purchased from Haematologic Technologies Inc (Vermont, USA). The following HemosIL^{TM} reagents were supplied by IL

Company (Bedford, USA): PT HemosIL^{TM} RecombiPlasTin 2G, aPTT HemosIL^{TM}, calcium chloride ($CaCl_2$) and HemosIL^{TM} SynthASil aPTT reagent , special test control level 2, normal control, Calibrated Plasma, Factor VIII Deficient Plasma and Factor Diluent. Precision Biologic Cryocheck abnormal 1 (Cryo Green) and 2 (Cryo Red) were purchased from Precision Biologic (Dartmouth, Canada). FVIII Deficient Plasma was obtained from Helena Biosciences Europe (Gateshead, UK). Phosphate buffered saline (PBS) was purchased from Microgen Bioproducts (Surrey, England). Tween-20 was supplied by Novara Group Ltd (Leicestershire, England). K-Blue Substrate and Red Stop Solution were supplied by Neogen Europe Ltd (Ayr, Scotland, UK). Pathway Diagnostic Normal Human Control Plasma was supplied by Technoclone Ltd (Dorking, UK). Polyclonal rabbit anti-Human von Willebrand factor (A0082) and Polyclonal rabbit anti-Human von Willebrand factor conjugated with horse radish peroxidise (anti-human von Willebrand factor/HRP, P0226) were supplied by Dako (Glostrup, Denmark). VWF reference standard (6th International Standard plasma for Factor VIII and VWF) was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). The PL reagents 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (phosphatidylserine), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (phosphatidylethanolamine) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (phosphatidyl choline) were obtained from Avanti Polar Lipids Inc. (Alabama, USA). TF was supplied by Siemens Healthcare (Marburg, Germany). Fluorogenic substrate Z-Gly-Gly-Arg-AML HCL was purchased from Bachem AG (Hauptstrasse, Switzerland). Bovine Serum Albumin (BSA), N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic Acid (HEPES) acid and HEPES salt were obtained from Sigma Chemical Company (Dorset, UK). $CaCl_2$ for CAT analyses was supplied by Prolabo (Geldenaaksehaan, Belgium). Thrombin calibrator

was supplied by Thrombinoscope BV (Maastricht, Netherland). Recombinant human FVIII (Advate) was obtained from Baxter HealthCare Corporation (Norfolk, UK). *Bcl*I and NEBuffer 3, T4 DNA Ligase, T4 DNA ligase buffer, pBR322/*Msp*I size markers and 1 kb ladder size standard were obtained from New England Biolabs Inc. (Hertfordshire, UK). AmpliTaq DNA Polymerase, PCR Buffer II and magnesium chloride ($MgCl_2$) were purchased from Roche Diagnostic Limited (West Sussex, UK). Bromophenol blue was purchased from BDH chemicals Ltd (Dorset, UK). Primers ID, IU, ED, Int1h-2F, Int1h-2R, 9F, 9cR and all primers for *F8* sequence analysis were obtained from Life Technologies Ltd (Manchester, UK). Centri-Sep 8-well strips and the ABI PRISM BigDye Terminator (BDT) Cycle sequencing Ready Reaction Kit were purchased from Applied Biosystems, Life Technologies Ltd (Paisley, UK).

2.3. Preparation of Platelet Poor Plasma

Patients: blood (2x5 mL) was collected into (i) sodium citrate anticoagulant (3.2 %, w/v) and (ii) sodium citrate/CTI (20 mmol/L) tris, 150 mmol/L NaCl, 11 mmol/L citrate, 50 µg/mL CTI, final concentrations in the collected blood). Controls: blood (2x5 mL) was collected into sodium citrate as above. PPP was prepared by double centrifugation of all anticoagulated samples at 2000 xg for 15 min. PPP and cell fractions were stored frozen at -80 ^{o}C and transported to the UK on dry ice for phenotypic and genetic analysis.

2.4. Phenotypic Parameters

The following phenotypic parameters were measured for citrated plasma from all 100 patients: PT, aPTT, fibrinogen, FVIII:C, FIX coagulant activity (FIX:C), FVIII inhibitor and VWF:Ag.

PT, aPTT, fibrinogen and FVIII:C were measured using an ACL-TOP 500 automated bench-top random access analyser (Instrumentation Laboratory Company, Bedford, USA). FVIII:C was measured with an automated 1-stage aPTT-based (non chromogenic) assay. Internal quality control was done for each test prior to analysis of experimental samples. For each phenotypic parameter, the reagents for analysis were prepared as follows:

2.4.1. PT

HemosIL^{TM} RecombiPlasTin 2G (supplied as "diluent reagent" and "PT reagent") was used. Diluent and PT reagent were allowed to equilibrate separately at RT for 15 min. Diluent (20 mL) was then added to PT reagent, the mixture was swirled gently and allowed to reconstitute for 20 min. The solution was stable for 10 days at 2-8 ^{o}C.

2.4.2. aPTT

HemosIL^{TM} $CaCl_2$ (0.02 mol/L) and HemosIL^{TM} SynthASil aPTT reagent were used. Both reagents were allowed to equilibrate at RT for 15 min. The stabilities were respectively 10 and 30 days for aPTT reagent and $CaCl_2$.

2.4.3. FVIII:C

HemosIL™ special test control level 2, HemosIL™ normal control, HemosIL™ FVIII Deficient Plasma and Helena Biosciences FVIII Deficient Plasma were each reconstituted in 1 mL of sterile water. Control reagents were kept at 15-25 °C for 30 min. Samples were stored frozen at -70 °C prior to analysis. Before use, samples were thawed at 37 °C for 5 min and then checked to ensure they were fully defrosted. The stabilities of the reconstituted reagents were as follows: HemosIL™ special test control level 2, 8 h at 15 °C or 24 h at -20 °C; PrecisionBiologic Cryocheck abnormal 1 (Cryo Green) and 2 (Cryo Red), 4 h at 15 °C; Helena Biosciences FVIII Deficient Plasma, 8 h at 2-6 °C or 4 h at 15 °C.

2.4.4. FVIII Bethesda

Helena congenital FVIII Deficient Plasma and Pathway Diagnostic Normal Human Control Plasma were reconstituted in 1 mL of sterile water at RT for 30 min. Test plasma was prediluted (1:1, v/v) in HemosIL™ Factor Diluent reagent. The diluted and neat plasma were respectively each mixed (1:1, v/v) with Pathway Diagnostic Normal Human Control Plasma to give a final volume of 200 µL. The mixture was incubated at 37 °C for 2 h. An inhibitor control (used to generate a reference curve) was prepared by adding 300 µL of Helena congenital FVIII Deficient Plasma into 300 µL Pathway Diagnostic Normal Human Control Plasma. The inhibitor control was also incubated at 37 °C for 2 h. After 2 h, a calibration curve was done prior to analysis of the samples. The stability of the Normal Human Control Plasma was 4 h at RT, 8 h at 2-8 °C and 1 month at -20 °C.

2.4.5. von Willebrand Factor Antigen Enzyme-Linked Immuno-Sorbent Assay

PBS (1x) was prepared by diluting stock solution (20 x) with water. PBS-Tween-20 (PBS-Tween) contained PBS (x1) and Tween-20 (0.1 %, v/v). PBS-Tween-BSA consisted of PBS (x1), Tween-20 (0.1 %, v/v/) and BSA (0.1 %, w/v). PBS, PBS-Tween and PBS-Tween-BSA were stored at 4 ^{o}C for several weeks.

Coating ELISA wells: polyclonal rabbit anti-Human von Willebrand Factor antibody (A0082) was diluted 1:1000 (v/v) in PBS and then 100 µL was added to each well of the ELISA plate. The plate was sealed and incubated overnight at 4 ^{o}C. The anti-VWF diluents were discarded thoroughly from the wells and 200 µL of PBS-Tween were added to each well. The plate was incubated at ambient temperature for 15 min to block the well walls. Following this, the wells were emptied and rinsed 3 times with PBS-Tween (200 µL). The serial dilutions for the standard curve were added in duplicate, and in triplicate for test plasma. The secondary antibody (polyclonal rabbit anti-Human von Willebrand Factor/HRP (P0226, 1:750 v/v in PBS-Tween-BSA, 50 µL) was then added. The plate was incubated at ambient temperature for 2 h. The plate contents were discarded and rinsed 3 times with 200 µL PBS-Tween. K-Blue substrate (100 µL) was added to each well, incubated at 37 ^{o}C for 5 min and then 50 µL of Red Stop Solution was added, mixed and incubated for 5 min at RT. The ELISA plate was read using a BioTek Synergy HT Plate reader (BioTek UK, Bedfordshire, UK).

The standard curve comprised serial dilutions (100, 50, 25, 12.5, 6.25, 3.125, 1.5625) for which the highest value corresponded to 5 µL reference plasma + 95 µL

PBS-Tween-BSA. The subsequent dilutions were done using PBS-Tween-BSA. Test plasma was diluted 200-fold using PBS-Tween-BSA.

2.4.6. Calibrated Automated Thrombography

Thrombin generation was measured according to the method of Hemker et al (127).

Preparation of PL vesicles: phosphatidylserine (120 μL of 25 mg/mL in chloroform), phosphatidylethanolamine (120 μL of 25 mg/mL in chloroform) and phosphatidylcholine (458 μL of 20 mg/mL in chloroform) were mixed and the mixture dried under an argon stream. Buffer A (HEPES (20 mmol/L), NaCl (140 mmol/L), pH7.35, 4 mL) was added, the PLs were allowed to dissolve and then vesicles prepared using a Mini Extruder according to manufacturer's instructions (Avanti Polar Lipids Inc, Alabama, USA). The final concentration of PL was 5 mmol/L and comprised a 1:1:3 (mole:mole:mole) mixture of phosphatidylserine:phosphatidylethanolamine:phosphatidylcholine.

Preparation of trigger: the contents of 1 vial of Innovin were reconstituted in 10 mL of water and the dissolved TF was then diluted 1:6 (v/v), followed by further dilution of a sub-aliquot 1:2 (v/v), both dilutions used buffer B (BSA (5 g/L) dissolved in buffer A). The final concentration of TF was 6 nmol/L (inferred from the serial dilutions). Trigger was prepared by mixing TF (6 nmol/L) and PL vesicles (5 mmol/L) in buffer B, to achieve a final concentration of 1 – 5 pmol/L TF (according to the experiment) and 4 μmol/L PL.

Preparation of fluorogenic substrate: Z-Gly-Gly-Arg-AML HCL was dissolved in dimethylsulphoxide to a concentration of 100 mmol/L and stored at -70 ^{o}C in glass vials. Fluorogenic buffer was prepared with BSA (60 mg/mL) in HEPES

buffer (8.21 mmol/L, pH 7.35). Substrate (60 µL of 100 mmol/L), fluorogenic buffer (2.1 mL, 20 mmol/L HEPES, pH7.35, 60 g/L BSA) and $CaCl_2$ (240 µL of 1 mol/L) were mixed ("FluCa mixture") immediately before use in an experiment.

Preparation of thrombin calibrator: the contents of 1 vial of lyophilised thrombin calibrator were reconstituted in 1 mL water to give a concentration of 640 nmol/L. The dissolved calibrator was stored frozen at -70 ^{o}C until use.

For CAT assays, 20 µL of TF trigger (1 pmol/L, 3 pmol/L or 5 pmol/L TF and 4 µmol/L PL) and 80 µL PPP were manually pipetted in triplicate into a 96 well round bottom microtiter plate. For each test plasma, a single calibrator well was also prepared containing thrombin calibrator (20 µL) and PPP (80 µL). To all wells, FluCa mixture (40 µL) was added to initiate both thrombin generation and calibrator activity. The fluorescence signal was measured for 120 min at 30 s intervals in a Fluoroskan Ascent micro plate fluorimeter, using Thrombinoscope Ascent software (version 3.0.0.29) (Thermo Electron Corporation, Vantaa, Finland).

2.5. Genetic Analysis

2.5.1. DNA Extraction

2.5.1.1. Rapid Extraction of DNA from Blood

Cell lysis buffer contained analar sucrose (11 %, w/v), tris-HCl pH 7.6 (1 mol/L), $MgCl_2$ (5 mol/L), Triton X100 (1 %, v/v) in distilled water. PBS (1x) was prepared by diluting stock solution (20x) with water. NaOH (35 mmol/L) and tris-

HCl (700 mmol/L) pH7.4 were made by diluting stock solution (1 mol/L) with water respectively. Sucrose solution 1 (33 %, w/v) and 2 (11 %, w/v) were prepared by adding analar sucrose to double distilled water. Thawed packed cells (50 µL) were added to 200 µL cell lysis buffer. The mixture was mixed thoroughly and gently with repeated uptake and evacuation of the pipette. Sucrose solution 1 (50 µL) was added to the base of tube followed by centrifugation at 10,000 xgfor 2 min. The supernatant was removed without disturbing the nuclei pellet which was then resuspended in 100 µL PBS (x1). Sucrose solution 2 (50 µL) was added to the base of the tube. The mixture was centrifuged at 10,000 xg for 2 min. The supernatant was discarded without disturbing the pellet of nuclei. NaOH (350 mmol/L, 50 µL) was added to the tube and the nuclei pellet resuspended thoroughly. The mixture was incubated at 95 ^{o}C for 8 min followed by brief centrifugation. Tris-HCl (700 mmol/L, 7 µL) was added and the mixture was vortexed briefly. The mixture was centrifuged at 10,000 xg for 2 min to pellet denatured protein and other debris. Supernatant (50 µL), which contained DNA, was transferred to a 0.5 mL Eppendorf tube and stored at -70 ^{o}C.

2.5.1.2. Rapid Extraction of High Molecular Weight DNA from Blood

Tris-EDTA-Citrate buffer (TEC) (10x) was prepared by adding tris-HCl (1 mol/L, pH7.4), EDTA (0.5 mol/L, pH8.0), Na_3Citrate (1 mol/L) to double distilled water. TEC-SDS buffer was prepared by adding TEC buffer (final concentration 1x) and SDS (final concentration 0.1 %, w/v) to double distilled water.

Thawed packed cells (500 µL) were added to 500 µL PBS (1x). The mixture was vortexed briefly and then centrifuged at 2800 xg for 5 min. The supernatant was

removed without disturbing the nuclei pellet. The nuclei pellet was washed by resuspension in PBS (1x, 500 µL) and then centrifugation was repeated as above. The supernatant was discarded without disturbing the nuclei pellet. The pellet was then frozen on dry ice for 1 min, followed by thawing at 55 ^{o}C for 1 min. Freeze-thawing was repeated two further times.

Saturated NaCl (100 µL) and chloroform (500 µL) were added and the mixture was inverted gently several times to create an emulsion, and then left for 5 min. The tube was then centrifuged at 10,000 xg for 5 min. The supernatant (300 µL) was decanted into 600 µL of 100 % (v/v) ethanol. The tube was inverted gently until the DNA precipitated. The mixture was centrifuged at 10,000 xg for 15 min to harvest the DNA pellet. The supernatant was removed thoroughly and then 25 µL of water was added and the DNA was left to dissolve at 37 ^{o}C for 1 h. The DNA solution was then vortexed gently to ensure uniformity, centrifuged very briefly and stored at -70 ^{o}C.

2.5.2.1. F8 Intron 22 Inversion Analysis Using Inverse Polymerase Chain Reaction

High molecular weight DNA was prepared as described earlier. The inverse polymerase chain reaction (PCR) was done in three stages: Stage 1: Restriction enzyme digestion. DNA (500 ng) was added directly into mineral oil (25 µL) and the volume made up to 15 µL by the addition of water. Restriction enzyme digestion mix (10 µL) comprising *Bcl*I (10 U), NEBuffer3 x10 (2.5 µL) and water was added directly into the DNA (final volume 25 µL). The mixture was incubated at 50 ^{o}C for 2h and then the digested DNA was transferred to a sterile tube. Stage 2: Ligation. The ligation master mix (225 µL) comprising T4 DNA ligase (1 U), T4 DNA ligase buffer x10 (25 µL) and water was added (final volume 250 µL). The mixture was

incubated at 15 ^{o}C overnight. The ligated DNA was transferred to a sterile tube and precipitated by adding 600 µL of ethanol (100 %, v/v) followed by 50 µL of NaCl (1.8 mol/L).The mixture was inverted gently several times and left at -20 ^{o}C for 5 min. The DNA was harvested at 10,000 xg for 10 min. The supernatant was removed and the pellet was allowed to air dry for 5 min. The pellet was reconstituted in 15 µL water and allowed to dissolve for a minimum of 5 min at room temperature. Stage 3: PCR amplification. Primers ID + IU + ED were used in multiplex to test each ligated product. Each primer was present at a final concentration of 1 µmol/L in the PCR. Ligated product (1 µL) was added to PCR mega mix (9 µL) comprising *Taq* DNA polymerase (5U), deoxynucleotide triphosphate (dNTPs) (100 µmol/L), $MgCl_2$ (1.5 mmol/L), *Taq* DNA polymerase buffer (1x) and water (5.17 µL). The PCR regimen comprised (94 ^{o}C for 5 min; 55 ^{o}C for 1 min; 72 ^{o}C for 1 min) x35 cycles. PCR product (5 µL) was analysed using agrose gel electrophoresis (2 % (w/v), 120 V, 80 mA). Products were visualised using ethidium bromide staining and UV light. The result was recorded using a Polaroid MP4 Land Camera (http://www.polaroid.co.uk/) and Fuji Film FP-3000 black and white film (Fujifilm UK Ltd, Bedford, UK). Amplification products of 487 bp and 559 bp were obtained for normal DNA and Inv22 respectively.

Table 2.1 Primers ID, IU and ED used for F8 Intron 22 Inversion.

Primer name	Primer Sequence (5`-3`)
ID	5`-ACATACGGTTTAGTCACAAGT-3`
IU	5`-CCTTTCAACTCCATCTCCAT-3`
ED	5`-TCCAGTCACTTAGGCTCAG-3`

2.5.2.2. Analysis of *F8* Intron 1 Inversion

High molecular weight DNA was prepared as described earlier. The following four primer pairs were used to test each DNA: Int1h-2F + Int1h-2R, 9F + 9cR, Int1h-2R + 9F, Int1h-2F + 9cR. Each primer was present at a final concentration of 1 µmol/L in the PCR.

Table 2.2 Primers Int1h-2F, Int1h-2R, 9F and 9cR used for analysis of F8 Intron 1 Inversion.

Primer name	Primer Sequence (5`→3`)
Int1h-2F	GGCAGGGATCTTGTTGGTAAA
Int1h-2R	TGGGTGATATAAGCTGCTGAGCTA
9F	GTTGTTGGGAATGGTTACGG
9cR	CTAGCTTGACTCCCTGTGG

PCR amplifications were done in a final volume of 25 µL and contained the following reagents: DNA (125 ng/µL), *Taq* DNA polymerase (5U), dNTPs (each at 100 µmol/L), $MgCl_2$ (1.5 mmol/L) and *Taq* DNA polymerase buffer (1x). The PCR regimen was (94 ^{o}C for 30 s; 65 ^{o}C for 60 s; 72 ^{o}C for 90 s) x30 cycles. PCR product (5 µL) and appropriate DNA size standard (1 kb ladder) were analysed using agrose gel electrophoresis (0.6 % w/v, 120 V, 40 mA). Products were visualised using ethidium bromide staining and UV light. The result was recorded using a Polaroid MP4 Land Camera and Fuji Film FP-3000 black and white film. The product profile indicated the genotype (Table 2.3).

Table 2.3 Result interpretation for F8 Intron 1 Inversion

Primer pair	Product	Genotype
Int1h-2F + Int1h-2R	~1 kb	Normal
9F + 9cR	~1.5 kb	
Int1h-2R + 9F	None	
Int1h-2F + 9cR	None	
Int1h-2F + Int1h-2R	None	Intron 1 inversion present
9F + 9cR	None	
Int1h-2R + 9F	~1.5 kb	
Int1h-2F + 9cR	~1 kb	

2.5.2.3. *F8* Sequence Analysis

The sequencing strategy was based on that of Keeney, S. (http://hadb.org.uk / WebPages /Database/Methods/pcr.html): 37 primer pairs were used to amplify the essential regions of *F8* and the products were then sequenced using common forward and reverse sequencing primers (N13F and N13R respectively) (Table 2.4).

Table 2.4 Primers used for amplification of F8.

Primer	PCR Primer sequences (5' - 3')	Product size (bp)
F8P2F	*gtagcgcgacggccagt*GAGCTCACCATGGCTACATTC	595
F8P2R	*cagggcgcagcgatgac*AATTTAAAACTATAAAGCGAGTCCTG	
F8P1F	*gtagcgcgacggccagt*GGACCTAGGCCATGGTAAAGA	634
F8P1R	*cagggcgcagcgatgac*TGCAGAGCATTTTAAGGAACTTT	
F8EX1F	*gtagcgcgacggccagt*TAGCAGCCTCCCTTTTGCTA	514
F8EX1R	*cagggcgcagcgatgac*CTAACCCGATGTCTGCACCT	
F8EX2F	*gtagcgcgacggccagt*CATTACTTCCAGCTGCTTTTTG	324
F8EX2R	*cagggcgcagcgatgac*TTTGGCAGCTGCACTTTTTA	
F8EX3F	*gtagcgcgacggccagt*GCATGCTTCTCCACTGTGAC	333
F8EX3R	*cagggcgcagcgatgac*GCCACCATTACAAAGCACAC	
F8EX4F	*gtagcgcgacggccagt*CATGTTTCTTTGAGTGTACAGTGG	406
F8EX4R	*cagggcgcagcgatgac*TTCAGGTGAAGGAACACAAATG	
F8EX5F	*gtagcgcgacggccagt*TCTCCTCCTAGTGACAATTTCC	293
F8EX5R	*cagggcgcagcgatgac*CCCATCTCCTTCATTCCTGA	
F8EX6F	*gtagcgcgacggccagt*GCGGTCATTCATGAGACACA	292
F8EX6R	*cagggcgcagcgatgac*CCGAGCTGTTTGTGAACTGA	
F8EX7F	*gtagcgcgacggccagt*TGTCCTAGCAAGTGTTTTCCATT	434
F8EX7R	*cagggcgcagcgatgac*AATGTCCCCTTCAGCAACAC	
F8EX8F	*gtagcgcgacggccagt*CACCATGCTTCCCATATAGC	518

F8EX8R	*cagggcgcagcgatgac*ATGGCTTCAGGATTTGTTGG	
F8EX9F	*gtagcgcgacggccagt*TTTGAGCCTACCTAGAATTTTTCTTC	334
F8EX9R	*cagggcgcagcgatgac*GGTATTTTAGAAACTCAAAACTCTCC	
F8EX10F	*gtagcgcgacggccagt*TTCTTGTTGATCCTAGTCGTTTT	284
F8EX10R	*cagggcgcagcgatgac*GCTGGAGAAAGGACCAACATA	
F8EX11F	*gtagcgcgacggccagt*CCCTTGCAACAACAACATGA	396
F8EX11R	*cagggcgcagcgatgac*TTTCTTCAGGTTATAAGGGGACA	
F8EX12F	*gtagcgcgacggccagt*TGCTAGCTCCTACCTGACAACA	332
F8EX12R	*cagggcgcagcgatgac*CATTCATTATCTGGACATCACTTTG	
F8EX13F	*gtagcgcgacggccagt*CATGACAATCACAATCCAAAATA	398
F8EX13R	*cagggcgcagcgatgac*CATGTGAGCTAGTGGGCAAA	
F8EX14AF	*gtagcgcgacggccagt*CTGGGAATGGGAGAGAACCT	601
F8EX14AR	*cagggcgcagcgatgac*ATGTCCCCACTGTGATGGAG	
F8EX14BF	*gtagcgcgacggccagt*GATCCATCACCTGGAGCAAT	633
F8EX14BR	*cagggcgcagcgatgac*GGGCCATCAATGTGAGTCTT	
F8EX14CF	*gtagcgcgacggccagt*AGCTCATGGACCTGCTTTGT	729
F8EX14CR	*cagggcgcagcgatgac*CATTCTCTTGGATTAATGTTTCCTT	
F8EX14DF	*gtagcgcgacggccagt*TCCAAGCAGCAGAAACCTATT	629
F8EX14DR	*cagggcgcagcgatgac*AGTAATGGCCCCTTTCTCCT	
F8EX14EF	*gtagcgcgacggccagt*GGATGACACCTCAACCCAGT	604
F8EX14ER	*cagggcgcagcgatgac*CCTTCCACGAGATCCAGATG	
F8EX14FF	*gtagcgcgacggccagt*TCCCTACGGAAACTAGCAATG	720

F8EX14FR	*cagggcgcagcgatgac*TCACAAGAGCAGAGCAAAGG	
F8EX15F	*gtagcgcgacggccagt*TGAGGCATTTCTACCCACTTG	333
F8EX15R	*cagggcgcagcgatgac*CCAAAAGTGGGAATACATTATAGTCA	
F8EX16F	*gtagcgcgacggccagt*CAGCATCCATCTTCTGTACCA	502
F8EX16R	*cagggcgcagcgatgac*AAAGCTTCTTATTGCACGTAGG	
F8EX17F	*gtagcgcgacggccagt*cAGGTTGGACTGGCATAAAAA	431
F8EX17R	*cagggcgcagcgatgac*CCCTGGATCAAGTCTCATTTG	
F8EX18F	*gtagcgcgacggccagt*TGGTGGAGTGGAGAGAAAGAA	396
F8EX18R	*cagggcgcagcgatgac*AGCATGGAGCTTGTCTGCTT	
F8EX19F	*gtagcgcgacggccagt*TTGGTATGTATCTCATGCTCATTTT	282
F8EX19R	*cagggcgcagcgatgac*GGAAGAAAGCTGTAAAGAAGTAGGC	
F8EX20F	*gtagcgcgacggccagt*TTTGAGAAGCTGAATTTTGTGC	262
F8EX20R	*cagggcgcagcgatgac*GAAGCATGGAGATGGATTCATTA	
F8EX21F	gtagcgcgacggccagtCCACAGCTTAGATTAACCTTTCTCA	295
F8EX21R	cagggcgcagcgatgacTGAGCTTGCAAGAGGAATAAGTAA	
F8EX22F	*gtagcgcgacggccagt*TCAGGAGGTAGCACATACAT	321
F8EX22R	*cagggcgcagcgatgac*GTCCAATATCTGAAATCTGC	
F8EX23F	*gtagcgcgacggccagt*TTGACAGAAATTGCTTTTTACTCTG	328
F8EX23R	*cagggcgcagcgatgac*TCCCCCAGTCTCAGGATAACT	
F8EX24F	*gtagcgcgacggccagt*ACTGAGGCTGAAGCATGTCC	284
F8EX24R	*cagggcgcagcgatgac*CCCAACCACTGCTCTGAGTC	
F8EX25F	*gtagcgcgacggccagt*TGGGAATTTCTGGGAGTAAATG	334
F8EX25R	*cagggcgcagcgatgac*AAGCTCTAGGAGAGGTGGTATTTTT	

F8EX26AF	*gtagcgcgacggccagt*CTGTGCTTTGCAGTGACCAT	591
F8EX26AR	*cagggcgcagcgatgac*TTCTACAACAGAGGAAGTGGTGA	
F8EX26BF	gtagcgcgacggccagtGGAGAAACCTGCATGAAAGC	630
F8EX26BR	*cagggcgcagcgatgac*TTGGCCATCACAAATTTCAA	
F8EX26CF	*gtagcgcgacggccagt*TGCAAATGTGCATTTTTCTGA	614
F8EX26CR	*cagggcgcagcgatgac*CCTCCAGCCCCCTTTACTAT	
F8EX26DF	*gtagcgcgacggccagt*CCACCCCCATAAGATTGTGA	614
F8EX26DR	*cagggcgcagcgatgac*CTGAAGAAACCAGCAGGAAAA	
F8EX26EF	*gtagcgcgacggccagt*CCCCAAAGGTGATATGGTTTT	264
F8EX26ER	*cagggcgcagcgatgac*TCAGTGTTCACATTTTTATTTCCA	
N13F	GTAGCGCGACGGCCAGT	N/A
N13R	CAGGGCGCAGCGATGAC	N/A

"F" and "R" in the primer names abbreviate "forward" and "reverse" respectively and define the primer pair used for each amplification. Italicised letters indicate N13F and N13R tails that were used for sequencing with primers N13F and N13R respectively. "N/A" abbreviates "not applicable." "bp" abbreviates base pair.

Sequence analysis of each test sample involved 37 PCR amplifications that included the genomic DNA and a further 12 reactions that used water instead of DNA (negative controls for contamination). The 12 negative control amplifications used the 37 primer pairs in multiplex as shown in Table 2.5.

Table 2.5 Primers pair used for negative control multiplex PCR

Negative Control Multiplex
F8EX14C+ F8EX8+ F8EX15+ F8EX20
F8EX14F+ F8EX1+ F8EX12
F8EX16+ F8EX14A+ F8EX23
F8EX14B+ F8EX7+ F8EX2
F8EX26B+ F8EX17+ F8EX22
F8EX14D+ F8EX4+ F8EX21
F8EX26C+ F8EX13+ F8EX5
F8EX26D+ F8EX11+ F8EX6
F8EX14E+ F8EX18+ F8EX10
F8P1+ F8EX9+ F8EX24
F8P2+ F8EX25+ F8EX19
F8EX26A+ F8EX3+ F8EX26E

Each control multiplex PCR in Table 2.5 contained both the upstream and downstream primers for the relevant exons. For example: F8Ex14C consisted of F8Ex14CF+F8Ex14CR.

For all amplifications, each primer was present at a final concentration of 1 µmol/L in the PCR. PCR amplifications were done in a final volume of 25 µL and contained the following reagents: DNA (125 ng/µL), *Taq* DNA polymerase (5U), dNTPs (each at 100 µmol/L), $MgCl_2$ (1.5 mmol/L) and *Taq* DNA polymerase buffer (1x). The PCR regimen comprised (94 ^{o}C for 30 s; 60 ^{o}C for 1 min; 72 ^{o}C for 1 min) x35 cycles.

The PCR product (25 µL) was purified using ethanol precipitation. NaCl (0.5 mol/L, 5 µL) and ethanol 100 % (v/v, 60 µL) were added to each well. The wells were sealed, the contents mixed by gentle inversion and left for 5 min at RT.

The 96 well plate was then centrifuged at 1780 xg for 30 min. The supernatant (85 µL) was removed and plate was kept at RT for 5 min to dry. Then 50 µL of water was added and left for 5 min at RT. Purified PCR product (2 µL) and appropriate DNA size standard (pBR322/*Msp*I) were analysed using agarose gel electrophoresis (2 % w/v, 450 V, 80 mA). Products were visualised using ethidium bromide staining and UV light. The result was recorded using a Polaroid MP4 Land Camera and Fuji Film FP-3000 black and white film.

For cycle sequencing, a main mix (8 µL) comprising BDT v3.1 Mix (2 µL, 2.5x stock), sequencing buffer (2 µL, 5x stock), sequencing primer N13 F or N13R (1 µL, 3.2 pmol/µL), and water (3 µL) was added directly to purified PCR product (2 µL). The cycle sequencing regimen comprised (96 ^{o}C for 3 min) then (96 ^{o}C for 30 s; 50 ^{o}C for 30 s; 60 ^{o}C for 4 min) x 25 cycles. Following cycle sequencing, reaction products were purified using Centri-Sep columns. Briefly, 7 µL of water was added to the cycle sequencing reaction products and then the entire 17 µL was gel filtered through a Centri-Sep column. The purified products were submitted to be sequenced to the Sequencing Core Facility, Central Biotechnology Services (CBS) of Cardiff University School of Medicine. The facility uses a 3130XL Applied Biosystems genetic analyser (Applied Biosystems, Warwickshire, UK). Sequencing data were analysed using Mutation Surveyor Software Version 4.0.6 (SoftGenetics, Philadelphia, USA).

Chapter 3

Recruitment of Cohorts

3.1. Recruitment of Cohorts

Two cohorts, each containing 100 individuals, were recruited in Pakistan. One cohort consisted of individuals who had previously been diagnosed with haemophilia A, whilst the other comprised random healthy controls. Health normal controls were recruited from different centres. Individuals were recruited from all five provinces of Pakistan (Punjab, Sindh, Baluchistan, Khyber Pakhtunkhwa, Gilgit Baltistan) (Figure 3.1). All relevant blood samples and clinical information (age, age at first bleed, family history and treatment history) were obtained at the time of recruitment.

3.2. Phenotypic Parameters

The PT, aPTT, fibrinogen, FVIII:C, FVIII inhibitor, VWF:Ag, FIX:C and ABO blood group determination were repeated in Cardiff (Table 3.1). A diagnosis of haemophilia A was confirmed for 92 individuals, whilst 7 were found to have haemophilia B and 1 was found not to have haemophilia. The 92 haemophilia A individuals were found to comprise the following severities: mild n=5 (5 %), moderate n=24 (26 %) and severe n=63 (69 %) (Table 3.1). An inhibitor

was detected in 4 (4 %) individuals; these were included in Table 3.1, however they were excluded from the analyses for all figures.

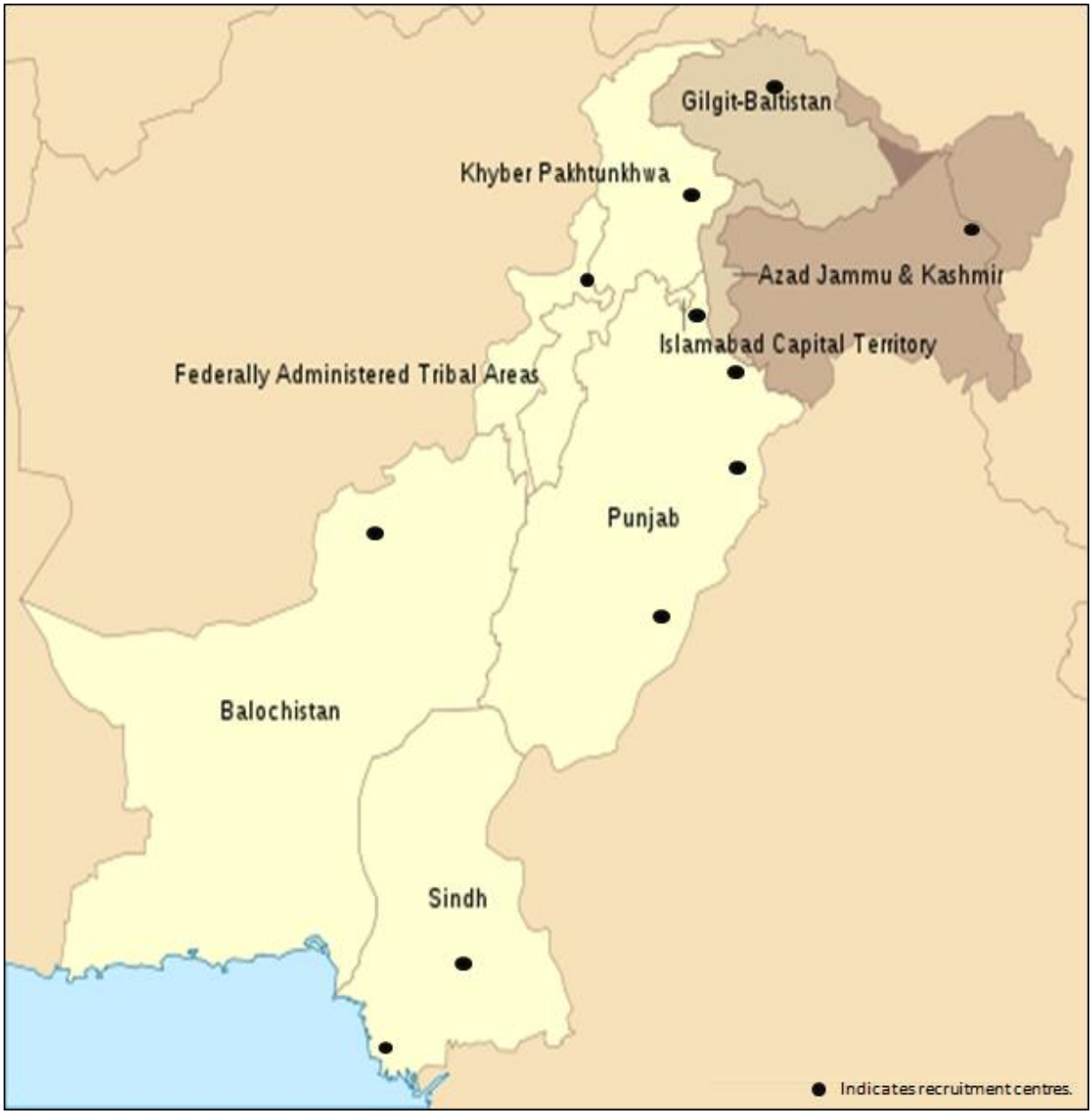


Figure 3.1: Map of Pakistan with location of recruitment centres. Pakistan is a sovereign country in South Asia, comprising five provinces – Punjab, Sindh, Baluchistan, Khyber Pakhtunkhwa, Gilgit Baltistan – as well as the Islamabad capital territory and the federally administered tribal areas in the northwest.

Table 3.1 Phenotypic data for haemophilia A individuals from Pakistan

Patient ID	PT (sec)	aPTT (sec)	FVIII:C (IU/Dl)	VWF:Ag (U/dL)	ABO Blood group	Patient ID	PT (sec)	aPTT (sec)	FVIII:C (IU/Dl)	VWF:Ag (U/dL)	ABO Blood group
1	11.6	71.1	2.2	107	O	51	12.3	84	1.2	68	AB
2	9.8	68.1	3.4	128	B	52	12.7	92.2	1.5	75	A
3	11.8	131.5	<1	337	B	53	11.1	118.5	<1	173	O
4	11.1	132.5	<1	181	O	54	11.4	91.7	<1	50	O
5	13.7	82.5	2.3	179	B	55	11.7	58.5	5	57	O
6	12.3	81.3	4.5	118	B	56	12.5	117.6	<1	155	B
7	11.1	97.2	1.3	28	B	57	12.8	114.4	<1	87	O
8	11.2	114.6	<1	55	AB	58	10.2	62.3	2.8	76	AB
9	13.1	115.8	<1	58	B	59	13.5	108.3	<1	148	B
10	12.2	129.9	<1	33	O	60	11	118.1	<1	61	O
11	10.5	59.9	3.1	41	B	61	9.6	101.9	<1	44	O
12	11.3	75.6	2.6	66	A	62	11.4	93	<1	186	AB
13	11.1	121.4	<1	29	A	63*	13.1	110.4	99	100	O
14	11.2	54.6	<1	39	B	64*	11.7	107.7	82.7	101	B
15	10.7	98.1	2.2	59	AB	65	12.8	47.3	<1	74	A
16	11.9	85.6	1.1	37	O	66	12.4	50	9.2	124	A
17	11.3	102.1	<1	76	B	67*	12.3	54.1	102	125	B
18	12.5	120.6	<1	46	O	68*	12.3	48	105	174	AB
19	11.8	127.8	2.7	132	O	69**	12.7	31.7	70	51	O
20*	13.6	156.4	57	121	B	70	12.4	101.1	<1	60	O
21	11.3	129.8	<1	34	O	71	10.7	119	<1	84	A
22	11	116.5	<1	29	O	72	9.3	126.5	<1	39	AB
23	10.9	78	5.1	36	B	73	16.2	107.1	<1	125	B
24	12.3	132.5	10.2	121	B	74	10	96.3	<1	81	B
25	11.4	122.5	<1	35	O	75	10.6	123.6	<1	88	O
26	10.1	146.3	<1	53	O	76	10.9	129.5	<1	149	A
27	10.8	112.4	<1	121	O	77	11.3	132.4	<1	82	A
28	9.9	130.9	<1	90	B	78	10.9	114.6	<1	97	B
29	11.6	144.5	<1	50	B	79	12.5	98.5	<1	92	O
30	11.6	92.6	<1	132	A	80	10.3	75.7	2.1	39	B
31	9.1	54.2	7.5	38	AB	81	12.8	134.4	<1	86	A
32	13.8	124.1	2.6	50	O	82	13.7	139.2	<1	69	A
33	12.6	84.7	<1	88	A	83	10.1	106.4	<1	122	O
34	12.1	104.5	<1	85	B	84	11	109	<1	183	B
35	13.7	48.6	17.7	38	O	85	10.7	107.7	<1	36	O
36	12.6	120.2	<1	111	B	86	13.1	71	2.1	77	A
37	11.4	85.3	1.7	60	O	87	11.3	133.3	<1	40	B
38	11.3	114.1	<1	63	B	88	12.2	91.4	2.2	127	A
39	10.2	142.4	3.5	55	O	89	10.8	98	<1	35	A
40	10	77.8	1.2	122	AB	90	10.2	118.9	<1	51	A
41	10.7	74.3	1.6	60	O	91	10.5	126.3	<1	67	A
42	11.2	136.7	<1	73	AB	92	10.7	68.6	2	171	B
43	10.7	135.8	<1	61	B	93	11.4	61.8	4.3	102	A

44	12	117.6	<1	38	B	94	10.6	135.4	<1	117	A
45	11.1	143	<1	93	B	95	10.4	92.7	<1	61	O
46	11.2	135.6	<1	93	A	96	10.8	132.7	<1	148	AB
47	11.3	148.8	<1	163	AB	97	11.1	115.2	<1	83	B
48	11	119.1	<1	97	B	98	10.5	115.4	<1	183	O
49	14.1	103.9	<1	29	O	99*	11.2	71.1	114	119	B
50	12.5	85	<1	52	B	100*	10.9	51.8	111	127	B

*Individuals with haemophilia B. ** Individual haemostatistically normal.

A minor proportion (19 %) of the cohort had minimal access to FVIII concentrate, the exact amount used is not known because accurate records were not available. Direct questioning of each individual suggested the FVIII concentrate usage was about once per year. Individuals with no access to FVIII concentrate (81 %) were treated intermittently with FFP. Furthermore, none of the individuals had access to physiotherapy support.

3.3. Clinical Findings

Individuals with mild haemophilia A presented with a first bleed at a similar age compared to severe (Table 3.2). For all severities, a high proportion of individuals first bled at circumcision, other types of first presentation were rarer: post traumatic bleed (7 %), bleeding from mouth (4 %), nose (3 %) and tongue (2 %) and joint pain (2 %). Eighty per cent of the individuals presented with a positive family history of bleeding. More than half of the individuals had ≥2 target joints, knee was the most affected followed by elbow and ankle (Figure 3.2). Although target joint was more prevalent among severe individuals, a high proportion of moderately and mildly affected cases also had at least one target joint. For all severities combined, 13 % of individuals had no affected joint but most of these cases were in their early years of life. Of the moderate individuals, 76 % had at least one affected joint compared to 90 % of severe cases. The proportion of knees as the

target joint was similar in severe and nonsevere individuals. Severe individuals had a higher proportion of elbows and a lower proportion of ankles as target joints compared to nonseveres. The findings for haemophilic arthropathy were similar to those for target joints.

Table 3.2 Characteristics of haemophilia A cohort from Pakistan

Parameter	Mild (n=5)	Moderate (n=24)	Severe (n=63)	All (n=92)
Age at recruitment (years)*	9 (8-40)	18.5 (1.5-50)	13 (1.5-47)	14 (1.5-50)
Individuals with no access to FVIII concentrate n (%)	3 (60 %)	17 (70 %)	50 (79 %)	70 (76%)
Individuals with minimal access to FVIII concentrate**	2 (40 %)	7 (30 %)	13 (21 %)	17 (19 %)
Age of first bleed (weeks)*	28 (20-288)	38 (1-192)	24 (1-192)	24 (1-288)
First bleed: circumcision	80 %	80 %	85 %	82 %
Other	20 %	20 %	15 %	18 %
Positive family history	80 %	88 %	72 %	80 %
Number of joints with haemophilic arthropathy:				
Knee	4 (40 %)	19 (40 %)	66 (52 %)	89 (48 %)
Elbow	3 (30 %)	12 (25 %)	50 (40 %)	65 (35 %)
Ankle	2 (20 %)	10 (21 %)	20 (16 %)	32 (17 %)
Total number of affected joints*	0 (0-3)	1 (0-3)	2 (0-6)	2 (0-6)
Individuals with no affected joint	2 (40 %)	4 (17 %)	7 (11 %)	13 (14 %)
Target joint: none	60 %	24 %	11 %	17 %
1	0 %	37 %	31 %	31 %
≥2	40 %	39 %	59 %	52 %
Target joint***				
Knee	3 (30 %)	23 (48 %)	67 (53 %)	93 (51 %)
Elbow	2 (20 %)	6 (13 %)	34 (27 %)	42 (23 %)
Ankle	3 (30 %)	10 (21 %)	16 (13 %)	29 (16 %)

*Values presented are median (range). ** The exact access to FVIII concentrate is not known because accurate records had not been kept, on average treatment with

FVIII concentrate was about once per year. ***Values presented are: the absolute number of the target joint and the proportion as a percentage of the total number of that joint in the relevant group. For example in severe individuals: 67 knee target joints in 63 individuals, percentage = (67/126) x 100 = 53 %.

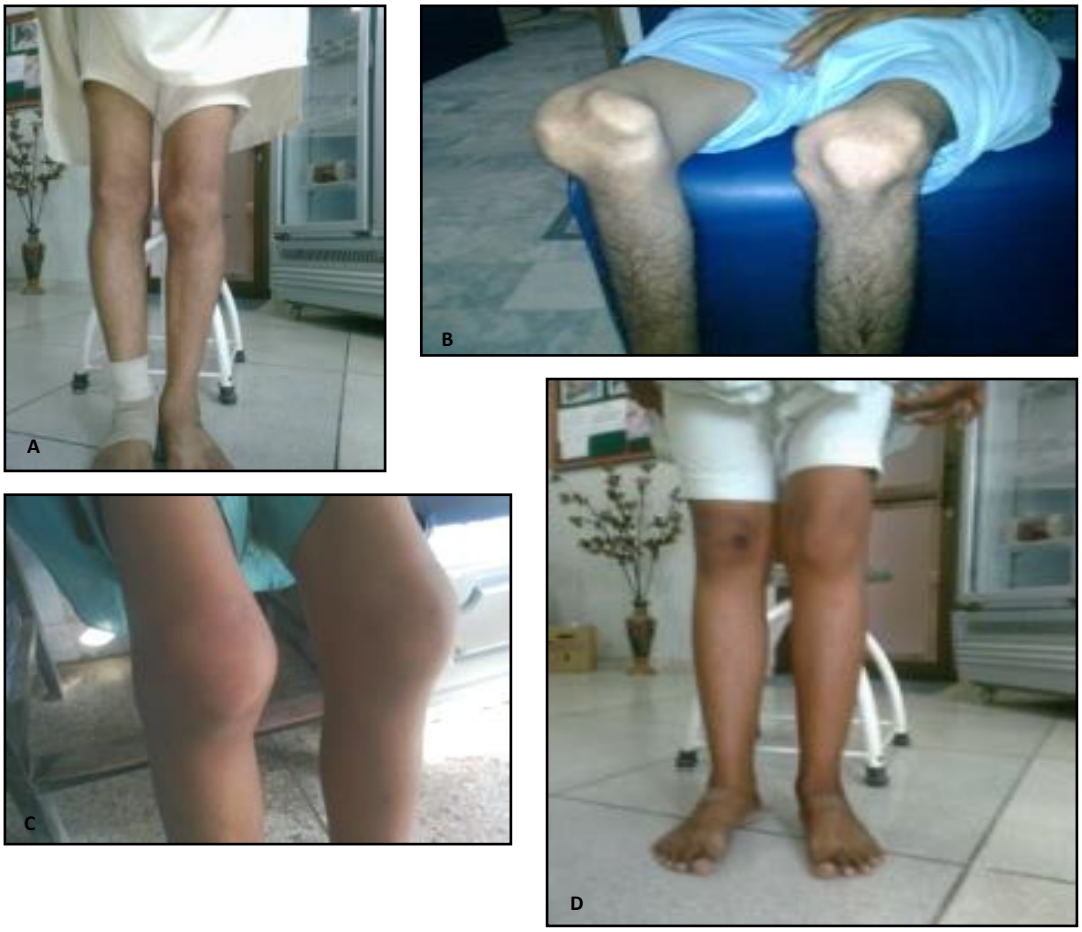


Figure 3.2: Joint arthropathy in haemophilia A individuals in Pakistan. (A) 18 -year-old man (severe), (B) 21-year-old man (severe), (C) 9-year-old boy (severe with inhibitors), (D) 41-year-old man (mild).

3.4. Discussion

In the present study, a cohort of 100 individuals – for whom a diagnosis of haemophilia A had been made in Pakistan – was recruited from the five provinces of the country. Confirmatory phenotypic testing in Cardiff revealed that 7 individuals in fact had haemophilia B and one person was unaffected.

Most of the individuals in this study had multiple joint involvements, including those with laboratory defined mild disease. Knees were the most frequently affected joints, followed by elbows and ankles. A number of factors might explain the more frequent involvement of these joints: first, the knee, elbow and ankle predominately move only in two directions compared with other joints that can move more freely; second, these three joints are not surrounded by muscles that may provide protection while moving. More than half of individuals had involvement of ≥ 2 joints; the number of joints involved increased with age and severity. In this cohort, about 78 % of individuals bled at circumcision, very few individuals gave a history of bleeds from nose, mouth, tongue or post-trauma. Overall, the bleeding history was less in mild individuals, greater in moderate individuals and greatest in severe. Individuals with severe haemophilia A and mild haemophilia had first bleeds at about the same age because these were induced by circumcision. These results are consistent with other international studies: a group from China reported that most severe haemophilia A individuals presented with a first bleed in their initial year of life (128), a group from Germany reported a similar finding that most individuals with haemophilia A have their first bleeding

episode within the first year of life (129). However, the studies cannot be compared because of the important effect that circumcision has had in the cohort from Pakistan.

The misdiagnosis of haemophilia revealed here-in may be due to a lack of relevant laboratory expertise and facilities in some parts of Pakistan. The misdiagnoses may have gone unnoticed because very few individuals had access to coagulation factor concentrate. Treatment, when it was accessible, was principally with FFP which contains both factor VIII and IX. Haemophilia expertise in Pakistan is localized principally to certain developed areas. Pakistan, a country with an approximate population of 176 million (World Health Organisation (WHO) 2011 data, URL http://www.who.int/gho/countries/pak.pdf) is one of several countries comprising the "Eastern Mediterranean region" as designated by the World Federation of Haemophilia (WFH) (URL http://www.who.int/gho/countries/pak.pdf). Pakistan is relatively less urbanized than other areas of this region, with 36 % of the population living in urban districts compared to the regional average of 50 % (WHO 2011 data, URL http://www.who.int/gho/countries/pak.pdf). Availability of medical services is limited in non-urban areas. There is a relatively lower rate of available health workforce: 8.1 physicians *per* 10,000 *capita* compared to 10.9/10,000 in the Eastern Mediterranean region, and similarly 5.6/ *per* 10,000 nurses/midwives compared to 15.6 *per* 10,000). The factor VIII concentrate usage in Pakistan is 0.01 U/head of population which is one of the lowest on the world (http://www1.efh.org/publications/files/pdf). Many people with haemophilia have limited access to clinicians and physiotherapists with expertise in haemophilia care.

The findings highlight the need for more wide spread expertise in the laboratory diagnosis and clinical management of haemophilia in Pakistan. Although these findings are consistent with those of other studies, the severity of clinical symptoms is greater overall in this cohort, possibly reflecting lack of access to on-demand or prophylatic treatment.

Chapter 4

Characterisation of *F8* defects in haemophilia A in Pakistan

4.1. Results

4.1.1. Characterization of Pathological Sequence Variants

The causative *F8* defect was investigated in the 92 individuals with haemophilia A. Initially, all of the severe haemophilia A individuals (n=63) were investigated for the Inv22 and Inv1 and the former was found to be present in 18 of the 63 (29 %) severe individuals, whilst the latter was present in 1/63 (1.6 %) (Table 4.2). *F8* sequence analysis, covering the essential regions of the gene (promotor, exons, splice junctions and 3' polyadenylation signal region), was then applied to the remaining severe individuals and to the mild and moderate cases (a total of 73 altogether). This identified the pathological sequence variant (or a candidate variant) in 62 cases, leaving 11 individuals in whom a causative gene defect was not found (Table 4.1).

Table 4.1 Genetic and phenotypic data for haemophilia A individuals from Pakistan.

Individual	FVIII:C (IU/dL)	Pathological Sequence Variant	HAMSTeRS
1	2.2	c.536C>G; p.(Ser179Cys)	N
2	3.4	c.5324T>G; p.(Leu1775Trp)	N
3	<1	c.5507G>A; p.(Trp1836Arg)	N
4	<1	Inv22	-
5	2.3	c.6187+1G>A	N
6	4.5	c.6406G>A; p.(Gly2136Arg)	Y
7	1.3	c.4099A>T; p.(Thr1367Ser)	N
8	<1	c.6972C>T; p.(Tyr2324*)	Y
9	<1	c.3385C>T; p.(Gln1129*)	N
10	<1	Inv22	-
11	3.1	No result	n/a
12	2.6	c.4379delA; p.(Asn1460Ilefs*5)	N
13[Inhib]	<1	del of Ex 15,16,17 & 18	Y
14	<1	Inv22	-
15	2.2	c.4379delA; p.(Asn1460Ilefs*5)	N
16	1.1	c.4379delA; p.(Asn1460Ilefs*5)	N
17	<1	c.1332delA; p.(Val445Serfs*37)	N
18	<1	c.1775delT; p.(Val592Alafs*68)	N
19	2.7	c.862delA; p.(Ile288Tyrfs*10)	N
21	<1	Inv22	-
22	<1	Inv22	-
23	5.1	c.3930T>A; p.(Tyr1310*)	N
24	10.2	c.601+17G>A	N

25	<1	c.1694G>A; p.(Gly565Glu)	N
26	<1	c.1694G>A; p.(Gly565Glu)	N
27	<1	c.4825dupA; p.(Thr1609Asnfs*4)	N
28	<1	Inv22	-
29	<1	No result	n/a
30	<1	c.-966G>T	N
31	7.5	c.7021G>A; p.(Glu2341Lys)	Y
32	2.6	c.1786T>C; p.(Ser596Pro)	Y
33	<1	c.1332A>C; p.(Lys444Asn)	Y
34	<1	c.3280G>T; p.(Glu1094*)	N
35	17.7	c.6047G>A; p.(Arg1216Gln)	N
36 [Inhib]	<1	c.5724G>A; p.(Trp1908*)	N
37	1.7	c.4379delA; p.(Asn1460Ilefs*5)	N
38	<1	No result	n/a
39	3.5	c.4430delA; p.(Glu1477fs*90)	N
40	1.2	c.4379delA; p.(Asn1460Ilefs*5)	N
41	1.6	No result	n/a
42	<1	c.2962-2963delAG; p.(Ser988Trpfs*2)	N
43	<1	c.2962-2963delAG; p.(Ser988Trpfs*2)	N
44	<1	c.195C>G; p.(Tyr65*)	Y
45	<1	c.195C>G; p.(Tyr65*)	Y
46 [Inhib]	<1	c.1332delA; p.(Val445Serfs*37)	N
47	<1	c.2686A>T; p.(Lys869*)	N
48	<1	Inv22	-
49	<1	c.1332delA; p.(Val445Serfs*37)	N
50	<1	c.1332delA; p.(Val445Serfs*37)	N
51	1.2	No result	n/a
52	1.5	No result	n/a
53	<1	c.1332delA; p.(Val445Serfs*37)	N
54	<1	c.5797G>T; p.(Glu1933*)	N

55	5	c.1898T>C; p.(Met633Thr)	N
56	<1	No result	n/a
57	<1	Inv22	-
58	2.8	No result	n/a
59	<1	c.1694G>A; p.(Gly565Glu)	N
60	<1	c.1365dupT; p.(Lys456fs*)	N
61	<1	Inv22	-
62	<1	c.5566T>G; p.(Tyr1856Asp)	N
65	<1	Inv22	-
66	9.2	No result	n/a
70	<1	c.205-206delCT; p.(Leu69Valfs*13)	N
71	<1	Inv22	-
72	<1	c.1576G>T; p.(Glu526*)	N
73 [Inhib]	<1	c.5866-5867dupAG; p.(Asp1956Glufs*75)	N
74	<1	c.1332delA; p.(Val445Serfs*37)	N
75	<1	c.1786T>C; p.(Ser596Pro)	Y
76	<1	c.1786T>C; p.(Ser596Pro)	Y
77	<1	c.1786T>C; p.(Ser596Pro)	Y
78	<1	c.1332delA; p.(Val445Serfs*37)	N
79	<1	Inv1	-
80	2.1	No result	n/a
81	<1	c.6869G>A; p.(Trp2290*)	Y
82	<1	c.6869G>A; p.(Trp2290*)	Y
83	<1	c.1063C>T; p.(Arg355*)	Y
84	<1	c.901C>T; p.(Arg301Cys)	Y
85	<1	Inv22	-
86	2.1	c.5999-11G>A	N
87	<1	Inv22	-
88	2.2	c.3637delA; p.(Ile1213Phefs*5)	N

89	<1	Inv22	-
90	<1	Inv22	-
91	<1	Inv22	-
92	2	c.6972C>T; p.(Tyr2324*)	Y
93	4.3	No result	n/a
94	<1	Inv22	-
95	<1	Inv22	-
96	<1	c.6972C>T; p.(Tyr2324*)	Y
97	<1	c.6972C>T; p.(Tyr2324*)	Y
98	<1	No result	n/a

Y and N respectively indicate mutation present or not listed (novel variant) in Haemophilia A Mutation, Structure, Test and Resource Site (HAMSTeRS). There were 30 novel variants, recurrence of 8 of these results in 43 instances of N in the table. "-" indicates known pathogenic change not listed in HAMSTeRS; Inv22 and Inv1 respectively abbreviate intron 22 and intron 1 inversion; "n/a" abbreviates "not applicable". [Inhib]Indicates individuals with FVIII inhibitors.

A diverse spectrum of mutations was found in the cohort and these comprised point mutations (n=39, including missense, nonsense, splice site and promoter region changes), inversions (n=19, Inv22 and Inv1) and frameshifts (n=22, deletions nucleotide duplications) (detailed in Table 4.1, summarized in Table 4.2). The most frequent mutation type overall was point mutation (42 % of individuals), followed by the Inv22 (20 % of all cases).

Table 4.2 Summary of F8 defects in a haemophilia A cohort from Pakistan.

Point mutation (n=39)				Inversion (n=19)		Frame Shift (n=22)		Deletion of exons (n=1)	No change (n=11)	Total Individuals (n=92)
Missense	Nonsense	Splice site	Promoter region	Inv22	Inv1	nucleotide deletion	nucleotide duplication			
18	17	3	1	18	1	19	3	1	11	92

Inv22 and Inv1 respectively abbreviate intron 22 and intron 1 inversions. “No change” indicates individuals for whom no mutation was detected.

Aside from the Inv22, 8 mutations were recurrent (Tables 4.1 and 4.3). Analysis of 7 intragenic single nucleotide polymorphisms (SNPs) showed that each recurrent mutation was associated with a specific haplotype (Table 4.3).

Table 4.3 Haplotypes found in recurrent mutations among individuals with haemophilia A from Pakistan.

Mutation	Individual ID Number	Haplotype						
		rs7058826	rs28370212	rs1800291	rs1800292	rs489352	rs1509787	rs1050705
		Intron 7	Exon 14	Exon 14	Exon 14	Intron 18	Intron 25	Exon 26
		c.1010-27G>A	c.3621C>T	c.3780C>G	c.3864A>C	c.5998+91T>A	c.6901-2607C>T	c.*1672A>G
c.1332delA	17	G	C	C	C	A	C	G
	46	G	C	C	C	A	C	G
	49	G	C	C	C	A	C	G
	50	G	C	C	C	A	C	G
	53	G	C	C	C	A	C	G
	74	G	C	C	C	A	C	G
	78	G	C	C	C	A	C	G
c.1786T>C	32	A	C	G	A	A	C	G
	75	A	C	G	A	A	C	G
	76	A	C	G	A	A	C	G
	77	A	C	G	A	A	C	G
c.1694G>A	25	G	C	C	A	T	C	A
	26	G	C	C	A	T	C	A
	59	G	C	C	A	T	C	A
c.195C>G	44	G	C	C	A	T	C	A
	45	G	C	C	A	T	C	A
c.2962-2693delAG	42	G	C	C	A	T	C	A
	43	G	C	C	A	T	C	A

c.4379delA	12	G	C	C	A	T	C	A
	15	G	C	C	A	T	C	A
	16	G	C	C	A	T	C	A
	37	G	C	C	A	T	C	A
	40	G	C	C	A	T	C	A
c.6869G>A	81	G	C	C	A	T	C	A
	82	G	C	C	A	T	C	A
c.6972C>T	8	G	C	C	A	T	C	A
	92	G	C	C	A	T	C	A
	96	G	C	C	A	T	C	A
	97	G	C	C	A	T	C	A

"rs" designations refer to reference SNP identity numbers in the international SNP data base (URL http:www.ncbi.nlm.nih.gov/snp/).

Table 4.4 Prediction of functional effect of the protein

Mutation	Polyphen-2 Score	SIFT Score
c.5362C>G; p.(Ser179Cys)	1	0
c.5324T>G; p.(Leu1775Trp)	1	0
c.5507G>A; p.(Trp1836Arg)	1	0
c.1694G>A; p.(Gly565Glu)	1	0
c.1898T>C; p.(Met633Thr)	0.993	0
c.5566T>G; p.(Tyr1856Asp)	1	0
c.4099A>T; p.(Thr1367Ser)	0.20	0.25

PolyPhen-2 scores: 1 = most damaging, 0 = least damaging. SIFT Human Protein scores: 0 = most damaging, 1 = least damaging.

Table 4.5 List a mixture of nationality and country names for noval mutation, the nationalities are:

Nationality	Noval Mutation (%)	References
Indian	37 %	(130)
Spanish	11 %	(131)
French	75 %	(132)
Spanish	32 %	(133)
American	38 %	(134)

Table 4.6 List a mixture of nationality and country names for Inv22, the nationalities are:

Nationality	Inv22 Mutation	References
Indian	51 %	(130)
Spanish	52 %	(133)
Italian	42 %	(135)
Mexican	45 %	(136)
Hungarian	50 %	(137)

4.2. Discussion

The causative gene defect was investigated in the 92 individuals with haemophilia A, a mutation or candidate mutation in *F8* was found in 81 of them. The sequence variants found were heterogeneous: point mutations (including missense, nonsense, splice site and promoter region changes), inversions (Inv22 and Inv1) and frameshifts (deletions and duplications). Point mutation (42 % of individuals) was the most frequent change found in this cohort, followed by Inv22 (20 % of cohort, 29 % of severe cases).

The lack of laboratory expertise and facilities for pheontypic diagnosis in Pakistan (Chapter 2, section 2.4.) is paralleled by an even greater insufficiency for genetic diagnosis. Although carrier status and prenatal diagnosis using molecular analysis are availible for thalassaemia, such services have not yet been adopted for haemophilia. Access to molecular diagnosis would allow women to establish their carrier status and make informed reproductive decisions. Families with a severe phenotype are particularly dependent on haemophilia care, and for these families carrier detection and genetic counselling are important, among the other factors. The genetic information generated in this study can be used within the relevant families for diagnosis of individuals with haemophilia A and for the carrier detection and prenatal diagnosis.

Research into *F8* defects has been undertaken by other investigators for haemophilia A individuals from Pakistan, however the cohort size was not large (n=56) and comprised an unknown complement of both Pakistani and Indian cases (138). The latter study predated the discovery of the Inv22 and focused specifically on potentially hypermutable CpG sites. The screening strategy led to the

identification of CG-transitions in three Pakistani individuals, but no other *F8* defects in this ethnic group were reported.

The study presented here-in identified 30 mutations that have not been previously reported (Table 4.1) and the frequency of noval mutation is similar with other nationality (Table 4.3). The novel mutations comprised 8 missense, 7 nonsense, 3 splice site, 1 promoter region, 3 single nucleotide duplications and 8 with nucleotide deletions (Table 4.1).

The novel variants can be divided into those with a high probability of pathology (nonsense, duplication, deletion and the splice site change c.6187+1G>A which destroys the conserved GT of the splice junction) (total = 19) and those for which a detrimental effect was uncertain (missense, promoter region and the other two splice site changes) (total = 11). The latter represent unclassified sequence variants. The possibility that these may be neutral SNPs present in the population was explored by screening for each in a cohort of 50 haemostatically normal males from Pakistan which was recruited from different region of the country. Ten of the unclassified variants were not found in the normal population, supporting a pathogenic role. The remaining unclassified variant (c.601+17G>A) was detected in one control sample. This sequence change was associated with mild haemophilia A (P24, Table 4.1) and its presence in the normal population may be explained by individuals with a low FVIII level who do not come to clinical attention.

The unclassified missense variants were assessed for potential pathological impact using two programs for the prediction of functional effects: Polyphen 2 uses features comprising the sequence, phylogenetic and structural information to assess the impact of the missense change on the structure and function of a protein (139)] (URL http://genetics.bwh.harvard.edu/pph2/); SIFT impact prediction is based on

the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences (140)] (URL http://sift.jcvi.org/). Each missense variant scored highly for predicted pathological effect in both programs (Table 4.4).

The above findings support a causative role for the novel missense variants in haemophilia A. In the case of c.1694G>A p.(Gly565Glu) and c.1786T>C p.(Ser596Pro), further support for a causative role arises from the observation that they are recurrent (Table 4.1). Indeed, 8 sequence variants characterised in this cohort were recurrent. The possibility that recurrence may be due to a founder effect was investigated by comparison of *F8* SNP haplotypes associated with each. A given recurrent mutation was found in association with an identical *F8* haplotype in all individuals in whom it occurred (Table 4.3), supporting the possibility of a founder effect.

A causative gene defect was not found in 11 individuals, a plausible explanation for this is that the defect occurs outside the *F8* regions screened. However, why this should occur so frequently is difficult to explain and may ultimately only be understood when the mutation(s) is(are) finally characterized.

This study of a large haemophilia A cohort from Pakistan showed a diverse spectrum of mutations, similar to studies done in other nationalities. Consistent with those studies, point mutations were the predominant gene defect in the cohort and, in severe individuals, the Inv22 showed a high frequency (Table 4.6). This cohort additionally provided 30 novel mutations thereby expanding the known molecular basis for hereditary haemophilia A.

Chapter 5

Haemophilia Joint Health Score and Gilbert Score

Recurrent joint bleeding in an individual with haemophilia A leads to joint damage which is associated with pain, loss of range of motion and function and physical and psychological destruction (141). Knees, elbows and ankles are the most commonly affected joint in haemophilia, about 80% of haemarthrosis occur in these joints (142).

Different joints score are used to monitor the joint health and evolution of treatment in haemophilic, HJHS and Gilbert scores are among them (142). Gilbert score mainly composed of four parameters pain, bleeding, physical examination and X-ray examination; it is the most widely used tool for the joint assessment for individuals with severe haemophilia A who have severe arthropathy. HJHS is an 11-items score, mainly composed for young children with minimal arthropathy or the group who have on prophylaxis.

There are certain limitations of the HJHS, this instrument is primarily designed for the individuals with milder arthropathy, therefore it may not be very useful in developing countries where prophylaxis is not available and majority of the haemophilia population are on-demand treatment.

In this study, both HJHS and Gilbert score were employed to assess the joint changes in individuals who had minimal access to replacement therapy and found a very close association between them. Preferably, both health scores are not performed in acute condition: in the presence of joint bleed, acute pain or in inflamed joint.

5.1. Results

5.1.1. Haemophilia A severity and joint health scores

HJHS and Gilbert score were determined for all recruited individuals. A strong correlation (r =0.97) was found between the scores in individuals with both severe and mild arthropathy (Figure 5.1).

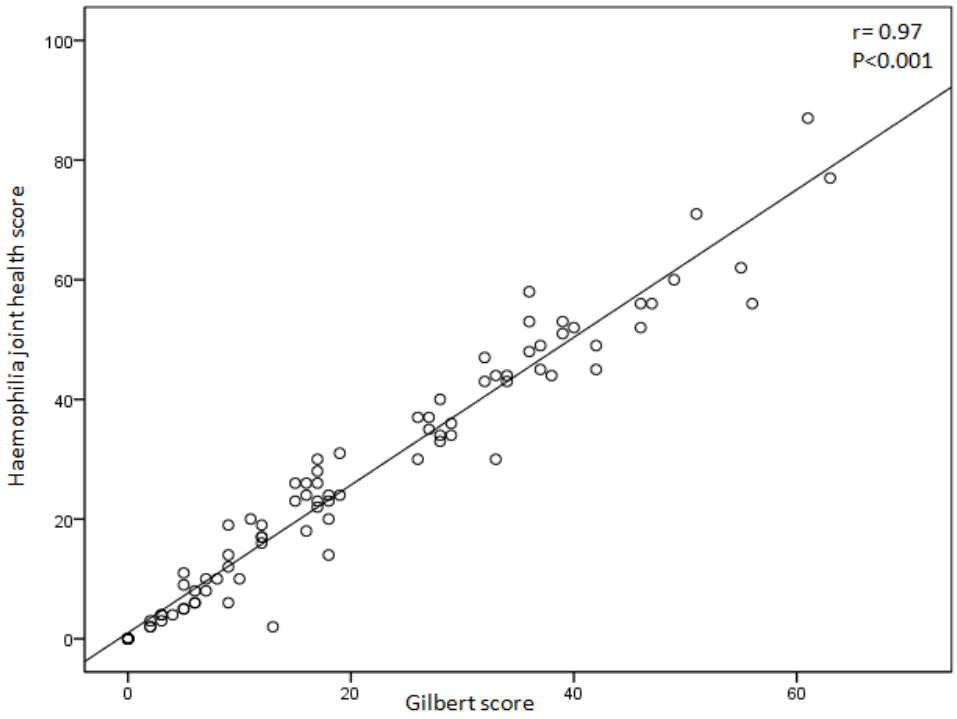


Figure 5.1: Correlation between HJHS and Gilbert score. The two measures of joint health score showed a strong positive correlation.

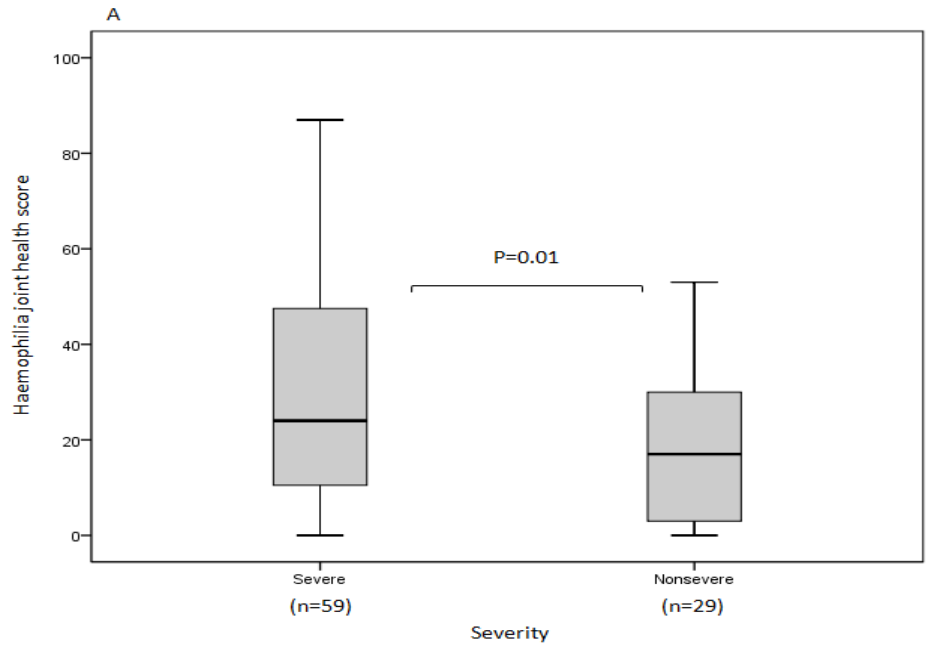
Both HJHS and Gilbert score were studied according to disease severity and found to be higher for severe compared with nonsevere individuals, this was statistically significant (Figure 5.2A and 5.2B). Despite this, many individuals with nonsevere disease had significant joint pathology. In the severe group, the median (range) for HJHS was 25 (0-87) and for Gilbert score was 19 (0-63) compared to nonsevere 17 (0-53) and 12 (0-40) respectively (Figure 5.2A and 5.2B). The nonsevere group comprised 5 individuals with mild haemophilia and 24 moderate cases, of which some also had significant arthropathy: median (range) of HJHS and Gilbert score were respectively 0 (0-52) and 0 (0-40) (mild group), 9 (0-53) and 14 (0-36) (moderate group).

5.1.2. Age and Joint Health Scores

Both HJHS and Gilbert score were studied according to developmental age group (<12 years, 12-16 years and >16 years) and across three groups, a statistically significant difference ($P \leq 0.001$) was observed. Inter-group comparisons showed that both scores were significantly different between individuals <12 years and the other age groups, whilst there was no significant difference in joint health scores between individuals 12-16 years compared with those >16 years (Figure. 5.3A and 5.3B).

In the age group <12 years, both scores were significantly different between severe and nonsevere groups; this difference was not seen in the older individuals

(Figure 5.4A and 5.4B). The median ages of the two groups were 13 years for severe and 16 years for nonsevere.


A
Haemophilia joint health score
100
80
60
40
20
0
P=0.01
Severe
(n=59)
Nonsevere
(n=29)
Severity

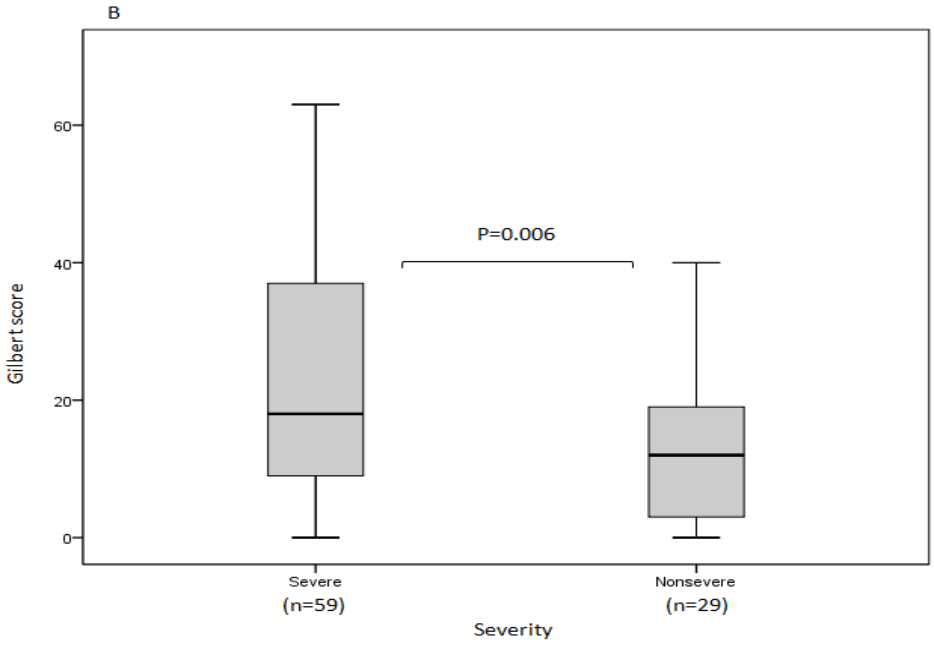

B
Gilbert score
60
40
20
0
P=0.006
Severe
(n=59)
Nonsevere
(n=29)
Severity

Figure 5.2: HJHS and Gilbert score according to haemophilia A severity. HJHS (Panel A) and Gilbert score (Panel B) both showed a statistically significant difference between severe and nonsevere individuals, however there was considerable overlap between both groups. Box-whisker plots show interquartile range and median value.

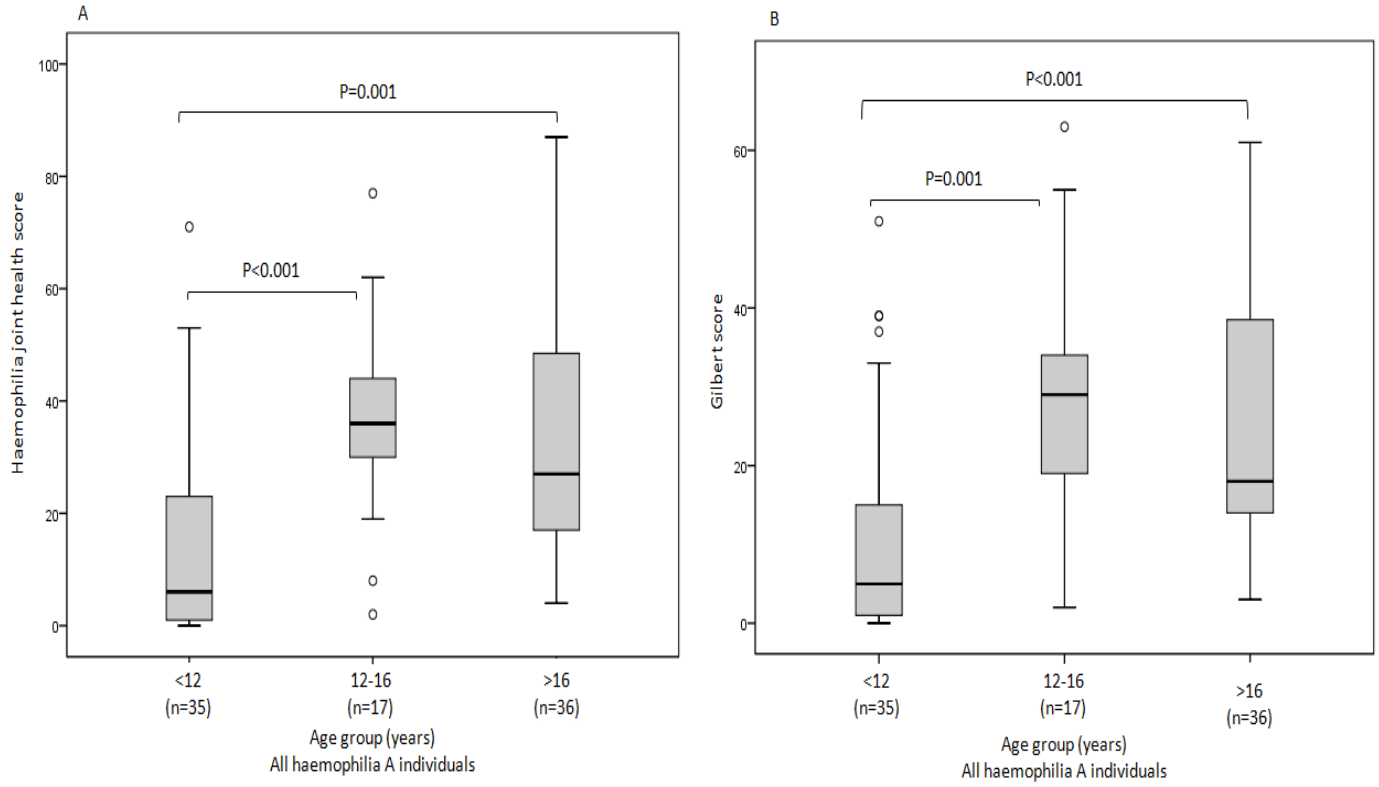


Figure 5.3: HJHS and Gilbert score according to age. HJHS (Panel A) and Gilbert score (Panel B) showed a statistically significant difference between <12 years and each of the other two age groups. There was no significant difference in the joint scores between 12-16 years and >16 years. “o” indicates an outlier.

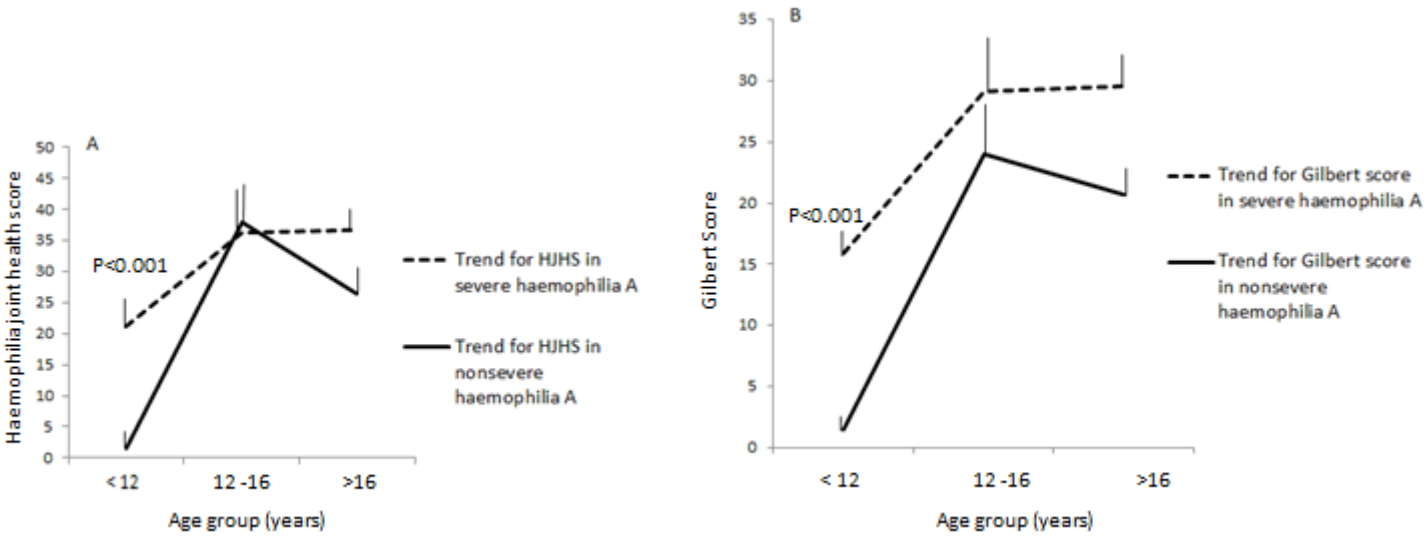


Figure 5.4: Trend of HJHS and Gilbert score according to haemophilia A severity and age. Both HJHS (Panel A) and Gilbert score (Panel B) were significantly different between severe and nonsevere groups in age <12 years, but not thereafter. The perpendicular lines indicate the standard error of the mean.

In the subset of individuals with severe haemophilia A, both scores gave a very similar picture to the above with regard to age, with the exception that both the HJHS and Gilbert Score were not significantly different between <12 years and 12-16 years (Figure 5.5A and 5.5B). Due to insufficient individuals in the nonsevere group, similar comparisons according to age were not possible.

When comparing the severe and nonsevere groups there was a marked difference in the < 12 year olds. At this age the severe individuals had evidence of significant arthropathy (median HJHS 12 and Gilbert score 11). In contrast, in the nonsevere individuals < 12 years the HJHS and Gilbert scores were low with a median of 1.5 and 1.5, respectively. This difference between severe and nonsevere individuals was not found in older as the joint scores in the severe group tended to plateau whilst in the nonsevere the joint scores progressed.

5.1.2. Causative Mutation and Joint Health Scores

In order to explore whether different mutation types underlying severe haemophilia A may have a differential effect on joint health, HJHS and Gilbert score were inspected for missense, nonsense, frameshift and Inv22 mutations (52 of the 59 severe, non-inhibitor individuals). There was no significant difference for either score across these mutation types or in pair-wise comparisons between them (Figure 5.6A and 5.6B).

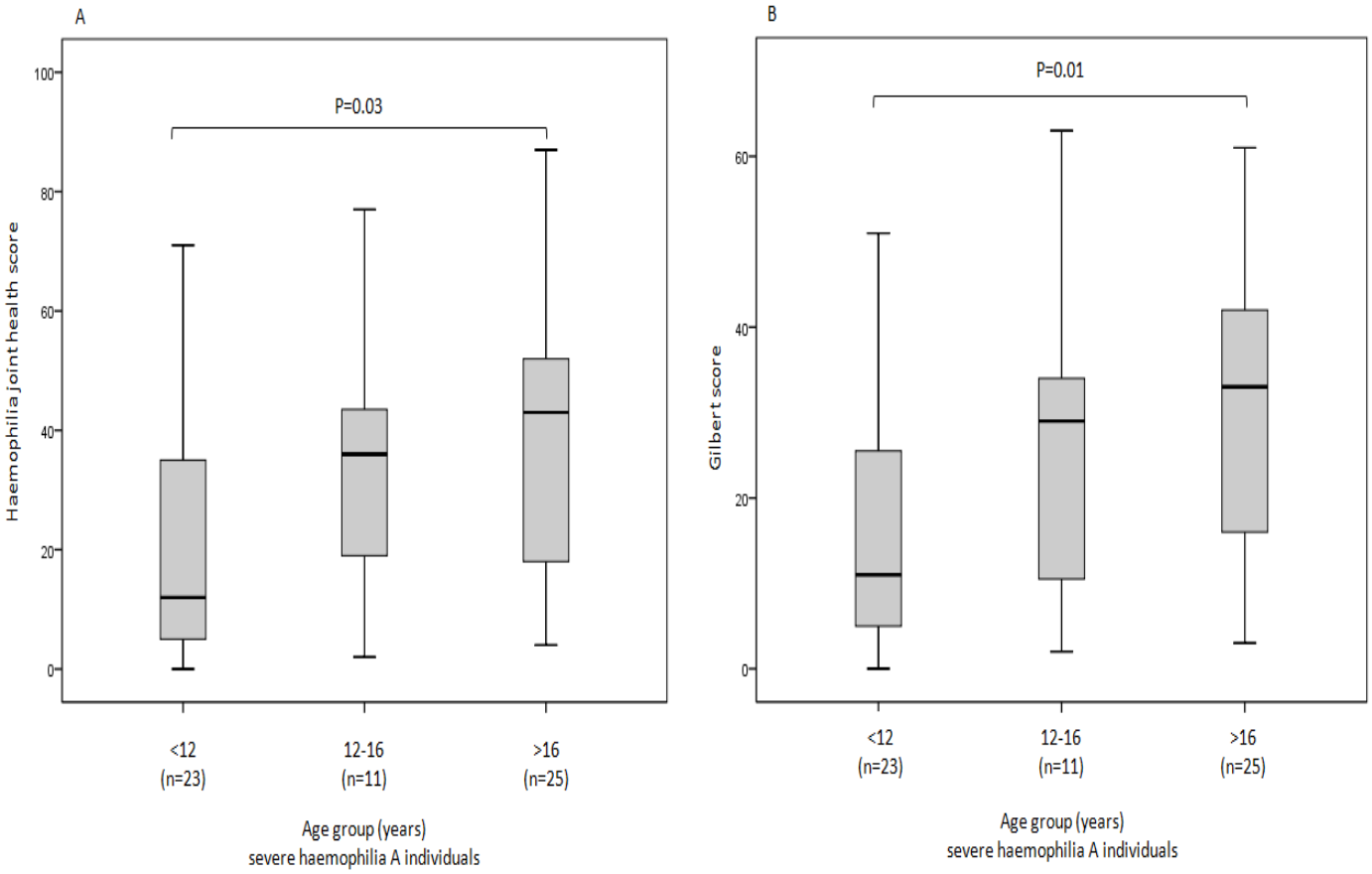


Figure 5.5: HJHS and Gilbert score according to age for severe haemophilia A individuals. Both HJHS (Panel A) and Gilbert score (Panel B) were significantly different between <12 years and >16 years but not between <12 years and 12-16 years (P~0.2 for each score).

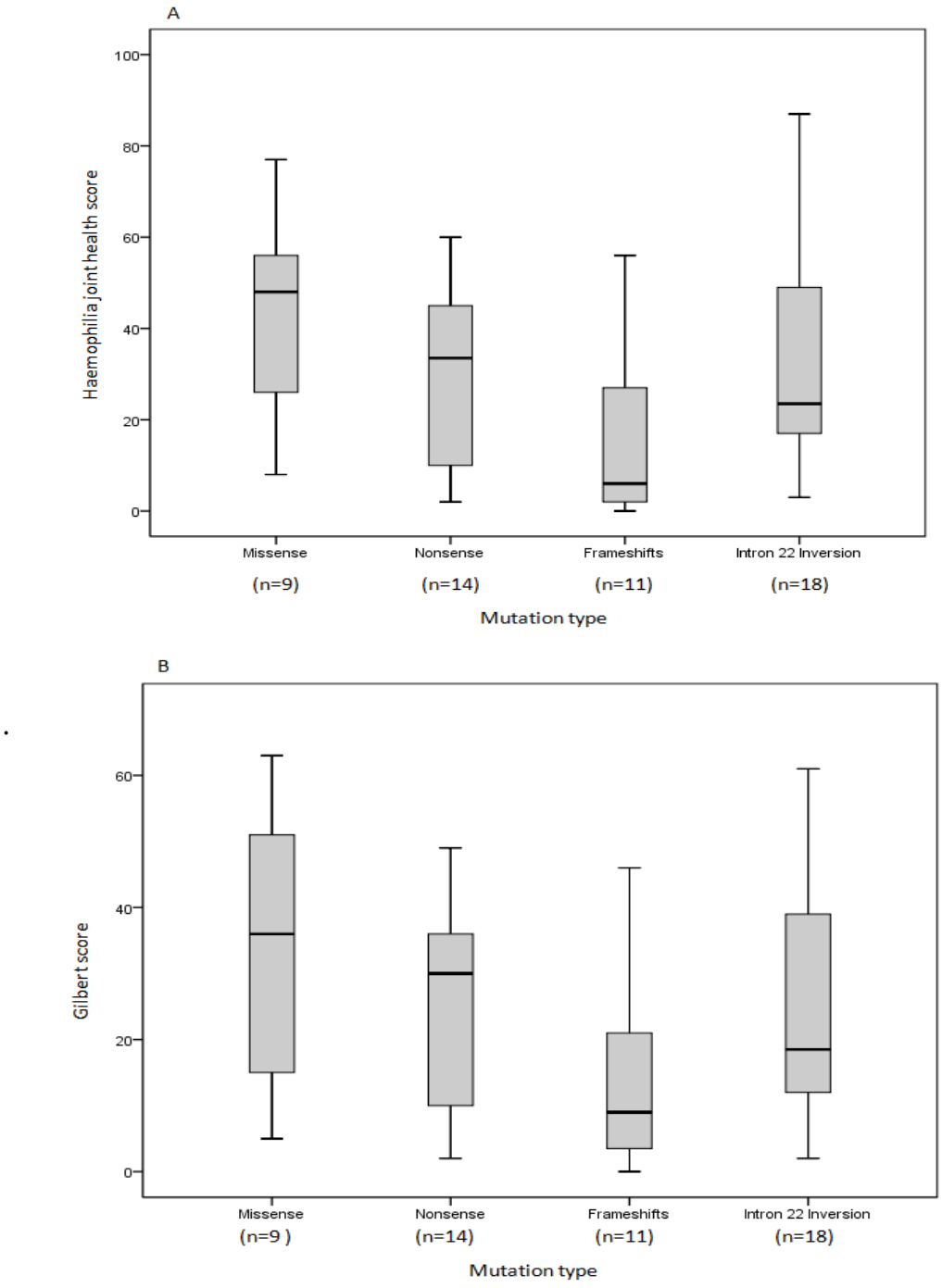


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Figure 5.6: HJHS and Gilbert score according to *F8* mutation type in severe haemophilia A. HJHS (Panel A) and the Gilbert score (Panel B) showed no statistical difference between any of the mutation types.

5.2. Discussion

Haemophilia A is associated with recurrent joint bleeding which leads to synovitis and debilitating arthropathy. Coagulation FVIII level is an important determinant of bleed number and development of arthropathy. This study investigated musculoskeletal status in individuals with haemophilia A who had minimal assess to both FVIII concentrate and multidisciplinary haemophilia care. HJHS and Gilbert score were found to be higher in severe compared with nonsevere individuals, consistent with international studies in which cohorts did have access to FVIII replacement: a Canadian study investigated 226 boys with mild, moderate or severe haemophilia (both A and B included) and found a higher HJHS in severe individuals. They also observed that HJHS was 97 % more efficient than the Gilbert score at differentiating severe haemophilia from mild and moderate, it was also noticed that HJHS was 74 % more efficient than Gilbert score at differentiating individuals treated with prophylaxis compared to on demand (143). Even though the individuals in those studies were on prophylaxis or on-demand therapy, the severity of haemophilia still played an important role in joint health, as found in our cohort which had minimal access to FVIII concentrate.

An important issue with regard to the joint score measurements is reproducibility. In keeping with studies such as those cited above, joint scores in the present investigation were measured on a single occasion only, however all were assessed by the same person throughout the study, thereby minimizing differences that could arise through subjectivity on the part of the investigator. Reproducibility

is, however, an issue in that, even with a sole investigator, joint measurements taken on different days for the same affected individual could differ according to the circumstances of the latter.

The progression of haemophilic arthropathy was investigated according to developmental age. Both joint health scores were significantly lower in younger individuals compared with older. Joint bleeds typically start between 1 and 3 years of age in children with haemophilia (144), especially those with severe disease, and this was found in the present study. This was reflected in the joint scores because most of the children aged less than 12 years had evidence of significant arthropathy. In contrast, individual with nonsevere disease had much lower joint scores at this age, probably because they were less prone to spontaneous and recurrent haemarthroses. These differences in joint scores between severe and nonsevere individual had narrowed and disappeared by the age of 12 years and old. This implies that factor VIII levels in the mild to moderate range cannot protect joints longer term if individuals have very limited access to factor VIII replacement therapy. The high proportion of individual with nonsevere disease who had target joints, as described in chapter 3 supports this concept. This has been seen by other groups (145, 146). In the absence of FVIII concentrate, there is little to prevent arthropathy appearing as early as the first decade in severely affected individuals. Our data suggest that haemophilic joint damage starts at an early age in severely affected individuals and progresses until the age of 12-16 years, by which time aggregative damage approaches maximum and little further progression in arthropathy is possible. In contrast, the low levels of factor VIII in mild and moderate disease can partially protect against arthropathy in young children but not old individual.

Several studies have reported a positive relationship between age and HJHS: a Lithuanian study (n=20) showed higher HJHS in older children (mean score = 24.5) compared with younger (mean score = 11.6) (147); a cohort of 39 individuals with haemophilia from Brazil showed a statistically significant positive correlation of Gilbert score with age (148). A group from London examined the influence of various factors – age, prophylaxis, history of high-titre inhibitors (HTI) and bleeding events on HJHS and found a positive association of HJHS with age. Furthermore, they observed that in the non-HTI group, HJHS was higher in those individuals where prophylaxis was started in late age group compared to other group where prophylaxis started in early years of life (149). Despite the fact that in each of these studies, the cohorts were either on prophylaxis or on-demand treatment, the joint health scores increased with age, however the magnitude of increase was not as great as observed in our cohort. The protection against joint pathology afforded by prophylaxis is not only a result of increasing the baseline/trough FVIII level, but also on intermittent peaks and appropriate physiotherapy. The progression of arthropathy with age is likely to be, at least in part, a result of inadequate access to FVIII replacement, and also due to a lack of access to specialised physiotherapy care. It is not possible to define what impact specialist physiotherapy would have had, but at a minimum it could be expected to help individuals cope better with their musculoskeletal disability and potentially improve joint function.

In this cohort, more than half of individuals had ≥ 2 joint involvement and the number of joints involved increased with age (Chapter 3, Table 3.2). Importantly, target joints were common in individuals with both moderate and mild haemophilia.

As shown the next chapter (Chapter 6, Figure 6.12), a negative correlation between TG and joint health scores was observed in nonsevere individuals (there was no correlation in the severe group). This suggests that the baseline FVIII level is more important than global haemostasis in predisposing an individual to arthropathy. Additionally, it is also plausible that, once joint damage is initiated, it becomes an on-going process in which TG has little (if any) role whilst, in contrast, factors relating to inflammation may come to the fore. Our data indicate that destructive changes occur before 12 years of age, from which it could be inferred that coagulant treatment early in life may be important to limit or prevent arthropathy as shown previously (150).

The comparison of HJHS and Gilbert score between different mutation types – missense, nonsense, frameshift and the intron 22 inversion – in severe haemophilia A showed no significant difference for either joint health score. Thus, although the mutation is the basis of the disorder, in severe individuals the nature of the gene defect does not correlate with the degree of arthropathy. This is consistent with the findings of Chapter 6 (151) in which it was demonstrated that TG does not appear to be influenced by the nature (missense or null) of the *F8* mutation.

This study provides the first data on musculoskeletal assessment using HJHS and Gilbert score in individuals with haemophilia A who have minimal access to FVIII replacement therapy. Pakistan, a country with an approximate population of 176 million (World Health Organisation (WHO) 2011 data, URL http://www.who.int/gho/countries/pak.pdf) has around 1,500 individuals diagnosed with haemophilia (A or B) (http://www.org/publications/files/pdf), a significant under-representation based upon the size of the populace with the expected number being about 17 500. The management of haemophilia in Pakistan has been limited until now, the *per capita* usage of FVIII concentrate is very low compared to other

countries in the same region ("Eastern Mediterranean", World Federation of haemophilia (WFH) designation). The WFH has established that 1 IU *per capita* of FVIII concentrate should be the minimum target to achieve the optimal survival within the haemophilia population. However, to preserve the joint function or achieve a good quality of life, a higher level of FVIII concentrate would be required. According to a WFH 2010 report, Pakistan has the lowest *per capita* usage of FVIII concentrate (0.01 IU/person) compared to the rest of the countries in this region (http://www1.efh.org/publications/files/pdf). The problems with management of haemophilia in Pakistan are insufficient awareness, inadequate diagnostic facilities, insufficient multidisciplinary teams (especially physiotherapy) and limited factor concentrates for treatment. Despite the fact that 80 % of the individuals had positive family history, due to the insufficient awareness of this disorder, parents circumcised their children in the early weeks of life resulting in bleeding in many cases. In Pakistan, priorities in establishing services for haemophilia should include the training of health care workers, setting up haemophilia care centres, establishing laboratories able to diagnose inherited bleeding disorders, introducing a registry, educating affected individuals and their families about the condition, providing low-cost factor concentrate and appropriate education regarding the management of joint bleeds.

In conclusion, this study of haemophilia A individuals from Pakistan who had minimal access to FVIII concentrate is the first to assess the correlation between HJHS and Gilbert score in such a cohort and demonstrated that the two scores correlate strongly. In addition, this study showed a relationship between the joint health scores and (i) severity of haemophilia A, and (ii) age. The study revealed that having a baseline FVIII above 1 IU/dL did not protect against severe musculoskeletal disability in older individuals. In the absence of proper treatment

(prophylaxis or on demand), individuals are at risk of target joints and severe arthropathy from their early years of life. Additionally, this study indicated that for individuals with severe haemophilia A, although the gene defect underlies the disorder, the nature of the defect has little influence on the severity of arthropathy. Thus, whether FVIII is produced but dysfunctional or not produced at all (respectively missense and null mutations), appears to have little bearing on the mechanisms that lead to arthropathy.

Chapter 6

Thrombin Generation

To measure the ability of plasma to generate thrombin, TG assay is used which investigate the propagation phase (where the bulk amount of thrombin is generated) and termination phase (where the thrombin generation is shut down by anticoagulant pathway), in contrast to PT and aPTT which investigate the initiation phase (where the trace amount of thrombin is generated) of cell based model of coagulation. Therefore, the resulting thrombogram reflects all pro- and anticoagulant reactions that regulate all the process involved in the formation and inhibition of thrombin (152).

In 1953, the first TG assay was introduced; thrombin was measured by subsampling method. Due to time consumption and technical difficulty, over time, subsampling method was replaced by chromogenic technique and as a result of successive improvement both techniques were replaced by CAT. Among several advantages of CAT assay, one of important fact is that this assay can be easily modified for specific purpose.

In CAT assay, low affinity fluorogenic substrate (Z-Gly-Gly-Arg-AMC) is used to continue monitor of thrombin activity in plasma. In this assay, plasma is triggered with an appropriate amount of TF and synthetic phospholipids vesicles PL. To correct the" inner-filter" effect, a known amount of substrate converting activity known as thrombin calibrator (α_2-macroglobulin) is used. The α_2-macroglobulin is a form of thrombin that cannot be inhibited by plasma protease

inhibitor. The assay is performed in a microtitre plate and fluoresce reading is automatically converted by using a reserved software. To minimize the artefacts introduced at the time of sample collection following parameters were taken: blood was collected using 21-gauge needle with minimum suction into Haematologic Technologies Inc blood collecting tubes containing sodium citrate/CTI (20 mmol/L) tris, 150 mmol/L NaCl, 11 mmol/L citrate, 50 µg/mL CTI. CTI collecting tubes were used for mesurment at 1 pmol/L, 3 pmol/L and 5 pmol/L. During the whole process, for a internal quality control pooled PPP from healthy individuals were run in triplicate.

Several studies have been described the correlation between TG parameters and the risk of bleeding or clotting disorders. In bleeding disorder like haemophilia, TG assay is well reported in PPP, this test is also helpful in monitoring the haemophilia treatment with inhibitor bypassing agents (153).

6.1. Result

6.1.1. Thrombin Generation Measurement Using Calibrated Automated Thrombography

CAT analysis was done for all haemophilia A individuals in order to investigate thrombin generation between and within severity. According to the analysis, the final concentration of TF trigger used was either 1 pmol/L alone, or 1 pmol/L, 3 pmol/L and 5 pmol/L. Different concentrations were used where appropriate, to ensure maximum sensitivity of the assay, especially in the case of

severe FVIII deficiency. Unless otherwise stated, the data refer to 1 pmol/L. For reference, the normal range for peak and VI in Cardiff for 1 pmol/L TF were 26-65.1 nmol/L and 3.1-15 nM/min respectively. A typical CAT analysis is shown in Figure 6.1, highlighting the different parameters of the thrombogram. In many cases in individuals with severe haemophilia the thrombin generation curve did not complete and, hence, the ETP could not be reported and therefore peak and VI are used.

6.1.2. Relationship between Parameters of Thrombin Generation

The relationship between different parameters of TG with 1 pmol/L of TF trigger was investigated in haemophilia A plasmas. Peak showed a moderate positive correlation with both time to peak (tt) and velocity index (VI) and this was statistically significant (Figure 6.2A and 2B). A statistically significant moderate positive correlation between lag time and tt peak was also found (Figure 6.2C).

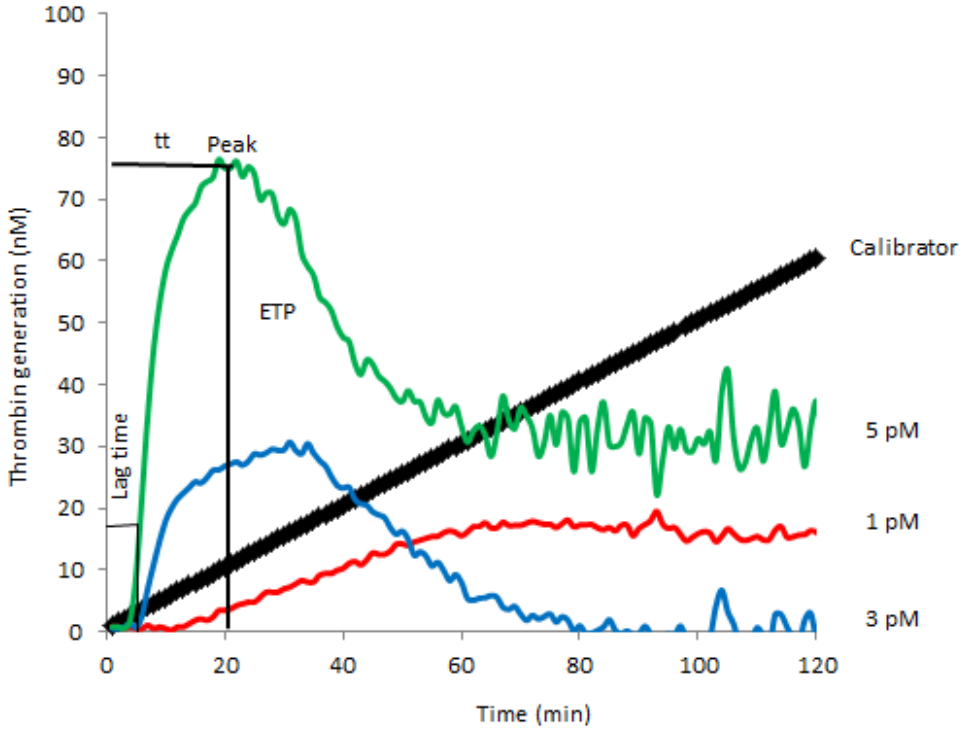

Figure 6.1: CAT analysis for Individual 24 (mild haemophilia A). Three different concentrations of TF (1 pmol/L, 3 pmol/L and 5 pmol/L) were used to trigger coagulation. The key measures of thrombin generation are indicated: lag time, peak height of TG, time to peak (tt) and ETP.

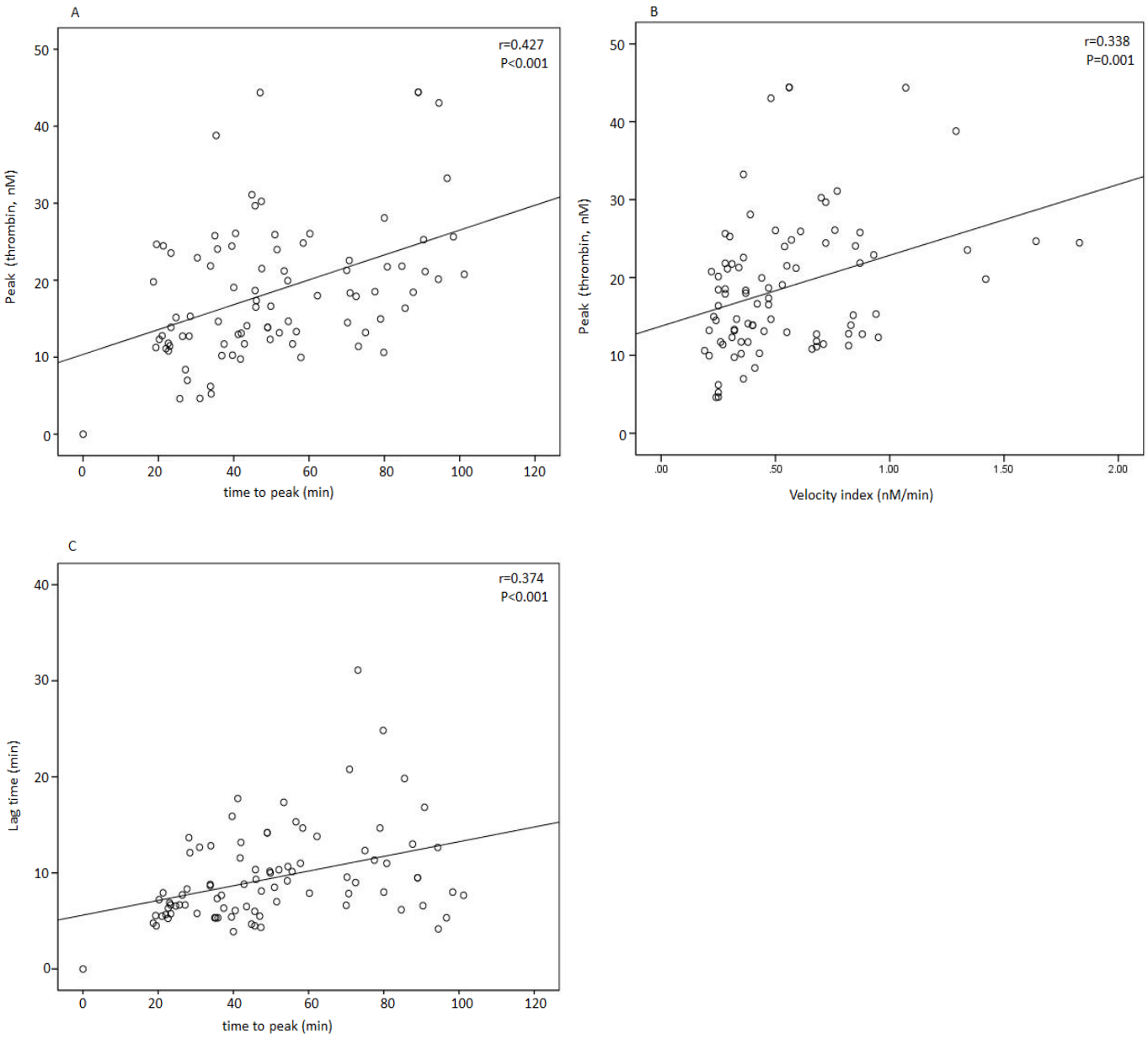

Figure 6.2: Correlation of thrombin generation parameters in haemophilia A plasmas. Positive correlations were found between peak and tt peak (Panel A), peak and VI (panel B), lag time and tt peak (Panel C). All correlations were moderate but statistically significant.

The possibility of correlation between peak and ETP was investigated. A large proportion of plasmas did not give an ETP at 1 and 3 pmol/L (the curve did not complete and no tail was obtained) (Figure 6. 3A,6.4A ETP = 0) and in the case of 5 pmol/L, a small number of plasma did not give ETP (Figure 6. 5A). Individuals with a high peak at 1 and 3 pmol/L were less likely to have a completed ETP than those with a lower peak. Although an incomplete ETP can not be given a value it is not appropriate to label them as 0. After the exclusion of individuals with "zero" ETP, a strong positive correlation was observed at 1 pmol/L (r = 0.92) between peak and ETP. Weaker but statistically significant correlations were also seen at 3 and 5 pmol/L (r = 0.74 and 0.64, respectively) (Figure 6.3B, 6.4B and 6.5B). Peak showed a wide range of values when the ETP was either 0 or measurable (>0) (Figure 6.3C, 6.4C and 6. 5C). Peak was significantly higher in individuals with unrecordable ETP compared with those who had a measurable ETP at 1 and 3 pmol/L but not 5 pmol/L (Figure 6. 3C,6. 4C and 6.5C).

6.1.3. FVIII:C and Thrombin Generation

Log FVIII:C did not correlate with Peak r=0.027 but there was a moderate positive correlation (r=0.33) with VI ($P<0.05$) (Figures 6.6A and 6.6B). This suggests that whilst VI is influenced by FVIII level in this cohort using the described reagents and triggers, the peak is not. The influence of FVIII on the VI is not large and the wide variation between VI at similar FVIII levels suggests that other coagulation factors and inhibitors are influencing the result. The peak thrombin does not correlate with FVIII level and the variability must, therefore, be related to the influence of other coagulation factors and inhibitors.

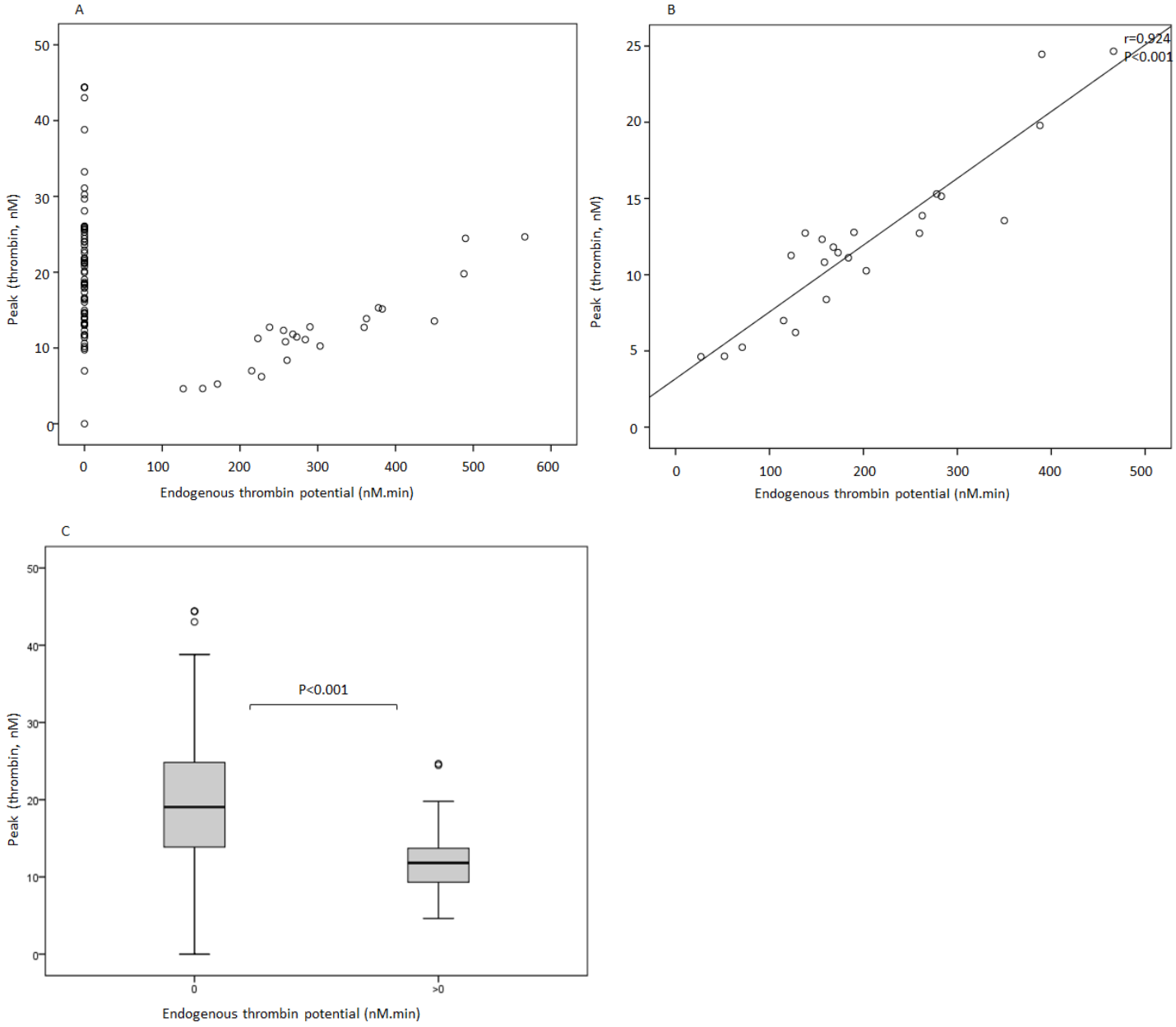

Figure 6.3: Peak thrombin generation in relation to ETP at 1pmol/L. Peak varied considerably irrespective of whether ETP was unrecordable (termed 0) or measurable (Panels A and C). Where ETP was measurable, it showed a strong positive correlation with peak (Panel B). "o" indicates an outlier.

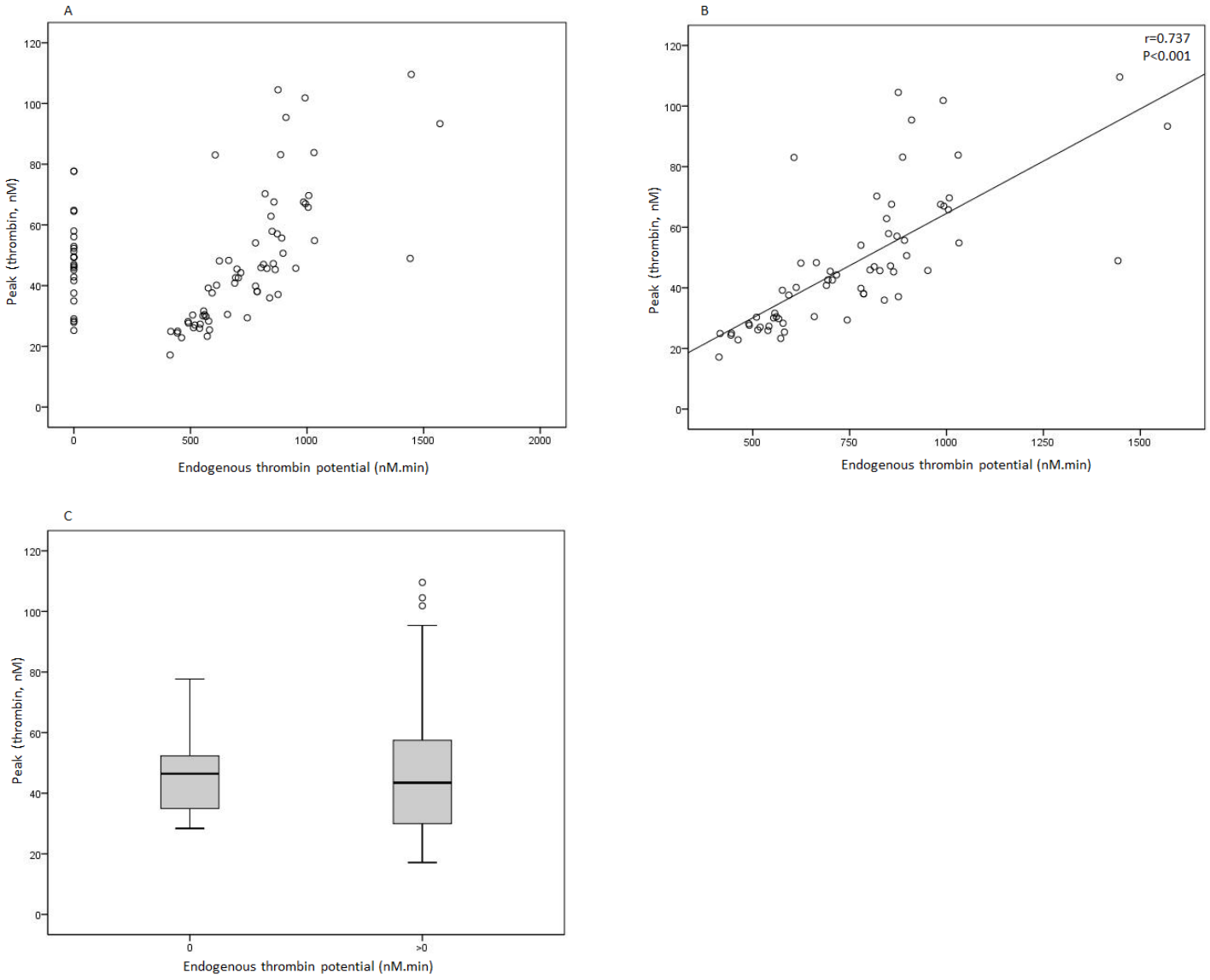

Figure 6.4: Peak thrombin generation in relation to ETP at 3pmol/L. Peak had a wide-range irrespective of whether ETP was recordable (termed 0) or measurable (Panels A and C). Where ETP was measurable, it showed a moderate positive correlation with peak (Panel B). "o" indicates an outlier.

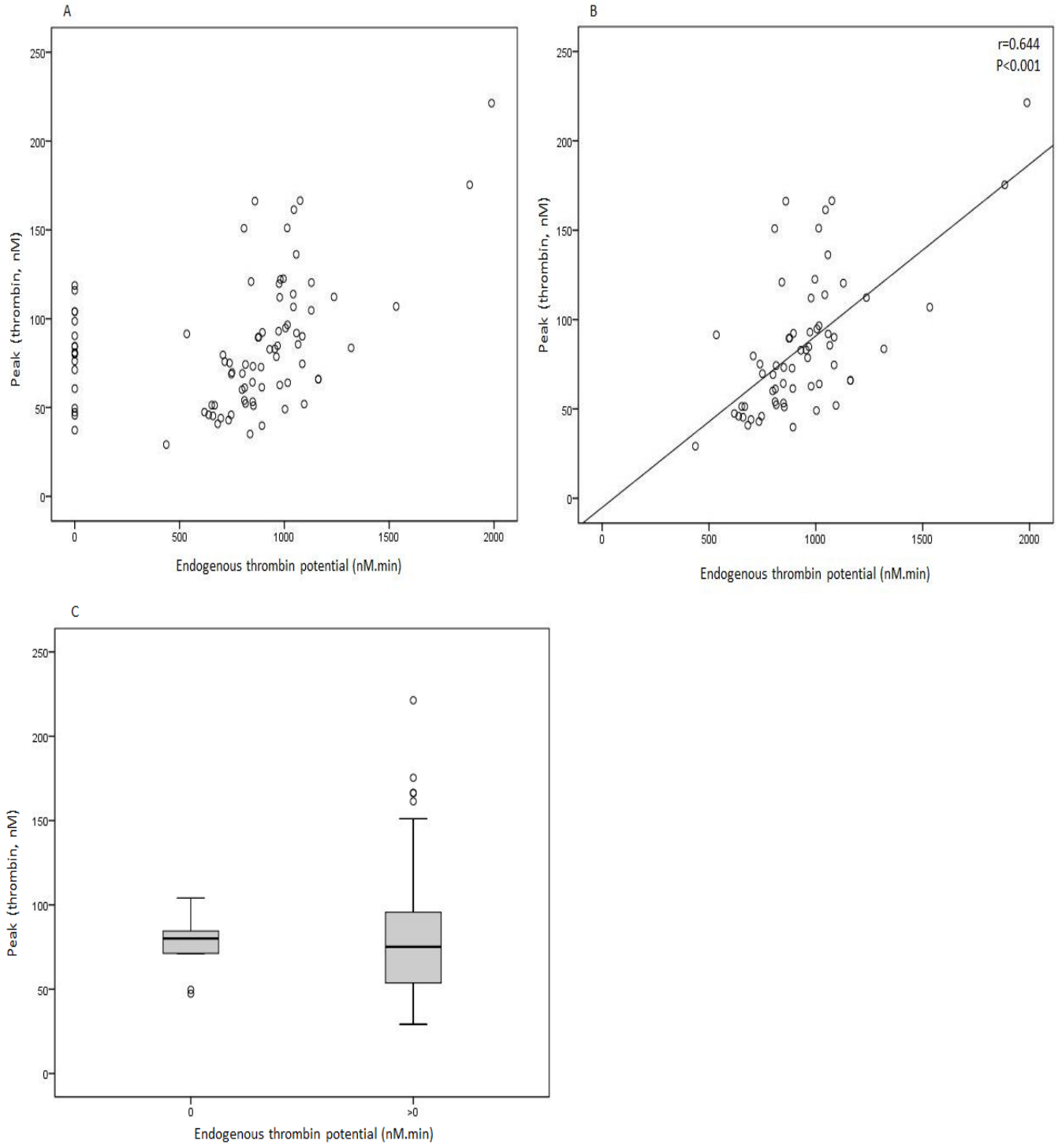

Figure 6.5: Peak thrombin generation in relation to ETP at 5pmol/L. Peak showed wide-range irrespective of whether ETP was unrecordable (termed 0) or measurable (Panels A and C). Where ETP was measurable, it showed a moderate positive correlation with peak (Panel B). "o" indicates an outlier.

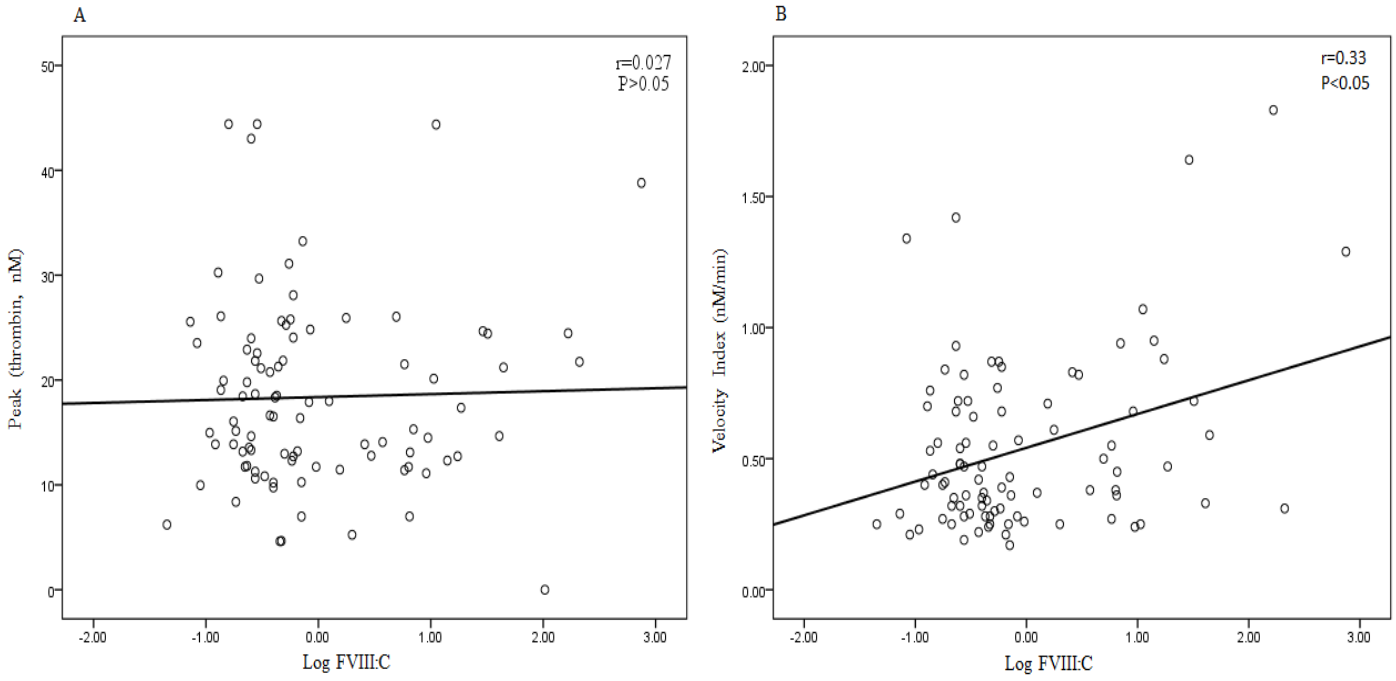

Figure 6.6: Correlation between LogFVIII:C and parameters of thrombin generation. LogFVIII:C (all severities included) showed no correlation with Peak (panel A), and a moderate positive correlation with VI (panel B).

6.1.4. Thrombin Generation and Severity of Haemophilia A

The parameters peak and VI varied widely in both severe and nonsevere haemophilia A individuals (Figure 6.7A and 6.7B) suggesting that haemostatic factors other than factor VIII were having a marked effect. Of note, peak and VI showed considerable overlap between severe and nonsevere individuals, and peak was negligible or zero for some nonseveres. There was a statistically significant difference between the VI of severe and nonsevere individuals but not peak thrombin. This is expected because FVIII correlated with VI but not peak.

Peak and VI were compared between severe and nonsevere groups, at 1, 3 and 5 pmol/L of TF trigger. Increasing the trigger TF concentration resulted in an increase in peak and VI for both groups of individuals as expected and this increment was statistically significant between each TF concentration (Figure 6.8). As TF concentration increased, the spread of values for peak and VI widened. At each concentration of TF, the absolute values for peak were similar in severe and nonsevere groups, likewise for VI (Figure 6.8).

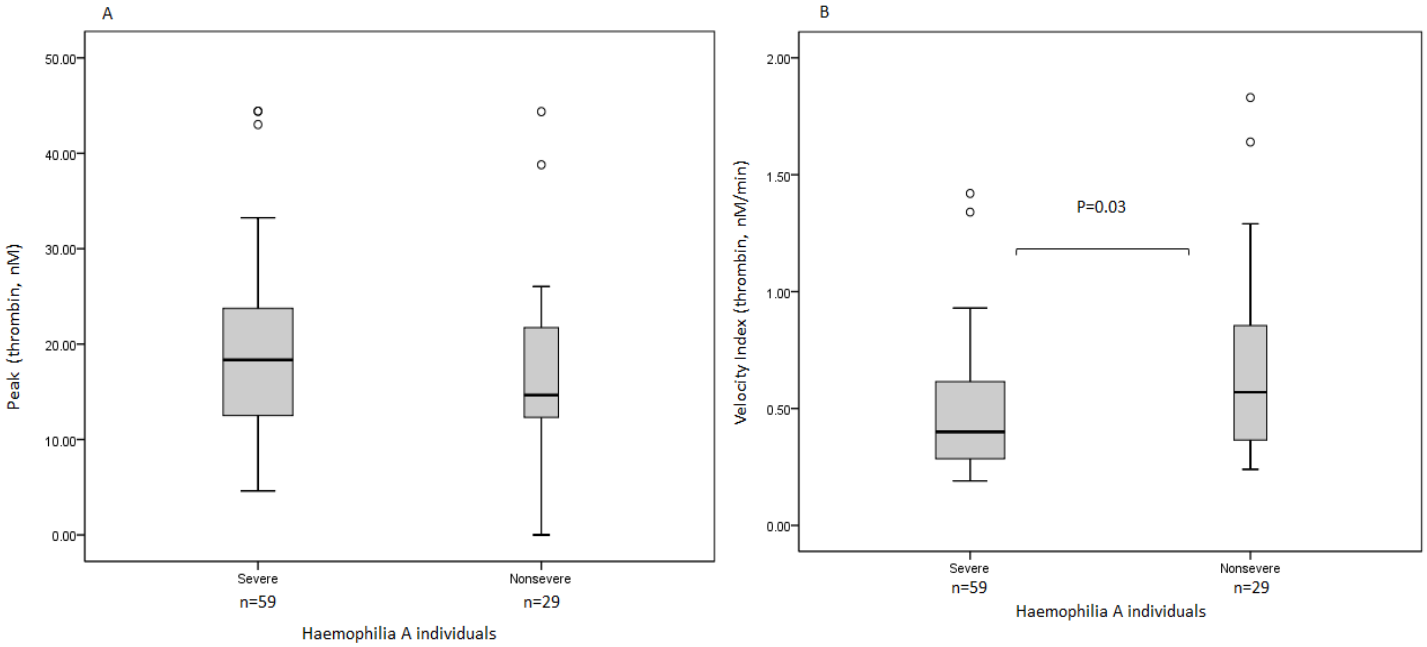

Figure 6.7: Comparison of thrombin generation parameters between severe and nonsevere haemophilia A individuals. Peak (panel A) and VI (panel B) each showed considerable overlap between severe and nonsevere groups. Of the two parameters, only VI was statistically different between the groups. “o” indicates an outlier.

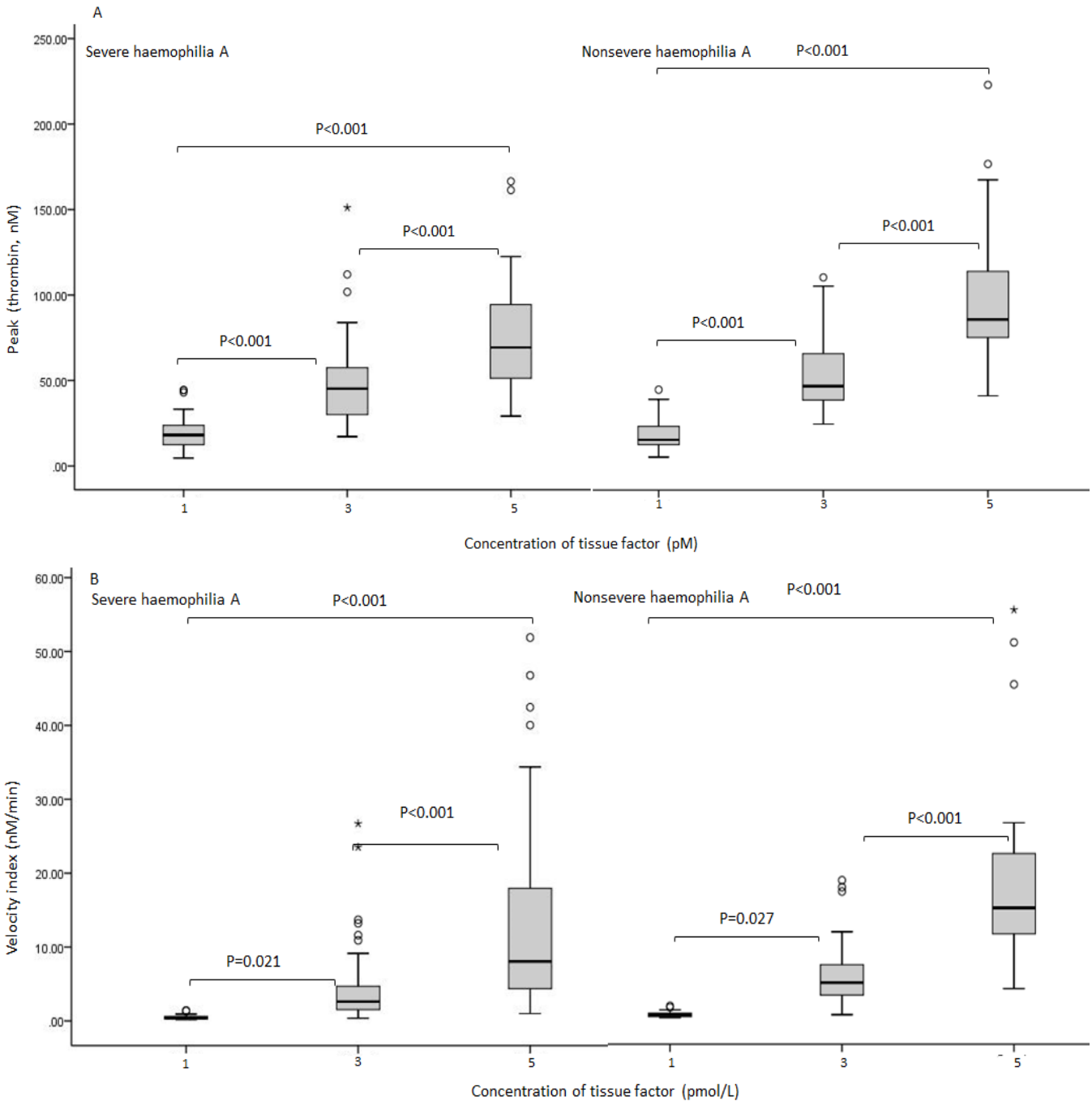

Figure 6.8: Peak and VI at different concentrations of TF trigger in severe and nonsevere haemophilia A plasmas. Peak (Panel A) and VI (Panel B) increased significantly with increasing concentrations of TF but the overlap between severe and nonsevere remained. "o" indicates an outlier.

6.1.5. Influence of added exogenous FVIII on thrombin generation

Plasmas from severe haemophilia A individuals (n=22) were used to investigate the effect of added exogenous FVIII on thrombin generation. Plasmas were spiked with recombinant human coagulation FVIII (Advate) at the following final concentrations: 1 %, 3 %, 5 %, 25 %, 50 % and 100 % (% relative to a normal activity of 100 %) and TG were monitored. Peak and VI rose as the concentration of FVIII increased. Within an individual a positive correlation (Table 6.1) was observed between logFVIII and peak and same was noticed with VI (Figure 6.9). The correlation between logFVIII and peak or VI ranged from very strong to moderate (Table 6.1). The mean r (SD) for peak was 0.70 (0.19) and for VI 0.77 (0.21). Figure 6.9 shows the relationship between FVIII and peak and VI in 8 representative individuals, there was wide variation in the slope of the lines suggesting that FVIII affected TG differently between them.

When the data for all individuals was pooled, a statistically significant difference was observed for both peak and VI across the FVIII range tested ($P<0.001$, ANOVA). However, despite significance across the FVIII range, there was no statistical difference between consecutive FVIII values either for peak or for VI (Figure 6.10A and 6.10B). At all concentrations of exogenous FVIII, VI was statistically different compared with control plasma. In notable contrast, peak showed no statistical difference for any FVIII level tested compared with control plasma.

Table 6.1 r value for peak and VI in severe haemophilia A plasma of added FVIII

Individual ID	r logFVIII and peak	r logFVIII and VI
P04	0.98	0.91
P08	0.96	0.96
P10	0.58	0.83
P18	0.53	0.85
P21	0.55	0.93
P29	0.65	0.9
P33	0.39	0.83
P34	0.87	0.86
P44	0.78	0.62
P57	0.67	0.65
P59	0.39	0.02
P74	0.97	0.66
P79	0.67	0.89
P83	0.68	0.67
P85	0.95	0.99
P87	0.78	0.59
P91	0.75	0.7
P94	0.44	0.93
P95	0.94	0.96
P96	0.88	0.93
P97	0.59	0.88
P98	0.53	0.8

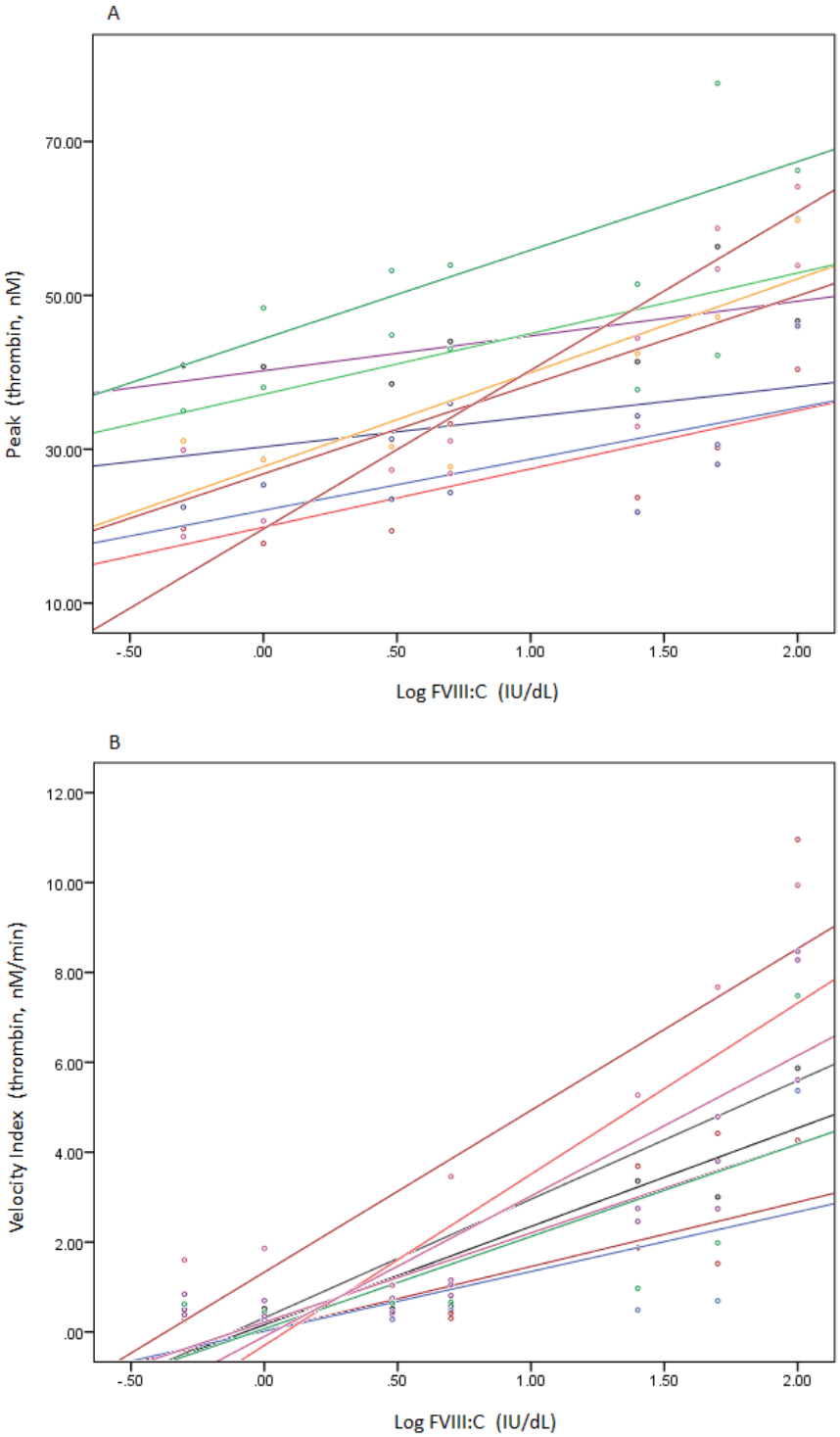

Figure 6.9: Effect of added FVIII on thrombin generation in severe haemophilia A plasma. The peak (Panel A) and VI (Panel B) plotted against Log FVIII:C. Each coloured line represented an individual patient. A positive correlation with wide inter- individual variability for both parameters was observed (table 6.1).

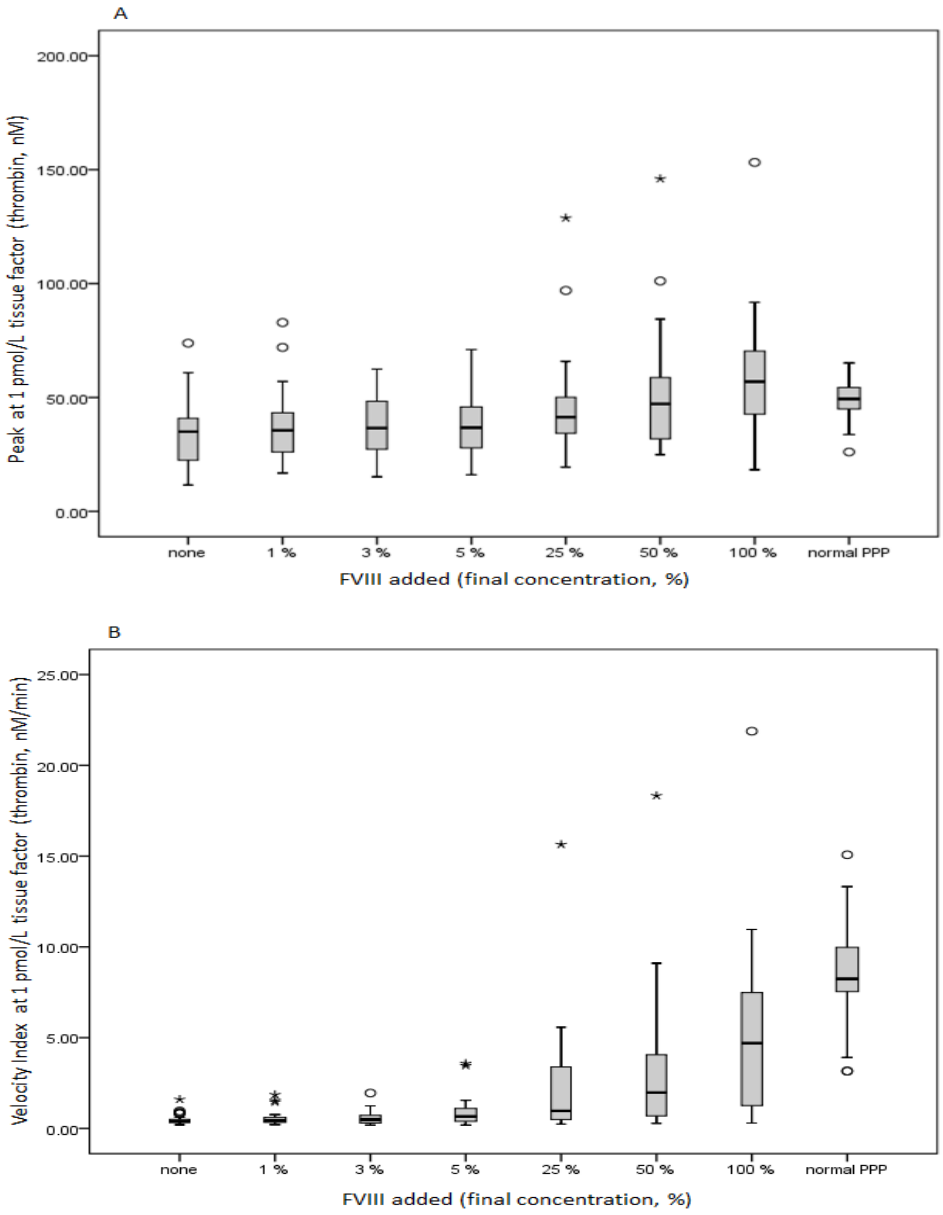

Figure 6.10: Effect of added FVIII on thrombin generation in severe haemophilia A plasma. Peak (Panel A) and VI (Panel B) were measured for different severe haemophilia A plasma samples (n=22) spiked with increasing amounts of recombinant FVIII. Both peak and VI increased as FVIII increased. For both peak and VI there was no statistically significant difference between consecutive FVIII values, however there was a significant increase across the FVIII range ($P<0.001$, ANOVA).

6.1.6. Comparison of Thrombin Generation and Causative Mutation

In order to investigate the possibility of association between TG parameters and different severe mutations, peak and VI were inspected according to mutation type in individuals with severe haemophilia A. Neither parameters showed any difference between severe missense mutations nor null defects (which comprised Inv22, frameshifts and nonsense changes collectively) (Figure 6.11A and 6.11B).

When peak and VI were inspected according to different severe mutation types (missense, nonsense, frameshifts, Inv22), there was no significant difference for either TG parameter between the various defects (Figure 6.12A and 6.12B).

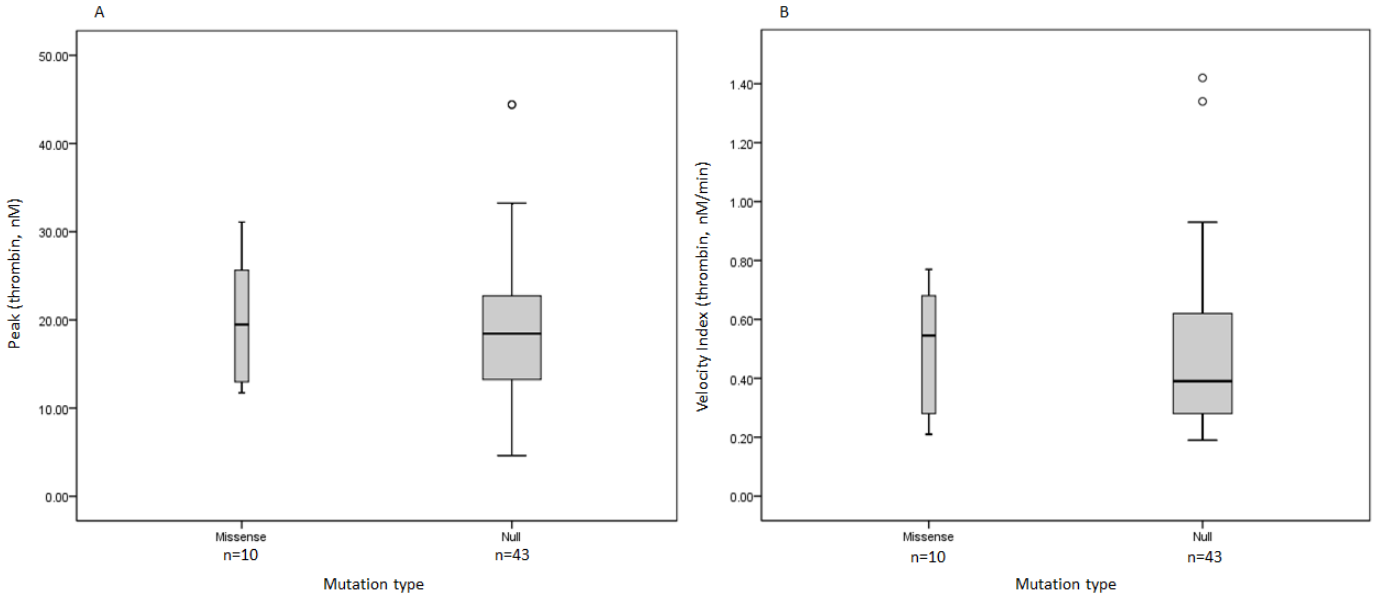

Figure 6.11: Peak and VI for missense and null mutations in severe haemophilia A. Peak (panel A) and VI (panel B) each showed no significant difference according to the presence of dysfunctional FVIII (missense) or absence of the coagulation factor (null). "o" indicates an outlier.

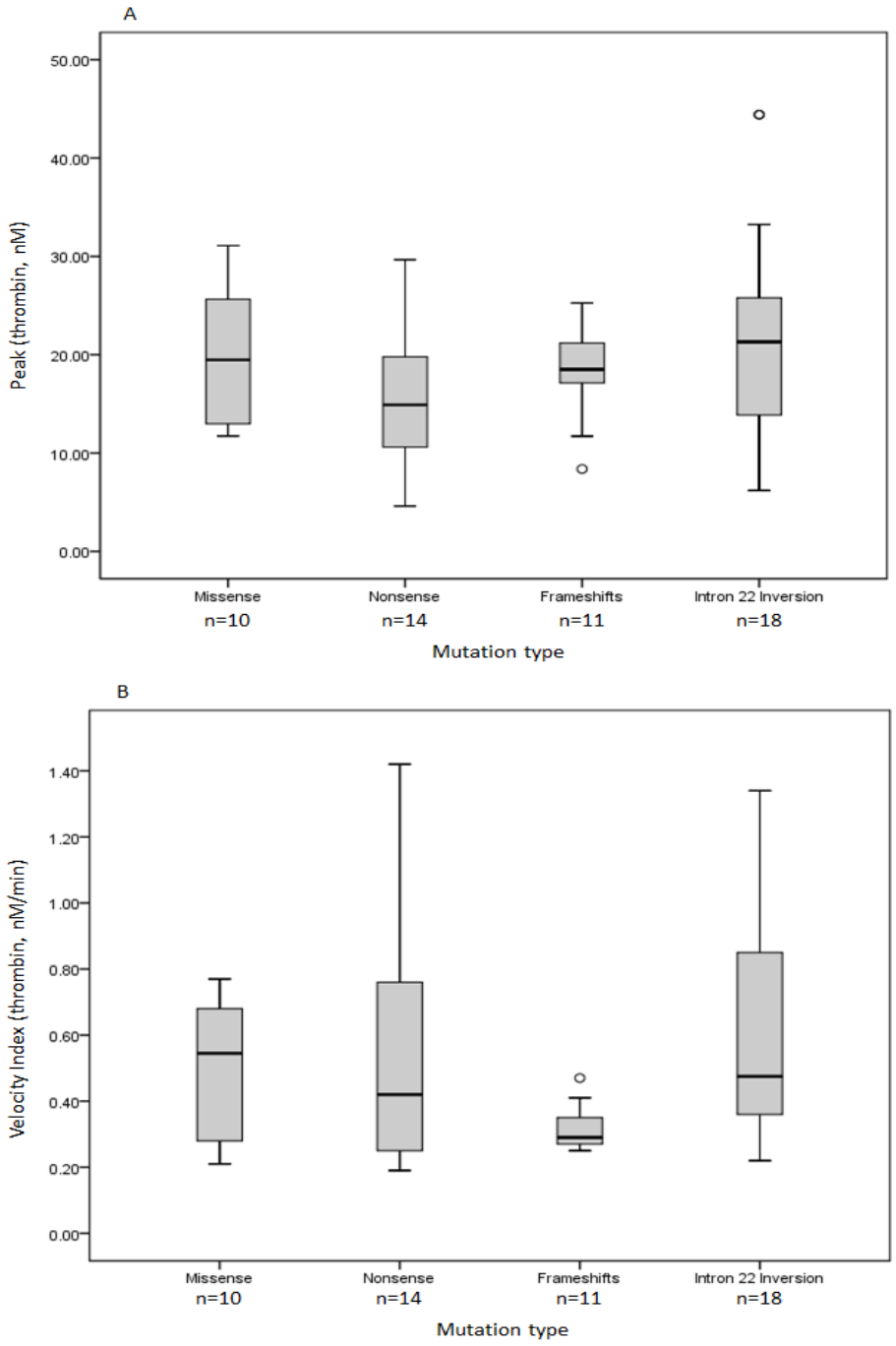

Figure 6.12: Comparison of thrombin generation parameters between *F8* mutation types in severe haemophilia A. Neither peak (panel A) nor VI (panel B) differed significantly between the various gene defects. "o" indicates an outlier.

6.1.7. Thrombin Generation and Joint Health Score

In order to investigate a possible relationship between *in vitro* TG and joint health, the parameter peak and VI were inspected according to HJHS and Gilbert score. For individuals with severe haemophilia A there was no correlation between joint score and peak (Figure 6.13A and 6.13B) whereas in nonsevere haemophilia A, peak showed a negative correlation with both scores (HJHS P=0.006; Gilbert score, P=0.01) (Figure 6.13C and 6.13D). VI showed no correlation with Gilbert score or HJHS in either severe or nonsevere groups in severe and HJHS in nonsevere group (Figure 14A-D). A negative correlation was observed between FVIII:C and joint health score in all individuals with haemophilia A (Figure 6.15) and the nonsevere group alone (figure 5.16). This was despite the peak not being correlated to factor VIII levels and possible suggests that differences in peak TG related to factors other than factor VIII might play a role in joint pathology.

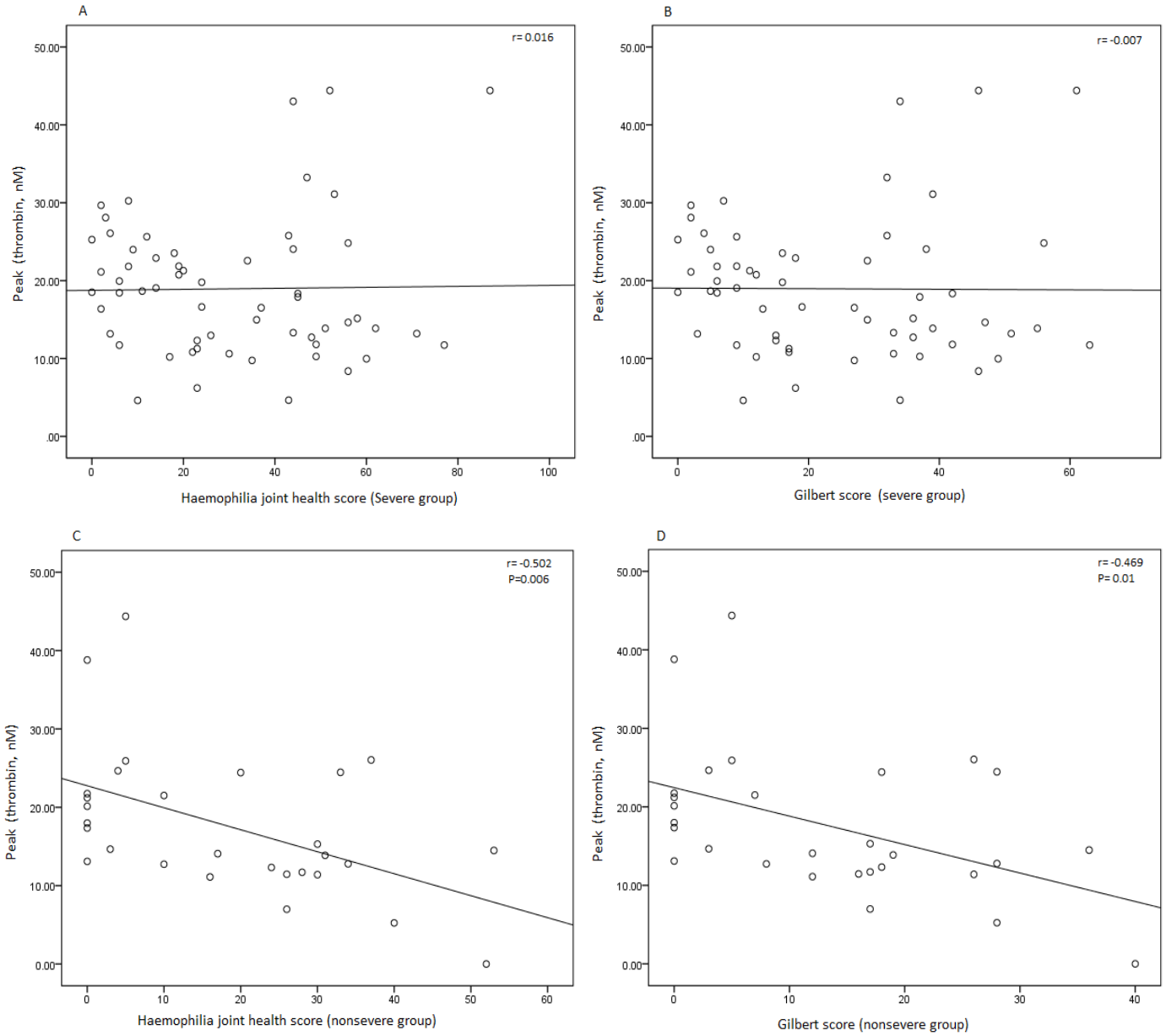

Figure 6.13: Correlation of HJHS and Gilbert score in severe (Panel 13A and Panel 13B) and nonsevere (Panel 13C and Panel 13D) individuals with haemophilia A. In nonsevere individuals, a significant correlation was observed between peak and each joint health score; this was not seen in severe individuals.

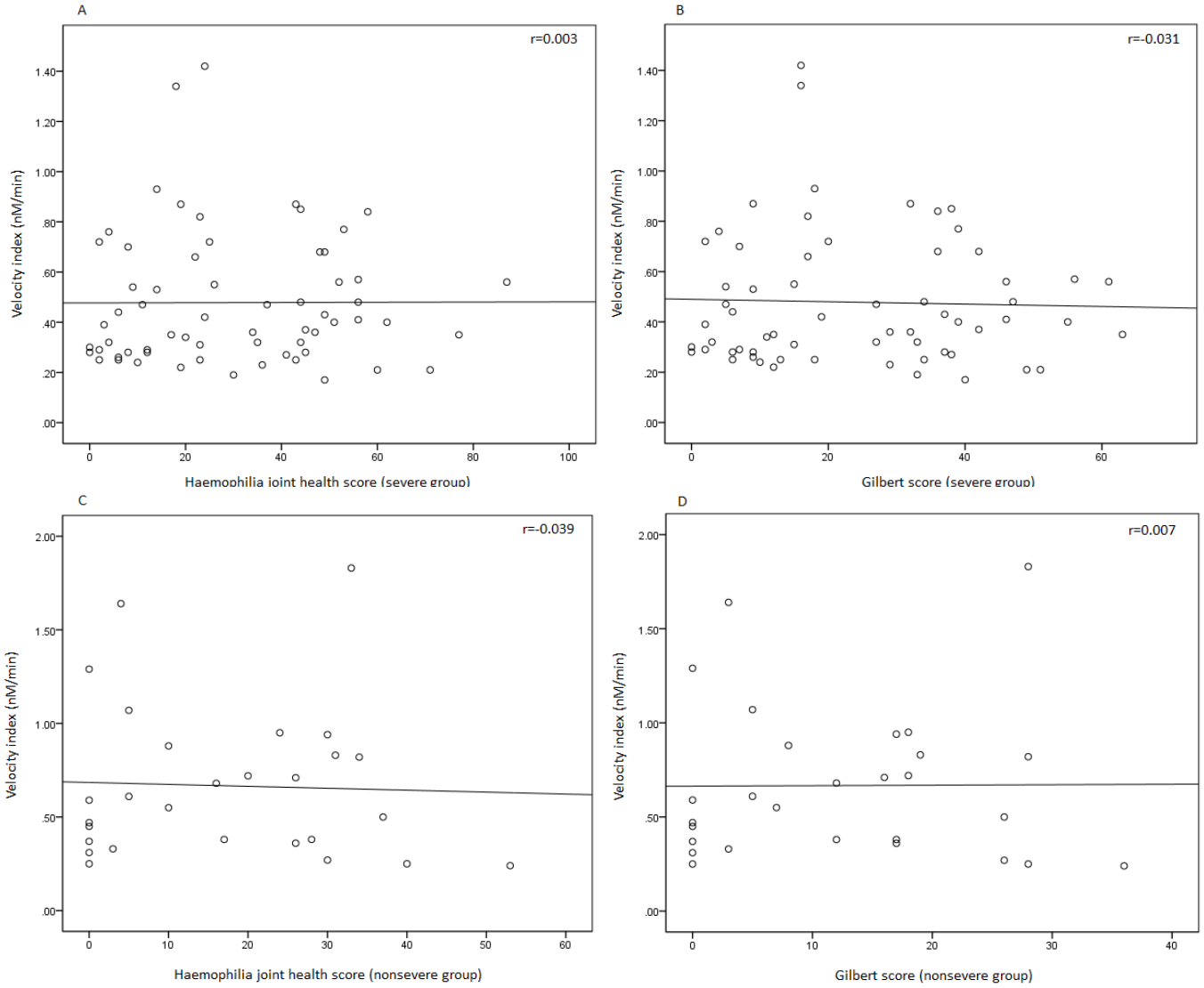

Figure 6.14: Correlation of HJHS and Gilbert score in severe (Panel 13A and Panel 13B) and nonsevere (Panel 13C and Panel 13D) individuals with haemophilia A. No significant correlations were seen.

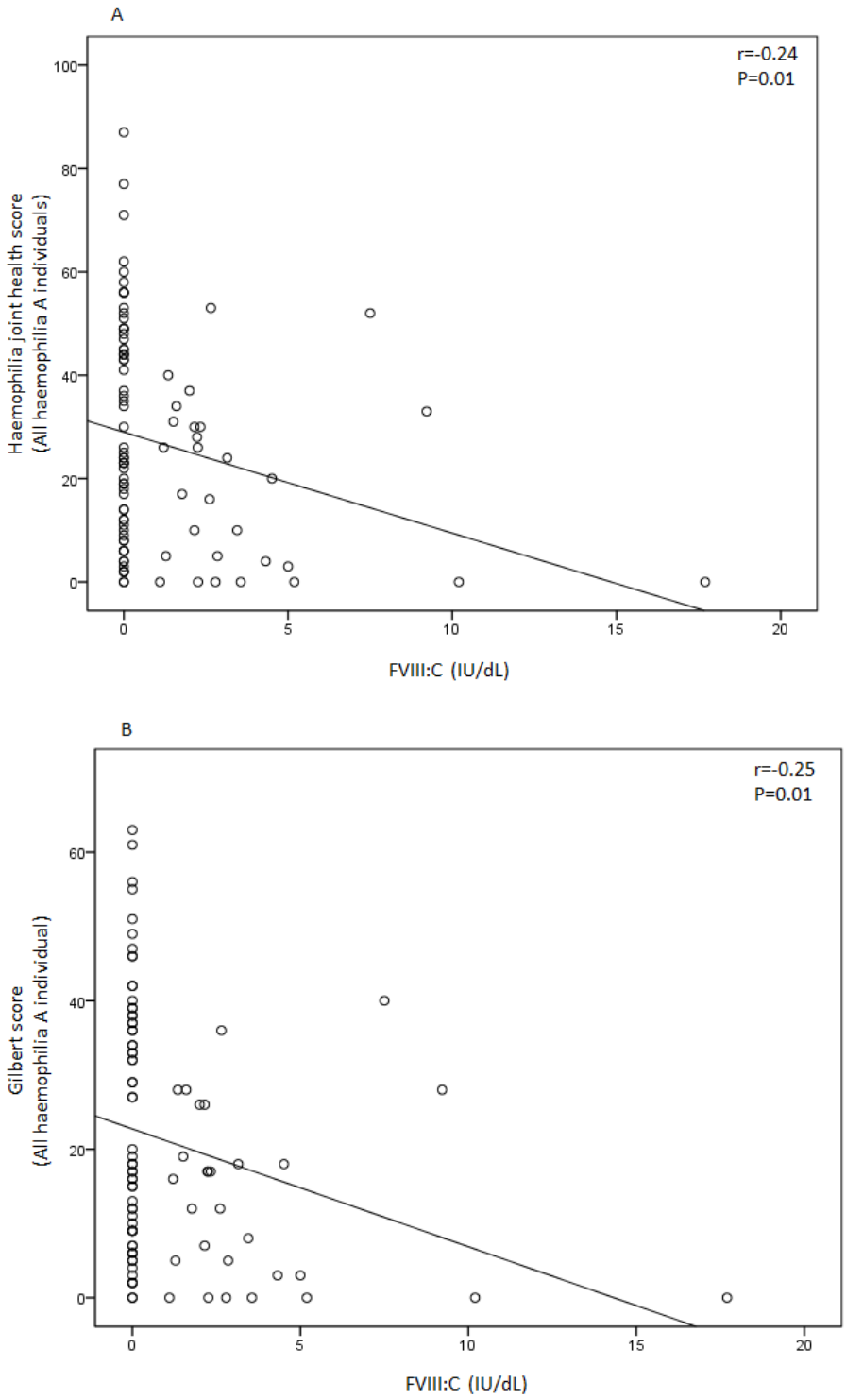

Figure 6.15: Correlation of HJHS and Gilbert score in (Panel A and Panel B) individuals with haemophilia A. A significant negative correlation was observed between FVIII:C and each joint health score.

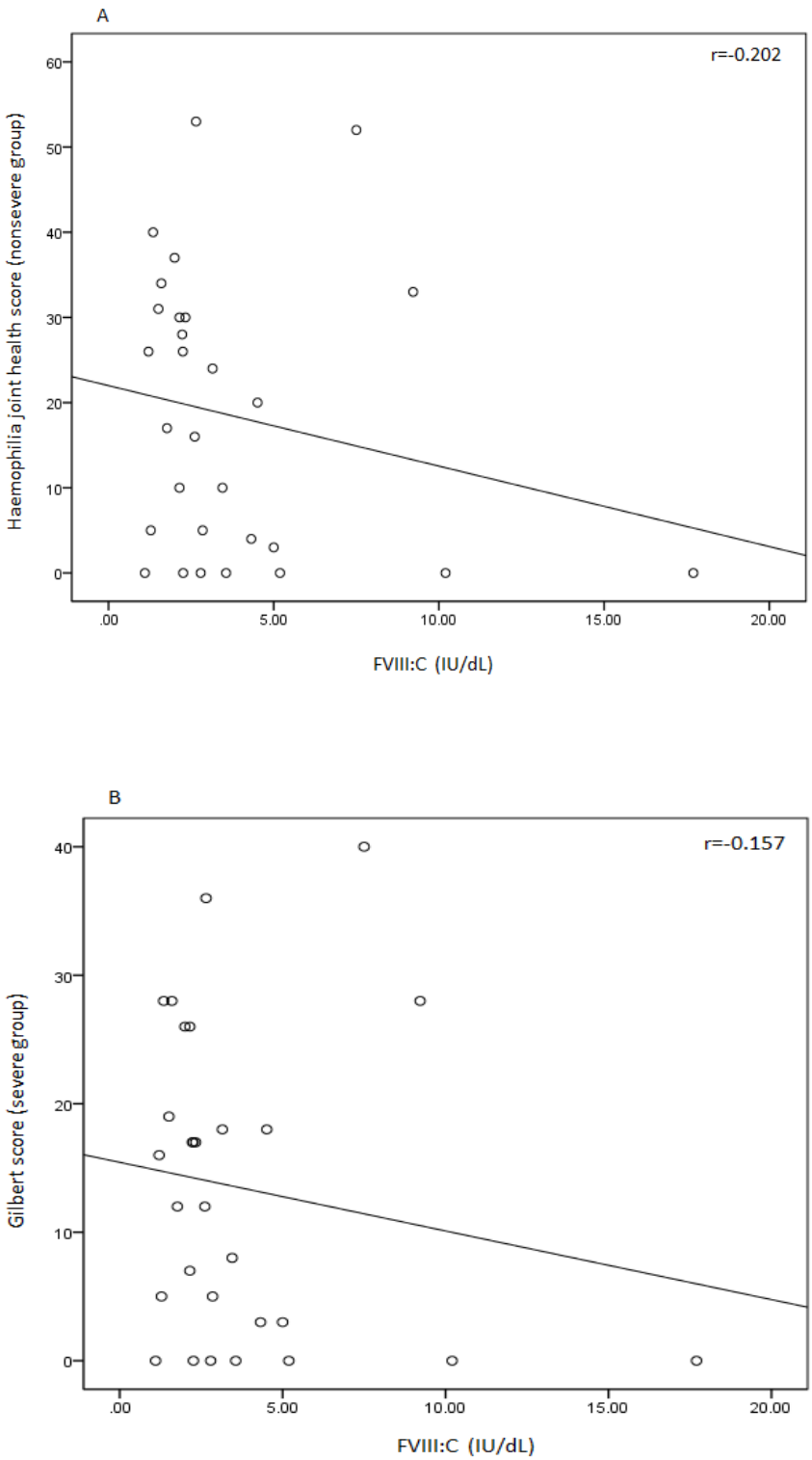

Figure 6.16: Correlation of HJHS and Gilbert score in (Panel A and Panel B) individuals with nonsevere haemophilia A. A negative correlation was observed between FVIII:C and each joint health score.

6.2. Discussion

Coagulation tests are the first line investigation of a suspected inherited bleeding disorder. The most commonly used tests are the PT and aPTT, both of which measure the time to clot formation after non-physiological activation of plasma. There has been much recent interest in assays that measure the integrated activity of all coagulation factors, such as TG tests. These measure the amount of thrombin produced in plasma or in whole blood after recalcification and triggering by the addition of activators such TF and PL. Various methods have been employed to measure the total TG capacity of the blood: sub-sampling method (122), chromogenic technique (123) and CAT (124).

Haemophilia is a disease characterised by a defect in the propagation phase of haemostasis. The initiation and amplification phase of TG depends on the concentration of different components, such as TF, TFPI, FII, FV, FVIII, FXI and phospholipid supplied by activated platelets. In general, the sensitivity of TG assays to the propagation phase through the tenase complex increases as TF concentration decreases. Excess of TF (>5 pmol/L) will cause prothrombinase formation to follow the initiation (extrinsic) pathway (120, 126) bypassing the tenase and propagation phase by overwhelming the action of TFPI. Decreased concentration of TF allows the assay to become sensitive to the propagation phase which includes FVIIIa and FIXa activation of FX because TFPI can regulated the TF/FVIIa complex (154).

Using 1 pmol/L TF trigger, peak was positively correlated with tt and VI. When the comparison was done between lag time and tt peak, a similar finding was observed. The ETP, peak height of TG and VI increased with increasing concentrations of TF trigger for nonsevere and severe haemophilia plasma. This was expected because, irrespective of the severity of FVIII deficiency, an increase in the concentration of TF will cause supra-physiological levels of activated FVII which overcome TFPI and drive activation of the prothrombinase complex without the need for the tenase to function correctly. The latter is effectively bypassed.

As anticipated, in FVIII deficient plasma, parameters of TG were strongly influenced by TF concentration. There was no recordable ETP with 1 and 3 pmol/L in most of the severe individuals (due to negligible FVIII: C) compared to nonsevere. Peak and VI were significantly increased when the concentration of TF increased from 1 pmol/L to 5 pmol/L in both severe and nonsevere. In most of the individuals, ETP was obtained with 5 pmol/L of TF suggesting that this concentration (and higher) is sufficient to trigger coagulation without contribution from the tenase complex (154).

A moderate – but significant – correlation was observed between log FVIII:C and VI, but not between the coagulation factor activity and peak. The latter observation is in contrast to the findings of Dargaud (154), who obtained a significant relationship between plasma FVIII/FIX and peak (and also between both coagulation factor activities and ETP or tt peak). A possible explanation is that the former study included a larger proportion of nonsevere individuals (50 %) compared with the study here-in (31 %), peak being more accurately determined for nonsevere than for severe plasma. Another likely explanation is that in this study different reagents were used and so even though we and Dargaud report using 1 pmol/L this is an arbitrary value based on dilution of different batches of innovin.

Also the phospholipid vesicles are likely to be different. Veer et al. showed the activity of different batches of calibrator and found a significant batch to batch difference in the measurement (155).

In this study, a comparsion of TG parameters was made between severe and nonsevere haemophilia A individuals. VI was significantly higher in nonsevere individuals compared with severe, however there was no difference between peak thrombin levels. This suggests that at low FVIII concentration, using the reagents described here, peak thrombin was not affected by FVIII whereas VI was. This is a suprising result which contrasts with other studies and is mostly due to the specific reagents used. Peak and VI were the more useful parameters for the determination of TG in this cohort because ETP was unrecordable in many cases. VI appeared to be more sensitive to low levels of FVIII than the peak in this assay, possibly because the former is related to the rate of TG. This effect may be specific to the reagents and assay used in this study and may not be replicated if a different trigger mix was used. The finding that peak varied widely at similar FVIII levels may allow it to be used to investigate the effect of other coagulation factors and inhibitors whereas results with VI, to some degree, may be confounded by FVIII.

At higher FVIII levels, ETP was measureable and could be used for the determination of TG. Lewis *et al*. (156) obtained similar findings when TG was measured in PRP and PPP in severe haemophilia A individuals and noticed that only the rate of TG was measurable in severe FVIII deficient plasma. Others found a significant relationship between FVIII:C and peak, ETP and VI (157) in contrast to the finding presented here for peak and ETP, again differences in reagents are the most like explanation. Others have also shown a correlation between PPP, TG and bleeding phenotype (158). The individuals included in that study had varying

baseline FVIII:C and it was concluded that clinical severity could be correlated with FVIII activity as well as TG parameters.

In addition to the general diffferences observed, a variation was noticed in both VI and peak thrombin for individuals with FVIII <1 IU/dL. This may reflect differences in levels of other pro- and anti-coagulant proteins and could, in part, explain the variation in clinically observed bleeding in severe haemophilia A individuals.

In the severe group, there was no significant difference for either VI or peak when missense and null mutations were compared. This suggests that the tenase complex malfunctions similarly in individuals with severe haemophilia A whether FVIII is absent altogether (null defects) or present but dysfunctional (missense changes). There was, however, a wide variation in TG comparing the null and missence groups suggsting that other factors play a similarly varied role in global haemostasis irrespective of the underlying FVIII mutation. Sangostino *et al*. obtained similar findings when comparing TG parameters between severe individuals with a typical bleeding tendency and those with an extremely mild clinical phenotype: using PPP, the TG parameters ETP, lag time, peak, time to peak and start tail were similar in mild and severe bleeders. In contrast, using PRP, higher ETP values were obtained for mild bleeders (other TG parameters were not increased). These researchers found that non-null mutations were a main determinant of bleeding tendency in severe haemophilia, and they proposed that this may be through marginally increased TG (reflected in the ETP) arising from very low activity of the dysfunctional coagulation factor (159).

Several different studies investigated correlation between *in vitro* TG and phenotypic measures of coagulation. One group showed a positive correlation

between ETP and Log FVIII:C in 39 individuals with haemophilia A (160). Veer *et al*. reported a similar finding and showed a correlation between TG parameters (ETP and peak) and FVIII:C (120). Some investigators reported the parameters of TG in PRP and suggested that results might vary from individual to individual due to the difference in platelet characteristics (161). The possibility that genetic factors such as factor V Leiden and the prothrombin gene variant could impact upon TG and hence phenotypic severity has been proposed (162) but not formally substantiated. Differences between the studies reported in the literature and this study may be related to differnces in access to FVIII concentrate.

A negative correlation between TG and joint health scores was observed in nonsevere individuals (there was no correlation in the severe group). This suggests that the baseline FVIII level is more important than global haemostasis in predisposing an individual to arthropathy in situations where access to FVIII concentrates is very limited. Additionally, it is also plausible that, once joint damage is initiated, it becomes an on-going process in which TG has little (if any) role whilst, in contrast, factors relating to inflammation may come to the fore. The potential effect of thrombin generation on joint score may have been masked by the effect of age and it would have been important to perform a multivariate analysis to establish whether these are independent risk factors. There were too few individuals in the study to allow this analysis to be done.

Taken together with the results presented in Chapter 5 , our data indicate that destructive changes occur <12 years, from which it could be inferred that coagulant treatment early in life may be important to limit or prevent arthropathy as

shown previously. The effect of global haemostasis is unclear and large cohorts of individuals need to be studied.

Chapter 7

General Discussion

Pakistan, a developing country with an approximate population of 176 million (World Health Organisation (WHO) 2011 data, URL http://www.who.int/gho/countries/pak.pdf) has around 1,500 individuals diagnosed with haemophilia (A or B) (http://www.org/publications/files/pdf). Most of the individuals with haemophilia A have minimal access to FVIII concentrate and are treated intermittently with FFP. Most of them also have no access to physiotherapy support. In addition to a paucity of clinical support, there is also a major lack of facilities to diagnose and research bleeding disorders in Pakistan. On the basis of these facts, the study presented within this thesis was designed, exploring clinical, genetic and phenotypic aspects of haemophilia A in Pakistan. A main aim of this study was to investigate an association between haemophilic arthropathy in individuals with minimal access to haemostatic replacement therapy, and to investigate thrombin generation in PPP in relation to the underlying *F8* defect.

One hundred apparently unrelated individuals, who had previously been diagnosed with hereditary haemophilia A, and 100 random healthy controls, were recruited from Pakistan. Confirmatory phenotypic testing in Cardiff revealed that 7 individuals had been misdiagnosed and in fact had haemophilia B, whilst one did not have haemophilia at all and was actually haemostatically normal and both joint health scores were 0. Such misdiagnoses represent an important finding within this study, demonstrating the need for improved diagnostic facilities in Pakistan. Possible explanations for misdiagnosis include minimal government spending (9.7

% of total budget: http://finance.punjab.gov.pk/system/files/2013-2014ABS.pdf) in health care sector, with priority given to economic affairs and housing (64.7% of total budget). Furthermore, most of the modern health services in Pakistan are provided in the private sector which is usually not accessible for a common man. Pakistan has multiple problems regarding management of haemophilia and insufficient awareness of the disorder within the population is among them. Direct questioning of each individual with haemophilia suggested that 80 % of them had a positive family history. However due to insufficient knowledge of this disorder, parents circumcised their children in the early weeks of life resulting in serious bleeding in many cases.

The 92 haemophilia A individuals within the affected cohort were categorized according to disease severity: mild n=5 (5 %), moderate n=24 (26 %) and severe n=63 (69 %). Four individuals with severe haemophilia A were found to have an inhibitor. A minor proportion (19 %) of them had minimal access to FVIII concentrate, the exact amount used is not known because accurate records were not available. Individuals with no access to FVIII concentrate (81 %) were treated intermittently with FFP. Furthermore, none of the individuals had access to proper physiotherapy support.

Ninety two individuals with haemophilia A were investigated for the causative gene defect. Out of those, a candidate mutation in *F8* was found in 80. The sequence variants obtained were heterogeneous: point mutations (missense, nonsense, splice site and promoter region changes), inversions (Inv22 and Inv1) and frameshifts (deletions and duplications). The most frequent variation was point mutation (42 % of individuals), followed by Inv22 (20 % of cohort, 29 % of severe cases). The results are consistent with the findings of other international studies, for example point mutations predominated in cohorts from Korea (40.9 %) (163)],

China (46 %) (164)] and India (43%) (130)] and, in these studies Inv22 was frequent among severe cases (Korea 39.5 %, Chinese 38.5 % , Taiwanese 38.7 %) (164-166)]. The frequency of Inv22 in this cohort is lower than for other studies, possibly a sampling artefact.

When the musculoskeletal status in individuals with haemophilia A who had minimal assess to multidisciplinary haemophilia care was investigated, it was found that multiple joint involvement was present in most cases and this involvement increased with age. Arthropathy was common in mild and moderate haemophiliacs as well as severely affected individuals demonstrating that low levels of FVIII do not protect against joint damage in many cases. Due to lack of awareness of this disease, many individuals with mild or moderate haemophilia A present quite late when their joints are already affected. Even after the diagnosis, due to the lack of appropriate management, their joint disease progresses similar to severe disease. Knees are the most commonly affected joint as compared to elbow and ankles. These findings are consistent with other studies which reported the knee to be the most affected joint (167). In this study, HJHS and Gilbert score were used to assess the joint health. This study showed that the two joint scores correlated well across the whole spectrum of severity. Both scores were found to be higher in severe individuals when compared with nonsevere. Similar findings were reported by Dutch study, they observed HJHS for 47 cases and concluded that individuals with mild or moderate haemophilia can be expected to have less joint damage (168). Furthermore, when HJHS and Gilbert score were studied according to developmental age, both scores were found to be lower in younger age group compared to older. In individuals not receiving FVIII concentrate, gross clinical findings of haemophilic arthropathy appeared in severe children during the first decade of life and in nonsevere individuals mainly during the second and

subsequent decades. This further emphasises that, irrespective of the severity of the disease, early and accurate diagnosis and access to factor concentrate is critical for good long term outcomes.

Both health scores were compared between different mutation types – missense, nonsense, frameshift and the intron 22 inversion – in individuals with severe haemophilia A, no significant difference was found between the gene defects. Although, on first principles, this result may have been anticipated, the data formally exclude the possibility that the presence or absence of FVIII protein *per se* in the tenase complex may subtly influence *in vitro* parameters of TG.

Our data suggest that haemophilic joint damage may begin at an early age and advances until the age of 12-16 years, by which time aggregative damage approaches maximum and little further development in arthropathy is possible. Although the *F8* defect is the basis of the disorder, the nature of the mutation does not appear to correlate with the arthropathy, at least in the case of severe FVIII deficiency.

TG was measured in all individuals with haemophilia A: TG parameters were studied according to disese severity and VI, but not peak, found to be higher in nonsevere individuals when compared with severe. In individuals with FVIII < 1 and > 1 IU/dL, variation was observed in TG parameters and this variation could be due to differences in the levels of pro- and anti-coagulant proteins between individuals. A group from Denmark showed similar findings and noticed a wide variation in TG parameters in individuals with severe haemophilia A (169). Similar differences were also obtained in other studies (170) and the authors concluded that

the combination of each individual coagulation factor (besides FVIII) determines each individual's baseline thrombin potential and may affect bleeding risk. It can be concluded that it is not one factor alone that contributes to overall TG dynamics, but it is the combination of different coagulation factors. With the same level of FVIII, individuals with procoagulant factors at the high end of the normal range and anticoagulant factors at the low end, the ability to generate thrombin will be faster than those who have lower than normal procoagulant levels and higher than normal anticoagulant levels. Therefore, an individual could potentially be at a haemostatic benefit over another even though the FVIII levels are equivalent. In addition to this, in individuals with severe haemophilia A, TG was investigated according to *F8* defect and no association was observed with any mutatuion type. This suggested that on average global haemostasis is similar in individuals with severe haemophilia A whether FVIII is absent altogether (null defects) or present but dysfunctional (missense changes), although there is wide inter-individual variation.

This study provides a rare opportunity for musculoskeletal assessment in a haemophilia population with minimal access to haemostatic replacement therapy. The data demonstrate a strong correlation between HJHS and Gilbert Score. Both scores are considered to be applicable to different age groups with different clinical severity: HJHS is considered more helpful for younger individuals aged between 4-18 years with mild joint impairment while the Gilbert score is considered more applicable for adults and older people with established arthropathy.

Further studies into the factors affecting haemophilic arthropathy in this population are required. Most importantly, it has not been possible to investigate the effect of global haemostasis fully because of the important and large effect of age.

Increasing the numbers of individuals and performing a multivariate analysis would be useful. In addition, it would be interesting to investigate the immune system in this group to establish whether some individuals are more prone to the inflammatory responses that result in severe arthropathy.

This study also provides 30 novel candidate mutations that have not been previously reported. The novel candidate mutations comprise 8 missense, 7 nonsense, 3 splice site, 1 promoter region, 3 single nucleotide duplications and 8 with deletions.

The novel variants those with a significant likelihood of pathology (nonsense, duplication, deletion and the splice site change c.6187+1G>A which destroys the conserved GT splice site donor motif) (total = 19) and those for which a detrimental effect was uncertain (missense, promoter region and the other two splice site changes) (total = 11). *In silico* analysis of the latter 11 variants gave results that strongly supported involvement in pathology.

In Pakistan, the genetic analysis of haemophilia is not available. Currently, there is a major insufficiency for genetic investigation of this disorder. Although carrier status and prenatal diagnosis using molecular analysis are available for thalassaemia and other congenital disorders, such services have not yet been implemented for haemophilia. Haemophilia care is important for the families with a severe phenotype, and for them genetic counselling as well as carrier detection is necessary.

In conclusion, the data generated in this study provide insight that addresses the hypothesis stated at the outset: “The severity of haemophilic arthropathy, in individuals with the same baseline FVIII level who are receiving minimal FVIII replacement, is affected by their global haemostatic potential despite the mutation underlying haemophilia A” (Chapter 1, section 1.7). The data suggest that haemophilia arthropathy correlate with disease severity and individual age; among the severe group there is no association between joint health score and either the underlying mutation or *in vitro* TG. Ultimately the study has too few individuals to address whether global haemostasis affects arthropathy because of the very marked effect of age. Further cohorts of minimally treated individuals of similar ages would be required to answer this question fully. This study contributes novel genetic and phenotypic data that broaden knowledge and understanding in the complex field of haemophilia A.

References

1. Stassen JM, Arnout J, Deckmyn H. The hemostatic system. Current medicinal chemistry. 2004;11(17):2245-60.
2. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? Blood. 2003;102(2):449-61.
3. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. Journal of thrombosis and haemostasis : JTH. 2003;1(7):1335-42.
4. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. Journal of thrombosis and haemostasis : JTH. 2005;3(8):1752-62.
5. Boylan B, Gao C, Rathore V, Gill JC, Newman DK, Newman PJ. Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets. Blood. 2008;112(7):2780-6.
6. Dawood BB, Wilde J, Watson SP. Reference curves for aggregation and ATP secretion to aid diagnose of platelet-based bleeding disorders: effect of inhibition of ADP and thromboxane A(2) pathways. Platelets. 2007;18(5):329-45.
7. Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. Arteriosclerosis, thrombosis, and vascular biology. 2008;28(3):403-12.
8. Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. Blood. 2006;108(6):1903-10.
9. Ono A, Westein E, Hsiao S, Nesbitt WS, Hamilton JR, Schoenwaelder SM, et al. Identification of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth. Blood. 2008;112(1):90-9.
10. Jackson SP. The growing complexity of platelet aggregation. Blood. 2007;109(12):5087-95.
11. Gao C, Boylan B, Bougie D, Gill JC, Birenbaum J, Newman DK, et al. Eptifibatide-induced thrombocytopenia and thrombosis in humans require FcgammaRIIa and the integrin beta3 cytoplasmic domain. The Journal of clinical investigation. 2009;119(3):504-11.
12. Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. Journal of thrombosis and haemostasis : JTH. 2006;4(2):312-9.
13. Michelson AD, Cattaneo M, Eikelboom JW, Gurbel P, Kottke-Marchant K, Kunicki TJ, et al. Aspirin resistance: position paper of the Working Group on Aspirin Resistance. Journal of thrombosis and haemostasis : JTH. 2005;3(6):1309-11.
14. Morrissey JH. Tissue factor: an enzyme cofactor and a true receptor. Thrombosis and haemostasis. 2001;86(1):66-74.
15. Aird WC. Endothelial cell heterogeneity. Cold Spring Harbor perspectives in medicine. 2012;2(1):a006429.

16. Caen J, Wu Q. Hageman factor, platelets and polyphosphates: early history and recent connection. Journal of thrombosis and haemostasis : JTH. 2010;8(8):1670-4.
17. Hoffman M. A cell-based model of coagulation and the role of factor VIIa. Blood reviews. 2003;17 Suppl 1:S1-5.
18. McMichael M. New models of hemostasis. Topics in companion animal medicine. 2012;27(2):40-5.
19. Smith SA. The cell-based model of coagulation. Journal of veterinary emergency and critical care (San Antonio, Tex : 2001). 2009;19(1):3-10.
20. Bajaj MS, Birktoft JJ, Steer SA, Bajaj SP. Structure and biology of tissue factor pathway inhibitor. Thrombosis and haemostasis. 2001;86(4):959-72.
21. Bouma BN, Meijers JC. Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). Journal of thrombosis and haemostasis : JTH. 2003;1(7):1566-74.
22. Dahlback B, Villoutreix BO. Molecular recognition in the protein C anticoagulant pathway. Journal of thrombosis and haemostasis : JTH. 2003;1(7):1525-34.
23. Hay CR. National Hemophilia Databases. Textbook of Hemophilia, Second Edition. 2010:415-9.
24. Poustka A, Dietrich A, Langenstein G, Toniolo D, Warren ST, Lehrach H. Physical map of human Xq27-qter: localizing the region of the fragile X mutation. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(19):8302-6.
25. Freije D, Schlessinger D. A 1.6-Mb contig of yeast artificial chromosomes around the human factor VIII gene reveals three regions homologous to probes for the DXS115 locus and two for the DXYS64 locus. American journal of human genetics. 1992;51(1):66-80.
26. Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, et al. Structure of human factor VIII. Nature. 1984;312(5992):337-42.
27. Eaton D, Rodriguez H, Vehar GA. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. Biochemistry. 1986;25(2):505-12.
28. Knobe KE, Villoutreix BO, Tengborn LI, Petrini P, Ljung RC. Factor VIII inhibitors in two families with mild haemophilia A: structural analysis of the mutations. Haemostasis. 2000;30(5):268-79.
29. Stoilova-McPhie S, Villoutreix BO, Mertens K, Kemball-Cook G, Holzenburg A. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. Blood. 2002;99(4):1215-23.
30. Saenko EL, Shima M, Gilbert GE, Scandella D. Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition. The Journal of biological chemistry. 1996;271(44):27424-31.
31. Neuenschwander PF, Jesty J. Thrombin-activated and factor Xa-activated human factor VIII: differences in cofactor activity and decay rate. Archives of biochemistry and biophysics. 1992;296(2):426-34.
32. Fay PJ. Regulation of factor VIIIa in the intrinsic factor Xase. Thrombosis and haemostasis. 1999;82(2):193-200.

33. Lapan KA, Fay PJ. Interaction of the A1 subunit of factor VIIIa and the serine protease domain of factor X identified by zero-length cross-linking. Thrombosis and haemostasis. 1998;80(3):418-22.
34. Ananyeva NM, Kouiavskaia DV, Shima M, Saenko EL. Catabolism of the coagulation factor VIII: can we prolong lifetime of f VIII in circulation? Trends in cardiovascular medicine. 2001;11(6):251-7.
35. Sarafanov AG, Ananyeva NM, Shima M, Saenko EL. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. The Journal of biological chemistry. 2001;276(15):11970-9.
36. Lakich D, Kazazian HH, Jr., Antonarakis SE, Gitschier J. Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. Nature genetics. 1993;5(3):236-41.
37. Antonarakis SE, Rossiter JP, Young M, Horst J, de Moerloose P, Sommer SS, et al. Factor VIII gene inversions in severe hemophilia A: results of an international consortium study. Blood. 1995;86(6):2206-12.
38. Levinson B, Kenwrick S, Lakich D, Hammonds G, Jr., Gitschier J. A transcribed gene in an intron of the human factor VIII gene. Genomics. 1990;7(1):1-11.
39. Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood. 2002;99(1):168-74.
40. Schwaab R, Oldenburg J, Tuddenham EG, Brackmann HH, Olek K. Mutations in haemophilia A. British journal of haematology. 1993;83(3):450-8.
41. Goodeve AC, Peake IR. The molecular basis of hemophilia A: genotype-phenotype relationships and inhibitor development. Seminars in thrombosis and hemostasis. 2003;29(1):23-30.
42. Morichika S, Shima M, Kamisue S, Tanaka I, Imanaka Y, Suzuki H, et al. Factor VIII gene analysis in Japanese CRM-positive and CRM-reduced haemophilia A patients by single-strand conformation polymorphism. British journal of haematology. 1997;98(4):901-6.
43. Liu ML, Nakaya S, Thompson AR. Non-inversion factor VIII mutations in 80 hemophilia A families including 24 with alloimmune responses. Thrombosis and haemostasis. 2002;87(2):273-6.
44. Pipe SW, Saenko EL, Eickhorst AN, Kemball-Cook G, Kaufman RJ. Hemophilia A mutations associated with 1-stage/2-stage activity discrepancy disrupt protein-protein interactions within the triplicated A domains of thrombin-activated factor VIIIa. Blood. 2001;97(3):685-91.
45. Youssoufian H, Antonarakis SE, Aronis S, Tsiftis G, Phillips DG, Kazazian HH, Jr. Characterization of five partial deletions of the factor VIII gene. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(11):3772-6.
46. Wehnert M, Herrmann FH, Wulff K. Partial deletions of factor VIII gene as molecular diagnostic markers in haemophilia A. Disease markers. 1989;7(2):113-7.
47. Lavergne JM, Bahnak BR, Vidaud M, Laurian Y, Meyer D. A directed search for mutations in hemophilia A using restriction enzyme analysis and denaturing gradient gel electrophoresis. A study of seven exons in the factor VIII gene of 170 cases. Nouvelle revue francaise d'hematologie. 1992;34(1):85-91.
48. Kazazian HH, Jr., Wong C, Youssoufian H, Scott AF, Phillips DG, Antonarakis SE. Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. Nature. 1988;332(6160):164-6.

49. Sukarova E, Dimovski AJ, Tchacarova P, Petkov GH, Efremov GD. An Alu insert as the cause of a severe form of hemophilia A. Acta haematologica. 2001;106(3):126-9.
50. Pergantou H, Matsinos G, Papadopoulos A, Platokouki H, Aronis S. Comparative study of validity of clinical, X-ray and magnetic resonance imaging scores in evaluation and management of haemophilic arthropathy in children. Haemophilia : the official journal of the World Federation of Hemophilia. 2006;12(3):241-7.
51. Hoyer LW. Hemophilia A. New England Journal of Medicine. 1994;330(1):38-47.
52. Ljung RC. Intracranial haemorrhage in haemophilia A and B. British journal of haematology. 2008;140(4):378-84.
53. Collins PW, Chalmers E, Hart DP, Liesner R, Rangarajan S, Talks K, et al. Diagnosis and treatment of factor VIII and IX inhibitors in congenital haemophilia: (4th edition). UK Haemophilia Centre Doctors Organization. British journal of haematology. 2013;160(2):153-70.
54. Chalmers E, Williams M, Brennand J, Liesner R, Collins P, Richards M. Guideline on the management of haemophilia in the fetus and neonate. British journal of haematology. 2011;154(2):208-15.
55. Jansen NW, Roosendaal G, Wenting MJ, Bijlsma JW, Theobald M, Hazewinkel HA, et al. Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2009;17(4):433-40.
56. Saxne T, Heinegard D. Cartilage oligomeric matrix protein: a novel marker of cartilage turnover detectable in synovial fluid and blood. British journal of rheumatology. 1992;31(9):583-91.
57. Garnero P, Christgau S, Delmas PD. The bisphosphonate zoledronate decreases type II collagen breakdown in patients with Paget's disease of bone. Bone. 2001;28(5):461-4.
58. Sowers MF, Karvonen-Gutierrez CA, Yosef M, Jannausch M, Jiang Y, Garnero P, et al. Longitudinal changes of serum COMP and urinary CTX-II predict X-ray defined knee osteoarthritis severity and stiffness in women. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2009;17(12):1609-14.
59. Cohen NP, Foster RJ, Mow VC. Composition and dynamics of articular cartilage: structure, function, and maintaining healthy state. The Journal of orthopaedic and sports physical therapy. 1998;28(4):203-15.
60. Parsons JR, Zingler BM, McKeon JJ. Mechanical and histological studies of acute joint hemorrhage. Orthopedics. 1987;10(7):1019-26.
61. Alsaleh G, Sparsa L, Chatelus E, Ehlinger M, Gottenberg JE, Wachsmann D, et al. Innate immunity triggers IL-32 expression by fibroblast-like synoviocytes in rheumatoid arthritis. Arthritis research & therapy. 2010;12(4):R135.
62. Maciejewska Rodrigues H, Jungel A, Gay RE, Gay S. Innate immunity, epigenetics and autoimmunity in rheumatoid arthritis. Molecular immunology. 2009;47(1):12-8.
63. Sharif SA, Du X, Myles T, Song JJ, Price E, Lee DM, et al. Thrombin-activatable carboxypeptidase B cleavage of osteopontin regulates neutrophil survival and synoviocyte binding in rheumatoid arthritis. Arthritis and rheumatism. 2009;60(10):2902-12.
64. Quayle JA, Adams S, Bucknall RC, Edwards SW. Cytokine expression by inflammatory neutrophils. FEMS immunology and medical microbiology. 1994;8(3):233-9.

65. Cascao R, Moura RA, Perpetuo I, Canhao H, Vieira-Sousa E, Mourao AF, et al. Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis. Arthritis research & therapy. 2010;12(5):R196.
66. Lubberts E, Koenders MI, van den Berg WB. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. Arthritis research & therapy. 2005;7(1):29-37.
67. Barsante MM, Cunha TM, Allegretti M, Cattani F, Policani F, Bizzarri C, et al. Blockade of the chemokine receptor CXCR2 ameliorates adjuvant-induced arthritis in rats. British journal of pharmacology. 2008;153(5):992-1002.
68. Pinto LG, Cunha TM, Vieira SM, Lemos HP, Verri WA, Jr., Cunha FQ, et al. IL-17 mediates articular hypernociception in antigen-induced arthritis in mice. Pain. 2010;148(2):247-56.
69. Akhavani MA, Madden L, Buysschaert I, Sivakumar B, Kang N, Paleolog EM. Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis. Arthritis research & therapy. 2009;11(3):R64.
70. Bosco MC, Delfino S, Ferlito F, Puppo M, Gregorio A, Gambini C, et al. The hypoxic synovial environment regulates expression of vascular endothelial growth factor and osteopontin in juvenile idiopathic arthritis. The Journal of rheumatology. 2009;36(6):1318-29.
71. Clavel G, Bessis N, Lemeiter D, Fardellone P, Mejjad O, Menard JF, et al. Angiogenesis markers (VEGF, soluble receptor of VEGF and angiopoietin-1) in very early arthritis and their association with inflammation and joint destruction. Clinical immunology (Orlando, Fla). 2007;124(2):158-64.
72. Dhaouadi T, Sfar I, Abelmoula L, Jendoubi-Ayed S, Aouadi H, Ben Abdellah T, et al. Role of immune system, apoptosis and angiogenesis in pathogenesis of rheumatoid arthritis and joint destruction, a systematic review. La Tunisie medicale. 2007;85(12):991-8.
73. Giatromanolaki A, Sivridis E, Maltezos E, Athanassou N, Papazoglou D, Gatter KC, et al. Upregulated hypoxia inducible factor-1alpha and -2alpha pathway in rheumatoid arthritis and osteoarthritis. Arthritis research & therapy. 2003;5(4):R193-201.
74. Murata M, Yudoh K, Masuko K. The potential role of vascular endothelial growth factor (VEGF) in cartilage: how the angiogenic factor could be involved in the pathogenesis of osteoarthritis? Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2008;16(3):279-86.
75. Paleolog EM, Miotla JM. Angiogenesis in arthritis: role in disease pathogenesis and as a potential therapeutic target. Angiogenesis. 1998;2(4):295-307.
76. Whitnall M, Howard J, Ponka P, Richardson DR. A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(40):14901-6.
77. Richardson DR, Kalinowski DS, Lau S, Jansson PJ, Lovejoy DB. Cancer cell iron metabolism and the development of potent iron chelators as anti-tumour agents. Biochimica et biophysica acta. 2009;1790(7):702-17.
78. Xu X, Sutak R, Richardson DR. Iron chelation by clinically relevant anthracyclines: alteration in expression of iron-regulated genes and atypical changes in intracellular iron distribution and trafficking. Molecular pharmacology. 2008;73(3):833-44.
79. Wang L, Johnson EE, Shi HN, Walker WA, Wessling-Resnick M, Cherayil BJ. Attenuated inflammatory responses in hemochromatosis reveal a role for iron in

the regulation of macrophage cytokine translation. Journal of immunology (Baltimore, Md : 1950). 2008;181(4):2723-31.
80. Walter PB, Macklin EA, Porter J, Evans P, Kwiatkowski JL, Neufeld EJ, et al. Inflammation and oxidant-stress in beta-thalassemia patients treated with iron chelators deferasirox (ICL670) or deferoxamine: an ancillary study of the Novartis CICL670A0107 trial. Haematologica. 2008;93(6):817-25.
81. Tacchini L, Gammella E, De Ponti C, Recalcati S, Cairo G. Role of HIF-1 and NF-kappaB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals. The Journal of biological chemistry. 2008;283(30):20674-86.
82. Tripathi L, Kumar P, Singhai AK. Role of chelates in treatment of cancer. Indian journal of cancer. 2007;44(2):62-71.
83. De Ponti C, Carini R, Alchera E, Nitti MP, Locati M, Albano E, et al. Adenosine A2a receptor-mediated, normoxic induction of HIF-1 through PKC and PI-3K-dependent pathways in macrophages. Journal of leukocyte biology. 2007;82(2):392-402.
84. Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. The Journal of clinical investigation. 2001;107(9):1055-61.
85. Grcevic D, Jajic Z, Kovacic N, Lukic IK, Velagic V, Grubisic F, et al. Peripheral blood expression profiles of bone morphogenetic proteins, tumor necrosis factor-superfamily molecules, and transcription factor Runx2 could be used as markers of the form of arthritis, disease activity, and therapeutic responsiveness. The Journal of rheumatology. 2010;37(2):246-56.
86. Walsh NC, Reinwald S, Manning CA, Condon KW, Iwata K, Burr DB, et al. Osteoblast function is compromised at sites of focal bone erosion in inflammatory arthritis. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2009;24(9):1572-85.
87. Valentino LA, Hakobyan N, Enockson C, Simpson ML, Kakodkar NC, Cong L, et al. Exploring the biological basis of haemophilic joint disease: experimental studies. Haemophilia : the official journal of the World Federation of Hemophilia. 2012;18(3):310-8.
88. Valentino LA. Blood-induced joint disease: the pathophysiology of hemophilic arthropathy. Journal of thrombosis and haemostasis : JTH. 2010;8(9):1895-902.
89. Gilbert MS. Characterizing the hemophilic pseudotumor. Annals of the New York Academy of Sciences. 1975;240(1):311-5.
90. Sancho FG. Experimental model of haemophilic arthropathy with high pressure haemarthrosis. International orthopaedics. 1980;4(1):57-62.
91. Steven MM, Yogarajah S, Madhok R, Forbes C, Sturrock R. Haemophilic arthritis. QJM. 1986;58(2):181-97.
92. Lereim P, Goldie I, Dahlberg E. Hardness of the subchondral bone of the tibial condyles in the normal state and in osteoarthritis and rheumatoid arthritis. Acta orthopaedica Scandinavica. 1974;45(4):614-27.
93. Kreuz W, Escuriola-Ettingshausen C, Funk M, Schmidt H, Kornhuber B. When should prophylactic treatment in patients with haemophilia A and B start?--The German experience. Haemophilia : the official journal of the World Federation of Hemophilia. 1998;4(4):413-7.

94. Doria AS. State-of-the-art imaging techniques for the evaluation of haemophilic arthropathy: present and future. Haemophilia : the official journal of the World Federation of Hemophilia. 2010;16 Suppl 5:107-14.
95. Melchiorre D, Linari S, Innocenti M, Biscoglio I, Toigo M, Cerinic MM, et al. Ultrasound detects joint damage and bleeding in haemophilic arthropathy: a proposal of a score. Haemophilia : the official journal of the World Federation of Hemophilia. 2011;17(1):112-7.
96. Hilliard P, Funk S, Zourikian N, Bergstrom BM, Bradley CS, McLimont M, et al. Hemophilia joint health score reliability study. Haemophilia : the official journal of the World Federation of Hemophilia. 2006;12(5):518-25.
97. Aledort LM, Haschmeyer RH, Pettersson H. A longitudinal study of orthopaedic outcomes for severe factor-VIII-deficient haemophiliacs. The Orthopaedic Outcome Study Group. Journal of internal medicine. 1994;236(4):391-9.
98. Feldman BM, Funk S, Lundin B, Doria AS, Ljung R, Blanchette V. Musculoskeletal measurement tools from the International Prophylaxis Study Group (IPSG). Haemophilia : the official journal of the World Federation of Hemophilia. 2008;14 Suppl 3:162-9.
99. Gilbert MS. Prophylaxis: musculoskeletal evaluation. Seminars in hematology. 1993;30(3 Suppl 2):3-6.
100. Heijnen L, de Kleijn P. Physiotherapy for the treatment of articular contractures in haemophilia. Haemophilia : the official journal of the World Federation of Hemophilia. 1999;5 Suppl 1:16-9.
101. van der Net J, Vos RC, Engelbert RH, van den Berg MH, Helders PJ, Takken T. Physical fitness, functional ability and quality of life in children with severe haemophilia: a pilot study. Haemophilia : the official journal of the World Federation of Hemophilia. 2006;12(5):494-9.
102. Bosch D. Haemophilia therapy: assessing the cumulative risk of HIV exposure by cryoprecipitate. Haemophilia : the official journal of the World Federation of Hemophilia. 1999;5(5):295-300.
103. Brooker M. Registry of clotting factor concentrates. World Federation of Hemophilia. 2008(6).
104. Stanworth SJ, Grant‐Casey J, Lowe D, Laffan M, New H, Murphy MF, et al. The use of fresh‐frozen plasma in England: high levels of inappropriate use in adults and children. Transfusion. 2011;51(1):62-70.
105. Berntorp E, Astermark J, Bjorkman S, Blanchette VS, Fischer K, Giangrande PL, et al. Consensus perspectives on prophylactic therapy for haemophilia: summary statement. Haemophilia : the official journal of the World Federation of Hemophilia. 2003;9 Suppl 1:1-4.
106. Coppola A, Franchini M, Tagliaferri A. Prophylaxis in people with haemophilia. Thrombosis and haemostasis. 2009;101(4):674-81.
107. Coppola A, Tagliaferri A, Di Capua M, Franchini M. Prophylaxis in children with hemophilia: evidence-based achievements, old and new challenges. Seminars in thrombosis and hemostasis. 2012;38(1):79-94.
108. Richards M, Williams M, Chalmers E, Liesner R, Collins P, Vidler V, et al. A United Kingdom Haemophilia Centre Doctors' Organization guideline approved by the British Committee for Standards in Haematology: guideline on the use of prophylactic factor VIII concentrate in children and adults with severe haemophilia A. British journal of haematology. 2010;149(4):498-507.

109. Astermark J. Overview of inhibitors. Seminars in hematology. 2006;43(2 Suppl 4):S3-7.
110. Franchini M, Frattini F, Crestani S, Bonfanti C. Alloantibodies in previously untreated hemophilia A patients: the role of environmental factors. Hematology (Amsterdam, Netherlands). 2013;18(4):183-90.
111. Biggs R, Macfarlane G. Human blood coagulation and its disorders: Blackwell Scientific Publications; 1962.
112. Biggs R, Douglas A. The thromboplastin generation test. Journal of clinical Pathology. 1953;6(1):23.
113. Pitney W, Dacie J. A simple method of studying the generation of thrombin in recalcified plasma: application in the investigation of haemophilia. Journal of clinical pathology. 1953;6(1):9.
114. Seegers WH, editor A personal perspective on hemostasis and thrombosis (1937-1981). Seminars in thrombosis and hemostasis; 1981.
115. Dieri RA, Peyvandi F, Santagostino E, Giansily M, Mannucci PM, Schved JF, et al. The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding. THROMBOSIS AND HAEMOSTASIS-STUTTGART-. 2002;88(4):576-82.
116. Keularts I, Hamulyak K, Hemker H, Béguin S. The Effect of DDAVP Infusion on Thrombin Generation in Platelet-rich Plasma of von Willebrand Type I and in Mild Hemophilia A Patients. THROMBOSIS AND HAEMOSTASIS-STUTTGART-. 2000;84(4):638-42.
117. Al Dieri R, Wagenvoord R, Van Dedem G, Béguin S, Hemker H. The inhibition of blood coagulation by heparins of different molecular weight is caused by a common functional motif−the C‐domain. Journal of Thrombosis and Haemostasis. 2003;1(5):907-14.
118. Beguin S, Kumar R, Keularts I, Seligsohn U, Coller B, Hemker H. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. Blood. 1999;93(2):564-70.
119. Gerotziafas GT, Depasse F, Busson J, Leflem L, Elalamy I, Samama MM. Towards a standardization of thrombin generation assessment: the influence of tissue factor, platelets and phospholipids concentration on the normal values of Thrombogram-Thrombinoscope assay. Thrombosis journal. 2005;3:16.
120. van Veen JJ, Gatt A, Bowyer AE, Cooper PC, Kitchen S, Makris M. Calibrated automated thrombin generation and modified thromboelastometry in haemophilia A. Thrombosis research. 2009;123(6):895-901.
121. Dargaud Y, Lienhart A, Negrier C. Prospective assessment of thrombin generation test for dose monitoring of bypassing therapy in hemophilia patients with inhibitors undergoing elective surgery. Blood. 2010;116(25):5734-7.
122. de Bosch NB, Mosesson MW, Ruiz-Saez A, Echenagucia M, Rodriguez-Lemoin A. Inhibition of thrombin generation in plasma by fibrin formation (Antithrombin I). Thrombosis and haemostasis. 2002;88(2):253-8.
123. Hemker HC, Wielders S, Kessels H, Beguin S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. Thrombosis and haemostasis. 1993;70(4):617-24.
124. Hemker HC, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. Pathophysiology of haemostasis and thrombosis. 2002;32(5-6):249-53.
125. van Veen JJ, Gatt A, Cooper PC, Kitchen S, Bowyer AE, Makris M. Corn trypsin inhibitor in fluorogenic thrombin-generation measurements is only

necessary at low tissue factor concentrations and influences the relationship between factor VIII coagulant activity and thrombogram parameters. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 2008;19(3):183-9.
126. van Veen JJ, Gatt A, Bowyer AE, Cooper PC, Kitchen S, Makris M. The effect of tissue factor concentration on calibrated automated thrombography in the presence of inhibitor bypass agents. International journal of laboratory hematology. 2009;31(2):189-98.
127. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiology of haemostasis and thrombosis. 2003;33(1):4-15.
128. Li H, Sun J, Zhou X, Liu Y, Song X, Ma Q. [Clinical predictors for the phenotypic heterogeneity of severe hemophilia A in China]. Nan fang yi ke da xue xue bao = Journal of Southern Medical University. 2013;33(3):424-7.
129. Pollmann H, Richter H, Ringkamp H, Jurgens H. When are children diagnosed as having severe haemophilia and when do they start to bleed? A 10-year single-centre PUP study. European journal of pediatrics. 1999;158 Suppl 3:S166-70.
130. Jayandharan G, Shaji RV, Baidya S, Nair SC, Chandy M, Srivastava A. Identification of factor VIII gene mutations in 101 patients with haemophilia A: mutation analysis by inversion screening and multiplex PCR and CSGE and molecular modelling of 10 novel missense substitutions. Haemophilia : the official journal of the World Federation of Hemophilia. 2005;11(5):481-91.
131. Venceslá A, Corral-Rodríguez MÁ, Baena M, Cornet M, Domènech M, Baiget M, et al. Identification of 31 novel mutations in the F8 gene in Spanish hemophilia A patients: structural analysis of 20 missense mutations suggests new intermolecular binding sites. Blood. 2008;111(7):3468-78.
132. Vinciguerra C, Zawadzki C, Dargaud Y, Pernod G, Berger C, Nougier C, et al. Characterisation of 96 mutations in 128 unrelated severe haemophilia A patients from France. Description of 62 novel mutations. THROMBOSIS AND HAEMOSTASIS-STUTTGART-. 2006;95(4):593.
133. Casaña P, Cabrera N, Cid AR, Haya S, Beneyto M, Espinós C, et al. Severe and moderate hemophilia A: identification of 38 new genetic alterations. Haematologica. 2008;93(7):1091-4.
134. Citron M, Godmilow L, Ganguly T, Ganguly A. High throughput mutation screening of the factor VIII gene (F8C) in hemophilia A: 37 novel mutations and genotype–phenotype correlation. Human mutation. 2002;20(4):267-74.
135. Acquila M, Pasino M, Lanza T, Bottini F, Boeri E, Bicocchi MP. Frequency of factor VIII intron 1 inversion in a cohort of severe hemophilia A Italian patients. Haematologica. 2003;88(5):ELT17-ELT.
136. Mantilla‐Capacho JM, Beltrán‐Miranda CP, Luna‐Záizar H, Aguilar‐López L, Esparza‐Flores MA, López‐Guido B, et al. Frequency of intron 1 and 22 inversions of factor VIII gene in Mexican patients with severe hemophilia A. American journal of hematology. 2007;82(4):283-7.
137. Andrikovics H, Klein I, Bors A, Nemes L, Marosi A, Váradi A, et al. Analysis of large structural changes of the factor VIII gene, involving intron 1 and 22, in severe hemophilia A. Haematologica. 2003;88(7):778-84.
138. Pattinson JK, Millar DS, McVey JH, Grundy CB, Wieland K, Mibashan RS, et al. The molecular genetic analysis of hemophilia A: a directed search strategy for

the detection of point mutations in the human factor VIII gene. Blood. 1990;76(11):2242-8.
139. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nature methods. 2010;7(4):248-9.
140. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nature protocols. 2009;4(7):1073-81.
141. Panicker J, Warrier I, Thomas R, Lusher J. The overall effectiveness of prophylaxis in severe haemophilia. Haemophilia : the official journal of the World Federation of Hemophilia. 2003;9(3):272-8.
142. Rodriguez‐Merchan E. Orthopaedic assessment in haemophilia. Haemophilia : the official journal of the World Federation of Hemophilia. 2003;9(s1):65-74.
143. Feldman BM, Funk SM, Bergstrom BM, Zourikian N, Hilliard P, van der Net J, et al. Validation of a new pediatric joint scoring system from the International Hemophilia Prophylaxis Study Group: validity of the hemophilia joint health score. Arthritis care & research. 2011;63(2):223-30.
144. Mancuso ME, Graca L, Auerswald G, Santagostino E. Haemophilia care in children--benefits of early prophylaxis for inhibitor prevention. Haemophilia : the official journal of the World Federation of Hemophilia. 2009;15 Suppl 1:8-14.
145. Roosendaal G, Jansen NW, Schutgens R, Lafeber FP. Haemophilic arthropathy: the importance of the earliest haemarthroses and consequences for treatment. Haemophilia : the official journal of the World Federation of Hemophilia. 2008;14 Suppl 6:4-10.
146. Manco-Johnson MJ, Abshire TC, Shapiro AD, Riske B, Hacker MR, Kilcoyne R, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. The New England journal of medicine. 2007;357(6):535-44.
147. Saulyte Trakymiene S, Ingerslev J, Rageliene L. Utility of the Haemophilia Joint Health Score in study of episodically treated boys with severe haemophilia A and B in Lithuania. Haemophilia : the official journal of the World Federation of Hemophilia. 2010;16(3):479-86.
148. Ferreira AA, Bustamante-Teixeira MT, Leite IC, Correa CS, Rodrigues Dde O, da Cruz DT. Clinical and functional evaluation of the joint status of hemophiliac adults at a Brazilian blood center. Revista brasileira de hematologia e hemoterapia. 2013;35(1):23-8.
149. Bladen M, Main E, Hubert N, Koutoumanou E, Liesner R, Khair K. Factors affecting the Haemophilia Joint Health Score in children with severe haemophilia. Haemophilia : the official journal of the World Federation of Hemophilia. 2013;19(4):626-31.
150. Collins PW, Blanchette VS, Fischer K, Bjorkman S, Oh M, Fritsch S, et al. Break-through bleeding in relation to predicted factor VIII levels in patients receiving prophylactic treatment for severe hemophilia A. Journal of thrombosis and haemostasis : JTH. 2009;7(3):413-20.
151. Khanum F, Collins PW, Harris RL, Bowen DJ. Characterization of F8 defects in haemophilia A in Pakistan: investigation of correlation between mutation type and the in vitro thrombin generation assay. Haemophilia : the official journal of the World Federation of Hemophilia. 2013.
152. Baglin T. The measurement and application of thrombin generation. British journal of haematology. 2005;130(5):653-61.

153. van Veen JJ, Gatt A, Makris M. Thrombin generation testing in routine clinical practice: are we there yet? British journal of haematology. 2008;142(6):889-903.
154. Dargaud Y, Beguin S, Lienhart A, Al Dieri R, Trzeciak C, Bordet JC, et al. Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. Thrombosis and haemostasis. 2005;93(3):475-80.
155. Van Veen J, Gatt A, Cooper P, Kitchen S, Makris M. Between‐batch variation of calibrator activity can significantly influence fluorogenic measurement of thrombin generation. Journal of Thrombosis and Haemostasis. 2006;4(11):2514-6.
156. Lewis SJ, Stephens E, Florou G, Macartney NJ, Hathaway LS, Knipping J, et al. Measurement of global haemostasis in severe haemophilia A following factor VIII infusion. British journal of haematology. 2007;138(6):775-82.
157. Al Hawaj MA, Martin EJ, Venitz J, Barrett JC, Kuhn JG, Nolte ME, et al. Monitoring rFVIII prophylaxis dosing using global haemostasis assays. Haemophilia : the official journal of the World Federation of Hemophilia. 2013;19(3):409-14.
158. Beltrán‐Miranda C, Khan A, Jaloma‐Cruz A, Laffan M. Thrombin generation and phenotypic correlation in haemophilia A. Haemophilia : the official journal of the World Federation of Hemophilia. 2005;11(4):326-34.
159. Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Garagiola I, et al. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. Journal of thrombosis and haemostasis : JTH. 2010;8(4):737-43.
160. Chantarangkul V, Clerici M, Bressi C, Giesen PL, Tripodi A. Thrombin generation assessed as endogenous thrombin potential in patients with hyper- or hypo-coagulability. Haematologica. 2003;88(5):547-54.
161. Siegemund T, Petros S, Siegemund A, Scholz U, Engelmann L. Thrombin generation in severe haemophilia A and B: the endogenous thrombin potential in platelet-rich plasma. Thrombosis and haemostasis. 2003;90(5):781-6.
162. Sumner WT, Monroe DM, Hoffman M. Variability in platelet procoagulant activity in healthy volunteers. Thrombosis research. 1996;81(5):533-43.
163. You CW, Son HS, Kim HJ, Woo EJ, Kim SA, Baik HW. Mutation analysis of factor VIII in Korean patients with severe hemophilia A. International journal of hematology. 2010;91(5):784-91.
164. Xue F, Zhang L, Sui T, Ge J, Gu D, Du W, et al. Factor VIII gene mutations profile in 148 Chinese hemophilia A subjects. European journal of haematology. 2010;85(3):264-72.
165. Hwang SH, Kim MJ, Lim JA, Kim HC, Kim HS. Profiling of factor VIII mutations in Korean haemophilia A. Haemophilia : the official journal of the World Federation of Hemophilia. 2009;15(6):1311-7.
166. Ma GC, Chang SP, Chen M, Kuo SJ, Chang CS, Shen MC. The spectrum of the factor 8 (F8) defects in Taiwanese patients with haemophilia A. Haemophilia : the official journal of the World Federation of Hemophilia. 2008;14(4):787-95.
167. Petrini P, Lindvall N, Egberg N, Blomback M. Prophylaxis with factor concentrates in preventing hemophilic arthropathy. The American journal of pediatric hematology/oncology. 1991;13(3):280-7.
168. Engelbert RH, Plantinga M, Van der Net J, Van Genderen FR, Van den Berg MH, Helders PJ, et al. Aerobic capacity in children with hemophilia. The Journal of pediatrics. 2008;152(6):833-8, 8 e1.

169. Sorensen B, Ingerslev J. Whole blood clot formation phenotypes in hemophilia A and rare coagulation disorders. Patterns of response to recombinant factor VIIa. Journal of thrombosis and haemostasis : JTH. 2004;2(1):102-10.
170. Gissel M, Whelihan M, Ferris L, Mann K, Rivard G, BRUMMEL‐ZIEDINS K. The influence of prophylactic factor VIII in severe haemophilia A. Haemophilia : the official journal of the World Federation of Hemophilia. 2012;18(2):193-9.

Annex

Table A1

Gilbert score

Individual ID		Elbow		Knee		Ankle		Total joint score	Individual ID		Elbow		Knee		Ankle		Total joint score
		Left	Right	Left	Right	Left	Right				Left	Right	Left	Right	Left	Right	
	Swelling	0	0	0	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	1	1	1	1	1	1			Muscle atrophy	0	0	1	1	1	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
1	Flexion loss	0	0	0	0	0	0		48	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	1	2	1	2	0	0			Joint pain	1	1	1	1	0	0	
	Strength	0	1	1	1	0	0			Strength	0	0	1	1	1	0	
	Total joint score	2	4	3	4	1	1	15+2=17		Total joint score	1	1	3	3	2	0	10+2=12
	Swelling	0	0	0	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	1	0	1	0	0	0	
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
2	Flexion loss	0	0	1	0	0	0		49	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	1	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	0	0	0			Joint pain	1	0	1	0	0	0	
	Strength	0	0	1	0	0	0			Strength	1	0	1	0	0	0	
	Total joint score	0	0	6	0	0	0	6+2=8		Total joint score	3	0	3	0	0	0	6
	Swelling	0	0	0	2	0	0			Swelling	1	0	1	0	1	0	
	Duration (swelling)	0	0	0	1	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	0	1	0	0			Crepitus on motion	0	0	1	0	0	0	
3	Flexion loss	1	0	0	2	0	0		50	Flexion loss	0	0	2	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	0	2	0	0			Joint pain	0	0	0	0	0	0	
	Strength	0	0	1	1	0	0			Strength	2	0	2	0	1	0	
	Total joint score	1	0	1	10	0	0	12+3=15		Total joint score	3	0	6	0	2	0	11+2=13
4	Swelling	1	0	0	0	0	0		51	Swelling	1	0	1	0	0	0	
	Duration (swelling)	1	0	0	0	0	0			Duration (swelling)	1	0	1	0	0	0	

	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	2	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	1	0	0	0	0	0	
	Flexion loss	3	0	0	0	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	2	1	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	1	1	0	1	0	0			Joint pain	1	0	0	0	0	0	
	Strength	1	1	0	1	0	0			Strength	1	0	0	0	0	0	
	Total joint score	9	3	0	3	0	0	15+1=16		Total joint score	5	0	4	0	0	0	9+1=10
	Swelling	0	1	0	0	0	0			Swelling	0	0	1	2	0	0	
	Duration (swelling)	0	1	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	1	0	0	
	Crepitus on motion	0	1	0	0	0	0			Crepitus on motion	0	0	1	1	0	0	
5	Flexion loss	0	0	3	3	0	0		52	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	1	1	1	0	0			Joint pain	0	0	1	1	0	0	
	Strength	0	0	1	1	0	0			Strength	0	0	3	3	1	1	
	Total joint score	0	4	5	5	0	0	14+3=17		Total joint score	0	0	7	8	1	1	17+2=19
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	1	1	1	1	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	0	0	
6	Flexion loss	0	3	0	0	0	0		53	Flexion loss	3	0	3	3	0	0	
	Extension loss	1	0	2	2	0	0			Extension loss	1	0	3	3	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	2	1	1	1	
	Strength	1	1	2	2	1	1			Strength	2	2	3	3	1	1	
	Total joint score	2	4	4	5	1	1	17+1=18		Total joint score	7	3	14	13	3	3	43+3=46
	Swelling	0	0	0	0	0	2			Swelling	0	1	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	1	1	0	0	0	0			Muscle atrophy	0	1	0	1	0	0	
	Crepitus on motion	0	0	0	0	0	1			Crepitus on motion	0	0	0	0	0	0	
7	Flexion loss	0	0	3	0	0	1		54	Flexion loss	1	1	0	0	0	0	
	Extension loss	1	1	0	0	0	0			Extension loss	0	0	1	0	0	0	
	Joint pain	0	0	0	0	0	2			Joint pain	0	1	0	1	0	0	
	Strength	2	2	1	1	0	4			Strength	0	1	0	1	0	0	
	Total joint score	4	4	4	1	0	9	22+4=28		Total joint score	1	5	1	3	0	0	10
8	Swelling	0	0	0	0	0	0		55	Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	

	Muscle atrophy	0	1	1	1	1	1			Muscle atrophy	0	1	0	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	1	0	0	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	3	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	0	1	1			Joint pain	0	1	0	0	0	0	
	Strength	0	0	1	1	1	1			Strength	0	1	0	0	0	0	
	Total joint score	0	2	6	2	3	3	16+1=17		Total joint score	0	3	0	0	0	0	3
	Swelling	2	0	0	0	0	0			Swelling	0	0	1	1	0	0	
	Duration (swelling)	1	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	2	0	2	0	1	0			Muscle atrophy	1	1	2	2	1	1	
	Crepitus on motion	1	0	1	0	0	0			Crepitus on motion	1	0	1	1	0	0	
9	Flexion loss	Ne	0	3	0	0	0		56	Flexion loss	0	0	2	2	1	0	
	Extension loss	Ne	0	1	0	0	0			Extension loss	0	0	1	1	0	0	
	Joint pain	2	0	2	0	1	0			Joint pain	0	0	1	1	0	0	
	Strength	4	0	4	1	4	0			Strength	1	1	4	4	1	1	
	Total joint score	12	0	13	1	6	0	32+2=34		Total joint score	3	2	12	12	3	2	34+3=37
	Swelling	1	0	0	0	0	2			Swelling	0	0	2	0	0	0	
	Duration (swelling)	1	0	0	0	0	1			Duration (swelling)	0	0	1	0	0	0	
	Muscle atrophy	0	0	1	0	0	1			Muscle atrophy	0	0	0	0	1	0	
	Crepitus on motion	0	0	0	0	0	1			Crepitus on motion	0	0	1	0	0	0	
10	Flexion loss	2	0	2	0	0	Ne		57	Flexion loss	0	0	3	0	0	0	
	Extension loss	0	0	0	0	0	Ne			Extension loss	0	0	2	0	0	0	
	Joint pain	1	0	0	0	0	2			Joint pain	0	0	1	0	0	0	
	Strength	1	0	0	0	0	3			Strength	0	0	3	0	2	0	
	Total joint score	6	0	3	0	0	10	19+0= 19		Total joint score	0	0	13	0	3	0	16+2=18
	Swelling	0	0	2	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	1	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	1	1	1	1	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
11	Flexion loss	0	0	0	0	0	0		58	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	1	1	1	1	0			Joint pain	0	0	1	1	0	0	
	Strength	0	0	2	0	1	1			Strength	1	0	1	1	0	0	
	Total joint score	0	2	8	2	3	1	16+2=18		Total joint score	1	0	2	2	0	0	5
12	Swelling	0	0	1	0	0	0		59	Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	1	0	0	0			Duration (swelling)	0	0	0	0	0	0	

	Muscle atrophy	0	0	1	1	1	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	1	0	0			Joint pain	1	1	0	1	0	0	
	Strength	0	0	1	1	0	0			Strength	0	0	0	0	1	1	
	Total joint score	0	0	6	3	1	0	10+2=12		Total joint score	1	1	0	1	1	1	5
	Swelling	0	0	1	1	0	0			Swelling	1	1	2	2	0	1	
	Duration (swelling)	0	0	1	1	0	0			Duration (swelling)	0	0	0	0	0	1	
	Muscle atrophy	0	0	2	1	0	0			Muscle atrophy	0	0	1	1	1	1	
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	1	1	0	0	
13 Inhib	Flexion loss	0	0	2	3	0	0		60	Flexion loss	2	0	3	3	0	1	
	Extension loss	0	0	0	0	0	0			Extension loss	0	2	2	0	0	1	
	Joint pain	1	1	1	1	0	0			Joint pain	1	1	1	1	1	1	
	Strength	0	0	1	1	0	0			Strength	1	2	3	3	1	2	
	Total joint score	1	1	9	8	0	0	19+1=20		Total joint score	5	6	13	11	3	8	46+3=49
	Swelling	0	0	2	2	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	1	1	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	1	1	2	2	1	1			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	1	1	0	0			Crepitus on motion	0	0	0	0	0	0	
14	Flexion loss	2	3	0	0	3	3		61	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	2	3	3	1	1			Extension loss	0	0	0	0	0	0	
	Joint pain	1	1	2	2	0	0			Joint pain	0	0	1	0	0	0	
	Strength	2	2	4	4	1	1			Strength	0	0	1	0	0	0	
	Total joint score	6	9	15	15	6	6	57+4=61		Total joint score	0	0	2	0	0	0	2
	Swelling	0	0	0	0	0	0			Swelling	2	2	2	2	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	1	2	2	0	1	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	1	1	
15	Flexion loss	0	0	0	0	0	0		62	Flexion loss	3	0	3	1	1	1	
	Extension loss	0	0	0	0	0	0			Extension loss	2	3	1	2	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	2	1	1	1	1	1	
	Strength	0	0	0	0	0	0			Strength	3	3	4	4	2	1	
	Total joint score	0	0	0	0	0	0	0		Total joint score	13	10	14	13	5	5	60+3=63
16	Swelling	0	0	0	0	0	0		65	Swelling	0	0	1	1	2	2	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	

	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	1	1	1	2	2	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	1	1	
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	1	2	3	3	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	1	1	3	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	1	1	
	Strength	0	0	0	0	0	0			Strength	1	1	3	3	3	3	
	Total joint score	0	0	0	0	0	0	0		Total joint score	3	3	9	10	13	15	53+2=55
	Swelling	0	0	1	0	0	0			Swelling	0	1	1	1	0	0	
	Duration (swelling)	0	0	1	0	0	0			Duration (swelling)	0	1	0	0	0	0	
	Muscle atrophy	1	0	1	1	0	0			Muscle atrophy	0	2	1	1	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	1	1	1	0	0	
17	Flexion loss	2	1	3	3	0	0		66	Flexion loss	0	3	0	0	0	0	
	Extension loss	3	1	1	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	1	1	1	1	1	1			Joint pain	0	1	1	1	0	0	
	Strength	3	0	2	2	1	1			Strength	1	3	3	3	0	0	
	Total joint score	10	3	10	7	2	2	34+3=37		Total joint score	1	11	7	7	0	0	26+2=28
	Swelling	0	2	2	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	1	1	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	1	1	0	0	0			Muscle atrophy	0	0	0	0	0	0	
18	Crepitus on motion	0	1	2	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	2	n/m	3	0	0	0		70	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	n/m	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	2	2	0	0	0			Joint pain	0	0	1	0	0	0	
	Strength	0	4	4	0	0	0			Strength	0	0	1	0	0	0	
	Total joint score	2	11	15	0	0	0	28+1=29		Total joint score	0	0	2	0	0	0	2
	Swelling	0	0	0	0	0	0			Swelling	0	2	0	3	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	1	1	2	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	1	0	1	0	0	
19	Flexion loss	0	0	0	0	0	0		71	Flexion loss	1	3	3	3	1	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	3	1	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	0	0	
	Strength	0	0	0	0	0	0			Strength	1	2	2	3	1	1	
	Total joint score	0	0	0	0	0	0	0		Total joint score	3	10	7	16	3	1	40+2=42
21	Swelling	0	0	1	1	0	0		72	Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	

	Muscle atrophy	1	1	1	1	0	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	1	1	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	3	0	2	2	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	2	0	1	2	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	1	1	1	1	0	0			Joint pain	0	0	1	1	0	0	
	Strength	2	2	4	4	0	0			Strength	0	0	1	1	0	0	
	Total joint score	9	4	11	12	0	0	36+3=39		Total joint score	0	0	2	2	0	0	4
	Swelling	0	0	0	0	0	0			Swelling	1	1	2	2	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	1	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	0	0	1	2	1	1	
22	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	0	0	
	Flexion loss	0	0	0	0	0	0		73 Inhib	Flexion loss	2	2	2	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	0	0	0	
	Joint pain	0	0	1	1	0	0			Joint pain	0	0	1	0	1	1	
	Strength	1	1	1	1	0	0			Strength	1	1	2	3	1	2	
	Total joint score	1	1	3	2	0	0	7+2=9		Total joint score	4	4	11	9	3	4	35+3=38
	Swelling	0	0	0	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
23	Flexion loss	0	0	0	0	0	0		74	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	0	0	0	0	0	0	
	Strength	0	0	0	0	0	0			Strength	0	0	0	0	0	0	
	Total joint score	0	0	0	0	0	0	0		Total joint score	0	0	0	0	0	0	0
	Swelling	0	0	0	0	0	0			Swelling	1	1	1	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	1	1	1	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	0	1	1	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	0	0	
24	Flexion loss	0	0	0	0	0	0		75	Flexion loss	0	0	3	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	3	0	0	0	1	1	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	0	0	
	Strength	0	0	0	0	0	0			Strength	3	2	2	1	1	1	
	Total joint score	0	0	0	0	0	0	0		Total joint score	10	5	10	4	2	2	33+3=36
25	Swelling	0	1	0	0	0	0		76	Swelling	1	1	2	3	0	0	
	Duration (swelling)	0	1	0	0	0	0			Duration (swelling)	1	1	1	0	0	0	

	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	1	1	2	1	1	
	Crepitus on motion	0	1	0	0	0	0			Crepitus on motion	0	0	1	1	0	0	
	Flexion loss	0	1	0	0	0	0			Flexion loss	0	0	3	0	1	1	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	3	3	1	1	
	Joint pain	0	1	0	0	0	0			Joint pain	0	0	2	2	0	0	
	Strength	0	2	0	0	0	0			Strength	1	1	3	4	2	2	
	Total joint score	0	7	0	0	0	0	7		Total joint score	4	4	16	13	5	5	47+4=51
	Swelling	0	0	0	0	0	0			Swelling	1	0	1	1	1	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	1	1	0	
	Muscle atrophy	0	0	0	0	1	1			Muscle atrophy	1	1	1	1	1	1	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
26	Flexion loss	0	0	0	0	1	1		77	Flexion loss	2	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	1	0	0	
	Joint pain	0	0	0	0	1	1			Joint pain	0	0	1	1	0	0	
	Strength	0	0	0	0	1	1			Strength	1	1	2	2	1	2	
	Total joint score	0	0	0	0	4	4	8+1=9		Total joint score	5	2	6	7	4	3	27+2=29
	Swelling	0	0	0	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
27	Flexion loss	0	0	0	0	0	0		78	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	1	0	0			Joint pain	0	0	0	0	0	0	
	Strength	0	0	1	1	0	0			Strength	0	0	0	0	0	0	
	Total joint score	0	0	2	2	0	0	4+1=5		Total joint score	0	0	0	0	0	0	0
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	1	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	1	1	1	
	Muscle atrophy	2	2	1	1	0	0			Muscle atrophy	1	1	2	1	1	1	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
28	Flexion loss	0	0	1	1	0	0		79	Flexion loss	2	1	3	0	0	0	
	Extension loss	1	2	0	0	0	0			Extension loss	0	0	0	0	1	1	
	Joint pain	1	0	1	1	1	0			Joint pain	0	0	0	0	0	0	
	Strength	4	4	2	2	1	1			Strength	1	1	3	2	2	1	
	Total joint score	8	8	5	5	2	1	29+3=32		Total joint score	4	3	10	5	6	4	32+2=34
29	Swelling	0	0	0	0	0	0		80	Swelling	2	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	1	0	0	0	1	0	

	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	0	0	1	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	1	1	0	0	0	0			Flexion loss	0	0	3	3	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	3	0	0	0	0	
	Joint pain	1	1	0	0	0	0			Joint pain	2	1	0	0	1	0	
	Strength	1	1	0	0	0	0			Strength	4	1	1	1	1	0	
	Total joint score	3	3	0	0	0	0	6		Total joint score	9	5	4	4	4	0	26
	Swelling	0	0	0	0	0	2			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	1			Duration (swelling)	2	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	1	1	0	0	0	0	
	Crepitus on motion	0	0	0	0	0	2			Crepitus on motion	0	0	0	0	0	0	
30	Flexion loss	0	0	0	0	0	1		81	Flexion loss	3	2	0	0	0	1	
	Extension loss	0	0	0	0	0	1			Extension loss	3	0	0	0	0	0	
	Joint pain	0	0	0	0	0	2			Joint pain	1	0	0	0	1	1	
	Strength	0	0	0	0	0	4			Strength	3	0	0	0	1	1	
	Total joint score	0	0	0	0	0	13	13+2=15		Total joint score	13	3	0	0	2	3	31+2=33
	Swelling	0	1	1	1	0	0			Swelling	0	1	3	2	0	0	
	Duration (swelling)	0	1	0	0	0	0			Duration (swelling)	0	1	0	0	0	0	
	Muscle atrophy	1	1	1	1	0	0			Muscle atrophy	0	0	2	2	1	1	
	Crepitus on motion	1	0	1	0	0	0			Crepitus on motion	1	1	0	0	0	0	
31	Flexion loss	N	0	3	3	1	0		82	Flexion loss	3	0	2	2	0	0	
	Extension loss	N	0	1	1	1	1			Extension loss	0	0	0	0	0	0	
	Joint pain	1	1	1	1	1	1			Joint pain	1	1	0	0	1	1	
	Strength	2	3	1	2	1	1			Strength	3	2	3	3	2	1	
	Total joint score	5	7	9	9	4	3	37+3=40		Total joint score	8	6	10	9	4	3	40+2=42
	Swelling	0	0	2	0	0	0			Swelling	0	0	1	2	1	1	
	Duration (swelling)	0	0	1	0	0	0			Duration (swelling)	0	0	0	0	0	1	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	1	2	2	2	1	1	
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
32	Flexion loss	0	1	3	3	1	1		83	Flexion loss	1	0	3	3	0	0	
	Extension loss	0	0	0	3	0	0			Extension loss	0	0	3	0	0	0	
	Joint pain	1	1	2	2	0	0			Joint pain	1	1	1	1	1	1	
	Strength	1	1	4	4	0	0			Strength	2	3	2	3	2	2	
	Total joint score	2	3	13	13	1	1	33+3=36		Total joint score	5	6	12	11	5	6	44+3=47
	Swelling	0	0	2	0	0	0			Swelling	0	0	1	1	1	1	
33	Duration (swelling)	0	0	0	0	0	0		84	Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	1	1	1	0	0	0			Muscle atrophy	2	2	11	0	1	1	

	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	2	3	3	0	0	0			Flexion loss	3	1	3	3	1	1	
	Extension loss	0	0	3	0	0	0			Extension loss	2	0	3	0	0	0	
	Joint pain	1	1	2	1	0	0			Joint pain	1	1	1	1	0	0	
	Strength	3	3	4	3	1	1			Strength	2	3	2	1	1	2	
	Total joint score	7	8	16	4	1	1	36+3=39		Total joint score	10	7	21	6	4	5	53+3=56
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	1			Muscle atrophy	0	0	2	1	1	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
34	Flexion loss	0	0	0	0	0	0		85	Flexion loss	0	1	3	3	1	1	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	1	1	
	Strength	0	0	0	0	0	1			Strength	1	0	2	3	1	1	
	Total joint score	0	0	0	0	0	2	2		Total joint score	2	2	9	9	4	3	29+3=32
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
35	Flexion loss	0	0	0	0	0	0		86	Flexion loss	0	0	1	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	0	0	1	0	0	0	
	Strength	0	0	0	0	0	0			Strength	0	0	2	0	0		
	Total joint score	0	0	0	0	0	0	0		Total joint score	0	0	6	0	0	0	6+1=7
	Swelling	0	0	1	2	0	0			Swelling	0	0	0	1	0	0	
	Duration (swelling)	0	0	1	1	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	1	0	1			Muscle atrophy	0	0	0	1	0	0	
	Crepitus on motion	0	0	0	1	0	0			Crepitus on motion	0	0	0	0	0	0	
36 Inhib	Flexion loss	1	1	2	2	1	1		87	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	3	3	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	2	1	1			Joint pain	0	0	1	1	0	0	
	Strength	0	0	3	4	1	1			Strength	1	1	0	1	0	0	
	Total joint score	1	1	12	16	3	4	37+3=40		Total joint score	1	1	1	4	0	0	7+2=9
	Swelling	0	0	0	1	0	0			Swelling	0	0	1	0	0	0	
37	Duration (swelling)	0	0	0	0	0	0		88	Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	1	1	0	0	

	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	1	3	0	0			Flexion loss	0	0	2	1	1	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	1	1	0	0	0	
	Joint pain	0	0	1	1	0	0			Joint pain	0	0	1	1	0	0	
	Strength	0	0	1	2	0	0			Strength	0	0	2	1	1	0	
	Total joint score	0	0	3	8	0	0	11+1=12		Total joint score	0	1	8	4	2	0	15+2=17
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	0	0	0	
	Muscle atrophy	1	0	0	0	1	0			Muscle atrophy	0	0	1	0	1	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
38	Flexion loss	0	0	0	3	0	0		89	Flexion loss	0	0	1	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	1	0	0	0	1	1			Joint pain	0	0	1	1	0	0	
	Strength	2	0	2	1	1	1			Strength	0	0	1	0	1	1	
	Total joint score	4	0	2	4	3	2	15+2=17		Total joint score	0	0	7	1	2	1	11+1=12
	Swelling	0	0	0	0	0	0			Swelling	0	0	2	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
39	Flexion loss	0	0	0	0	0	0		90	Flexion loss	0	0	2	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	2	0	1	1	
	Strength	0	0	0	0	0	0			Strength	1	1	1	0	1	1	
	Total joint score	0	0	0	0	0	0	0		Total joint score	2	2	10	0	2	2	18
	Swelling	0	0	1	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	1	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	1	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
40	Flexion loss	0	0	0	0	1	1		91	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	1	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	0	1	0			Joint pain	0	0	1	0	0	0	
	Strength	0	0	3	0	2	0			Strength	0	0	1	0	0	0	
	Total joint score	0	0	8	0	5	1	14+2=16		Total joint score	0	0	2	0	0	0	2+1=3
	Swelling	0	0	1	0	0	0			Swelling	1	0	0	2	0	0	
41	Duration (swelling)	0	0	1	0	0	0		92	Duration (swelling)	0	0	0	1	0	0	
	Muscle atrophy	2	0	2	0	0	0			Muscle atrophy	2	0	0	1	0	0	

	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	1	0	0	1	0	0	
	Flexion loss	3	0	3	0	0	0			Flexion loss	0	0	0	3	0	0	
	Extension loss	3	0	0	0	0	0			Extension loss	2	0	0	0	1	0	
	Joint pain	0	0	1	0	1	0			Joint pain	1	0	0	2	0	0	
	Strength	4	0	3	0	1	0			Strength	3	0	0	2	1	1	
	Total joint score	12	0	12	0	2	0	26+2=28		Total joint score	10	0	0	12	2	1	25+1=26
	Swelling	0	0	1	0	0	0			Swelling	0	0	0	0	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
42	Flexion loss	0	0	0	0	1	0		93	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	1	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	0	1	0	1	0			Joint pain	0	0	1	0	0	0	
	Strength	0	0	2	0	1	0			Strength	0	0	1	0	0	0	
	Total joint score	0	0	5	0	4	0	9+2=11		Total joint score	0	0	2	0	0	0	2+1=3
	Swelling	0	0	0	0	0	0			Swelling	0	0	2	1	1	1	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	0	0	1	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	2	2	1	1	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
43	Flexion loss	0	0	0	0	0	0		94	Flexion loss	0	2	3	3	1	1	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	3	3	1`	0	
	Joint pain	1	1	0	0	0	0			Joint pain	0	0	1	1	1	0	
	Strength	1	1	0	0	0	0			Strength	0	0	3	3	1	1	
	Total joint score	2	2	0	0	0	0	4		Total joint score	0	2	16	13	6	5	42+4=46
	Swelling	0	0	1	1	0	0			Swelling	0	2	1	2	1	1	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	1	0	1	1	0	
	Muscle atrophy	0	1	1	1	0	0			Muscle atrophy	1	0	1	1	1	1	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	1	0	0	
44	Flexion loss	3	3	0	0	0	0		95	Flexion loss	3	3	1	0	1	1	
	Extension loss	0	3	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	1	2	2	0	0			Joint pain	0	0	1	2	1	0	
	Strength	0	3	4	4	0	0			Strength	1	0	2	2	1	1	
	Total joint score	3	11	8	8	0	0	30+3=33		Total joint score	5	6	6	9	6	4	36+2=38
	Swelling	0	0	0	1	0	0			Swelling	0	0	1	2	1	1	
45	Duration (swelling)	0	0	0	0	0	0		96	Duration (swelling)	0	0	1	1	1	1	
	Muscle atrophy	0	0	1	1	1	1			Muscle atrophy	0	1	1	2	1	1	

	Crepitus on motion	0	0	1	1	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	3	0	0	0			Flexion loss	0	0	1	1	0	0	
	Extension loss	0	0	3	0	0	0			Extension loss	0	0	0	0	1	1	
	Joint pain	0	0	0	1	0	0			Joint pain	0	1	1	1	0	0	
	Strength	0	0	3	4	1	1			Strength	1	1	3	2	1	2	
	Total joint score	0	0	11	8	2	2	23+4=27		Total joint score	1	3	8	9	5	6	32+4=36
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	0	0	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	0	0	0	0	
	Muscle atrophy	0	1	0	1	0	0			Muscle atrophy	0	0	1	1	0	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
46 Inhib	Flexion loss	0	0	0	0	0	0		97	Flexion loss	0	0	3	2	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Joint pain	0	1	0	1	0	0			Joint pain	0	0	0	0	0	0	
	Strength	0	1	0	1	0	0			Strength	0	0	1	2	1	1	
	Total joint score	0	3	0	3	0	0	6+1=7		Total joint score	0	0	6	6	1	1	14+2=16
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	0	1	1	
	Duration (swelling)	0	0	0	0	0	0			Duration (swelling)	0	0	1	0	0	0	
	Muscle atrophy	1	1	0	0	0	0			Muscle atrophy	1	1	1	0	1	0	
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
47	Flexion loss	0	0	0	0	0	0		98	Flexion loss	0	0	1	1	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	1	0	0	0	1	0	
	Joint pain	1	1	0	0	0	0			Joint pain	0	0	1	0	1	1	
	Strength	1	1	0	0	0	0			Strength	2	1	3	0	1	1	
	Total joint score	3	3	0	0	0	0	6		Total joint score	4	2	9	1	5	3	24+3=27

Table A2

Haemophilia joint health score (2.1)

Individual ID		Ankle		Knee		Elbow		Total score	Individual ID		Ankle		Knee		Elbow		Total score
		Left	Right	Left	Right	Left	Right				Left	Right	Left	Right	Left	Right	
1	Swelling	0	0	0	0	0	0		48	Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	1	1	1	1	1	1			Muscle atrophy	1	0	1	1	0	0	
	Axial alignment	1	1	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	0	0	0	1	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	1	1	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	1	2	0	1			Joint pain	0	0	1	1	1	1	
	Strength	0	0	1	1	0	1			Strength	1	0	1	1	0	0	
	Gait	1	1	1	2					Gait	1	2	1	1			
	Joint total	3	3	6	8	1	3	24+2=26		Joint total	3	2	4	4	1	1	15+2=17
2	Swelling	0	0	0	0	0	0		49	Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	0	0	1	0	1	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	1	0	0	0			Flexion loss	0	0	0	0	0	0	0
	Extension loss	0	0	1	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	1	0	0	0			Joint pain	0	0	1	0	1	0	
	Strength	0	0	1	0	0	0			Strength	0	0	1	0	1	0	
	Gait	0	0	1	1					Gait	0	0	0	0			
	Joint total	0	0	7	1	0	0	8+2=10		Joint total	0	0	3	0	3	0	6
3	Swelling	0	0	0	2	0	0		50	Swelling	1	0	1	0	1	0	
	Duration swelling	0	0	0	1	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	0	0	1	0	
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	1	0	0			Crepitus on motion	0	0	0	0	1	0	
	Flexion loss	0	0	1	2	1	1			Flexion loss	0	0	2	0	0	1	
	Extension loss	0	0	1	1	0	0			Extension loss	0	0	0	0	0	0	16+2=18

	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	0	2	0	0			Joint pain	0	0	0	0	0	0		
	Strength	0	0	1	1	0	0			Strength	1	0	2	0	2	0		
	Gait	1	1	1	2					Gait	1	0	2	0	0			
	Joint total	1	1	5	14	1	1	23+3=26		Joint total	3	0	7	0	5	1		
	Swelling	0	0	0	0	1	0			Swelling	0	0	1	0	1	0		
	Duration swelling	0	0	0	0	1	0			Duration swelling	0	0	1	0	1	0		
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	2	0	0	0		
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	1	0		
4	Flexion loss	0	0	0	0	2	0		51	Flexion loss	0	0	0	0	0	0		
	Extension loss	0	0	1	1	2	1			Extension loss	0	0	0	0	0	0		
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	0	0	1	1			Joint pain	0	0	0	0	1	0		
	Strength	0	0	0	1	1	1			Strength	0	0	0	0	1	0		
	Gait	0	0	1	1					Gait	0	0	1	0	0			
	Joint total	0	0	2	4	8	3	17+1=18		Joint total	0	0	4	0	5	0	9+1=10	
	Swelling	0	0	0	0	0	1			Swelling	0	0	1	2	0	0		
	Duration swelling	0	0	0	0	0	1			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	1	1	1	0	0		
	Axial alignment	1	0	1	1					Axial alignment	1	1	1	1				
	Crepitus on motion	0	0	0	0	0	1		52	Crepitus on motion	0	0	1	1	0	0		
5	Flexion loss	0	0	3	3	1	1			Flexion loss	0	0	1	0	0	0		
	Extension loss	0	0	0	1	1	1			Extension loss	0	0	0	0	0	0		
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	1	1	0	1			Joint pain	0	0	1	1	0	0		
	Strength	0	0	1	1	0	0			Strength	1	1	3	3	0	0		
	Gait	3	0	1	1					Gait	1	1	2	2				
	Joint total	4	0	7	8	2	6	27+3=30		Joint total	3	4	11	11	0	0	29+2=31	
	Swelling	0	0	0	0	0	0			Swelling	0	1	1	1	0	0		
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	1	1	1	1	0	0		
	Axial alignment	0	0	0	0					Axial alignment	1	1	1	1				
6	Crepitus on motion	0	0	0	0	0	0		53	Crepitus on motion	0	0	1	1	0	0		
	Flexion loss	0	2	0	2	0	0			Flexion loss	0	0	3	3	2	0		
	Extension loss	2	0	0		0	0			Extension loss	0	0	2	2	1	0		
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		

	Joint pain	0	0	0	0	0	0			Joint pain	1	1	2	1	1	1	
	Strength	1	1	2	2	1	1			Strength	1	1	3	3	2	2	
	Gait	1	1	1	1					Gait	1	1	3	3			
	Joint total	4	4	3	6	1	1	19+1=20		Joint total	5	6	17	16	6	3	53+3=56
	Swelling	0	2	0	0	0	0			Swelling	0	0	0	0	0	1	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	1	1	1			Muscle atrophy	0	0	0	1	1	1	
	Axial alignment	1	1	0	0	0	O			Axial alignment	0	0	0	0			
	Crepitus on motion	0	1	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
7	Flexion loss	0	1	3	0	0	0		54	Flexion loss	0	0	0	0	1	1	
	Extension loss	0	0	0	0	1	1			Extension loss	0	0	0	0	0	1	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	2	0	0	0	0			Joint pain	0	0	0	0	0	1	
	Strength	0	4	1	1	2	2			Strength	0	0	0	1	0	1	
	Gait	2	2	3	2					Gait	0	0	0	0			
	Joint total	3	13	8	4	4	4	36+4=40		Joint total	0	0	0	2	2	6	10
	Swelling	0	0	0	0	0	0			Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	1	1	1	1	0	1			Muscle atrophy	0	0	0	0	0	1	
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
8	Flexion loss	0	0	1	1	0	1		55	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	1			Instability	0	0	0	0	0	0	
	Joint pain	1	1	1	1	0	0			Joint pain	0	0	0	0	0	1	
	Strength	1	1	1	1	0	0			Strength	0	0	0	0	0	1	
	Gait	1	1	1	1					Gait	0	0	0	0			
	Joint total	4	4	6	6	0	2	22+1=23		Joint total	0	0	0	0	0	3	3
	Swelling	0	0	0	0	2	0			Swelling	0	0	1	1	0	0	
	Duration swelling	0	0	0	0	1	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	1	0	2	0	2	0			Muscle atrophy	1	1	2	2	1	1	
	Axial alignment	0	0	1	0					Axial alignment	0	0	1	1			
9	Crepitus on motion	0	0	1	0	1	0		56	Crepitus on motion	0	1	1	1	1	0	
	Flexion loss	Ne	0	2	0	0	0			Flexion loss	0	1	2	2	0	0	
	Extension loss	Ne	0	2	0	0	0			Extension loss	1	0	1	1	0	0	
	Instability	0	0	1	1	1	0			Instability	0	0	1	1	0	0	
	Joint pain	1	0	2	0	2	0			Joint pain	0	0	0	1	0	0	

	Strength	4	0	4	0	4	0			Strength	1	1	4	4	1	1	
	Gait	2	0	2	2					Gait	1	1	2	3			
	Joint total	8	0	17	3	13	0	41+2=43		Joint total	4	5	15	17	3	2	46+3=49
	Swelling	0	2	0	0	1	0			Swelling	0	0	2	0	0	0	
	Duration swelling	0	1	0	0	1	0			Duration swelling	0	0	1	0	0	0	
	Muscle atrophy	0	1	1	0	0	0			Muscle atrophy	1	0	0	0	0	0	
	Axial alignment	0	1	0	0					Axial alignment	0	0	1	0			
	Crepitus on motion	0	1	0	0					Crepitus on motion	0	0	1	0	0	0	
10	Flexion loss	0	Ne	2	0	2	0		57	Flexion loss	0	0	3	0	0	0	
	Extension loss	0	Ne	0	0	0	0			Extension loss	0	0	1	0	0	0	
	Instability	0	0	0	0	1	0			Instability	0	0	0	0	0	0	
	Joint pain	0	2	0	0	1	0			Joint pain	0	0	1	0	0	0	
	Strength	0	3	0	0	1	0			Strength	2	0	3	0	0	0	
	Gait	0	2	0	1					Gait	1	1	2	1			
	Joint total	0	13	3	1	7	0	24+0=24		Joint total	4	1	15	1	0	0	21+2=23
	Swelling	0	0	2	0	0	0			Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	1	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	1	0	1	1	0	1			Muscle atrophy	0	0	0	0	0		
	Axial alignment	0	0	1	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
11	Flexion loss	0	0	0	0	0	0		58	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	1			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	1	0	1	0	0	1			Joint pain	0	0	1	1	0	0	
	Strength	1	1	2	0	0	0			Strength	0	0	1	1	1	0	
	Gait	1	1	2	1		2			Gait	0	0	0	0			
	Joint total	4	2	11	2	0	3	22+2=24		Joint total	0	0	2	2	1	0	5
	Swelling	0	0	1	0	0	0			Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	1	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	1	0	1	1	0	0			Muscle atrophy	0	0	0	0	0	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0			
12	Crepitus on motion	0	0	1	0	0	0		59	Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	1	1	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	1	1	0	0			Joint pain	0	0	1	1	1	1	
	Strength	0	0	1	1	0	0			Strength	1	1	0	1	1	1	

	Gait	0	0	1	1					Gait	0	0	0	0				
	Joint total	2	1	7	4	0	0	14+2=16		Joint total	1	1	1	2	2	2	9	
	Swelling	0	0	1	1	0	0			Swelling	0	1	2	2	1	1		
	Duration swelling	0	0	1	1	0	0			Duration swelling	0	1	0	0	0	0		
	Muscle atrophy	0	0	2	1	0	0			Muscle atrophy	1	1	1	1	0	0		
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0				
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	1	1	0	0		
13 Inhib	Flexion loss	0	0	2	2	0	0		60	Flexion loss	0	1	3	3	2	1		
	Extension loss	0	0	1	0	0	0			Extension loss	0	1	2	1	2	2		
	Instability	0	0	0	1	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	1	1	1	1			Joint pain	1	1	1	1	1	1		
	Strength	0	0	1	1	0	0			Strength	1	2	3	3	1	2		
	Gait	1	1	1	1					Gait	1	1	2	3				
	Joint total	1	1	11	9	1	1	24+1=25		Joint total	4	9	15	15	7	7	57+3=60	
	Swelling	0	0	2	2	0	0			Swelling	0	0	0	0	0	0		
	Duration swelling	0	0	1	1	0	0			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	1	1	2	2	1	1			Muscle atrophy	0	0	0	0	0			
	Axial alignment	1	1	1	1					Axial alignment	0	0	0	0				
14	Crepitus on motion	0	0	1	1	0	0			Crepitus on motion	0	0	0	0	0	0		
	Flexion loss	1	1	0	1	2	3		61	Flexion loss	0	0	0	0	0	0		
	Extension loss	3	3	3	3	1	2			Extension loss	0	0	0	0	0	0		
	Instability	0	0	1	1	1	1			Instability	0	0	0	0	0	0		
	Joint pain	0	0	2	2	1	1			Joint pain	0	0	1	0	0	0		
	Strength	1	1	4	4	2	2			Strength	0	0	1	0	0	0		
	Gait	4	4	4	4					Gait	0	0	1	0				
	Joint total	11	11	21	22	8	10	83+4=87		Joint total	0	0	3	0	0	0	3	
	Swelling	0	0	0	0	0	0			Swelling	1	0	2	2	2	2		
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	1	2	2	1	1		
	Axial alignment	0	0	0	0					Axial alignment	1	1	1	0				
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	1	1	1	1	0	0		
15	Flexion loss	0	0	0	0	0	0		62	Flexion loss	1	1	2	1	3	0		
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	2	2	3		
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	3	1		
	Strength	0	0	0	0	0	0			Strength	2	1	4	4	3	3		
	Gait	0	0	0	0					Gait	2	1	3	3				

	Joint total	0	0	0	0	0	0	0		Joint total	10	7	17	16	14	10	74+3=77
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	2	2	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	1	1	1	2	2	
	Axial alignment	0	0	0	0					Axial alignment	0	0	1	1			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	1	1	
16	Flexion loss	0	0	0	0	0	0		65	Flexion loss	0	0	1	2	3	3	
	Extension loss	0	0	0	0	0	0			Extension loss	1	0	1	1	1	3	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	1	1	
	Strength	0	0	0	0	0	0			Strength	1	1	3	3	3	3	
	Gait	0	0	0	0					Gait	1	0	2	2			
	Joint total	0	0	0	0	0	0	0		Joint total	5	3	12	13	13	15	61+2=62
	Swelling	0	0	1	0	0	0			Swelling	0	0	1	1	0	1	
	Duration swelling	0	0	1	0	0	0			Duration swelling	0	0	0	0	0	1	
	Muscle atrophy	0	0	1	1	1	0			Muscle atrophy	0	0	1	1	0	2	
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	0	1	
17	Flexion loss	0	0	3	3	2	1		66	Flexion loss	0	0	0	0	0	2	
	Extension loss	0	0	1	0	2	1			Extension loss	0	0	0	0	0	1	
	Instability	0	0	1	0	1	0			Instability	0	0	0	0	0	0	
	Joint pain	1	1	1	1	1	1			Joint pain	0	0	1	1	0	1	
	Strength	1	1	2	2	3	0			Strength	0	0	3	3	1	3	
	Gait	1	1	2	1					Gait	0	0	2	2			
	Joint total	3	3	14	9	10	3	42+3=45		Joint total	0	0	9	9	1	12	31+2=33
	Swelling	0	0	2	0	0	2			Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	1	0	0	1			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Axial alignment	1	1	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	2	0	0	1			Crepitus on motion	0	0	0	0	0	0	
18	Flexion loss	0	0	2	0	1	Ne		70	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	Ne			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	2	0	0	2			Joint pain	0	0	1	0	0	0	
	Strength	0	0	4	0	0	4			Strength	0	0	1	0	0	0	
	Gait	1	1	1	1					Gait	0	0	0	0			
	Joint total	2	2	16	2	1	10	33+1=34		Joint total	0	0	2	0	0	0	2
19	Swelling	0	0	0	0	0	0		71	Swelling	0	0	0	3	0	2	

	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	2	0	1		
	Axial alignment	0	0	0	0					Axial alignment	1	1	1	1				
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	1	0	1		
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	3	3	1	2		
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	3	0	0		
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	0	0	0	0			Joint pain	0	0	1	1	1	1		
	Strength	0	0	0	0	0	0			Strength	1	1	2	3	1	2		
	Gait	0	0	0	0					Gait	1	1	2	2				
	Joint total	0	0	0	0	0	0	0		Joint total	3	3	10	19	3	9	47+2=49	
	Swelling	0	0	1	1	0	0			Swelling	0	0	0	0	0	0		
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	0	0	1	1	1	1			Muscle atrophy	0	0	0	0	0	0		
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0				
	Crepitus on motion	0	0	1	1	0	0			Crepitus on motion	0	0	0	0	0	0		
21	Flexion loss	0	0	2	2	3	0		72	Flexion loss	0	0	0	0	0	0		
	Extension loss	0	0	1	2	2	0			Extension loss	0	0	0	0	0	0		
	Instability	0	0	1	1	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	1	1	1	1			Joint pain	0	0	1	1	0	0		
	Strength	0	0	4	4	2	2			Strength	0	0	1	1	0	0		
	Gait	1	1	3	3					Gait	0	0	0	0				
	Joint total	1	1	16	17	9	4	48+3= 51		Joint total	0	0	2	2	0	0	4	
	Swelling	0	0	0	0	0	0			Swelling	0	0	2	2	1	1		
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	1	1	0	0		
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	1	1	1	2	0	0		
	Axial alignment	0	0	0	0					Axial alignment	0	0	1	1				
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	0	0		
22	Flexion loss	0	0	1	0	1	1		73 Inhib	Flexion loss	0	0	2	0	2	2		
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	0	0	0		
	Instability	0	0	1	0					Instability	0	0	1	1	0	0		
	Joint pain	0	0	1	1	0	0			Joint pain	1	1	0	0	0	0		
	Strength	1	1	1	1	0	0			Strength	1	2	2	3	1	1		
	Gait	1	1	2	2					Gait	1	1	2	3				
	Joint total	2	2	7	4	1	1	17+2=19		Joint total	4	5	14	13	4	4	44+3=47	
23	Swelling	0	0	0	0	0	0		74	Swelling	0	0	0	0	0	0		
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0		

	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	0	0	0	0	0	0	
	Strength	0	0	0	0	0	0			Strength	0	0	0	0	0	0	
	Gait	0	0	0	0					Gait	0	0	0	0			
	Joint total	0	0	0	0	0	0	0		Joint total	0	0	0	0	0	0	0
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	0	1	1	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	1	0	1	1	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	1	1	0	
24	Axial alignment	0	0	0	0					Axial alignment	1	0	1	1			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	1	0	0	
	Flexion loss	0	0	0	0	0	0		75	Flexion loss	0	0	3	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	1	3	0	
	Instability	0	0	0	0					Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	1	1	
	Strength	0	0	0	0	0	0			Strength	1	1	2	1	3	2	
	Gait	0	0	0	0	0	0			Gait	2	1	2	3			
	Joint total	0	0	0	0	0	0	0		Joint total	5	3	14	9	9	5	45+3=48
	Swelling	0	0	0	0	0	1			Swelling	0	0	2	3	1	1	
	Duration swelling	0	0	0	0	0	1			Duration swelling	0	0	1	0	1	1	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	1	1	2	1	1	
	Axial alignment	0	0	0	0					Axial alignment	1	1	1	1			
	Crepitus on motion	0	0	0	0	0	1			Crepitus on motion	0	0	1	1	0	0	
25	Flexion loss	0	0	0	0	0	1		76	Flexion loss	1	1	3	0	0	0	
	Extension loss	0	0	0	0	1	0			Extension loss	1	1	3	3	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	1	1	0	0	
	Joint pain	0	0	0	0	0	1			Joint pain	0	0	2	2	0	0	
	Strength	0	0	0	0	0	2			Strength	2	2	3	4	1	1	
	Gait	0	0	0	0					Gait	3	3	2	4			
	Joint total	0	0	0	0	1	7	8		Joint total	9	9	20	21	4	4	67+4=71
	Swelling	0	0	0	0	0	0			Swelling	1	0	1	1	1	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	1	0	1	1	0	0	
26	Muscle atrophy	1	1	0	0	0	0		77	Muscle atrophy	1	1	1	1	1	1	
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	

	Flexion loss	1	1	0	0	0	0			Flexion loss	0	0	0	0	2	1		
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	1	0	0		
	Instability	0	1	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	1	1	0	0	0	0			Joint pain	0	0	1	1	0	0		
	Strength	1	1	0	0	0	0			Strength	1	2	2	2	1	1		
	Gait	1	1	0	0					Gait	1	1	2	2				
	Joint total	5	6	0	0	0	0	11+1=12		Joint total	5	4	8	9	5	3	34+2=36	
	Swelling	0	0	0	0	0	0			Swelling	5	4	8	9	5	3		
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0		
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	0	0	0	0		
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0				
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0		
27	Flexion loss	0	0	0	0	0	0		78	Flexion loss	0	0	0	0	0	0		
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0		
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0		
	Joint pain	0	0	1	1	0	0			Joint pain	0	0	0	0	0	0		
	Strength	0	0	1	1	0	0			Strength	0	0	0	0	0	0		
	Gait	1	1	1	1					Gait	0	0	0	0				
	Joint total	1	1	4	4	0	0	10+1=11		Joint total	0	0	0	0	0	0	0	
	Swelling	0	0	0	0	0	0			Swelling	1	0	1	1	0	0		
	Duration swelling	0	0	0	0	0	0			Duration swelling	1	1	1	1	0	0		
	Muscle atrophy	0	0	1	1	2	2			Muscle atrophy	1	1	2	1	1	1		
	Axial alignment	1	1	1	1					Axial alignment	0	0	1	1				
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0		
28	Flexion loss	0	0	1	1	0	0		79	Flexion loss	0	0	3	0	2	1		
	Extension loss	0	0	0	1	1	2			Extension loss	1	1	1	0	1	0		
	Instability	0	0	1	1	1	1			Instability	0	0	0	0	0	0		
	Joint pain	1	0	1	1	1	1			Joint pain	0	0	0	0	0	0		
	Strength	1	1	2	2	4	4			Strength	2	1	3	2	1	1		
	Gait	1	1	2	2					Gait	1	1	2	2				
	Joint total	4	2	9	10	9	10	44+3=47		Joint total	7	5	14	8	5	3	42+2=44	
	Swelling	0	0	0	0	0	0			Swelling	0	0	0	0	2	0		
	Duration swelling	0	0	0	0	0	0			Duration swelling	1	0	0	0	1	0		
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	1	0	0	0	0	0		
29	Axial alignment	0	0	0	0				80	Axial alignment	0	0	0	0				
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0		
	Flexion loss	0	0	0	0	1	0			Flexion loss	0	0	3	3	0	0		
	Extension loss	0	0	0	0	1	0			Extension loss	0	0	0	0	0	3		

	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	1	1			Joint pain	1	0	0	0	2	1	
	Strength	0	0	0	0	1	1			Strength	1	0	1	1	4	1	
	Gait	0	0	0	0					Gait	1	1	0	1			
	Joint total	0	0	0	0	4	2	6		Joint total	5	1	4	5	9	5	29+1=30
	Swelling	0	2	0	0	0	0			Swelling	0	0	0	0	0	0	
	Duration swelling	0	1	0	0	0	0			Duration swelling	0	0	0	0	2	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	0	0	1	1	
	Axial alignment	0	1	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	2	0	0	0	0			Crepitus on motion	0	0	0	0	1	0	
30	Flexion loss	0	1	0	0	0	0		81	Flexion loss	0	2	0	0	3	2	
	Extension loss	0	1	0	1	0	0			Extension loss	0	0	0	0	3	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	2	0	0	0	0			Joint pain	1	1	0	0	1	0	
	Strength	0	4	0	0	0	0			Strength	1	1	0	0	3	0	
	Gait	1	2	1	1					Gait	1	2	1	1			
	Joint total	1	16	1	3	0	0	21+2=23		Joint total	3	6	1	1	14	3	28+2=30
	Swelling	0	0	1	1	0	1			Swelling	0	0	3	2	0	1	
	Duration swelling	0	0	0	0	0	1			Duration swelling	0	0	0	0	0	1	
	Muscle atrophy	0	0	1	1	1	1			Muscle atrophy	1	1	2	2	0	0	
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	1	0	0	1			Crepitus on motion	0	0	0	0	0	0	
31	Flexion loss	1	0	3	3	N	1		82	Flexion loss	0	0	2	2	3	0	
	Extension loss	1	1	1	1	N	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	1	0	1	0			Instability	0	0	0	0	0	0	
	Joint pain	1	1	1	1	1	1			Joint pain	1	1	0	0	1	1	
	Strength	1	1	1	2	2	3			Strength	2	1	3	3	3	2	
	Gait	2	2	2	1					Gait	1	1	1	2			
	Joint total	6	5	13	11	5	9	49+3=52		Joint total	5	4	11	11	7	5	43+2=45
	Swelling	0	0	2	0	0	0			Swelling	1	1	1	2	0	0	
	Duration swelling	0	0	1	0	0	0			Duration swelling	0	1	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	1	1	2	2	1	2	
	Axial alignment	0	0	1	1	0	0			Axial alignment	1	1	1	1			
32	Crepitus on motion	0	0	1	0	0	0		83	Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	1	1	3	3	0	1			Flexion loss	0	0	3	3	1	0	
	Extension loss	0	0	0	3	0	0			Extension loss	0	0	2	0	0	0	
	Instability	0	0	1	1	0	0			Instability	0	0	0	0	0	0	

	Joint pain	0	0	2	2	0	1			Joint pain	1	1	1	1	1	1	
	Strength	1	1	4	4	1	1			Strength	2	2	2	3	2	3	
	Gait	2	2	2	4	1	1			Gait	1	0	2	3			
	Joint total	4	4	17	19	2	4	50+3=53		Joint total	7	6	14	15	5	6	53+3=56
	Swelling	0	0	2	0	0	0			Swelling	1	1	1	1	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	0	1	1			Muscle atrophy	1	1	1	0	2	2	
	Axial alignment	0	0	1	1					Axial alignment	1	1	1	1			
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
33	Flexion loss	1	1	3	1	2	2		84	Flexion loss	0	0	3	3	3	1	
	Extension loss	0	0	3	0	0	0			Extension loss	0	0	2	0	2	0	
	Instability	0	0	1	1	O	O			Instability	0	0	0	0	0	0	
	Joint pain	0	0	2	1	1	1			Joint pain	0	0	1	1	1	1	
	Strength	1	1	4	3	3	3			Strength	1	2	2	1	2	3	
	Gait	1	1	3	2					Gait	1	2	3	3			
	Joint total	3	3	21	9	7	7	50+3=53		Joint total	5	7	14	10	10	7	53+3=56
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	1	0	0	0	0			Muscle atrophy	1	0	2	1	0	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	1	1			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
34	Flexion loss	0	0	0	0	0	0		85	Flexion loss	1	0	3	3	0	1	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	1	1	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	1	1	1	1	1	1	
	Strength	0	1	0	0	0	0			Strength	1	1	2	3	1	0	
	Gait	0	0	0	0					Gait	1	2	1	3			
	Joint total	0	2	0	0	0	0	2		Joint total	5	4	13	14	2	2	40+3=43
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	0	0	0	
	Axial alignment									Axial alignment	0	0	0	0			
35	Crepitus on motion	0	0	0	0	0	0		86	Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	0	0	0	0			Flexion loss	0	0	1	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	0	0	1	0	0	0	

	Strength	0	0	0	0	0	0			Strength	0	0	2	0	0	1	
	Gait	0	0	0	0					Gait	0	0	1	1			
	Joint total	0	0	0	0	0	0	0		Joint total	0	0	7	1	0	1	9+1=10
	Swelling	0	0	1	2	0	0			Swelling	0	0	0	1	0	0	
	Duration swelling	0	0	1	1	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	1	1	1	0	0			Muscle atrophy	0	0	0	1	0	0	
	Axial alignment	1	0	0	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	1	0	0			Crepitus on motion	0	0	0	0	0	0	
36 Inhib	Flexion loss	1	1	2	2	1	1		87	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	3	3	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	1	1	1	2	0	0			Joint pain	0	0	1	1	0	0	
	Strength	1	1	3	4	0	0			Strength	0	0	0	1	1	1	
	Gait	1	1	2	3					Gait	1	1	1	2			
	Joint total	5	5	14	20	1	1	46+3=49		Joint total	1	1	2	6	1	1	12+2=14
	Swelling	0	0	0	1	0	0			Swelling	0	0	1	0	0	1	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	0			Muscle atrophy	0	0	1	1	0	2	
	Axial alignment	0	0	1	1					Axial alignment	0	0	1	1			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
37	Flexion loss	0	0	1	2	1	1		88	Flexion loss	0	0	2	1	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	1	0	0	1	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	1	1	0	0			Joint pain	0	0	1	1	0	1	
	Strength	0	0	1	2	0	0			Strength	0	0	2	1	0	2	
	Gait	0	0	1	1					Gait	0	1	2	2			
	Joint total	0	0	5	9	1	1	16+1=17		Joint total	0	1	11	7	0	7	26+2=28
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	1	0	0	0	
	Muscle atrophy	1	0	0	0	1	0			Muscle atrophy	1	0	1	0	0	0	
	Axial alignment	0	0	1	1					Axial alignment	1	0	1	1			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
38	Flexion loss	0	0	0	2	0	0		89	Flexion loss	0	0	1	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	1	0	0	0	
	Joint pain	1	1	0	0	1	0			Joint pain	0	0	1	1	0	0	
	Strength	1	1	2	1	2	0			Strength	1	1	1	0	0	0	

	Gait	1	1	1	1					Gait	0	1	1	1			
	Joint total	4	3	4	5	4	0	20+2=22		Joint total	3	2	10	3	0	0	18+1=19
	Swelling	0	0	0	0	0	0			Swelling	0	0	2	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	1	0	0	0	
	Muscle atrophy	0	0	0	0	0	0			Muscle atrophy	0	0	1	0	0	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
39	Flexion loss	0	0	0	0	0	0		90	Flexion loss	0	0	2	0	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	0	0			Joint pain	0	0	2	0	1	1	
	Strength	0	0	0	0	0	0			Strength	0	0	1	0	1	1	
	Gait	0	0	0	0					Gait	0	0	0	0			
	Joint total	0	0	0	0	0	0	0		Joint total	0	0	10	0	2	2	14
	Swelling	0	0	1	0	0	0			Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	1	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	1	0	0	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Axial alignment	1	1	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	0	0	0	
40	Flexion loss	1	1	0	1	0	0		91	Flexion loss	0	0	0	0	0	0	
	Extension loss	0	0	1	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	1	0	1	0	0	0			Joint pain	0	0	1	0	0	0	
	Strength	2	0	3	0	0	0			Strength	0	0	1	0	0	0	
	Gait	1	1	2	1					Gait	0	0	1	0			
	Joint total	7	3	11	3	0	0	24+2=26		Joint total	0	0	3	0	0	0	3+1=4
	Swelling	0	0	1	0	0	0			Swelling	0	0	0	2	1	0	
	Duration swelling	0	0	1	0	0	0			Duration swelling	0	0	0	1	0	0	
	Muscle atrophy	0	0	2	0	2	0			Muscle atrophy	0	1	0	1	2	0	
	Axial alignment	0	0	1	1					Axial alignment	1	1	1	1			
	Crepitus on motion	0	0	1	0	0	0			Crepitus on motion	0	0	0	1	1	0	
41	Flexion loss	0	0	3	0	2	0		92	Flexion loss	0	0	0	3	1	0	
	Extension loss	0	0	1	0	2	0			Extension loss	1	0	0	0	2	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	1	0	1	0	1	0			Joint pain	0	1	0	2	1	0	
	Strength	1	0	3	0	4	0			Strength	1	1	0	2	3	1	
	Gait	1	1	2	2					Gait	1	0	1	1			

	Joint total	3	1	16	3	9	0	32+2=34		Joint total	4	4	2	14	11	1	36+1=37
42	Swelling	0	0	1	0	0	0		93	Swelling	0	0	0	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	0	0	0	0	0	0	
	Axial alignment	1	1	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	1	0	0	0	0	0			Flexion loss	0	0	0	0	0	0	
	Extension loss	1	0	1	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	1	0	1	0	0	0			Joint pain	0	0	1	0	0	0	
	Strength	1	0	2	0	0	0			Strength	0	0	1	0	0		
	Gait	1	1	2	2					Gait	0	0	1	0			
	Joint total	6	2	8	2	0	0	18+2=20		Joint total	0	0	3	0	0	0	3+1=4
43	Swelling	0	0	0	0	0	0		94	Swelling	1	1	2	1	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	1	0	0	0	
	Muscle atrophy	0	0	1	0	0	0			Muscle atrophy	1	1	2	2	0	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	1	0	0	0	
	Flexion loss	0	0	0	0	1	0			Flexion loss	1	1	3	3	0	2	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	3	3	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	1	1			Joint pain	1	0	1	1	0	0	
	Strength	0	0	0	0	1	1			Strength	1	1	3	3	0	0	
	Gait	0	0	0	0					Gait	1	1	3	3			
	Joint total	0	0	1	0	3	2	6		Joint total	6	5	19	16	0	2	48+4=52
44	Swelling	0	0	1	1	0	0		95	Swelling	1	1	1	2	0	2	
	Duration swelling	0	0	0	0	0	0			Duration swelling	1	0	0	1	0	1	
	Muscle atrophy	0	0	1	1	0	1			Muscle atrophy	1	0	1	1	1	0	
	Axial alignment	0	0	1	1					Axial alignment	0	0	0	0			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	1	0	0	
	Flexion loss	0	0	1	1	3	3			Flexion loss	1	1	3	3	1	Ne	
	Extension loss	0	0	0	0	0	2			Extension loss	0	0	0	0	0	Ne	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	2	2	0	1			Joint pain	1	0	1	2	0	0	
	Strength	0	0	4	4	0	3			Strength	1	1	2	2	1	0	
	Gait	1	1	3	3					Gait	1	1	2	3			
	Joint total	1	1	13	13	3	10	41+3=44		Joint total	7	4	10	15	3	3	42+2=44
45	Swelling	0	0	0	1	0	0		96	Swelling	1	1	1	2	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	1	1	1	1	0	0	

	Muscle atrophy	1	1	1	1	0	0			Muscle atrophy	1	1	1	2	0	1	
	Axial alignment	0	0	1	1					Axial alignment	0	1	1	1			
	Crepitus on motion	0	0	1	1	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	3	0	0	0			Flexion loss	0	0	1	2	0	0	
	Extension loss	0	0	3	0	0	0			Extension loss	1	1	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	1	0	0			Joint pain	0	0	1	1	0	1	
	Strength	1	1	3	4	0	0			Strength	1	2	3	2	1	1	
	Gait	2	2	3	2					Gait	1	2	2	3			
	Joint total	4	4	14	11	0	0	33+4=37		Joint total	6	9	11	14	1	3	54+4=58
	Swelling	0	0	0	0	0	0			Swelling	0	0	1	1	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	0	0	0	0	
	Muscle atrophy	0	0	0	1	0	1			Muscle atrophy	0	0	1	1	0	0	
	Axial alignment	0	0	0	0					Axial alignment	0	0	1	1			
	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
46 Inhib	Flexion loss	0	0	0	0	0	0		97	Flexion loss	0	0	3	2	0	0	
	Extension loss	0	0	0	0	0	0			Extension loss	0	0	0	0	0	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	1	O	1			Joint pain	0	0	0	0	0	0	
	Strength	0	0	1	1	0	1			Strength	0	0	1	2	1	1	
	Gait	1	1	1	1					Gait	1	1	2	2			
	Joint total	1	1	2	4	0	3	11+1=12		Joint total	1	1	9	9	1	1	22+2=24
	Swelling	0	0	0	0	0	0			Swelling	1	1	1	0	0	0	
	Duration swelling	0	0	0	0	0	0			Duration swelling	0	0	1	0	0	0	
	Muscle atrophy	0	0	0	0	1	1			Muscle atrophy	1	0	1	0	1	1	
	Axial alignment	0	0	0	0					Axial alignment	0	0	1	1			
47	Crepitus on motion	0	0	0	0	0	0			Crepitus on motion	0	0	0	0	0	0	
	Flexion loss	0	0	0	0	0	0		98	Flexion loss	0	0	1	1	0	0	
	Extension loss	0	0	0	0	1	1			Extension loss	1	0	0	0	1	0	
	Instability	0	0	0	0	0	0			Instability	0	0	0	0	0	0	
	Joint pain	0	0	0	0	1	1			Joint pain	1	1	1	0	0	0	
	Strength	0	0	0	0	1	1			Strength	1	1	3	0	2	1	
	Gait	0	0	0	0					Gait	1	1	2	3			
	Joint total	0	0	0	0	4	4	8		Joint total	6	4	11	5	4	2	32+3=35

Table A3

Thrombin Generation data at different concentration of tissue factor trigger

	Thrombin generation at 1 pmol/L of tissue factor					Thrombin generation at 3 pmol/L of tissue factor					Thrombin generation at 5 pmol/L of tissue factor				
	Lagtime (min)	ETP (nM.min)	Peak (nM)	ttpeak (min)	Velocity Index (nM/min)	Lagtime (min)	ETP (nM.min)	Peak (nM)	ttpeak (min)	Velocity Index (nM/min)	Lagtime (min)	ETP (nM.min)	Peak (nM)	ttpeak (min)	Velocity Index (nM/min)
Normal range	0-31.11	0-566	0-44.41	0-101.22	0.17-1.83	2-13.33	0-1570	17.15-109.57	5.11-102.51	0.37-26.70	1.67-5.17	0-1988	29.17-221.35	3.83-51.00	1-55.41
Individual															
1	8.33	215	6.99	27.67	0.36	2.67	576	39.22	9.33	5.89	2.11	707	79.63	5.67	22.37
2	13.67	238	12.73	28.17	0.88	2.67	664	48.31	11.89	5.24	2.11	967	84.77	9.44	11.56
3	17.75	0	12.97	41.17	0.55	3.67	561	30.45	15.67	2.54	3.08	0	80.99	9.92	11.84
4	5.75	0	23.53	23.33	1.34	2.75	845	62.85	10.42	8.19	2.25	978	112.02	7.08	23.19

5		12.11	378	15.30	28.44	0.94	4.11	779	54.07	14.22	5.35	2.89	814	74.26	10.44	9.84
6		5.42	0	24.43	39.50	0.72	3.17	910	95.39	8.50	17.90	2.50	859	166.22	5.50	55.41
7		12.83	171	5.24	34.00	0.25	3.78	578	28.32	16.33	2.26	3.00	682	40.84	11.83	4.63
8		5.56	223	11.26	19.33	0.82	2.78	820	70.25	9.22	10.91	2.33	1013	151.11	5.89	42.45
9		12.67	152	4.65	31.00	0.25	3.56	510	30.32	12.78	3.29	2.78	800	69.22	7.78	13.84
10		10.00	0	16.62	49.83	0.42	3.22	891	55.68	11.33	6.87	2.56	1015	96.56	8.33	16.73
11		7.22	256	12.32	20.22	0.95	2.89	624	48.16	10.11	6.67	2.44	738	75.09	7.67	14.36
12		5.67	284	11.11	22.00	0.68	2.78	694	42.62	12.11	4.57	2.11	972	92.95	7.56	17.06
13	Inhib	5.83	450	13.55	24.67	0.72	3.78	785	41.47	18.11	2.89	3.11	835	55.45	14.33	4.94
14		9.50	0	44.41	89.00	0.56	3.00	0	77.68	17.67	5.30	2.33	0	104.02	11.00	12.00
15		13.17	0	13.10	42.00	0.45	3.33	593	37.60	12.42	4.14	2.67	850	73.23	7.83	14.19
16		13.80	0	17.99	62.23	0.37	4.50	700	45.46	14.18	4.70	3.44	1005	94.77	9.20	16.45
17		9.00	0	17.90	72.49	0.28	3.44	0	52.93	48.61	1.17	2.67	1128	104.74	7.28	22.72
18		7.85	0	22.56	70.67	0.36	3.25	0	46.45	101.94	0.47	2.87	1043	106.66	7.28	24.19
19		12.65	0	20.13	94.36	0.25	3.54	0	64.82	102.51	0.65	2.48	715	75.85	7.56	14.93
21		14.18	0	13.87	49.00	0.40	4.40	786	38.04	18.11	2.77	3.34	1162	65.96	12.84	6.94
22		8.81	0	21.85	33.84	0.87	3.54	985	67.55	10.92	9.15	2.96	1129	120.36	7.37	27.29
23		17.35	0	21.20	53.41	0.59	3.82	803	45.91	12.84	5.09	3.25	0	118.74	8.62	22.11
24		11.00	0	21.74	80.78	0.31	2.67	716	44.27	11.33	5.11	2.50	955	82.99	8.00	15.09
25		4.33	0	30.25	47.33	0.70	2.33	828	45.68	12.33	4.57	1.83	874	89.55	7.00	17.32
26		8.00	0	25.64	98.33	0.28	3.00	490	28.17	14.89	2.37	2.44	734	42.93	12.67	4.20
27		6.00	0	18.66	45.67	0.47	3.44	413	17.15	19.33	1.08	2.89	852	51.06	11.33	6.05
28		5.33	0	33.24	96.67	0.36	2.33	0	64.46	20.67	3.51	2.00	747	68.85	7.56	12.38
29		9.17	0	19.94	54.33	0.44	3.50	0	56.09	22.17	3.00	2.50	981	122.16	6.50	30.54
30		10.17	0	12.31	49.67	0.31	3.67	840	35.96	17.67	2.57	3.33	1094	51.97	14.78	4.54
31		0.00	0	0.00	0.00		3.17	519	27.05	16.00	2.11	2.56	746	45.94	13.56	4.18
32		9.56	0	14.49	70.22	0.24	2.78	554	30.07	13.78	2.73	2.44	619	47.42	10.44	5.93
33		4.67	0	31.10	44.83	0.77	2.22	858	67.57	10.00	8.69	2.00	841	120.90	5.89	31.08
34		4.50	0	29.67	45.67	0.72	2.33	1030	83.83	8.67	13.22	2.00	1075	166.50	5.56	46.77
35		5.33	0	38.80	35.33	1.29	2.67	875	104.50	8.22	18.83	2.00	807	150.90	5.33	45.32
36	Inhib	11.17	0	6.98	53.33	0.17	4.00	462	17.97	18.56	1.23	3.33	665	31.01	14.33	2.82
37		6.50	0	14.08	43.50	0.38	2.78	813	46.99	10.00	6.51	2.22	1085	90.13	6.22	22.53
38		6.33	259	10.82	22.67	0.66	2.67	855	47.27	13.00	4.58	2.33	1086	74.60	10.00	9.73
39		9.33	0	17.35	46.00	0.47	2.83	445	25.01	14.33	2.17	2.33	811	61.13	9.67	8.33
40		6.89	273	11.45	23.00	0.71	2.78	705	42.59	13.56	3.95	2.33	962	78.61	9.11	11.59
41		5.50	290	12.78	21.00	0.82	2.89	993	66.98	11.11	8.15	2.33	1058	91.95	8.56	14.76

42	6.63	0	21.28	70.00	0.34	3.45	0	51.30	23.52	2.56	2.62	0	98.62	19.34	5.90
43	10.14	0	11.72	55.62	0.26	4.12	0	25.24	40.41	0.70	2.95	836	35.10	14.82	2.96
44	15.32	0	13.32	56.62	0.32	4.29	539	25.94	19.84	1.67	3.95	893	39.80	17.50	2.94
45	10.34	0	16.52	45.86	0.47	2.32	0	37.55	47.42	0.83	2.09	975	119.73	6.55	26.85
46 Inhib	7.17	0	25.56	95.83	0.29	2.83	0	54.59	14.17	4.81	2.17	929	103.07	5.83	28.16
47	6.17	0	21.82	84.67	0.28	4.17	0	45.19	20.17	2.82	2.17	0	84.36	9.50	11.51
48	7.67	0	10.21	36.83	0.35	3.17	542	27.32	13.33	2.69	2.67	0	37.30	12.83	3.67
49	13.00	0	18.44	87.67	0.25	4.50	0	49.30	43.50	1.26	3.00	0	80.44	22.33	4.16
50	19.83	0	16.37	85.50	0.25	13.33	0	27.90	59.33	0.61	5.17	0	45.61	51.00	1.00
51	8.50	0	25.92	50.93	0.61	3.49	1032	54.84	14.68	4.90	2.82	1237	112.28	8.50	19.77
52	6.67	363	13.87	23.33	0.83	3.67	1442	48.95	14.00	4.74	2.67	1533	106.93	8.17	19.44
53	6.67	261	8.38	27.17	0.41	4.11	582	25.42	21.67	1.45	2.83	815	52.28	15.00	4.30
54	6.67	127	4.62	25.67	0.24	2.89	462	22.85	15.00	1.89	2.33	437	29.17	11.44	3.20
55	10.67	0	14.66	54.50	0.33	3.67	557	31.64	15.67	2.64	2.67	892	61.42	10.33	8.02
56	15.89	303	10.26	39.67	0.43	3.89	513	26.13	19.11	1.72	3.00	653	51.37	11.44	6.09
57	8.67	228	6.21	33.83	0.25	4.00	876	37.09	14.17	3.65	3.00	1318	83.58	7.50	18.57
58	5.50	0	44.37	47.00	1.07	2.44	1570	93.35	12.67	9.13	2.00	1884	175.41	8.78	25.87
59	7.00	0	23.98	51.50	0.54	3.17	1007	69.68	9.17	11.61	2.50	995	122.53	6.67	29.38
60	11.00	0	9.97	57.83	0.21	3.33	659	30.50	12.50	3.33	2.67	1016	63.98	7.50	13.25
61	8.00	0	28.09	80.00	0.39	2.83	0	49.47	34.67	1.55	2.33	0	115.98	7.33	23.20
62	8.83	0	11.73	42.83	0.35	3.33	744	29.40	17.00	2.15	2.83	849	53.28	10.83	6.66
65	14.18	0	13.87	49.00	0.40	4.40	786	38.04	18.11	2.77	3.34	1162	65.96	12.84	6.94
66	7.93	490	24.46	21.29	1.83	3.14	887	83.14	10.15	11.86	2.36	1056	136.23	7.48	26.61
70	16.83	0	21.12	90.83	0.29	5.67	0	41.60	40.17	1.21	4.17	0	60.66	37.33	1.83
71	5.26	268	11.81	22.62	0.68	2.81	690	40.83	15.28	3.27	2.70	667	51.29	13.38	4.80
72	6.09	0	26.08	40.49	0.76	2.75	0	52.30	33.31	1.71	1.92	798	60.06	11.82	6.07
73 Inhib	9.04	0	16.05	68.60	0.27	4.03	613	29.58	15.16	2.66	3.42	1481	42.42	13.77	4.10
74	6.59	0	25.26	90.42	0.30	2.81	0	58.00	32.42	1.96	2.25	848	64.24	11.60	6.87
75	7.71	360	12.72	26.41	0.68	3.59	779	39.85	16.39	3.11	2.92	978	62.70	12.94	6.26
76	12.33	0	13.20	75.00	0.21	3.33	572	23.32	17.33	1.67	2.78	809	54.05	10.33	7.16
77	14.67	0	14.97	79.00	0.23	4.17	0	45.91	55.83	0.89	3.00	0	76.14	24.00	3.63
78	11.33	0	18.51	77.50	0.28	4.00	0	46.97	76.00	0.65	2.67	0	71.23	20.17	4.07
79	4.17	0	43.02	94.50	0.48	2.00	606	83.04	5.11	26.70	1.83	0	80.05	3.83	40.03
80	31.11	0	11.40	73.11	0.27	2.50	1447	109.57	8.83	17.31	2.33	1988	221.35	6.67	51.00
81	24.83	0	10.61	79.83	0.19	8.50	0	29.09	46.00	0.78	5.00	0	49.61	38.17	1.50
82	20.78	0	18.34	70.89	0.37	7.22	0	34.96	101.00	0.37	4.33	0	47.34	32.33	1.69

83	5.33	0	14.64	35.89	0.48	2.67	567	29.83	15.33	2.36	2.33	697	44.04	13.22	4.04
84	14.67	0	24.82	58.44	0.57	2.00	612	40.14	10.33	4.82	1.67	750	69.73	7.22	12.56
85	5.33	0	25.78	35.00	0.87	3.11	863	45.29	14.78	3.88	2.78	1003	49.11	13.78	4.46
86	8.11	0	21.50	47.44	0.55	3.78	952	45.72	15.56	3.88	2.89	1065	85.59	8.56	15.10
87	3.89	0	19.05	40.00	0.53	2.33	850	57.89	6.56	13.69	1.78	534	91.45	4.44	34.38
88	6.33	0	11.71	37.44	0.38	2.33	444	24.40	12.11	2.49	2.11	639	45.90	9.11	6.56
89	7.67	0	20.76	101.22	0.22	3.11	0	42.85	31.56	1.51	2.67	0	90.32	9.33	13.56
90	5.78	0	22.90	30.33	0.93	3.33	416	24.92	16.50	1.89	2.00	889	72.76	11.00	8.08
91	10.33	0	13.17	52.11	0.32	4.17	0	28.38	42.17	0.75	2.89	659	45.33	14.33	3.96
92	7.89	0	26.04	60.22	0.50	3.00	491	27.66	15.33	2.24	2.33	930	82.79	9.33	11.83
93	4.50	566	24.66	19.50	1.64	2.67	1005	65.80	10.17	8.77	2.50	1042	113.89	7.00	25.31
94	9.50	0	44.41	89.00	0.56	3.00	0	77.68	17.67	5.30	2.33	0	104.02	11.00	12.00
95	7.33	0	24.05	35.67	0.85	3.00	897	50.66	13.50	4.82	2.67	878	89.63	8.33	15.84
96	6.56	383	15.15	24.67	0.84	3.00	872	57.05	14.33	5.04	2.33	895	92.29	9.11	13.61
97	4.78	488	19.79	18.67	1.42	3.00	991	101.84	7.33	23.52	3.33	1045	161.38	6.44	51.89
98	11.56	0	9.76	41.78	0.32	5.67	0	49.30	24.67	2.59	3.56	0	84.54	16.44	6.56

"0" is an assignment when no ETP was obtained, as described in the text (Chapter 6, section 6.1.2)