

Detection and Prediction of Factor VIII Antibody Formation in Congenital and Acquired Haemophilia A

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Detection and Prediction of Factor VIII Antibody
Formation in Congenital and Acquired
Haemophilia A

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BSc(Hons) MB BS MRCP

Submitted in partial fulfilment of the requirements of the
Degree of Doctor of Philosophy

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January 2016

Statement of Originality

I, Paul Andrew Batty, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

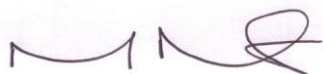
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A handwritten signature in purple ink, appearing to read 'P. A. Batty', with a stylized flourish at the end.

Date: 24th January 2016

Details of Collaborations

Testing of clinical samples (Nijmegen-Bethesda assay and Factor VIII ELISA) as part of Chapters 6 and 7 were performed as part of routine care within specialist haemostasis laboratories at The Royal London Hospital and St Thomas' Hospital. I designed and performed all analyses within these studies. I performed all subsequent experimental work optimising pre-analytical heat treatment incubation conditions.

The epitope mapping work described in Chapter 8, involved a collaboration with the Department of Biological Sciences and Institute of Molecular and Structural Biology, Birkbeck, University of London (Dr Adrian Shepherd, Reader in Computational Biology and Stuart Skelton, PhD Candidate) and the Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre, Milan, Italy (Prof Flora Peyvandi, Dr Elisa Mancuso and Dr Elena Santagostino). Local approvals were obtained and clinical samples collected by Dr Elisa Mancuso and her team. Samples were robotically loaded onto the Pepscan array by staff within Pepscan, Lelystad, Netherlands. Normalisation of solvent accessibility, distances between the alpha carbons and threading within pGENTHREADER were performed by Stuart Skelton. I designed, performed all analyses and interpreted all structural data from these experiments.

RNA extractions within Chapter 9, were performed within the Genome Centre Core Facility Charterhouse Square, London. I designed this study, collected all samples and performed all data analyses.

Details of Publications

The following publications have arisen from this thesis:

- 1) Batty P, Platton S, Bowles L, Pasi KJ, Hart DP. Pre-analytical heat treatment and a FVIII ELISA improve Factor VIII antibody detection in acquired haemophilia A. *Br J Haematol* 2014; 166(6):953-956.
- 2) Batty P, Palmer B, Chalmers E, Hay CR, Liesner R, Rangarajan S et al. A national survey of immunosuppression strategies for acquired haemophilia A. *Haemophilia* 2015; 21(1):e73-e76.
- 3) Batty P, Moore GW, Platton S, Maloney JC, Palmer B, Bowles L et al. Diagnostic accuracy study of a factor VIII ELISA for detection of factor VIII antibodies in congenital and acquired haemophilia A. *Thromb Haemost* 2015; 114(4):804-811.

The following abstracts (poster, P and oral communications, OC) have arisen from this thesis:

- 1) Batty P, Skelton S, Shepherd AJ, Hart DP. Amino acid sequence epitope mapping of four factor VIII monoclonal antibodies. *J Thromb Haemost* 2013; 11(Supplement S2):579. *ISTH, Amsterdam, 2013 (P)*
- 2) Batty P, Moore GW, Platton S, Maloney JC, Bowles L, Pasi J et al. Immunological detection of factor VIII antibodies in congenital and acquired haemophilia A. *J Thromb Haemost* 2013; 11(Supplement S2):930. *ISTH, Amsterdam, 2013 (P)*
- 3) Batty P, Platton S, Hart DP. The Bethesda assay in isolation significantly underestimates the presence of factor VIII antibodies in acquired haemophilia A. *Br J Haematol* 2013; 161(Supplement S1):13. *BSH, Liverpool, 2013 (OC)*
- 4) Batty P, Rangarajan S, Austin S, Khair K, Millar C, Stümpel J et al. Uptake and timing of inhibitor screening in non-severe hemophilia A: results of a pan-London evaluation. *Haemophilia* 2014; 20(Supplement S3):48. *WFH, Melbourne, 2014 (P)*
- 5) Batty P, Collins PW, Chalmers E, Hay CR, Liesner R, Palmer B et al. A UKHCDO National Survey of the management of acquired haemophilia A. *Haemophilia* 2014; 20(Supplement S2):31. *EAHAD, Brussels, 2014 (P)*

- 6) Batty P, Skelton S, Shepherd AJ, Hart DP. Amino acid sequence epitope mapping of anti-factor VIII monoclonal antibodies. *Br J Haematol* 2014; 165(Supplement S1):8. *BSH, Birmingham, 2014 (OC)*
- 7) Batty P, Skelton S, Shepherd AJ, Mancuso ME, Santagostino E, Peyvandi F et al. High throughput amino acid sequence epitope mapping of inhibitory antibodies in severe hemophilia A. *J Thromb Haemost* 2015; 13(Supplement S2):135. *ISTH, Toronto, 2015 (OC)*
- 8) Batty P, Mein C, Wozniak E, Hart DP. Reliable RNA retrieval from low-volume paxgene tubes in simulated "real world" next generation sequencing (RNA-SEQ): Gena-05 substudy pilot. *J Thromb Haemost* 2016; 13(Supplement S2):365. *ISTH, Toronto, 2015 (P)*
- 9) Batty P, Platton S, Hart DP. Optimisation of Pre-Analytical Heat Treatment for Factor VIII Antibody Detection. *Haemophilia* 2016; 22(Supplement S2):88. *EAHAD, Malmö, 2016 (P)*

Abstract

Factor VIII (FVIII) is a co-factor in the haemostatic system required for fibrin-rich clot formation. Inherited *F8* gene defects result in haemophilia A (HA), one of the commonest inherited bleeding disorders. Acquired FVIII defects (acquired haemophilia A, AHA) occur through auto-antibody formation. FVIII antibodies (allo and auto-antibodies) are the greatest challenges facing the haemophilia treating physician. Prediction of risk of antibody formation is based on genetic and environmental factors. There is incomplete understanding of the total immune response to FVIII due to limitations in current laboratory methodology used for FVIII antibody testing.

The aim of this work was to assess clinical practices, laboratory methodology and high-throughput approaches to further characterise the immune response to FVIII. The key findings are as follows:

- 1) Sub-optimal compliance with targeted inhibitor screening following FVIII treatment was seen in non-severe HA in London haemophilia centres.
- 2) A national survey of AHA demonstrated heterogeneity in the management of immunosuppression and testing.
- 3) A FVIII ELISA was specific with a high negative predictive values for FVIII antibody detection in routine practice.
- 4) Pre-analytical heat treatment prior to antibody testing, improved sensitivity for auto-antibody detection and a systematic evaluation optimised incubation conditions for this modification.
- 5) A novel re-usable high-throughput peptide microarray, characterised B-cell epitopes of monoclonal and polyclonal FVIII antibodies, demonstrating immunodominant epitopes in regions of functional or structural importance.
- 6) A modified low volume RNA sample tube demonstrated feasibility for transcriptome analysis in patients with severe haemophilia A, providing a repository of transcriptome data for developing understanding of the allo-immune response to FVIII.

Heterogeneity in clinical and laboratory practices limits interpretation of data from observational studies of FVIII antibodies. Improvements in detection and characterisation of FVIII antibodies, may further advance understanding of the total immune response to FVIII identifying biomarkers for risk stratification.

Dedication

This thesis is dedicated to Sofia, Alex, Cristina, Angela and Philip

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I am hugely fortunate to have had the opportunity to collaborate with a number of excellent academic units both nationally and internationally. The Computational Biology team at Birkbeck University, in particular Adrian Shepherd and Stuart Skelton have provided endless encouragement for the epitope mapping work. Stuart has taught me how to use many *in-silico* methodologies and always found time to explain these simply. The UKHCDO Inhibitor Working Party, in particular Prof Peter Collins, has been instrumental in the development of the national survey presented in this work and also the development of ideas surrounding inhibitor testing. Collaboration with the team working at the Milan haemophilia centre, in particular Flora Peyvandi and Marcin Gorski, on the B-cell epitope mapping work has been an amazing experience. I am also grateful for the teams at all of the London haemophilia centres for their help with data collection within the Pan-London audit. The support and statistical guidance offered by Ben Palmer at the National Haemophilia Database has been invaluable and I am grateful for the time he has taken to guide me over the last few years. I am grateful for the support of Chaz Mein and his team at the Genome Centre who have guided me through the RNA-Seq work. I am also grateful to all of the volunteers and patients who provided samples, with whom none of this work would have been possible.

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Finally, this work would not have been possible without the support, love and patience provided by my family from Sofia, Alex, Cristina and my parents who have allowed me to pursue these dreams.

List of Abbreviations

aa	Amino acids
ABR	Annualised bleed rate
AHA	Acquired haemophilia A
ANOVA	Analysis of variance
APC	Activated Protein C
aPTT	Activated partial thromboplastin time
ASA	Accessible surface area
ASGRP	Asialoglycoprotein receptor
BCR	B-cell receptor
BDD	B domain deleted
bFVIII:C	Baseline FVIII:C
BHK	Baby hamster kidney
bp	Base pairs
BSA	Bovine serum albumin
CANAL	Concerted action on neutralizing antibodies in severe haemophilia
CBA	Classical Bethesda assay
CCC	Comprehensive care centre
CCL3L1	Chemokine (C-C motif) ligand 3-like 1
CCL3L3	Chemokine (C-C motif) ligand 3-like 3
CD	Cluster of differentiation
ChBA	Chromogenic Bethesda assay
CHO	Chinese hamster ovary
CI	Confidence interval
CLEC4M	C-type lectin domain family 4 member M
CLIPS	Chemical linkage of peptides onto scaffolds
CPA	Clinical pathological accreditation
CR	Complete response
CTLA4	Cytotoxic T-lymphocyte-associated protein 4

CXCL7	Chemokine (C-X-C motif) ligand 7
CXCL9	Chemokine (C-X-C motif) ligand 9
CXCL11	Chemokine (C-X-C motif) ligand 11
DC	Dendritic cell
DDAVP	1-deamino-8-d-arginine vasopressin
Del	Deletion
DNA	Deoxyribonucleic acid
DOR	Diagnostic odds ratio
DSSP	Dictionary of protein secondary structure
EACH2	European acquired haemophilia registry
ECAT	European concerted action on thrombosis foundation
ED	Exposure days
ELISA	Enzyme linked immunosorbent assay
EQA	External quality assurance
EUHASS	European haemophilia safety surveillance
Ex	Exon
F8DP	Factor VIII deficient plasma
Fab	Fragment antigen binding
Fc	Fragment crystallisable region
FEIBA	Factor VIII inhibitor bypassing activity
FLI	Fluorescence-based immunoassays
FMOC	Fluorenylmethyloxycarbonyl
FVIII	Factor VIII
FVIII(a)	Factor VIII (activated)
FVIII:C	Factor VIII coagulant activity
FIX(a)	Factor IX (activated)
FX(a)	Factor X (activated)
GEE	Generalised estimating equations
GTH	Gesellschaft für thrombose-und hämostaseforschung

HA	Haemophilia A
HEK	Human embryonic kidney
HIGS	Hemophilia inhibitor genetic Study
HIPS	Hemophilia inhibitor PUP Study
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HR	Hazard ratio
HRes	High resolution epitope
HSPG	Heparin sulphate proteoglycans
HTC	Haemophilia treatment centre
IB	Immunoblotting
Ig	Immunoglobulin
IL	Interleukin
INSIGHT	International study on etiology of inhibitors in patients with a moderate or mild form of hemophilia A, influences of immuno Genetic & hemophilia treatment factors
IP	Immunoprecipitation
IQR	Interquartile range
ISTH	International society on thrombosis and haemostasis
ITI	Immune tolerance induction
LR	Likelihood ratio
LRP	LDL receptor related protein
LRes	Low-resolution epitope
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mRNA	messenger RNA
miRNA	micro RNA
MIBS	Malmö international brother Study
MNAR	Missing not at random
MR	Macrophage mannose receptor

NASCOLA	North American specialized coagulation laboratory association
NBA	Nijmegen-Bethesda Assay
NGS	Next generation sequencing
NNA	Non-neutralising antibody
NPV	Negative predictive value
OD	Optical density
OR	Odds ratio
pdFVIII	Plasma derived FVIII
PG	PAXgene
PHT	Pre-analytical heat treatment
PPV	Positive predictive value
PR	Partial response
QMUL	Queen Mary University London
qPCR	Quantitative polymerase chain reaction
rBDD-FVIII	Recombinant B domain deleted Factor VIII
RCSB	Research collaboratory for structural bioinformatics
rFVIIa	Recombinant activated Factor VII
rFL-FVIII	Recombinant full-length Factor VIII
rFVIII	Recombinant Factor VIII
RIN	RNA integrity number
RISE	Response to DDAVP In mild hemophilia A patients, in search for determinants
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RODIN	Research Of determinants of Inhibitor development
RR	Relative risk
RT	Room temperature
SACHA	Surveillance des auto anti-Corps au cours de L'hémophilie acquise
SCARA5	Scavenger receptor class A member 5
SD	Standard deviation

SE	Standard error
Siglec5	Sialic acid binding IgG-like lectin 5
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
SPR	Surface plasmon resonance
SSC	Scientific and standardization committee
STAB2	Stabilin-2
STARD	Standards for the reporting of diagnostic accuracy studies
TCR	T-cell receptor
T _H	Helper T-cell
T _{reg}	Regulatory T-cell
TNF- α	Tissue necrosis factor alpha
UK	United Kingdom
UKHCDO	United Kingdom haemophilia centre doctors' organisation
UKNEQAS	UK national quality external assessment scheme
USA	United States of America
VWF	von Willebrand factor
WFH	World federation of hemophilia

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Chapter 1: Introduction

1.1 Factor VIII Structure and Function

1.1.1 Cell Based Model of Haemostasis

The process of haemostasis is a tightly regulated chain of events that results in clot formation at the site of vascular injury. This system restricts clot formation to these sites and prevents inappropriate clot formation (thrombosis). Haemostasis has traditionally been divided into two distinct (primary and secondary), but overlapping phases. Primary haemostasis, results in vasoconstriction and formation of a platelet rich plug with platelet binding to exposed collagen. Secondary haemostasis occurs through a series of proteolytic reactions at the surface of tissue-factor (TF) bearing cells and activated platelets, resulting in formation of a stable fibrin rich clot. This process is currently summarised in a cell-based model¹, occurring over three phases: initiation, amplification and propagation (Figure 1.1). This series of proteolytic reactions results in the formation of a thrombin (Factor IIa) burst, which cleaves fibrinogen to fibrin¹. Fibrin forms a mesh-like network allowing stable clot formation, which is further mediated by Factor XIII (FXIII). Inherited or acquired deficiencies of these pro-coagulant constituents (factors), result in the development of a bleeding phenotype.

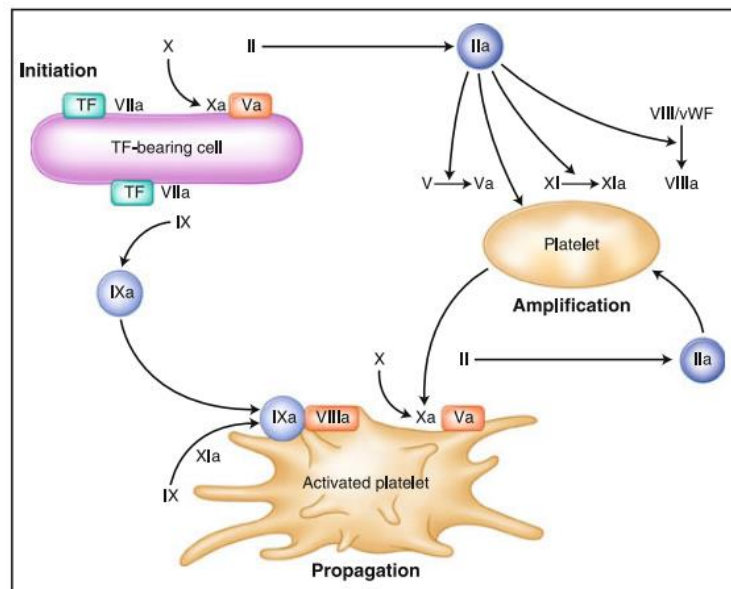


Figure 1.1: Cell Based Model of Haemostasis, consisting of three phases: initiation, amplification and propagation. This involves a series of proteolytic reactions occurring on the surface of TF expressing cells and platelets. The result of activation of this pathway is the production of thrombin (Factor IIa), which causes cleavage of fibrinogen to fibrin. Image reproduced from Woodruff et al. with the permission of the publisher².

Factor VIII (FVIII) is an important co-factor in this system, which upon activation increases the catalytic activity of activated Factor IXa (FIXa) in activating Factor X (FX), as part of the tenase complex (Figure 1.1). Understanding of the structure and function of this co-factor has progressed greatly since it was first characterised in 1984^{3;4}, through study of natural and artificial mutations and more recently through crystalline structures⁵⁻⁹. Advances in understanding of structural and functional properties of FVIII have resulted directly in improvements in the care of patients with inherited defects (haemophilia A) in FVIII function.

1.1.2 Factor VIII Gene (*F8*) and Sites of Synthesis

The FVIII gene (*F8*) is located on the distal part of the long arm of the X chromosome (Xq28) from base pair (bp) positions 154835788 to 155026940, covering 191153 base pairs (bp). The *F8* gene consists of 26 exons (69-3106bp) with a coding sequence of approximately 9000bp³. Although expression of FVIII has been demonstrated in many tissues including the spleen, placenta, kidney and lymph nodes¹⁰⁻¹², the liver represents the principal site of FVIII synthesis. This was identified, firstly through studies looking at mRNA expression and subsequently through animal model perfusion and transplantation studies¹³. This was confirmed *in-vivo* in patients with haemophilia A following liver transplantation resulting in normalisation of FVIII levels¹⁴. There has been controversy surrounding the predominant hepatic cellular source of FVIII with studies demonstrating mRNA expression in both hepatocytes and liver sinusoidal endothelial cells¹⁵. Recent data from a murine knockout model¹⁶ and from purified human liver sinusoidal endothelial cells¹⁷ has demonstrated that hepatic sinusoidal endothelial cells are the primary source of FVIII biosynthesis.

1.1.3 Factor VIII Structure and Domain Organisation

The *F8* gene encodes a multi-domain protein of 2351 amino acids (aa) (267kDa), comprising of a mature protein of 2332aa and signal protein of 19aa^{3;4}. The mature FVIII protein consists of 3 A domains (~370aa), 2 C domains (~160aa) and a B domain (908aa). These six domains are ordered as follows, A1-A2-B-A3-C1-C2 (Figure 1.2), forming a heavy (A1-A2-B) and light chain (A3-C1-C2) (Table 1.1). Sub-domains containing negatively charged amino acid residues are located at the carboxy-terminal ends of the A1 and A2 domains (a1 and a2) and the amino-terminus of the A3 domain (a3) (Figure 1.2 & Table 1.1). These regions also contain multiple sulphated tyrosine residues and are thought to play key roles in the function of FVIII.

Within FVIII structure there is inter-domain homology. The A domains share 31% homology with each other and with the A domains of hephaestin, ceruloplasmin and Factor V^{3;18}. The

C domains, share approximately 40% homology with each other and the C domains of Factor V. Sequence homology is also seen between the C domains of FVIII and those in milk fat globule-EGF factor 8 (MFGE8) and discoidin I ^{3;19;20}.

Domain	Legacy Position (aa)	HGVS Position (aa)	Length	Exons
Signal	-18 – 0	1 – 19	19	1
A1	1 – 336	20 – 355	336	1 – 8
a1	337 – 372	356 – 391	36	
A2	373 – 710	392 – 729	338	8 – 14
a2	711 – 740	730 – 759	30	
B	741 – 1648	760 – 1667	908	14
a3	1649 – 1689	1668 – 1708	41	14 – 19
A3	1690 – 2019	1709 – 2038	330	
C1	2020 – 2172	2039 – 2191	153	20 – 23
C2	2173 – 2332	2192 – 2351	160	24 – 26

Table 1.1: FVIII domain organisation, indicating start and finish positions within the mature in-active protein ^{6;15}.

The B domain shares no substantial homology with other known protein sequences or with the FVIII B domains found in other species ³. This domain, despite its length, is encoded by a single un-interrupted exon (exon 14). The B domain plays no significant role in the haemostatic function of FVIII and is cleaved upon activation of FVIII ²¹. This domain plays important intracellular roles (processing / trafficking) and protecting FVIII from premature activation or clearance from the circulation ²².

FVIII requires a number of co-ordinating metal ions to maintain its structural integrity and function ²³, which have been further characterised within the crystal structures of B domain deleted FVIII molecules. The first of these structure (resolution 3.98 Å) consists of heavy chain residues 1-740, a short linker sequence 741-754 and light chain 1649-2332 ⁶. The structure is poorly ordered in the heavy chain residues 17-43, 211-223, 334-376, 714-754 and light chain residues 1649-1690 and 1714-1724. The second (resolution 3.70 Å), presents a fully active (B domain deleted) hetero-dimeric structure of FVIII. This is poorly ordered over sequence positions 34-38, 213-227, 335-336 and a 155 long residue sequence spanning the end of the A2/a2 domain through the truncated B domain and the final three residues of the C2 domain ⁷. These models contain predicted copper (2 ions) and calcium (1-2 ions) ions ^{6;7}. The first copper ion position is modelled within the A3 domain, co-ordinated by His1954, His2005 and Cys2000. The second lies within the A1 domain, co-ordinated by His267, His315 and Cys310. The first calcium ion observed in both structures is modelled in the A1 domain, co-ordinated

by Glu110, Asp116, Asp125, Asp126, Lys107 and Glu122^{6,7}. The second is seen in the A2 domain co-ordinated by Asp538 and Asp542 in the first model only⁷.

1.1.4 Intra-Cellular Processing and Post Translational Modification

Prior to secretion into the circulation, the newly synthesised single chain FVIII polypeptide undergoes a number of post-translational modifications (Figure 1.2B)^{15;22;23}. Following synthesis, FVIII is translocated to the Endoplasmic Reticulum (ER) where the signal peptide (19aa) is cleaved. Glycosylation (N-linked) of asparagine residues occurs at consensus sequences (Asn-Xxx-Ser/Thr). Twenty five, potential N-linked glycosylation sites have been identified, of which 21 have been identified as carrying oligosaccharides. The majority of these (n=17) are located within the B domain, with additional sites seen in the A1 (n=2), A3 (n=1) and C1 domains (n=1)^{15;22;24}. For those seen in the A1 and C1 domains, high mannose glycans have been characterised at positions, Asn239 and Asn2118 respectively. Within the ER, disulphide bond formation occurs, with eight disulphide bonds being present; two in each A domain and one in each C domain¹⁵. Within the ER, the N-glycosylated FVIII undergoes folding, a process facilitated by a number of intercellular chaperones, including calnexin and calreticulin. If properly folded, FVIII leaves the ER in coated vesicles, which when uncoated form the Endoplasmic Reticulum-Golgi intermediate compartment (ERGIC). This process requires the trans-membrane mannose-binding lectin 1 (LMAN) and the luminal multiple coagulation factor deficiency protein 2 (MCFD2). This pathway is involved in transportation of both FV and FVIII from the ER to the Golgi, with mutations in LMAN resulting in combined deficiency in FV and FVIII²⁵. In the Golgi, further modifications occur which includes sulphation of tyrosine residues at six positions (346, 664, 719, 721, 723 and 1680), O-linked glycosylation (≥ 7 B domain sites)²⁶ and further modification of the N-linked oligosaccharides. Finally, prior to secretion, variable cleavage of the heavy chain occurs at the B-a3 domain junction (Arg1648) and within the B domain (Arg1313). This cleavage process is thought to occur through the action of an unknown protease from the protease furin/PACE (paired basic amino acid cleavage enzyme) family, based on an Arg-Xxx-Xxx-Arg motif seen at these cleavage points²³.

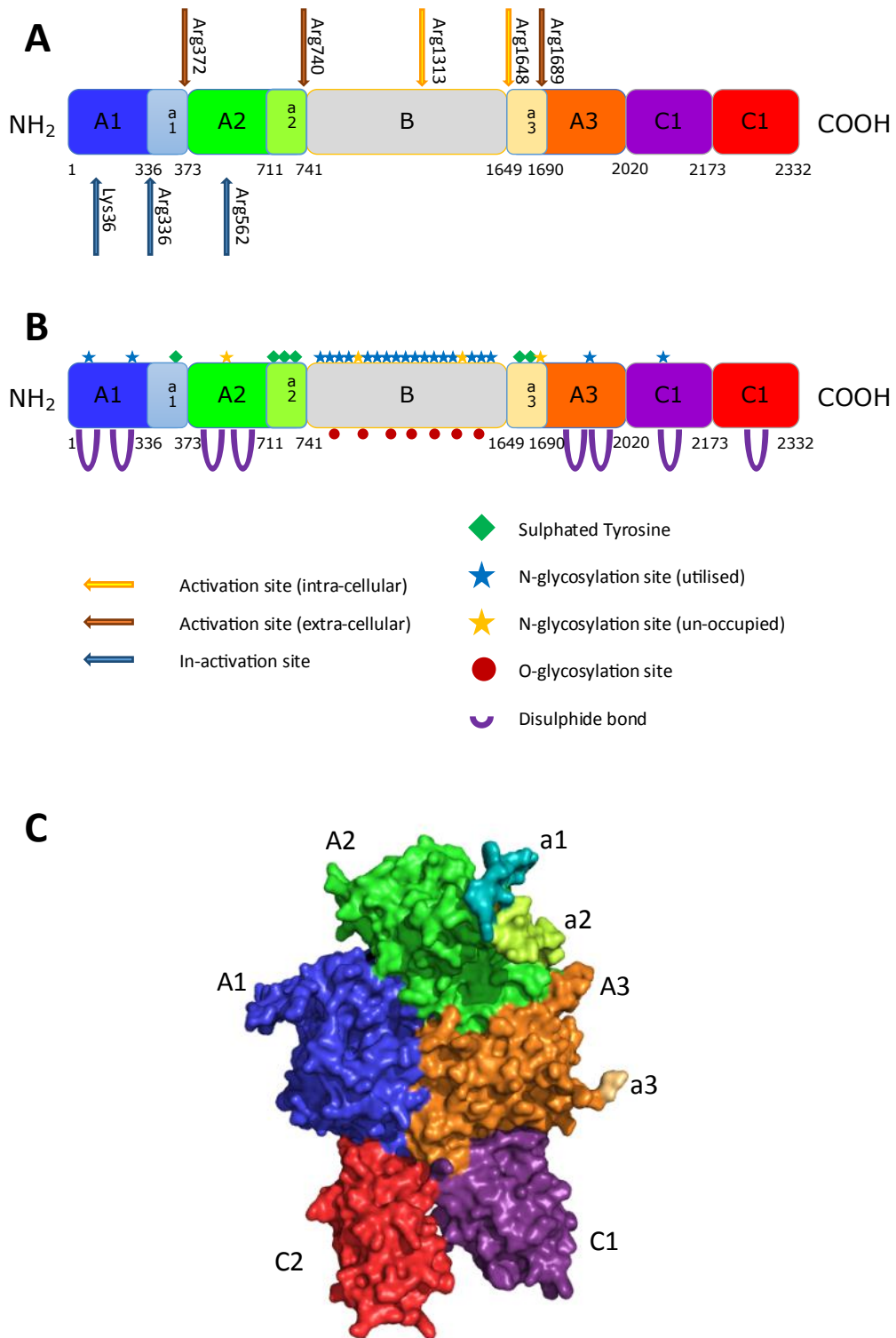


Figure 1.2: Factor VIII Structure and Domain Organisation. A: Two dimensional representation of FVIII demonstrating sites of activation and inactivation. B: Two dimensional representation of FVIII with domain related post-translational modification. C: Three dimensional crystal structure of B-domain depleted Factor VIII (2R7E)⁷ showing domain organisation, created using the PyMOL Molecular Graphics System.

1.1.5 Extra-Cellular Interaction with von Willebrand Factor

FVIII is then secreted into the circulation as a hetero-dimer consisting of a variably processed heavy chain (~90-200kDa) and light chain (~80kDa). These two chains are non-covalently bound with metal-ion dependent linkage between the A1 and A3 domains. Once in the circulation, FVIII rapidly associates with high affinity ($k_d \sim 0.3nM$) to a chaperone molecule, von Willebrand factor (VWF), with one molecule of FVIII binding to one monomer of the VWF multimer. VWF is a large multimeric glycoprotein (250kDa) secreted from the Weibel-Palade bodies of vascular endothelial cells (ECs). This interaction occurs through non-covalent binding between the a3, C1 and C2 domains of FVIII with the D'D3 domains of VWF^{27;28}, with the a3-VWF interaction demonstrating 8 fold greater binding affinity²⁹. The sequence positions of the interaction between FVIII and VWF have previously been mapped to positions 1649-1689 (a3), 2181-2243 (C2) and 2248-2312 (C2)²⁷. This interaction has recently been further characterised by electron microscopy and hydrogen-deuterium mass spectrometry in a recombinant FVIII-Fc fusion protein complexed to the VWF D'D3 domain. This demonstrated binding of VWF to the a3 (Val1670-Asp1678), A3 (Val1857-Asn1861 and Glu1875), C1 (Trp2062-Ser2069; Thr2086-Ser2095 and Ser2157-Leu2166) and C2 domains (Gln2235-Thr2244) of FVIII³⁰. Formation of the VWF-FVIII complex, directs FVIII to sites of vascular injury through the involvement of VWF in primary haemostasis³¹. The presence of VWF, increases the stability of the interaction between the FVIII heavy and light chains, increases FVIII half-life ($t_{1/2}$) and prevents premature tenase complex involvement and inactivation by activated Factor Xa (FXa) and activated Protein C (APC)³²⁻³⁶. This complex formation is thought to play a role in one of the mechanisms of FVIII cellular uptake²⁶.

1.1.6 Activation of Factor VIII

FVIII requires proteolytic activation to fulfil its co-factor role of enhancing the activity of FIXa within the tenase complex³⁷. This process occurs via cleavage of FVIII at residues Arg372, Arg740 and Arg1689 which are located at the junctions of the a1-A2, a2-B and a3-A3 domains respectively³⁸. This proteolysis has been shown *in-vitro* to be mediated by thrombin and FXa³⁷. There is sequential cleavage occurring firstly at Arg740, followed by cleavage at Arg372, with the catalytic activity being approximately 20 fold higher for the first cleavage site. The presence of VWF increases cleavage efficiency at Arg372 by 8 fold, although the mechanism via which this is mediated is not clear. This is followed by cleavage at Arg1689 resulting in dissociation of FVIII from VWF. Although thrombin and FXa share cleavage points on FVIII, the mechanism behind these events appear different. Sulphation of tyrosine residues close

to these cleavage sites appears to be important for this process when mediated by thrombin, but not for FXa. Point mutation converting Tyrosine to Phenylalanine at positions 346 and 1664 suggest that sulphation at these points accelerates thrombin but not FXa mediated cleavage³⁹. Experiments using thromboplastin activated plasma *in-vitro* in the presence of a thrombin specific inhibitor (hirudin) have demonstrated that thrombin and not FXa is responsible for FVIII activation⁴⁰. Alongside this, FXa requires the presence of an anionic phospholipid surface for efficient cleavage of FVIII, which is blocked when FVIII is associated to VWF³⁸.

1.1.7 Role of Activated FVIII Within the Tenase Complex

Thrombin cleavage at these three positions (Arg372, Arg740 and Arg1689) results in the removal of the a3 and B domains and formation of activated FVIII (FVIIIa). Removal of the a3 domain causes conformational change within the C2 domain, which is relatively flexible within the light chain^{6;7}. This facilitates release of VWF, increasing the affinity of FVIIIa for phospholipid membranes (e.g. activated platelet) and increases the activity of FVIII via a further unknown mechanism³⁷. Following extrusion of these regions, FVIIIa forms a heterotrimer comprised of the A1, A2 and A3-C1-C2 regions. FVIIIa is then able to act as a tri-directional magnet for formation of the tenase complex⁴¹. The first stage of this process occurs through high affinity binding of FVIIIa to the phospholipid membrane via hydrophobic and electrostatic interactions between residues in the C2 domain and the phospholipid membrane³⁷. These hydrophobic interactions are mediated by residues Met2199, Phe2200, Val2223 and Lys2252 which penetrate the lipid bi-layer and electrostatic interactions by Arg2215, Arg2220, Lys2227 and Lys2249, respectively⁴². Thrombin cleavage within the heavy chain at positions 372 and 740 results in the exposing of cryptic sites allowing interaction between FVIIIa with FIXa. These sites of interaction with FIXa have been modelled to residues within the A2/a2 (484-509, 558-565 and 707-714)⁴³ and A3 domain (1811-1819)^{6;44}. Finally, interaction between FVIIIa and FX is mediated by residues within the a1 domain (positions 337-372)⁴⁵.

1.1.8 Factor VIII Inactivation and Clearance

Following activation of FVIII, there is rapid loss of activity. This process of inactivation is both active (proteinase mediated) and passively mediated. Passive inactivation occurs through spontaneous dissociation of the A2 domain^{46;47}. Proteinase mediated inactivation occurs via the action of APC, FXa, and thrombin, with APC likely representing the key enzyme in this

process (Figure 1.2A). APC cleaves the A1 domain at Arg336 and the A2 domain at Arg562⁴⁸. Factor Xa has a dual role in the lifecycle of FVIII, firstly in the activation of FVIII and subsequent inactivation of FVIIIa. Cleavage points for Factor Xa have been identified within the A1 domain at Lys36 and Arg336, the latter a position shared with APC (Figure 1.2A)^{38;49}.

The mechanism and location for clearance of the inactive and breakdown products of FVIII is poorly understood. It has recently been proposed that FVIII, may be cleared in the presence of VWF or via uptake of free FVIII by Kupffer cells and hepatocytes respectively⁵⁰. A number of scavenger receptors have been implicated in this process including the low-density lipoprotein receptor related protein-1 (LRP1)³⁵, low-density lipoprotein receptor (LRP), asialoglycoprotein receptor (ASGRP), macrophage-mannose receptor type 1 (MMR/CD206), heparin sulphate proteoglycans (HSPG), sialic acid binding IgG-like lectin 5 (Siglec5), scavenger receptor class A member 5 (SCARA5), stabilin-2 (STAB2) and the C-type lectin domain family 4 member M (CLEC4M)⁵¹.

1.2: Congenital Defects of Factor VIII Function (Haemophilia A)

1.2.1 History of Haemophilia

The term haemophilia (love of blood), was first attributed to Friedrich Hopff in his dissertation of 1828: "*Haemophilie oder Über die Hämphilie oder die erbliche Anlage zu tödlichen Blutungen*" (On haemophilia or hereditary predisposition to fatal bleeding)⁵². Haemophilia has been used to describe two X-linked recessive inherited bleeding disorders, haemophilia A and B, which occur through defects in the *F8* and *F9* genes respectively. There have been descriptions of inherited bleeding disorders thought to represent haemophilia within the classical literature (Talmud) since the 2nd century AD^{53;54}. These reports recognised familial inheritance, stating some families had "loose blood" whereas others had blood that is "held fast"⁵². The first clear genealogical descriptions likely to be due to haemophilia appeared in the medical literature at the turn of the 19th century⁵². One of the first of these was presented by John Conrad Otto in 1803, describing a family with a hereditary haemorrhagic disposition spanning three generations in New Hampshire, USA^{52;55}. The extensive genealogy text (The Treasury of Human Inheritance) by Bulloch and Fildes in 1911, collated published (and unpublished) cases from the late 19th and early 20th century⁵⁶. This important early descriptive text provided details on clinical presentation and inheritance of this disorder prior to the availability of more advanced diagnostic techniques. Haemophilia is commonly referred to as the Royal Disease, with Queen Victoria being a carrier of

haemophilia. This resulted in male descendants being affected with haemophilia in the Russian, Spanish and German royal families⁵⁷. Perhaps the most well described of these descendants is the Tsarevich Alexei, the son of the Russian Tsar Nicholas II and his consort Alexandra, who was cared for by the mystic Grigori Rasputin. Genotype analysis of historical samples of members of the Romanov family has recently revealed a mutation in the *F9* gene, showing that Victoria's kindred were affected by the less common haemophilia B⁵⁸.

1.2.2 Prevalence of Haemophilia A

Haemophilia A represents one of the commonest inherited bleeding disorders affecting males of all countries and race, forming the focus of this thesis^{59;60}. Survey data collected by the World Federation of Haemophilia (WFH) has reported a mean prevalence of 8.4 per 100,000 males worldwide, with higher prevalence seen in higher income countries compared to the rest of the world (12.8 versus 6.6 per 100,000 males)⁵⁹. Differences were also seen between countries of similar financial status. Lower prevalence was seen for lower income countries which is likely to result from impaired access to effective diagnostic and treatment regimens, meaning patients with milder phenotypes are not diagnosed and those with more severe phenotypes die at a younger age. In the United Kingdom (UK) there are 5686 patients registered with haemophilia A, of whom 3077 (54.1%) received treatment between 2013-2014 (Table 1.2)⁶¹.

1.2.3 Diagnosis and Classification of Haemophilia A

International guidelines classify severity of haemophilia A as being either mild, moderate or severe based on the residual functional FVIII protein detected by a clotting based assay of FVIII activity (FVIII:C) (Table 1.2)^{62;63}.

Severity	FVIII:C (IU/dL)	Registered patients (UK)	Age at diagnosis (range)	Age at first joint bleed (range)	Phenotype
Severe	<1	2002	0.8 (0-5)	1.9 (1.2-3.0)	Spontaneous joint and muscle bleeds
Moderate	1-5	512	1.8 (0-27)	6.7 (3.7-23.9)	Occasional spontaneous bleeding
Mild	5-40	3172	2.3 (0-36)	14.2 (7.3-28.2)	Spontaneous bleeding rare

Table 1.2: Classification of haemophilia A and associated clinical features. FVIII:C levels in bold indicate the international diagnostic criteria as set out by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis⁶³⁻⁶⁸.

These laboratory cut-offs for defining severity are based on cohort studies from the late 1950s^{62;69}, with bleeding severity generally relating to the level of residual FVIII:C⁶⁶. Patients with severe haemophilia A have undetectable residual FVIII:C (<1IU/dL)⁷⁰. Without treatment patients with severe haemophilia A experience spontaneous bleeding into the joints and soft tissues, which progresses to a chronic disabling arthropathy by the second decade⁶⁹. Patients with non-severe (mild and moderate) haemophilia A generally bleed only following trauma or surgery (Table 1.2)⁶⁵. As the FVIII:C increases there is a reduction in the annual number of joint bleeds with distinctions seen between severe and non-severe haemophilia A. In a study of 377 patients with haemophilia A, the percentage of patients who had never suffered a joint bleed was 54%, 20% and 6% for mild, moderate and severe haemophilia respectively. In this study, in patients with FVIII:C levels >12IU/dL, the number of joint bleeds per year was approximately 0 (Figure 1.3)⁶⁶. There is however heterogeneity in the laboratory cut-offs used to define severity within the published literature, with some reports (particularly those from the UK) using a FVIII:C <2IU/dL and <50IU/dL to classify severe and mild haemophilia A, respectively⁶⁴. These variations in classification of mild haemophilia A are discussed in more detail later in this work and could directly impact on outcomes described in clinical studies.

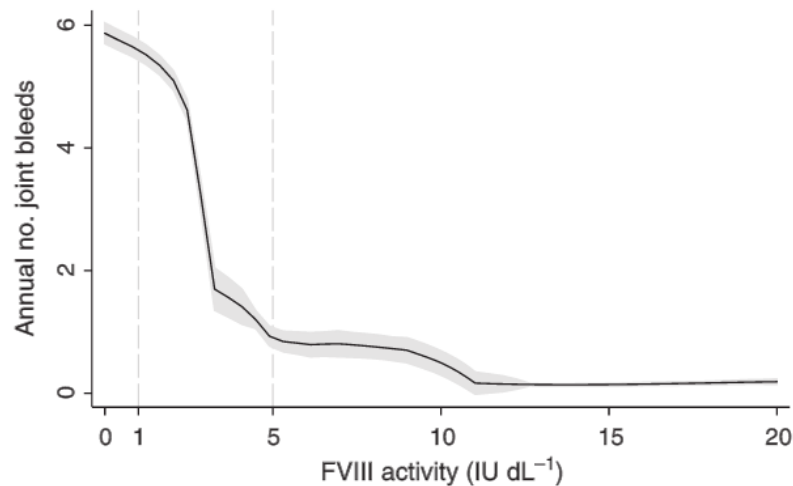


Figure 1.3: Relationship between baseline FVIII:C level and annual number of joint bleeds. Dotted lines represent divides between severe and moderate (FVIII:C 1IU/dL) and moderate and mild (FVIII:C 5IU/dL) haemophilia A. Clear distinctions in annual joint bleed rates are seen between severe and non-severe haemophilia A patients. Figure reproduced from Den Uijl et al. 2011 with the permission of the publisher⁶⁶.

1.2.4 Genetics of Haemophilia A

Haemophilia A results from an inherited mutation in the *F8* gene resulting in deficiency in functional FVIII. Further sub-classification can also be made based on whether the FVIII antigen (FVIII:Ag) is detectable or not. Patients can then be classified as being either cross reactive material (CRM) positive, where there is considerable amounts of FVIII:Ag present but this is dysfunctional, or CRM negative where the FVIII:Ag is not detectable. FVIII:Ag does not currently form part of routine laboratory testing and reporting of this is subsequently variable in the international databases⁷¹. Lack of functional FVIII results in impaired thrombin generation at platelet surfaces and the inability to form a stable fibrin clot. There is also a reduction in the activation of FXIII and Thrombin Activatable Fibrinolysis Inhibitor (TAFI) resulting in impaired clot stability and fibrinolysis⁷²⁻⁷⁵.

All types of genetic mutations (including missense, non-sense, splice site changes/variants, deletions, insertions and inversions) have been described as being haemophilia causative for both severe and non-severe haemophilia. There are currently two online, open-access reference databanks of *F8* mutations, the Factor VIII Variant/Mutation Database (formerly the Haemophilia A Mutation, Structure, Test and Resource Site, HAMSTeRS / HADB) and the Centers for Disease Control and Prevention Hemophilia A Mutation Project (CHAMP). These have collated information regarding reported *F8* mutations and associated clinical phenotype and list 2107 (HADB) and 2537 (CHAMP) unique *F8* mutations^{71,76}.

Severe haemophilia A results from a mutation in the *F8* gene leading to an absence of functional FVIII. The commonest mutation seen in this group is an inversion mutation involving intron 22, affecting around half of patients (42-47%)⁷⁷⁻⁸¹. This causes translocation of exons 1-22 away from exons 23-26, as a result of homologous recombination, with the *F8A* gene within intron 22 and another *F8A* gene. Interestingly, despite this mutation resulting in CRM negativity in the peripheral blood, recent data has demonstrated intracellular FVIII^{82;83}. Mutations resulting in non-severe haemophilia A lead to reduced amounts of secreted functional FVIII, with the commonest genetic abnormalities being missense mutations (non-synonymous single nucleotide polymorphisms, SNPs)⁷¹. Dependent on the location of these mutations, these may have a number of potential structural and functional consequences⁸⁴. FVIII missense mutations form an interesting sub-group of haemophilia-causing mutations, forming the commonest mutation seen in the HADB (66.5%; 1341/2015)⁷¹, with some being well tolerated resulting in mild haemophilia, whilst others are intolerant resulting in severe

haemophilia. Phenotypic variability is described for the same *F8* mutation between individuals (e.g. Arg593Cys, FVIII:C <1-38%) and for different amino acid substitutions at the same residue (e.g. Leu308Val has a FVIII:C 15-34%, whereas Leu308Pro a FVIII:C<1%)^{71;85}. A number of factors could cause variance in FVIII:C seen for the same mutations, including inter-laboratory variability (coefficient of variation 20.5-23.3%)⁸⁶, discrepancies seen in FVIII:C depending on the laboratory testing methodology assay (one stage or chromogenic assay)⁸⁷⁻⁹¹ or be a result of reporting error when using a centralised database. Recent *in-silico* studies have evaluated *F8* SNPs which may allow prediction of deleterious, functional and phenotypic effects⁹²⁻⁹⁵. Although current classification is based on FVIII:C, with increased understanding of effect of *F8* mutations on other clinical factors, e.g. severity, response to treatment and immunogenicity, further genomic classification may be of use within the clinic.

1.2.5 Treatment of Haemophilia A

The aim of treatment in haemophilia A is to restore FVIII:C to a sufficient level to allow stable clot formation. Replacement therapy has progressed from early usage of blood transfusions⁹⁶, through use of fresh frozen plasma (1950s-60s), to cryoprecipitate (1965)⁹⁷ and FVIII concentrates from the early 1970s^{52;98}. In developed countries, the standard of care is the intravenous infusion of FVIII concentrate⁶⁷, although in countries of more limited resources, cryoprecipitate, fresh frozen plasma and even whole blood continue to provide a source of FVIII replacement⁶⁷. Treatment can be given either “on-demand” to treat or prevent (e.g. peri-operatively) bleeding, or as “prophylaxis” to prevent spontaneous joint bleeding. The duration and dosage of FVIII treatment required is dependent on the location and severity of bleeding or type of surgery. The duration of treatment is often summarised by the number of exposure days (ED), defined by a day in which one or more FVIII infusions has been given to a patient^{99;100}. An intravenous infusion of 1IU/kg of FVIII concentrate elevates plasma FVIII:C by approximately 2IU/dL, with infused FVIII having a half-life of 8-12 hours¹⁰¹.

Modern FVIII concentrates are derived either from plasma donation (plasma derived, pdFVIII) or are cell-line derived (recombinant, rFVIII). In the UK, rFVIII concentrates are recommended as the product of choice¹⁰², with national switching from pdFVIII occurring in 1998 for children <16 years and in 2003 for all other patients, following concerns regarding prion and viral safety¹⁰³. Recombinant FVIII concentrates, are derived from non-human cell lines (baby hamster kidney, BHK or Chinese Hamster Ovary, CHO) and may vary dependent on whether the encoded FVIII protein contains the B domain or not (full-length, FL or B domain deleted,

BDD). Differences in the primary cellular sources (animal and tissue) result in differences in post-translational modifications of rFVIII compared to human FVIII¹⁰⁴; although the significance of this is not clear. Further categorisation of rFVIII is made based on the presence of animal/human products stabilisation within the final formulation (first, second and third generation rFVIII)¹⁰². Advances in the manufacture of FVIII concentrates, firstly in pdFVIII (viral inactivation) and later with the development of rFVIII products has resulted in safe and efficacious treatment for persons with haemophilia. The greatest problem currently facing haemophilia treating physicians in developed nations is the occurrence of inhibitory antibodies (inhibitors) to FVIII.

An alternative approach, for management of bleeding or surgery in patients with non-severe haemophilia A, is the use of a synthetic antidiuretic hormone analogue, desmopressin (1-deamino-8-d-arginine vasopressin, DDAVP). DDAVP, acts on extra-renal arginine vasopressin V2 receptors causing release of VWF from endothelial cells and a subsequent release of FVIII from an unknown cellular source. In responsive DDAVP patients this results in a 2-6 fold¹⁰⁵, rise in endogenous FVIII:C. This approach can allow avoidance or reduction in exposure to FVIII concentrate, which could potentially be protective from the immune complications of FVIII treatment.

1.3: Acquired Defects of Factor VIII Function (Acquired Haemophilia A)

1.3.1 Introduction

Acquired Haemophilia A (AHA) is a rare acquired severe bleeding disorder (incidence 1 in 1.48 million/year) resulting from auto-antibody formation to FVIII¹⁰⁶. AHA mainly affects the elderly, with a median age at onset of 73.9 years¹⁰⁷. Incidence appears to increase further with age, reaching 14.7 per million/year in people >85 years of age¹⁰⁶⁻¹⁰⁸. A second, smaller peak in incidence is seen in younger women (median 34 years), relating to pregnancy^{107;109}. This most frequently presents in the post-partum period (21-120 days post-partum) and is estimated to affect 1 in 350,000 births in the UK^{106;108;109}. The occurrence of AHA in children (<16 years) is rare (0.045 per million/year)^{106;108;110}. Almost half of AHA cases are associated with an underlying disorder; including malignancy (11.8%), auto-immune disease (11.6%), pregnancy (8.4%), drugs (3.4%), skin disorders (1.4%), infection (3.8%) and blood transfusion (0.8%)¹⁰⁷. The pathological mechanisms underlying auto-antibody formation to FVIII are poorly understood. It is likely that this results from a loss in tolerance to FVIII (self). Given such complex aetiologies and rarity of this disorder there is limited investigation at a cellular

level of these immune mechanisms. Genetic associations relating to the underlying HLA genotype and a polymorphisms in the cytotoxic T lymphocyte antigen-4 genes (see Section 1.4.3 and 1.4.4) involved in the immune response have been characterised ^{111;112}.

Diagnosis is made generally made following a patient presenting with new onset bleeding and subsequently being found to have an inhibitory antibody to FVIII on laboratory testing. AHA often present to a general physician or surgeon, rather than directly to a haemostasis specialist, which may result in a delay in diagnosis or treatment ¹¹³. Rarely, AHA may be diagnosed on routine blood testing (2/149 patients) ^{108;110}. Even within a haemophilia comprehensive care centre the number of cases seen per year may be low ^{106;110;114;115}. A single centre experience over 28 years (1970-1998), described only 24 patients (0.9 cases/year) ¹¹⁴. Similarly, in a recent survey of clinicians in Germany, Austria and Switzerland, the median number of patients treated per year was 1.8 (range 0-10). As such, patients with AHA may often be managed by clinicians with limited personal experience of treatment of this disorder ^{110;115}. Treatment approaches are likely to be influenced by previous experience, local protocols or recently published reports. Within the recent literature, there have been a number of large observational studies that have provided important new data on the natural history of this rare disorder ^{106;107;116-118}.

1.3.2 Clinical Features of Acquired Haemophilia A

Bleeding is the presenting feature in the majority (94.6%) of patients with AHA which is often spontaneous (77.4%) or following a haemostatic challenge (trauma 8.4%, surgery 8.2% and peri-partum 3.6%) ¹⁰⁷. Of these episodes, 70.3% were severe and a similar proportion (70.5%) required treatment with a haemostatic agent ¹⁰⁷. The sites of bleeding seen in AHA differ from those seen in congenital haemophilia A, with subcutaneous (53.3%), deep muscle/retroperitoneal (50.2%) and mucosal bleeding (respiratory, gastrointestinal, urogenital) (31.6%) representing the commonest sites. Joint bleeding (4.9%) and intra-cerebral bleeding (1.1%) are less common ¹⁰⁶. Although most patients have only one episode of bleeding at presentation, a third (33.5%, n=159) will have more than one bleeding episode (median 28 days) ¹⁰⁷.

1.3.3 Treatment of Acquired Haemophilia A

The management of AHA involves therapy to treat bleeding followed by immunosuppression aimed at inhibitor eradication. Agents used in the treatment of bleeding are similar to those used in the treatment of patients with congenital haemophilia A with allo-antibodies to FVIII

(discussed in Section 1.5). Given the high mortality and morbidity associated with AHA without treatment (see below), there is consensus that all patients should receive immunosuppression with the goal of inhibitor eradication ^{113;119;120}. Although spontaneous remission has been reported in 25-35.5% patients in two reports from the 1980s, these findings have not been confirmed in larger cohorts ^{110;121}. The first of these studies, a case series of 215 patients, reported spontaneous disappearance of inhibitory antibodies in 11/31 patients (35.5%), receiving supportive care only (transfusion or FVIII) after 14 months (range 1-156 months). Of the remaining patients receiving supportive care alone, 8 died (median 12 months) and 10 had an inhibitor present at 36 months. Outcomes were better in patients who received treatment in comparison to those that did not ¹¹⁰. There is no reporting of morbidity in patients who received supportive care or immunosuppression in this study. A second single centre ten year experience (1975-1985) reported 16 patients receiving no or minimal immunosuppression, reporting spontaneous remission in up to 25% of patients (n=4; no treatment=2, minimally treated=2). Within this cohort significant morbidity due to bleeding was seen, although interestingly in some patients the bleeding phenotype appeared to ameliorate with time even in the presence of residual antibody ¹²¹. In more recent reports, patients who have not received immunosuppression are limited to those in whom treatment was felt inappropriate and poorer outcomes have been described ^{106;122-124}.

Treatment with immunosuppression leads to improvement in outcomes and remission in a substantial proportion of patients, although the optimal first line treatment is not clear ¹⁰⁸. There have been no adequately powered interventional studies in AHA, with all outcome data being derived from observation studies. The largest of these studies, is the recently reported European Acquired Haemophilia Registry (EACH2), which presented data on 331 patients with AHA. In this study, the most commonly used regimens for immunosuppression, were a corticosteroid (e.g. prednisolone) in isolation (43%, n=142) or combined with a cytotoxic agent (e.g. cyclophosphamide) (15%, n=83) ¹¹⁶. The presenting features of AHA including underlying disorder, bleeding and inhibitor titre may influence clinicians' treatment decisions ¹¹⁵. This could result in different immunosuppressive approaches being used in different patient groups, such as those presenting in the post-partum period or elderly.

One small prospective interventional study has been conducted in AHA investigated safety and efficacy of steroids (prednisolone) and cyclophosphamide in isolation or in combination ¹²⁵. All patients (n=31) were treated initially with single agent prednisolone and after 3 weeks, those who had not responded (n=21) were then randomised to either continuing steroids

(n=4), single agent cyclophosphamide (n=6) or steroids and cyclophosphamide (n=10). Complete response (CR) rates seen in these three groups were 75%, 50% and 50%, respectively with no difference between the treatment arms. This study was underpowered to demonstrate differences between the treatment arms and managed to accrue only 31 (target=100) patients over a 5 year period. A recent comparison of 25 clinical studies showed similar response rates for steroids (72%) compared to steroids in combination with a cytotoxic agent (78%)¹⁰⁸. If only studies that included both treatment arms were considered the response rates seen were 76% (68/90) and 78% (109/139), respectively¹⁰⁸. In a meta-analysis of 20 studies, although higher CR rates were seen for steroids and cyclophosphamide compared to steroids alone (89% versus 70%), overall survival was similar between both treatment groups, which was attributed to increased mortality from enhanced immunosuppression¹²³. In the EACH2 study, the CR rates (inhibitor undetectable, FVIII:C >70IU/dL and immunosuppression stopped) for those treated with steroids alone (n=142) compared to steroids and cyclophosphamide (n=83), were 58% and 80% respectively. Patients treated with steroids and cyclophosphamide, obtained CR faster (HR=2.36; 95%CI 1.49-3.74, p<0.001) and were more likely to obtain a stable CR (i.e. no relapse reported) than those treated with steroids alone (OR=3.25; 95%CI 1.51-6.69, p<0.003). Of note, the occurrence of adverse events was higher in patients treated with steroids and cyclophosphamide (41%) compared to those treated with steroids alone (25%). Despite improved CR rates in patients treated with steroids and cyclophosphamide, the final outcomes (being alive and inhibitor free at final follow-up) were similar in both groups (67% steroids alone versus 62% steroids and cyclophosphamide). This suggests that although patients treated with steroids and cyclophosphamide achieve higher rates of stable CR this is not associated with improved long term outcomes and at the cost of higher iatrogenic morbidity¹¹⁶. Other approaches for inhibitor eradication have been described in smaller patient groups, including immune tolerance induction (Section 1.5.7), mycophenolate mofetil, ciclosporin and usage of an anti-CD20 monoclonal (rituximab) which are discussed in more detail in the context of the data presented in Chapter 5.

There is less data surrounding response to immunosuppression in patients presenting with AHA relating to pregnancy. The EACH2 study presents one of the largest reports to date, with 42 patients, demonstrating complete response rates of 74%¹⁰⁹. In patients with available data, these were treated with steroids, either in isolation (n=27) or combined with another

agent (cyclophosphamide=6, intravenous immunoglobulin=4 or rituximab=2). All patients were alive at final follow-up and 86% were in complete remission.

1.3.4 Outcome Measures in Acquired Haemophilia A

Recent studies in the management of AHA have highlighted some of the difficulties in the management of this rare condition. Despite recent improvements in the treatment of this disorder, it continues to be associated with significant mortality (27.9% of patients in the EACH2 cohort) ¹⁰⁷. In this study, equal numbers of patients died as a result of immunosuppressive therapy (16%, n=16) as those who died from bleeding (16%, n=16) ¹⁰⁷. Patients treated with these modalities required substantial periods of time on immunosuppression, with the median time to stopping of immunosuppression being 108 days (IQR 55-208) for steroids alone and 74 days (IQR 52-151) for steroids and cyclophosphamide. Immunosuppression resulted in at least one adverse event in 25% and 41% of patients treated with steroids or steroids and cyclophosphamide, respectively ¹¹⁶. Of patients who obtained CR, 18% (steroids alone) and 12% (steroids and cyclophosphamide) relapsed. Current recommendations for screening for relapse suggests laboratory testing monthly for the first 6 months, then every 2-3 months up to one year and twice yearly after one year ¹¹⁹. Greater clarity in terms of the optimal way of testing for early relapse is required to reduce morbidity in this group of patients.

1.3.5 Prediction of Risk in Acquired Haemophilia A

Given the high morbidity, mortality and associated hospital stay associated with AHA, it would be desirable to provide risk stratification to identify patients in whom early escalation of immunosuppression may be required or, conversely, who could be treated with a less intensive approach to minimise toxicity from treatment. There have been a number of reports looking into factors associated with poorer outcomes, with two meta-analyses reporting in 2003 ¹²³ and 2009 ¹²⁶. The first of these included 20 studies (1985-2002) and a case-series from the authors' centre, comprising 249 patients (234 included) ¹²³. In the univariate analysis, failure to achieve CR, age >65 years, related diseases (malignancy v post-partum), inhibitor titre at diagnosis <10BU/mL, were significantly associated with poorer overall and disease specific survival. No significant difference was seen for sex, FVIII:C (2%) or choice of immunotherapy. In multivariate analysis, only achievement of CR, was seen to affect both overall and disease specific survival. A second meta-analysis (1985-2008), assessed 32 studies (359 patients) which included ≥ 5 patients ¹²⁶. Within the univariate

analysis, age, sex, underlying autoimmune disease, peri-partum period and inhibitor titre had no significant effect on obtaining CR. Patients with an underlying malignancy were less likely to achieve CR. Patient treated with immunosuppression were more likely to obtain a CR compared to those that did not receive treatment and those receiving immunosuppression were less likely to die. Older patients, those with underlying malignancy and those with a persistent inhibitor were more likely to die. The effect of survival from obtaining CR was only significant if this was achieved within 150 days. Multivariate analysis demonstrated that immunosuppression was associated with increased prediction of CR, with combination chemotherapy having the lowest odds of persistent disease.

More recent evaluation of prognosis has been performed within the EACH2¹⁰⁷ and GTH-AH 01/2010¹¹⁸ studies. Within the EACH2 study, multiple step-wise regression demonstrated older age, lower presenting haemoglobin, malignancy and failure to clear the auto-antibody as being independent predictors of death. Gender, initial FVIII:C and inhibitor titre did not affect outcome. Within this cohort, patients with pregnancy related AHA had improved survival than other patients included in this study. In the GTH-AH 01/2010 study, patients with a presenting FVIII:C <1IU/dL achieved the end point of partial remission (PR) less frequently¹¹⁸. Within this study a subset of patients presenting with FVIII:C >1IU/dL and inhibitor titre <20BU/mL, demonstrated faster achievement of a PR in <21 days (OR=11.2; p<0.0001). These findings suggest that it may be possible to select intensification of treatment early to improve outcomes in those at higher risk of not obtaining remission and to use less intensive approaches for those predicted to have better outcomes with the aim of minimising toxicity.

To allow provision of clearer guidance in the management of AHA, there is a need to define the optimal first line management, laboratory follow-up and response criteria. These criteria also tend to be based on measurement of patient's FVIII:C for which a patient's normal baseline would not be known and at present rely on usage of a functional clotting based inhibitor assay (discussed in Section 1.5 and 1.6). There is a lack of evidence to guide how immunosuppression should be reduced and stopped. Studies that could stratify patients into high or low risk groups, could allow more personalised approaches to management that might improve rates of treatment related morbidity. Finally, no studies to date have looked at the impact in terms of quality of life and effects of functional capacity in this group of patients.

1.4: Factor VIII and the Immune System

1.4.1 Introduction

The immune response to infused exogenous FVIII in patients with congenital haemophilia is seen as an example of a classical allo-immune response, mediated by CD4+ T-cells. In patients with severe haemophilia A, it is perhaps surprising only 30% of patients develop inhibitory antibodies in the absence of circulating FVIII to impart pre-existing tolerance. As a monogenic disorder, haemophilia offers a good model for the investigation of the immune response to an infused protein therapeutic for understanding the process of development of anti-drug antibodies. There have been many advances in the understanding of the immune response to FVIII over the last decade, the results of which have been elegantly outlined in a number of recent reviews ^{41;127-130}. T-cell (CD4+) dependence was first noted in HIV infected haemophilia patients with inhibitors, who developed a decline in their inhibitor titre and loss of anamnesis as their CD4 count fell, with progression to the acquired immunodeficiency syndrome (AIDS) ¹³¹. On commencement of anti-retroviral therapy (HAART), with a subsequent rise in their CD4 counts, these patients again produced inhibitors. Further evidence of T-cell dependence has been derived from studies in murine haemophilia models which have demonstrated somatic hypermutation and class switching for inhibitory FVIII antibodies ¹³²⁻¹³⁵.

1.4.2 Antigen Presenting Cells and Factor VIII Cellular Uptake

Following infusion, FVIII must undergo a number of steps prior to being presented to the immune system. Antigen presentation to the immune system is mediated by antigen presenting cells (APCs) consisting of dendritic cells (DCs), macrophages and B-cells (lymphocytes). Cellular uptake of foreign protein by APCs occurs via receptor mediated endocytosis, phagocytosis and macropinocytosis ¹³⁶. Experimental data from haemophilia mice (exon 16 deleted) has demonstrated that the main site of FVIII uptake into APC is the spleen (marginal zone) ¹³⁷. Following intravenous injection of FVIII, preferential co-localisation of FVIII occurred within metallophilic (MOMA-1 +ve) macrophages and to a lesser extent in CD11c +ve DCs and marginal zone (MARCO +ve) macrophages. Splenectomy or depletion of these cells *in-vivo* resulted in a reduction in the immune response to FVIII ¹³⁷. The precise nature and mechanisms of receptor mediated cellular uptake of FVIII is incompletely understood ^{130;138}. The macrophage mannose receptor (MR) forms one mechanism for cellular uptake interacting with mannose ending glycans in the A1 (Asn239)

and C1 (Asn2118) domains (Figure 1.2A). Blockade of these mannose mediated interactions, results in partial reduction in FVIII endocytosis and resultant T-cell activation in immature cultured human DCs ¹³⁹. Knockdown of MR (small interfering RNA, siRNA), however did not affect FVIII endocytosis suggesting the involvement of other mechanisms ¹⁴⁰. Other uptake mechanisms include the LDL receptor related protein (LRP), which interacts with FVIII via the A2, A3 and C1 (Lys2092 and Phe2093) domains. This receptor has, however, not been shown to have a role in FVIII uptake by DCs ¹⁴¹. Recently published experiments, using a C1 domain anti-FVIII antibody (KM33), which interacts with the C1 domain (Arg2090, Lys2092 and Phe2093), demonstrated inhibition of endocytosis of FVIII by DC (human and murine) *in-vitro* ¹⁴². Infusion of modified FVIII with alanine substitutions at these positions in a mouse model resulted in a reduced immune response. Although KM33 shares an epitope on FVIII with LRP, this does not appear to represent a key pathway of FVIII uptake into DCs ¹⁴³. Other receptors (ASGRP, HSPG, Siglec5, SCARA5, STAB2 and CLEC4M) have recently been found to play a role in FVIII uptake, although the full mechanism for this process remains to be identified.

1.4.3 Antigen Presentation and the Major Histocompatibility Complex

Following internalisation of FVIII into APCs, this undergoes proteolytic cleavage in the endosomal compartments. Endogenous and exogenous peptide fragments are then presented to the immune system via the major histocompatibility complex (MHC) or human leukocyte antigen (HLA). The MHC genes located on chromosome 6, encode the MHC class I (HLA-A, -B and -C) and II (HLA-DR, -DP and -DQ) molecules in humans. MHC class I and II present peptide fragments to CD8+ and CD4+ T-cells, respectively. Extracellular peptide fragments are loaded into the peptide binding groove of MHC Class II (≥ 13 aa) resulting in formation of the MHC-peptide complex. The MHC-peptide complex is then transported to the cell membrane for presentation to helper T-cells. Activation of naïve CD4+ depends on a number of “signals”, with the first being recognition of peptide antigens within the MHC-peptide complex by the T-cell receptor (TCR). Increased expression of MHC-peptide complexes is induced by maturation of naïve dendritic cells, a process which requires the presence of danger signals ^{144;145}. These danger signals may be provided by endogenous (e.g. co-stimulatory molecules or cytokines) or exogenous (e.g. pathogen derived products) stimuli. The precise nature of danger signals in response to FVIII are however unclear. *In-vitro* data demonstrated that neither FVIII alone (inactive or thrombin activated), nor complexed with VWF is able to modulate maturation of human monocyte derived DCs or stimulate autologous or allogenic T-cells ¹⁴⁶. Although a murine haemophilia model suggested thrombin

may provide this danger signal *in-vivo*¹⁴⁷, equal immunogenicity was seen by other investigators for wild type FVIII and two FVIII molecules (Arg372Ala/Arg1689Ala and Val634Met) devoid of pro-coagulant activity, suggesting immunogenicity is independent of FVIII haemostatic function¹⁴⁸. The environmental context at the time of FVIII infusion could potentially provide these danger signals, although data (*in-vitro* and *in-vivo*) to support this hypothesis is currently lacking^{99;100;149}.

1.4.4 Co-Stimulatory Signalling

Interaction between the MHC-peptide complex and the TCR alone is insufficient to induce differentiation of naïve CD4+ T cells. Co-stimulatory signalling is required, which includes interaction between CD40 and CD80/86 (B7-1/B7-2) on the surface of APC and CD40 ligand (CD40L) and CD28 on the surface of naïve helper T-cells (CD4+). Interaction of the TCR with the MHC class II complex causes up-regulation of CD40L, which binds CD40 causing further expression of B7, CD40 and MHC Class II. Negative regulation of co-stimulation occurs through the CD28 homologue, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152) which binds to B7-1 and B7-2 inhibiting signalling via the TCR. The importance of these co-stimulatory signalling pathways has been demonstrated in murine haemophilia models. Targeted disruption of the B7/CD28 co-stimulatory (B7-2^{-/-} knockout) signalling or injection of a murine CTLA-4 immunoglobulin blocked the primary immune response in the haemophilia mice¹⁵⁰. Blockade of the CD40/CD40L interaction also prevented induction of neutralising antibodies on exposure to human FVIII, with suppression of FVIII specific T-cell responses¹⁵¹. The third signal required for maturation of the follicular T-cell is promoted by cytokines which results in formation of helper (effector) T-cell (T_H1, T_H2 or T_H17) or regulatory T-cells (T_{reg}). Cytokine secretion from both T_H1 and T_H2 cells then promotes B-cell differentiation and anti-FVIII antibody synthesis¹⁵².

1.4.5 B-Cell Activation and Antibody Secretion

B-cells form the precursor to antibody secreting plasma cells, a process requiring T-cell help. Antigenic epitopes recognised by the B-cell receptor result in antigen internalisation via the B-cell receptor complex. These undergo internal processing and are presented in complex with MHC Class II to CD4+ effector T-cells. A naïve B-cell can then be activated if the MHC-class II-peptide complex is recognised by an effector T-cell that responds to the same antigen (linked recognition). Secondary signals are also required to promote differentiation of naïve B-cells, with interaction between CD40L and CD40 on the B-cell surface as well as various

other cytokines (including IL-4, IL-5, IL-6 and IL-10) promoting B-cell activation and proliferation. The importance of these co-stimulatory pathways has been seen through blockade of these pathways *in-vitro* and *in-vivo*. Anti-CD40L, anti-B7-1 or anti-B7-2 monoclonal antibodies and a CTLA4-Fc have all been shown to prevent re-stimulation and differentiation of memory B-cells *in-vitro* and *in-vivo* (exon 17 disrupted haemophilia A mice)¹⁵³. In the primary response, activation of B-cells results in differentiation into antibody secreting short-lived plasma cells. A proportion of these B-cells then translocate to the spleen and lymph nodes. In these organs, proliferation occurs in the germinal centres. Affinity maturation occurs in the germinal centres via somatic hypermutation and clonal selection. High affinity B-cells presenting a MHC-peptide complex on the surface that can then be recognised by CD4+ T-cells in an antigen specific manner by their T-cell receptor, with up-regulation of CD40L and cytokine secretion (IL-4). This leads to B-cell clonal expansion and isotype switching. These cells then go on to form antibody secreting cells or memory cells which remain resident in the germinal centres of the secondary lymphoid organs. On re-exposure to antigen, these memory cells are re-activated. The profile of FVIII antibodies seen in haemophilia, although showing physiological profiles of IgG, demonstrated higher amounts of the IgG1 and IgG4 subclasses. Class switching to IgG4 is a process seen as further evidence of the requirement for T-cell help.

1.5 Clinical and Laboratory Aspects of Factor VIII Antibodies

1.5.1 Clinical Aspects of Inhibitory Antibodies to Factor VIII

Antibody formation to FVIII in patients with congenital haemophilia and acquired haemophilia A is one of the greatest clinical challenges facing haemophilia treating physicians. These are associated with morbidity, mortality, impairment in quality of life and cost. Laboratory testing and quantification of these antibodies is based on a functional haemostatic assay (Nijmegen-Bethesda Assay, NBA), detecting antibodies with inhibitory capacity (inhibitors). These are sub-classified as being either low-titre (inhibitors titre <5BU/mL, despite repeated FVIII challenge and a lack of anamnesis) or high-titre (inhibitor titre >5BU/mL at any time)⁶³. Inhibitory antibodies represent only a proportion of the immune response to FVIII, with the total immune response being largely unknown. Much of current understanding of mechanism of action, testing methodology and inhibitor risk factors are derived from patients with severe haemophilia A. There are however, differences in the clinical and immunological contexts in which patients with severe and non-severe haemophilia A receive treatment with FVIII (discussed in Chapter 4). Similarly, the immune

processes and antibody characteristics seen in patients with acquired haemophilia A differ from those seen in congenital haemophilia. Despite commonality of the target antigen (FVIII) in these three settings, there are limitations in extrapolation of laboratory and clinical data.

1.5.2 Severe Haemophilia A

Patients with severe haemophilia A frequently require FVIII treatment within the first year of life (median 9.8 months, IQR 5.3-13.5). Understanding of the epidemiology and environmental risk factors for inhibitor formation in severe haemophilia A has progressed greatly within the last 8 years through large international cohort studies of previously untreated patients (PUPs). The first of these, the CANAL (Concerted Action on Neutralizing Antibodies in severe haemophiLia), reported on 366 consecutive patients with moderate-severe haemophilia (FVIII:C <2IU/dL), born between 1990-2000¹⁰⁰. The second of these, the Research Of Determinants of INhibitor development (RODIN) study, evaluated 606 severe haemophilia A PUPs born between 2000-2010^{149;154}. Within the RODIN study, there was a cumulative inhibitor incidence of 32.0% (high-titre 22.2%), occurring at median age of 15.5 months (14.5ED)¹⁴⁹. In patients who developed an inhibitor, 50% had developed this within 15EDs^{99;100}, with these findings being similar to previously published observational studies^{99;100}. The formation of inhibitors beyond 50EDs is less frequent, with a reported incidence of 3 per 1000 treatment years (95%CI 1-4) in a recent meta-analysis of 33 studies (4323 patients, 43 de-novo inhibitors)¹⁵⁵. Data published from a large cohort of UK patients, with severe haemophilia A alive and treated between 1990 and 2009, has suggested a bimodal distribution in inhibitor incidence¹⁵⁶. An initial peak in incidence was seen in those <5 years (64.29/1000 treatment years) and a second peak seen in those ≥60 years (10.49/1000 treatment years), compared to those aged 10-49 years (5.31/1000 treatment years, p=0.01)¹⁵⁶. This second peak remained significant even with adjustment for HIV seropositivity. Interestingly in this cohort, inhibitor incidence described in previously treated patients (PTPs) is higher than has been reported in other studies¹⁵⁵.

The formation of inhibitors in patients with severe haemophilia A impairs adequate delivery of prophylaxis and results in significant morbidity. There are varying reports of the effect of inhibitor formation on mortality. Although a UK report (1977-1999) demonstrated no significant difference in mortality in inhibitor patients¹⁵⁶, recent data from the US has suggested a 70% increased risk (OR=1.7, 95%CI 1.2-2.5) in severe haemophilia A patients with a current inhibitor compared to those without (including those who had undergone

successful ITI)¹⁵⁷. Inhibitor formation also negatively impacts on quality of life¹⁵⁸, physical activity¹⁵⁹, caregiver burden^{160;161} and is associated with significant cost relating to both delivery of treatment for bleeding and therapies directed at suppression of inhibitors.

1.5.3 Non-Severe Haemophilia A

The cumulative incidence of inhibitors in non-severe haemophilia is lower (3-13%) than is seen in patients with severe haemophilia A. Inhibitors in these patients however, still represented a substantial proportion (28%) of inhibitors reported in the UK between 1/1990 and 1/1997⁶⁴. Until recently, much of the understanding of inhibitor formation in non-severe haemophilia was derived from small cohort studies and case series^{64;162-165}. Recent reports from the “INTERNATIONAL Study on etiology of inhibitors in patients with a moderate or mild form of hemophilia A, influences of Immuno Genetic & Hemophilia Treatment factors” (INSIGHT)¹⁶⁶⁻¹⁷⁰ and European Haemophilia Safety Surveillance (EUHASS)^{171;172} studies are starting to provide more data in this area.

Inhibitor formation in patients with non-severe haemophilia A occurs later in life^{64;166;172-175}, at a median age of 46 years (IQR 18-65) and after 28 ED (IQR 12-71)¹⁶⁶. Although the majority of inhibitors occurred within the first 50ED, in the INSIGHT (69.5%, 41/59)¹⁶⁶ and EUHASS (72%, 28/39)¹⁷² studies respectively, there was continued inhibitor occurrence beyond 100EDs. In the EUHASS study, 15% of inhibitors occurred beyond 100ED. Within the INSIGHT study the cumulative inhibitor incidence was 3.5% at 20ED; 6.7% at 50ED; rising to 13.3% at 100ED. Within this study a substantial proportion (517/1112) of patients were minimally treated (<20ED), with 68% of patients receiving <50ED, so there was only a small number of patients remained under observation by 100ED (297 patients at 75ED reducing to 33 patients at 100ED)¹⁶⁶. Given the infrequent nature of exposure over many years there may also be limitations in the recall of accurate ED data in non-severe haemophilia A.

Some of the clinical difficulties specific to inhibitors in non-severe haemophilia A were first highlighted in a case-series (n=26) reported in the late 1990s⁶⁴. Alongside causing inhibition of infused exogenous FVIII, cross-reactivity with endogenous FVIII was seen in 24/36 patients which resulted in a fall in baseline FVIII:C (median pre: 8IU/dL to post: 1IU/dL) and change in bleeding phenotype (22/26)⁶⁴. Similar findings have been reported in the INSIGHT study, with a fall in FVIII:C in 58% (n=34) of inhibitor patients and change in bleeding phenotype in 51% (n=30). Sixteen patients (27%) had a fall in FVIII:C to ≤1%. Only 11 (19%) of inhibitors were detected by routine testing. Inhibitor formation is also associated with increased

morbidity^{64;170} and mortality^{64;168}. There was a ten-fold increase in annualised bleeding rate (pre: 0.095, IQR 0.02-0.42 and post 1.1, IQR 0.1-2.5) following inhibitor formation in the INSIGHT cohort¹⁷⁰. The all-cause mortality rate was also five-fold higher (n=16, age-adjusted mortality rate ratio 5.6) in inhibitor patients and 7 patients died from inhibitor related bleeding¹⁶⁸.

1.5.4 Risk Factors for Inhibitor Formation in Congenital Haemophilia A

The ability to predict whether patients with haemophilia will develop an immune response to FVIII could help personalise management. Risk factors that have been identified to date relate either to the context that FVIII is infused (environmental), are inherited (genetic) or the approaches (methodology and timing) to inhibitor testing¹⁷⁶.

1.5.4.1 Severe Haemophilia A

Genetic Risk Factors in Severe Haemophilia

In severe haemophilia A, the strongest predictor of risk of inhibitor formation is the underlying *F8* gene mutation¹⁷⁷. Patients with large deletions and nonsense mutations have higher risk of inhibitor development than those with inversion mutation, OR=3.6 (95%CI 2.3-5.7) and OR=1.4 (95%CI 1.1-1.8), respectively. The risk of inhibitor formation is less in patients with small deletions/insertions, OR=0.5 (95%CI 0.4-0.6) and missense mutations, OR=0.3 (95%CI 0.2-0.4)¹⁷⁷. A family history of inhibitor formation is associated with increased risk of inhibitor formation, with an inhibitor incidence reported at 48% (95%CI 35-62) in patients with a positive family history and 15% (95%CI 11-21%) in those with a negative family history (RR=3.2, 95%CI 2.1-4.9) in one large study¹⁷⁸.

Increased incidence of inhibitor formation has been described in patients of Hispanic and African origin compared to those of Caucasian origin in a number of studies, although the underlying genetic mechanism for this are not clear¹⁷⁸⁻¹⁸⁰. One mechanism that has been proposed is that there is a mismatch between wild type FVIII seen in these patients in contrast to that infused as part of replacement therapy. Four nonsynonymous single-nucleotide polymorphisms (SNPs) were identified in the *F8* gene in a study of healthy individuals (n=137), resulting in six haplotypes, which have been denoted H1-H6^{179;181}. These vary at four positions in the A2, B and C2 domains, and have the following residues represented: H1 (Arg484, Arg776, Asp1241, Met2238), H2 (Arg484, Arg776, Glu1241, Met2238), H3 (Arg484, Arg776, Glu1241, Val2238), H4 (His484, Arg776, Glu1241, Met2238),

H5 (Arg484, Arg776, Asp1241, Val2238) and H6 (Arg484, Gly776, Glu1241, Met2238). The H1 haplotype forms the predominant haplotype in Caucasian individuals, occurring in around 93% with the remaining 7% having the H2 haplotype. More diversity in wildtype haplotypes is seen in black individuals, with the following haplotype frequencies being seen: H1 (35%), H2 (37%), H3 (22%), H4 (4%) and H5 (1%). It was proposed, that a mismatch between the wild-type FVIII haplotypes (H3-4) seen in black patients and those found in recombinant FVIII products (H1-2) resulted in increased inhibitor formation. In this study (n=78) the prevalence of inhibitors was higher in black patients with H3-H4 haplotypes in comparison to those with *F8* H1-H2 haplotypes (OR=3.6; 95%CI 1.1-12.3; p=0.04)¹⁷⁹. These findings are however controversial and have not been replicated by other investigators^{180;182-185}. Further study in this area is required and the genetic mechanisms for these differences in inhibitor incidence are likely to be far more complex than relating to a single *F8* SNP.

Given the requirement for T-cell (CD4+) help in the process of inhibitor development, one might expect MHC class II alleles to be associated with an increased risk of inhibitor formation¹⁷⁸⁻¹⁸⁰. Studies in patients with severe haemophilia A, with the intron 22 inversion mutation have not demonstrated strong associations for any MHC class II haplotype and inhibitor formation, although HLA-DQA0102, -DQB0602 and -DR15 have been described to occur more frequently in patients with inhibitors^{186;187}. This potentially only forms a weak determinant of risk, given the vast number of potential T-cell epitopes that could be expressed on MHC in patients with null mutations. Alternatively, due to presence of intracellular FVIII in patients with intron 22 inversion⁸², this may allowed development of tolerance. Earlier investigations into polymorphisms in genes involved in the immune response showed a modest increase in risk for polymorphism in the IL-10 and TNF- α genes and decreased risk for mutations in the CTLA-4 gene¹⁸⁸⁻¹⁹². More recent work has identified a SNP within the FCGR2A, which encodes the low affinity Fc gamma receptor (Fc γ R), to be associated with increased risk of inhibitor formation (OR per H-allele=1.8; 95%CI 1.1-2.9)¹⁹³.

A recently published large international study (n=833), using a microarray approach to investigate 13331 SNPs in 1081 primary immune response / immune modifying genes has not confirm these findings, for polymorphisms in the IL-10, TNF- α and CTLA-4 genes¹⁹⁴. This study however identified 13 SNPs associated with either increased (n=5) or reduced risk (n=8) of inhibitor formation, including mutations the genes for CD44, CSF1R, DOCK2, MAPK9, and IQGAP2¹⁸³. There has also been one case-control small (n=20) study evaluating gene expression profiles (RNA) using a microarray comparing patients with inhibitors (n=9) to

controls (n=11) ¹⁹⁵. Downregulation of Chemokine (C-C motif) ligand 3-like 1 and 3 (CCL3L1 and CCL3L3), Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) PPBP (CXCL7), Interleukin-8 (IL8) and upregulation of Chemokine (C-X-C motif) ligand 9 (CXCL9) and Chemokine (C-X-C motif) ligand 11 (CXCL11) were seen in patients with inhibitors. These have all previously been shown to be involved in the regulation of the immune response ¹⁹⁵. Although these findings provide interesting preliminary data, this study is severely limited by a small sample size, poor case-control matching and lack of clarity in the sampling methodology. Further study of changes in mRNA expression in patients with inhibitors may provide a dynamic biomarker for prediction of risk, which is discussed in more detail in Chapters 9 and 10.

Environmental Risk Factors in Severe Haemophilia

FVIII administration alone is insufficient to provide danger signals for the development of an immune response. As such environmental factors relating to context of treatment could provide differential risk. Characterisation of these factors is important as this could allow modification of risk or future alternate treatment strategies in those who are known to have high baseline risk due to genetic factors. The main risk factors identified from observation studies relate to intensity of early exposure to FVIII. Intensive treatment for bleeding or surgery (HR=2.0; 95%CI 1.3-3.0) and high dose FVIII treatment (HR=2.3; 95%CI 1.0-4.8) has been associated with increased risk of inhibitor formation at the start of treatment ¹⁴⁹. Although age at first treatment, in a number of studies, was found to be a risk factor for inhibitor formation, data in the CANAL study has found that this directly related to treatment intensity ¹⁰⁰. No association with inhibitor risk has been seen relating to vaccination ^{196;197}, breast-feeding ^{100;196}, inter-current infection ^{196;198} or treatment for central nervous system bleeding. Within both the CANAL (RR=0.4; 95%CI 0.2-0.8) ¹⁰⁰ and RODIN (HR=0.6; 95%CI 0.47-0.99) ¹⁴⁹ studies, usage of prophylaxis was associated with a significant reduction in risk of inhibitor. The Kaplan-Meier curve of inhibitor incidence within the RODIN study, however, demonstrated no significant difference in risk comparing those on prophylaxis and on-demand during the first 20ED, but after this time point there is a marked reduction in the inhibitor incidence in those on prophylaxis ¹⁴⁹. Data from a large cohort of severe haemophilia patients from the UK has suggested that there is increased inhibitor risk occurring with age ≥ 60 years (discussed above) ¹⁹⁹. The underlying reasons for this requires further study, but could relate to treatment related factors (e.g. major surgery) or immunosenescence. There has been great discussion and controversy, within the recent literature regarding

immunogenicity relating to different FVIII concentrates which represents the most (if not only) modifiable risk factor in the treatment of patients with severe haemophilia A. There have been conflicting reports of inhibitor risk relating firstly to the source of FVIII concentrate, whether from a plasma derived or recombinant source^{154;176;200-202}. There have also been reports of differences in immunogenicity within FVIII sources, relating to VWF content (pdFVIII)^{154;203} and for BDD-FVIII^{155;204} and second generation rFL-FVIII concentrates (rFVIII)^{154;205-208}. There is clinical equipoise surrounding immunogenicity relating to FVIII concentrates and further study is required. There have also been concerns that switching between FVIII concentrates may be associated with increased inhibitor incidence, although this was not seen in a recent prospective report from the UK relating to product switching from rFL-FVIII concentrates to a recombinant B domain deleted FVIII concentrate (rBDD-FVIII)²⁰⁹.

1.5.4.2 Non-Severe Haemophilia A

Risk factors for inhibitor formation in non-severe haemophilia A are less clear, due to a paucity and difficulty in construction of large studies in these patients.

Genetic Risk Factors in Non-Severe Haemophilia

Similar to findings seen in severe haemophilia A, the causative *F8* mutation appears to influence risk of inhibitor formation^{64;166;210;211}. Following on from earlier reports of increased incidence of inhibitor formation relating to a number of specific *F8* point mutations, the INSIGHT study has provided an extensive analysis of risk relating to underlying *F8* mutation. In this study, mutations associated with increased incidence of inhibitor formation (risk at 50ED) were: Arg593Cys (18.3%), Asn618Ser (2.9%), Asp2074Gly (21.2%), Arg2150His (12.2%), Arg2159Cys (39.4%) and Trp2229Cys (41.7%)¹⁶⁶. A study of 1135 patients with missense mutations has also recently described significantly increased inhibitor risk where there is a substitution of an amino acid belonging to a different physiochemical class (inter-class) compared to a switch within the same class (intra-class)²¹¹. In this analysis the most significant risk (p=0.048) occurred when the substitution involves a small/hydrophobic (class I) amino acid. This study consisted of patients with all severities of haemophilia and of the patients with inhibitors, 42% (15/36) had severe haemophilia. A previous *in-silico* study of missense mutations showed a trend towards increased frequency of a switch to different group in those with severe 78.2% (240/307) compared to mild haemophilia 71.4% (235/329)

($p=0.05$)⁹⁵, although this effect is small. Whether these inter-class switches result in a change in phenotype, which is more marked for changes from a class I amino acid, is not known.

A small study of mild haemophilia A patients ($n=45$) with the Arg593Cys mutation did not show any statistically significant HLA associations in patients with inhibitors ($n=7$) compared to those without ($n=38$)²¹². There is no data relating to whether ethnicity or *F8* haplotype are risk factors for inhibitor formation in non-severe haemophilia A. Similar to studies in severe haemophilia, this is complicated by low inclusion/recruitment rates of non-Caucasian patients in clinical studies. The INSIGHT Study only included 14/1112 (1.3%) African-American patients. Similarly, there is no data on the effect of FVIII haplotype, family history or SNPs in genes involved within the immune response. These areas are of interest for further research, as differences between endogenous and exogenous FVIII or in the immune response could be more permissive to presentation of novel T-cell epitopes to the immune system, which is discussed in more detail in Chapter 4.

Environmental Risk Factors in Non-Severe Haemophilia

Environmental risk factors for inhibitor formation described in patients with non-severe haemophilia A include surgery (in patients with a high risk mutation) and intense exposure to FVIII^{173;174;213}. A case-control study performed by the Division of Blood Disorders of the Centers for Disease Control and Prevention has recently reported evaluating inhibitor risk in 98 patients (cases=36, controls=62). There was a strong association with intensive FVIII treatment, which was greater in those >30 years (OR 13.54), in comparison to those <30 years. Within this analysis ethnicity, FVIII:C 1 to <2%, <50ED prior, young age (<5 years) at first FVIII treatment and rFVIII usage were not significant. The incidence of the Arg593Cys mutation was more common in cases than controls (19.4% v 4.8%, $p=0.03$), but interestingly this lost significance within the multivariate analysis. Preliminary results (307 patients with 77 inhibitor cases) from the INSIGHT case-control study were presented in abstract format in 2013¹⁶⁷. After adjusting for potential confounding variables (ethnicity, mild/moderate haemophilia, family history and age), neither treatment intensity, for moderate (3-4ED) or major (≥ 5 ED) bleeding nor surgery were found to be independently associated with inhibitor formation compared to minor bleeding or absence of surgery respectively. Similar to observations seen within PTPs in severe haemophilia A, there has been reported increased inhibitor incidence occurring with age¹⁷⁴, with a trend towards increasing risk of inhibitor development in those >60 years (OR=1.8; 95%CI 0.79-4.17) within this preliminary analysis.

Whether this represents changes in the aging immune system or represents more intensive FVIII exposure occurring later in life due to factors such as surgery is not clear. The final analysis of the INSIGHT case-control study is awaited, which may provide further detail of environmental risk in these patients.

The risk relating to different FVIII concentrates in patients with non-severe haemophilia A has recently been evaluated within the first four years of the EUHASS registry¹⁷². This data reported on 7,969 patients with non-severe haemophilia A, with a total of 37 inhibitors and an inhibitor rate of 0.43/100 treatment years. Inhibitor rates were similar between different concentrate brands and classes. The inhibitor rate for pdFVIII concentrates was 0.14/100 years (95%CI 0.14-0.42) and 0.52/100 years (95%CI 0.36-0.73) for rFVIII concentrates, with overlapping confidence intervals.

1.5.5 Timing of Inhibitor Testing in Haemophilia A

In severe haemophilia A, as most inhibitors occur early in the course of treatment (50% within the first 15ED), the aim of inhibitor testing is to detect emerging inhibitors to allow commencement of inhibitor eradication therapy with immune tolerance induction⁹⁹. Based on clinical trial data, this has allowed the development of guidance suggesting that severe haemophilia A patients should be tested every 3EDs (or every 3 months) in the first 20EDs, every 3-6 months until 150ED and then 1-2 times/year²¹⁴. Alongside this, patients undertaking a product switch should be tested prior to and twice in the 6 months following switching²¹⁴. Actual, "real-world" timing of inhibitor testing undertaken in the PUP setting is not clear, although recently published data from the CANAL and PedNet studies has provided some important descriptive data in this area²¹⁵. Within the most recent birth cohort (2005-2009, n=305) in these studies, there was a median of 5 inhibitor tests (IQR 3-8) performed within the first 50ED²¹⁵. There was increased intensity of screening seen with time comparing the first 1990-1994 (1.9 tests/year) and last 2005-2009 (4.3 tests/year) birth cohorts²¹⁵. These studies²¹⁵, and a previous meta-regression¹⁷⁶, suggest that increased inhibitor testing is associated with an increased incidence of inhibitor formation, primarily through detection of low-titre inhibitors²¹⁵.

In non-severe haemophilia A, there is a paucity of data to provide evidence-based guidance on the timing and frequency of inhibitor testing. Published guidance in the UK advises inhibitor screening following episodes of perceived "high-risk" exposure to FVIII, based on consensus opinion and previously published case-series and cohort studies. These suggest all

patients treated with FVIII should be tested annually and following intensive exposure ($\geq 5ED$), surgery or any FVIII exposure in patients with “high-risk” *F8* gene mutation ²¹⁴. There is no data, to define current practices for inhibitor screening in non-severe haemophilia A as has been described in patients with severe haemophilia ²¹⁵.

1.5.6 Treatment of Bleeding in the Presence of Inhibitory Antibodies

Treatment options for managing bleeding in haemophilia A patients with inhibitors, include usage of FVIII concentrates or “bypassing agents”. FVIII concentrates may be of use in the treatment of bleeding in patients with low-titre antibodies, where it may be possible to saturate the inhibitor to provide adequate FVIII levels for haemostasis, although this approach may risk an amnestic response. Patients with high-titre antibodies, or in whom bleeding is unresponsive to high doses of FVIII, are treated with bypassing agents. Bypassing agents used in the UK include Factor Eight Inhibitor Bypassing Activity (FEIBA[®]) and recombinant activated factor VII (rFVIIa or NovoSeven[®]). These two agents appear to have similar efficacy for the treatment of bleeding based on data from two randomised studies ^{216;217}, although further study is required in this area ²¹⁸. Interestingly, patients with inhibitors may display different responses to the two bypassing agents, which may be seen on *in-vitro* spiking experiments, which some groups have used to guide management of patients with inhibitors requiring surgery ²¹⁹. Bypassing agents are recommended as the first line choice for the treatment of severe bleeding in AHA ¹¹⁹. Although no direct studies of these two agents have been performed to date in AHA, both are thought to be similarly efficacious. Within the EACH2 study the efficacy of rFVIIa and FEIBA[®] for control of bleeding events was 91.8%, with propensity score matching demonstrating equal efficacy for these approaches (OR=1; 95%CI 0.23-4.44; p=1) ²²⁰.

1.5.7 Treatment Aimed at Inhibitor Eradication

The mainstay of treatment aimed at inhibitor eradication in patients with severe haemophilia A is through immune tolerance induction (ITI) ²¹⁴, although other approaches have been described (reviewed in Laros-van Gorkom et al. ²²¹). ITI involves regular infusion of FVIII either alone or in combination with immune modulatory treatment with the aim of restoring “tolerance” to FVIII. The three main approaches used have included the Van Creveld (low dose FVIII), Bonn (high dose FVIII) and Malmö (high dose FVIII and immune modulation) protocols ²²². Despite the usage of this treatment in patients with inhibitors for more than 30 years, there is still very little information, regarding the cellular mechanism behind this

process²²³. Proposed mechanisms include development of anti-idiotypic antibodies which antagonise the epitope recognition Fab site of the inhibitory antibody and inhibition of differentiation of FVIII specific B cells. This does not, however, appear to cause deletion of FVIII specific T cells²²³.

Multiple studies have been carried out investigating the response to immune tolerance induction in patients with severe haemophilia A, with response rates seen to ITI from national and international registry data ranging from 51-76%, using varying ITI dosing protocols^{222;224-228}. The International-ITI study has given more insights into the question of dosing in ITI²²⁹. This study was a prospective multicentre randomised controlled study comparing a low dose (50IU/kg, 3 times a week) to a high dose (200IU/kg daily) ITI regimen in 115 patients with severe haemophilia A with inhibitor. Overall, ITI was successful in 69.7% (n=46) of patients and a partial response seen in a further 4.5%. Although success rates did not differ between the two arms of this study, time taken to reach all criteria for success (inhibitor negativity, normal recovery and tolerance) was significantly shorter in patients treated on the high dose regimen. Alongside this the rate of bleeding was higher in patients treated with a low dose regimen in comparison to a high dose regimen, prompting premature cessation of the study. The time to achievement of tolerance on ITI is variable between studies. Usage of protocols including immunomodulation such as the Malmö protocol reported median times of 1 month, although at the expense of inpatient treatment and exposure to cytotoxic agents²³⁰. Times to response in the five registry studies reported range from 7.6 to 16.3 months, with similar findings seen in the international ITI study (high dose 14.2 months and low dose 16.4 months)^{222;224-229}. The undertaking of ITI is a great commitment for patients and their family and is associated with significant cost. An estimate of overall costs incurred per patient on ITI were between 2.4 (low dose) and 3.4 (high dose) million Euros²³¹. The international ITI study has formed the basis for current guidance in the management of immune tolerance induction in patients with inhibitor in the United Kingdom²¹⁴. Following on from this guidance a national protocol has recently been proposed by the UK Haemophilia Centre Doctors' Organisation (UKHCDO) Inhibitor and Paediatric Working Parties, for guidance for initiation, dosing, monitoring and weaning of ITI in patients with severe haemophilia A²³². This now advised commencement of ITI immediately, in the presence of an inhibitor detectable by the Nijmegen-Bethesda assay on more than one occasion, interfering with prophylaxis or treatment of bleeds, with gradation of dosing for initiation dependent on the inhibitor titre.

Data in patients with non-severe haemophilia A has suggested that these patients have a poorer response to ITI ^{64;214}. In one case-series, describing eight patients treated with various different ITI regimens, a complete success was seen in two, partial response in four and no response in two patients. In the INSIGHT study, inhibitor disappearance occurred in 70% (51/73) of patients who underwent observation and 75% (21/28) of those treated with inhibitor eradication therapy (n=28) ¹⁶⁹. A variety of eradication therapies were used including ITI (n=16), immunosuppression (n=5) or a combination of ITI and immunosuppression (n=7). The median time to inhibitor disappearance, in those observed and treated with inhibitor eradication therapy was 9 (IQR 3-16) and 15 (IQR 7-38) months respectively. Patients were more likely to be treated with inhibitor eradication therapy if there was a fall in FVIII to ≤ 1 IU/dL (46% v 17%) or when treated in a smaller haemophilia treatment centre (53% v 22%). Patients treated with ITI were younger (median 13 years) than those that did not receive eradication therapy (median 42 years) and those receiving immunosuppression were older (median 69 years) than those receiving other eradication therapies. The authors suggest that there has been a change in practice with time, with eradication therapy not being administered before 1990, although this only constituted 4 patients in the study. When looking at the response to treatment (or observation) this will be dependent on whether the patient is re-challenged to FVIII (sustained response). The sustained response rates were 60% (21/35) and 83% (10/12) in those who were observed and treated with inhibitor eradication therapy. Although 65% of the total cohort were re-challenged with FVIII no details are provided as to the timing and methodology for inhibitor testing to confirm sustained response. Although there was no difference in the sustained response rate for use of inhibitor eradication therapy for low-titre antibodies (unadjusted relative probability=0.8, 95%CI 0.4-1.8), inhibitor eradication was more likely to be associated with sustained response for high-titre antibodies (unadjusted relative probability=2.3, 95%CI 1.3-4.3). A small survey (n=39, response rate 22.9%) of treatment approaches in adult non-severe haemophilia A patients from the USA demonstrated similar heterogeneity in the approaches to immunosuppression depending on the clinical scenario ²³³. In asymptomatic patients with a low-titre inhibitor, 77% (n=30) of respondents opted for observation alone, with only 18% (n=7) taking a similar approach in patients with a high-titre inhibitor who was bleeding. Although the most common approach for inhibitor eradication in those with a high-titre inhibitor and bleeding was to commence ITI once the titre was < 10 BU/mL, there were a variety of other approaches selected including use of rituximab and steroids (methylprednisolone). Although interpretation of these survey data is limited by a low

response rate, differences in approach to management of inhibitors in the non-severe setting based on the presenting features have been characterised both in this survey ²³³ and the INSIGHT study ¹⁶⁹, which directly affect interpretation of outcomes. There are still many questions in the optimal approach to management of inhibitors in patients with non-severe haemophilia A and further prospective study is required.

1.5.8 Classification and Physiology of Factor VIII Antibodies

Although inhibitors form an important class of clinically relevant FVIII antibodies, these represent only a proportion of the true immune response to FVIII, which also includes antibodies without direct inhibitory capacity (non-neutralising, NNA). Clinical studies investigating FVIII antibodies in congenital and acquired haemophilia have primarily defined end-points using a functional haemostatic assay for antibody characterisation, resulting in incomplete understanding of the true humoral response to FVIII. The clinical significance however of non-neutralising and proteolytic antibodies is not fully understood due to a lack of prospective studies studying long term outcomes in patients with these antibodies. Lack of characterisation of the complete immune response could impair the significance of clinical study findings, resulting in heterogeneity of risk factors and treatment responses described.

1.5.9 Inhibitory Factor VIII Antibodies (Inhibitors)

An inhibitor is an antibody directed towards either endogenous (allo-immune) or exogenous (allo- or auto-immune) FVIII, detected using a functional clotting based assay. Circulating anticoagulants, reducing the effect of early FVIII replacement therapies in haemophilia and in previously normal individuals, were first described over 70 years ago ²³⁴⁻²³⁷. Inhibitory antibodies are time and temperature dependent, which forms the basis of functional laboratory assays used in their detection. Inhibitors in congenital and acquired haemophilia A, are polyclonal immunoglobulins consisting almost exclusively of the IgG isotype ^{238;239}, although rarely, anti-FVIII IgA and IgM has been seen in patients with acquired haemophilia A ²⁴⁰. These inhibitors have been shown to be predominantly of the IgG1 and IgG4 subclasses ^{135;152;241}. Contribution of the IgG2 subclass is variable between reports and IgG3 is rarely seen ^{134;241}. A recent study has suggested that patients with low-titre antibodies are mainly of the IgG1 subclass and those with high-titre antibodies of the IgG4 subclass ^{241;242}. Whether differences in outcomes exist in those with IgG1 and IgG4 FVIII antibodies is not clear and requires further study. This is currently being evaluated prospectively in patients with severe

haemophilia A in the Hemophilia Inhibitor Previously Untreated Patients Study (HIPS, ClinicalTrials.gov Identifier: NCT01652027).

1.5.10 Mechanisms of Factor VIII Inactivation and Inhibitor Epitopes

The mechanism via which inhibitors act, will be dependent on its epitope (antigenic determinant) to FVIII. Dominant domain-based epitopes have been described in patients with congenital haemophilia A within the A2 and C2 domains (68%) and a3 region (46%)^{243;244}. FVIII auto-antibodies are directed towards the A1, A2 or C2 domains^{245;246}. One of the main mechanism via which inhibitory antibodies act is via steric hindrance. This could result in inhibition of the interaction between FVIII and VWF, phospholipid membranes, FVIII activation/inactivation by thrombin or FXa, interaction within the tenase complex with FIXa and FX and impairment in hetero-trimer formation. Other proposed mechanisms include increased clearance and through direct catalytic activity. Given the polyclonal nature of this immune response, it is likely that FVIII antibodies may have action via a number of these mechanisms. A change in epitope profile with time (epitope spreading) may potentially occur in patients with FVIII antibodies^{148;247}. This results in the development of an immune response against distinct and non-cross reactive epitopes from the original epitope, which has been seen as part of the normal immune response to infection and in autoimmune disease²⁴⁸. A number of techniques have been applied to investigate the epitope of anti-FVIII antibodies from both patients and monoclonal antibodies. These techniques have included immuno-precipitation, phage displayed peptides, solid phase peptide synthesis (SPPS) / Spot techniques and more recently an immunoassay utilising Luminex[®] techniques²⁴⁷ which will be discussed in more detail in Chapter 8. The majority of these techniques are not suitable for use in routine clinical practice due to labour intensity and sample volume requirements. Alongside this, many of these techniques are unable to identify conformational (or discontinuous) epitopes for antibodies. There is as yet no high-throughput approach for the investigation of amino acid sequence epitopes for use in patients with FVIII antibodies.

1.5.11 Kinetics of Inhibitor Activity

Inhibitors can be further classified based on their inactivation kinetics as being type I or II inhibitors. Type I inhibitors cause complete (>98%) inactivation of FVIII:C with a linear relationship between antibody concentration and log-residual FVIII:C. These antibodies are time and concentration dependent and potentially saturable in the presence of sufficient FVIII:C^{249;250}. Type II inhibitors demonstrate an inability to completely inactivate FVIII:C even

when undiluted, with a non-linear (complex kinetics) relationship between antibody concentration and log-residual FVIII:C. Repeated additions of FVIII, will therefore be required before saturation of the antibody is reached²⁵⁰⁻²⁵². Patients with severe haemophilia A are thought to primarily display type I antibodies^{250;253} and those with non-severe and acquired haemophilia A to display either type I or II inhibitors^{250;253}. Although the biological mechanism for these differences is not known, two potential hypotheses have been proposed²⁵³. The first of these is that the B-cell epitope formed by these different types of antibody may be different. It has been suggested that VWF may affect the kinetics of inactivation, with one study showing that most type II inhibitors showed partial inactivation only in the presence of VWF²⁵⁰, although more detailed studies of the binding epitopes of type I and II inhibitors have not been carried out to date. A second mechanism may relate to antibody binding affinity, with type I inhibitors having higher affinity than type II inhibitors²³⁹. It is interesting to note that clinically the bleeding pattern seen in patients with type I inhibitors, which tends to involve bleeding into joint or intra-organ varies from that seen in those with type II where bleeding is predominantly involves the soft tissue²³⁹. The mechanism for these differences is not apparent and is likely more complex than purely a function of the antibodies kinetics. Some studies have suggested that type II inhibitors may be more transient and immune tolerance induction may be more likely to be successful compared to type I inhibitors²⁵⁴.

1.5.12 Non-Neutralising Antibodies

Antibodies without detectable inhibitory capacity have been seen in patients with haemophilia A as well as in haemostatically normal individuals. There has been great variability in the reported prevalence (12-54%) of these antibodies within the literature, between studies and the assay used for their detection²⁵⁵⁻²⁶². In a recent study of 210 patients with haemophilia A, 18.1% of patients demonstrated non-neutralising antibodies²⁶¹. Within this study the prevalence of non-neutralising antibodies was not influenced by disease severity, age, *F8* mutation or FVIII concentrate (pdFVIII v rFVIII or FL-FVIII v BDD-FVIII). The majority of antibodies were directed against the heavy chain (73.7%) or both the heavy and light chains (13.2%) with only 13.2% being directed against the light chain alone. Of these antibodies 7/38 (18.4%) were directed to the B domain of FVIII²⁶¹.

The clinical relevance of this class of FVIII antibody is not clear. It has been hypothesised that NNAs may form immune complexes resulting in accelerated clearance of infused FVIII from

the circulation²⁶³. This theory might explain abnormal pharmacokinetic responses (impaired *in-vivo* recovery or increased clearance) in the absence of detectable inhibitory antibodies, although strong data to support this is lacking. The most widely cited support for this theory comes from a small study of severe (n=9) and non-severe haemophilia (n=14) haemophilia A patients²⁵⁶. In this group of patients 39% (9/23) were found to have non-neutralising antibodies using an in-house ELISA. The investigators studied the half-life of three patients (one with no detectable antibody, one with low-titre and one with high-titre non-neutralising antibodies) following an infusion of 30IU/kg of FVIII. The fall to baseline FVIII:C levels was seen after 36, 24 and 12 hours in these three patients, respectively. This has been interpreted by the investigators of indicating a reduced half-life in the patient with a high-titre non-neutralising antibody. Although an interesting observation, there are a number of limitations to this. Firstly, significant inter-individual variability is seen in FVIII pharmacokinetics in patients with haemophilia, making comparison of these variable difficult²⁶⁴. No historical pharmacokinetic data is available for these patients to demonstrate a true reduction in half-life. Finally, the presence of residual FVIII in the sample of this patient may have masked the presence of a low-titre inhibitory antibody (discussed in Section 1.5 and Chapter 7)²⁵⁶. Other studies looking at NNAs have failed to demonstrate an effect of these antibodies on FVIII pharmacokinetics²⁶⁵⁻²⁶⁷.

One study has looked at the longitudinal significance of non-neutralising antibodies in congenital haemophilia A. This study looked at the occurrence and natural history of non-neutralising antibodies in 78 patients (severe=74, moderate=4) over a four year period. In this cohort, ten patients (12.8%) developed non-neutralising antibodies, which were transient and no patients went on to develop inhibitory antibodies²⁶². Interestingly, the incidence of bleeding events was lower in patients that developed non-neutralising antibodies compared to those that did not. The study population, however, consisted entirely of previously treated patients who were mainly receiving continuous prophylaxis (89.7%). Given that dosing of prophylaxis is similar to that seen in low dose immune tolerance induction protocols (Van Creveld), these patients may have been tolerised by virtue of being on prophylaxis.

FVIII antibodies have been described in normal individuals as well as in patients with acquired or congenital haemophilia. A study of 500 healthy blood donors demonstrated the presence of FVIII antibodies (“natural antibodies”) in 17% (85/500) samples that had been heat treated (56°C for 60 minutes) prior to being tested with the Bethesda assay²⁶⁸. In these samples the

inhibitory activity ranged from 0.4-2.0BU/mL (low-titre), with an even distribution of antibody positivity in male and female donors. The divide between whether an antibody is a non-neutralising or “natural antibody” is not clear and clear antibody negativity on testing prior to first receiving FVIII treatment would be required to ensure that the measured antibody is truly a response to infused FVIII. Whether an antibody is classified as being non-neutralising or not is also affected by the sensitivity of the assay used to describe its functional activity. Differences in the characteristics of non-neutralising antibodies have been seen in patients without inhibitor history or healthy controls compared to those with inhibitors. Although, IgG1 and IgG4 were characterised in patients with FVIII inhibitors, there was a complete absence of IgG4 in those without an inhibitor history or healthy controls ²⁴². No correlation was seen between FVIII antibody (IgG1 and IgG3) presence and age or sex in the healthy controls. The binding affinity of FVIII antibodies seen in patients with inhibitors is substantially higher (100 fold) in comparison to healthy individuals and those without inhibitors ²⁶⁹. These findings are in keeping with the process of affinity maturation and class switching that has previously been characterised. Whether low affinity, non-neutralising antibodies may provide a biomarker for the development of a more established immune response, is not clear and requires further study in a well-constructed prospective collaborative study, with adequate centralised FVIII antibody testing.

1.6: Laboratory Detection of Factor VIII Antibodies

1.6.1 Functional (Haemostatic) Assays for the Detection of FVIII Antibodies

Recognition of the occurrence of inhibitory antibodies to FVIII has been described in the literature in both patients with congenital and acquired haemophilia A for greater than 70 years ²³⁴⁻²³⁷. The gold standard laboratory test for the detection and quantification of FVIII antibodies is a functional assay (Nijmegen Bethesda assay, NBA) ²⁷⁰. The principle underlying this (and related assays) assay, is that the FVIII antibody presence and inhibitory potency is calculated based on the degree of inhibition of FVIII:C when incubated in a known quantity of FVIII. These assays are therefore only able to detect FVIII antibodies with direct inhibitory capacity. Despite widespread usage of these assays in routine clinical practice, none have been systematically evaluated or reported following standard criteria, such as those recommended by the “STAndards for the Reporting of Diagnostic accuracy studies” (STARD) initiative ²⁷¹. Evaluation of assay performance to assess the presence of FVIII antibodies is limited by the lack of a pathological (e.g. histological sample) gold standard for comparison. This comparison is also limited by incomplete understanding of the significance of FVIII

antibodies with weak or no inhibitory capacity. Further, when evaluating inhibitors, no formal evaluation of what defines a positive or negative assay has been performed, with cut-offs based on consensus opinion ²⁷⁰. In this section the principles, evolution, clinical utility and limitations of these assays will be reviewed.

1.6.2 Classical Bethesda Assay

The classical Bethesda assay (CBA) which forms the basis for modern inhibitor testing was developed from consensus opinion at a meeting of American Haematologists at the Division of Blood Diseases and Resources, National Heart and Lung Institute in Bethesda, Maryland in 1974 ^{272;273}. Prior to this, there was great variation in inhibitor testing methodology and quantification, potentially impacting on management if a patient were to be treated at two centres ²⁷³. The CBA formed a uniform assay technique using standardised (consensus) units to facilitate comparison between centres and clinical studies for use in patients with severe haemophilia A ²⁷³. This assay involves incubation of test and control samples at 37°C for two hours in stoppered tubes (Figure 1.5). The test mixture consisted of one part citrated test plasma, with an equal part of normal pooled plasma and the control mixture, one part normal pooled plasma with one part imidazole buffer/NaCl (pH 7.4). Following sample incubation the FVIII:C is measured and the percentage residual FVIII:C found in the test sample relative to the control sample is then used to calculate the inhibitor titre. If no inhibitor is present then the expected residual FVIII:C, compared to the control mixture, would be 100%. A FVIII:C residual of 50% is equal to One Bethesda Unit / millilitre (1BU/mL) of inhibitor activity. Based on the residual FVIII:C, the inhibitor titre can be extrapolated from a reference curve (semi-logarithmic) of residual factor VIII (log scale) against inhibitor titre (linear scale) ²⁷³. A line is then drawn between points corresponding to 100% residual / 0 BU/mL and 50% residual / 1BU/mL (Figure 1.4)²⁷⁴. The inhibitor titre can then be read from this graph, where the FVIII residual lies between 25-75%. If the residual FVIII:C is <25% then serial doubling dilutions of the patient sample mixed with the incubation mixture are tested until the residual lies within this range. If the residual is >75% and there is clinical suspicion of inhibitor presence usage of a more sensitive assay is suggested. Calculation of the inhibitor titre can also be made using the linear regression equation formed by this curve ²⁷⁵: $INH = [(2 - \log_{10}R)/0.30103] \times D$; where INH Inhibitor titre (BU/mL); R=residual FVIII percentage and D=dilution factor.

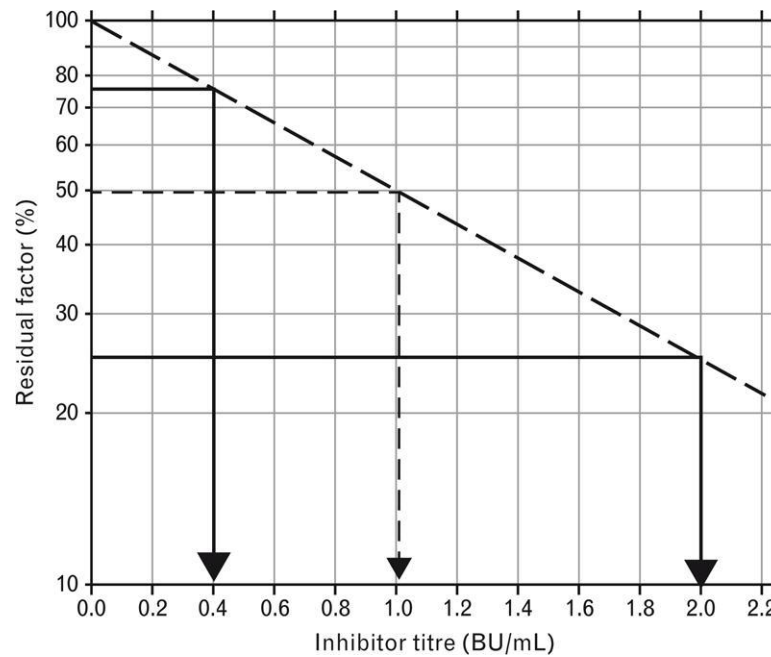


Figure 1.4: Reference Curve for the Bethesda Assay. Reproduced from Kershaw et al. 2012, with the permission of the publisher ²⁷⁴.

1.6.3 Modifications to the Bethesda Assay

Modification 1: Nijmegen-Bethesda Assay

The CBA, despite bringing about a standardised approach for inhibitor testing in severe haemophilia A lacked specificity, particularly around the lower range of inhibitor activity ²⁷⁶. Observations of potential false positives arising from the CBA with insufficient buffering or variable protein concentrations ²⁷⁷ resulted in the optimisation of this assay to improve testing specificity (Nijmegen-Bethesda assay, NBA) (Figure 1.5) ^{274;276;278}. This included buffering of the normal pooled plasma (test and control) with 0.1M imidazole to stabilise the pH at 7.4 and replacement of imidazole buffer in the control mixture with FVIII deficient plasma to give comparable protein concentrations in both mixtures. A comparison of the two assays (CBA and NBA) was performed using samples from patients with and without inhibitors. In patients with severe (n=10) or non-severe haemophilia A (n=22) with no inhibitor history, the inhibitory activity found was to be 0.0BU/mL compared to 0.5-0.9BU/mL using the NBA and CBA, respectively. Similar inhibitor titres were seen comparing the results of both assays in patients with an inhibitor history on treatment (n=6) and in those with high-titre inhibitors (n=7) ²⁷⁶.

Modification 2: 4% Bovine Albumin

Cost issues relating to replacement of the imidazole buffer with FVIII deficient plasma in the control mixture have been suggested as one reason for less than universal adoption of the NBA ²⁷⁹. The same investigators, who described the NBA have evaluated usage of 4% bovine serum albumin (BSA) with or without VWF, in substitution for FVIII deficient plasma in the control. This was first evaluated in spiking experiments of purified anti-FVIII:C IgG combined with plasma from a single patient with severe haemophilia A, titrated to an inhibitor titre of 1.5BU/mL with assays performed in quadruplicate ²⁷⁹. This showed good agreement between the results of the inhibitor assay using congenital FVIII:C deficiency plasma (F8DP) or 4% BSA, with or without the addition of VWF (1.61, 1.59 and 1.63BU/mL, respectively). This preliminary analysis was confirmed in samples from 6 patients with known inhibitor presence ²⁷⁹. A validation of this modification has been recently presented, comparing usage of F8DP with 4% BSA, in 59 samples (35 patients) with known FVIII inhibitors, demonstrating good agreement between the results of the two assays, especially in those of low-inhibitory capacity (<2BU/mL) ²⁸⁰.

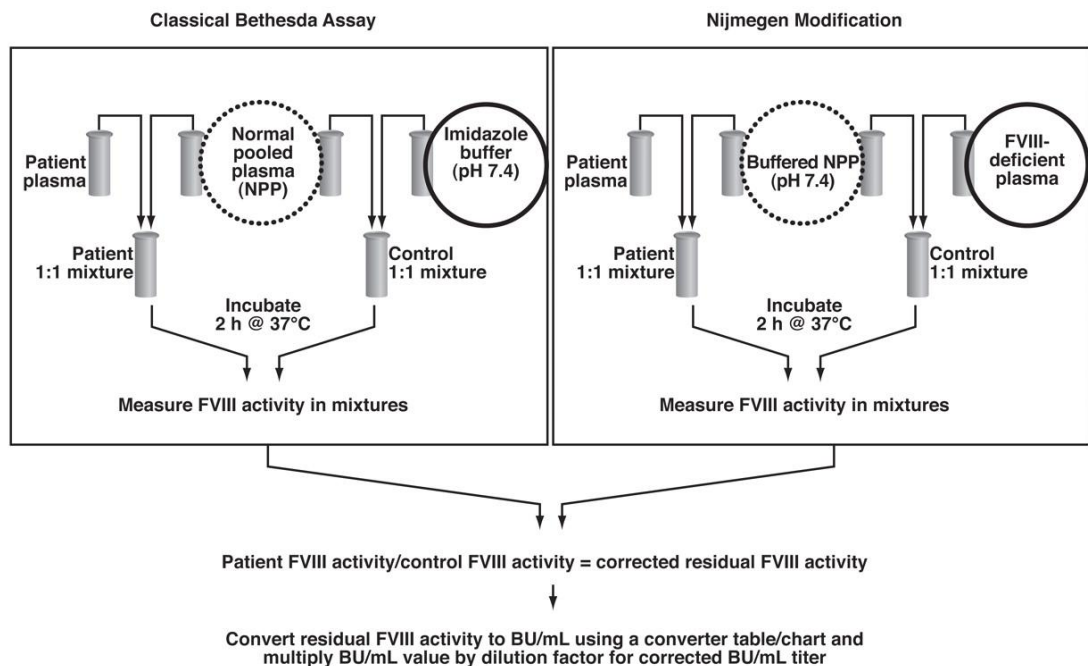


Figure 1.5: Comparison of the Classical Bethesda (CBA) and Nijmegen Bethesda assays (NBA). Reproduced from Peerschke et al. with permission of the publisher ²⁷⁸.

1.6.4 Validation of the Nijmegen-Bethesda Assay

The CBA and NBA have been compared in an inhibitor surveillance study during switching from pdFVIII to rFVIII in Canada in the early 1990s²⁸¹. This study included 877 samples tested in a single central reference laboratory. In the first year of this study all samples were monitored using the CBA and from the second year onwards all samples were tested in parallel. A total of 877 samples (233 prior to conversion) were tested using a consensus cut-off of ≥ 0.5 BU/mL for the assays.

	CBA +ve	CBA -ve
NBA +ve	34	1
NBA -ve	13	829

Table 1.3: Assessment of the classical Bethesda assay (CBA) in comparison to the Nijmegen-Bethesda assay (NBA) in a large Canadian population of haemophilia A patients on product switching²⁸¹.

There were 35 samples positive by NBA, with all except one of these being positive by the CBA. There were 13 samples that were positive by the CBA and negative using the NBA, all of which were of low-titre (0.5-0.8BU) (Table 1.3). No significant difference was seen in inhibitor titres comparing the CBA and NBA (mean \pm SD: CBA 15.5 \pm 34.6 v 33.4 \pm 69.7, $p < 0.05$). All 31 “grey-zone” samples (> 0 BU/mL to < 0.5 BU/mL) detected using the CBA were negative using the NBA.

1.6.5 Assay Positivity using the Classical and Nijmegen Bethesda Assays

When the Bethesda assay was first described, no cut-off between positivity and negativity was assigned, with this being left to individual laboratories to assign^{273;276}. Prior to the introduction of the NBA the range of cut-offs seen in one report was between 0 and 0.8BU/mL²⁷⁶. In the Canadian switching study which compared the CBA and NBA in parallel, a survey of centres involved, showed, although approximately 50% (9/19) of centres used a cut-off of > 0.5 BU/mL, there was a wide range from > 0 BU/mL to > 1.9 BU/mL^{281;282}. This study also showed the difficulties that arise in samples that they defined as being in a “grey-zone” of < 0.5 BU/mL, with these samples being positive by one assay and negative by another assay. A recently published study, using centralised inhibitor testing in 710 samples from patients with or without an inhibitor history described a cut-off of ≥ 0.5 BU/mL, based on comparative data of inhibitor titres seen in both groups²⁸³. There has also been a consensus statement in 2007, suggesting usage of a threshold of ≥ 0.6 BU/mL²⁷⁰. As part of the EUHASS study, data on

methodology of inhibitor testing and local assay cut-offs is being collected which will give further information into current inhibitor testing practices ^{171;284}.

1.6.6 Application of Functional Assays in Clinical Practice and Trials

Functional inhibitor assays give a quantitative description of inhibitory capacity, facilitating comparison of results between clinical studies, at least for type I inhibitors. The results from these assays have been used to guide treatment and dosing practices in patients with inhibitors. For example, patients with low-titre inhibitors (<5BU/mL) are generally treated with high dose FVIII treatment as a first line treatment for bleeding ^{214;285}. For type I inhibitors the assay result can be used to make a mathematical approximation of the FVIII dose required to saturate an inhibitor^{101;285;286}. An example of a formula used for dosing based on the inhibitor titre from the Bethesda assays is as follows: FVIII Dose = (40 X Body Weight (kg) x BU) + required therapeutic dose (in absence of an inhibitor) ¹⁰¹. As well as this, various studies in congenital and acquired haemophilia A have attempted to risk stratify patients based upon their inhibitor titre. Studies of patients undergoing ITI have used the results of the Bethesda assay to classify patient as being good or poor risk (i.e. those likely to undergo successful ITI) ^{226;227}. All of the large international studies investigating environmental and genetic risk factors have only used a functional assay to determine their end-point of antibody development to FVIII ^{99;100;149;154;166;178;183;188-191;194;287}. The true incidence of antibody formation will be underestimated in these patient groups and this may impair the detection of significance of weak risk factors.

1.6.7 Pre-Analytical Variables and Assay Limitations

A number of pre-analytical variables may affect the results that are obtained using the Bethesda assay. A blinded study of laboratories from the Royal College of Pathologists of Australasia Quality Assurance Program looked at the performance of 42 laboratories has shown that a number of factors, including heparin, EDTA and lupus anticoagulants may be result in false positives using this assay ^{288;289}. The Bethesda assay was developed for quantification of type I inhibitory antibodies and will underestimate the inhibitory capacity of antibodies with complex kinetics and cannot detect antibodies without direct inhibitory capacity. This may have implications if this assay is used in dosing of FVIII replacement therapy or for risk stratification based on the inhibitor titre in acquired or non-severe haemophilia A. Further study is required to define the optimal strategies for testing for these different classes of FVIII antibodies. The commonest pre-analytical variable in routine testing

will be presence of FVIII in samples which will be discussed in the following section and Chapters 6 and 7.

1.6.8 Presence of Residual Factor VIII:C in Test Samples

The principle of the Bethesda assay (CBA and NBA) is based around the presence of equal amounts of FVIII:C in both the test and control samples prior to sample incubation. This relies on the fact that the patient's sample does *not* contain FVIII:C, i.e. a patient with severe haemophilia A, who has received no recent treatment. There are however a number of circumstances in which inhibitor testing may be required where samples will contain FVIII:C including:

- 1) Severe haemophilia A patients who have received FVIII treatment (prophylaxis, ITI or post-infusion)
- 2) Non-severe haemophilia A
- 3) Acquired haemophilia A (diagnosis or upon recovery)

Laboratories may take a number of approaches when performing inhibitor testing in the presence of pre-analytical FVIII:C; including testing without adjustment, rejection of samples in which FVIII:C is present ²⁹⁰, or usage of a modification to adjust for this. Although a number of modifications ²⁹¹ have previously been described, it is unclear whether these forms part of routine testing:

- 1) *In-vitro* correction: FVIII is added to the control sample, providing equal FVIII:C in both the control and test samples. This requires testing the FVIII:C of each sample prior to performing an inhibitor assay.
- 2) *In-silico* correction: A mathematical correction for residual FVIII:C present in test sample prior to testing can be calculated using the formula: $RES_{corr} = RES_{test} \times [100 + (100 \times FVIII_{pre}/D)]^{274}$, where RES_{test} = FVIII:C residual in the test sample, $FVIII_{pre}$ = FVIII:C present within the sample prior to testing and D = dilution factor. This also requires knowledge of the FVIII:C present in samples prior to inhibitor testing
- 3) Pre-analytical heat treatment: This involves sample incubation (56-58°C) prior to inhibitor testing to denature residual FVIII:C ²⁹². This provides a more standardised approach to sample preparation and does not require knowledge of FVIII:C in each test sample prior to testing. The modification is based on experimental data from the early 1970s, prior to standardisation of inhibitor testing ²⁹². Despite increased usage of this modification in recent studies ^{283;290;293-297}, varying incubation conditions have

been described and these have not been systematically evaluated using current laboratory methodology. A detailed discussion of this modification is provided in Chapter 7.

1.6.9 Inter-Laboratory Assay Variability and External Quality Assurance (EQA)

Similar to other areas of laboratory practice there are a number of national and international external quality assurance (EQA) schemes which include modules for the testing of FVIII antibodies. These EQA schemes include the UK National Quality External Assessment Scheme (UKNEQAS), European Concerted Action on Thrombosis Foundation (ECAT), World Federation of Hemophilia (WFH) and North American Specialized Coagulation Laboratory Association (NASCOLA). All of these projects are aimed at standardisation of laboratory practices in haemostasis. Functional assays used in the detection of FVIII antibodies have shown significant inter-laboratory variability as reported by two EQA exercises^{298;299}. The first of these was carried out over a 13 year period (60-120 centres) by the UKNEQAS scheme, which reported a co-efficient of variation of 33-106% in samples from congenital haemophilia with an inhibitor²⁹⁸. The second study carried out over a 3 year period (100-170 laboratories) by the ECAT scheme reported variation of 28-52%, with slightly lower variation for the NBA (39%) compared to the CBA (45%)²⁹⁹. In both surveys the variation described would have influenced patient management in some centres^{298;299}. Differences in the assay components, for example the source of plasma, were seen to influence the measured inhibitor titre between centres²⁹⁸.

1.6.10 Immunological Assays for the Detection of Factor VIII Antibodies

Given the limitations of functional inhibitor assays there is a requirement for more sensitive assays to detect FVIII antibody presence. Immunological based assays for antibody detection are commonplace within clinical haematology, transfusion and immunology laboratories. These approaches could enable description of the totality of the humoral response to FVIII and are likely to be less affected by the pre-analytical limitations of functional inhibitor assays. A number of techniques have been described in clinical and research settings, including enzyme linked immunosorbent assays (ELISA), fluorescence-based immunoassays (FLI), and immunoprecipitation (IP)^{259-261;266;300-303}. The most commonly used immunological based technique is the ELISA, developed from radio-immunoassays in the early 1970s^{304;305}. Despite a number of reports of commercial and research laboratory ELISAs for FVIII antibody

detection, it is unclear what role this test has in current clinical and laboratory practice³⁰⁰⁻³⁰³.

1.6.11 Laboratory Based ELISA

Two large studies have compared the use of a research laboratory based FVIII ELISA with the Bethesda assay^{302;306}. The first of these studies used a H1 haplotype rFVIII (Helixate®, CSL Behring, Haywards Heath, UK) as the ELISA plate antigen³⁰¹. Samples from 312 patients with haemophilia A (inhibitor positive=24 and negative=288) were tested in parallel using this ELISA and CBA in parallel, demonstrating a sensitivity of 100% and specificity of 97.8%³⁰⁶. The second study also used a H1 haplotype rFVIII (Kogenate®, Bayer Healthcare, Pittsburg, USA) as the ELISA plate antigen, with various secondary antibodies to assess Ig subclasses and IgG isotypes³⁰². Samples for 60 patients with severe haemophilia A (inhibitor positive, n=30 and inhibitor negative n=30) and healthy controls (n=30) were tested, demonstrating significant correlation between the optical density for Ig, IgG and IgG4 and the Bethesda assay³⁰².

1.6.12 Anti-Factor VIII ELISA Kit (GTI Diagnostics / Immucor)

A commercially available solid phase indirect ELISA kit has been available for qualitative and quantitative detection of FVIII antibodies since the late 1990s. This ELISA utilises a recombinant full-length FVIII as its plate antigen, with earlier versions using Recombinate® (Baxter Healthcare, Deerfield, USA) and current kits using Kogenate® (Bayer Healthcare, Pittsburg, USA)³⁰⁷ corresponding to H2 and H1 haplotypes of wild type FVIII respectively. Two medium sized studies have evaluated usage of the earlier version of this assay^{300;303}. The first of these studies looked at 131 samples (93 patients; 92 congenital HA and 1 acquired haemophilia A) over a period of 18 months (inhibitor sample prevalence, 32.8%), testing samples in parallel using the ELISA and the New Oxford Assay³⁰⁰. On the basis of the New Oxford Assay being a “gold standard” this study gave an unadjusted sensitivity of 97.7%, specificity of 78.4%, negative predictive value of 98.6% and positive predictive value of 68.9%. There were 19 false positives for the ELISA, which were felt to represent non-neutralising antibodies. Only moderate correlation was seen between the ELISA and functional assay ($r=0.68$). The largest study published to date looking at the use of this FVIII ELISA reviewed the results of 246 samples (176 patients) tested over an 18 month period³⁰³. This study looked only at samples that had tested positive by the Bethesda (CBA or NBA) assay (i.e. 100% prevalence). Of these samples, 235/246 were also positive by the ELISA,

corresponding to an unadjusted sensitivity of 95.5%, although with a lack of negative samples no further assessment of assay performance is possible. Strong correlation ($r=0.82$) was seen between the ELISA optical density and log-Bethesda titre³⁰³. Data from these studies showing high assay sensitivity could be interpreted as suggesting a role for this assay as a confirmatory test for FVIII antibody presence, although this has not been evaluated as part of routine practice in a large unselected cohort.

Chapter 2: Hypotheses and Aims

There is incomplete understanding of the total immune response to FVIII in both congenital and acquired haemophilia A, due to limitations in the current methodology used for FVIII antibody testing. Although standardisation of these approaches to testing since the 1970s has allowed comparison of results between clinical studies in severe haemophilia A, this has skewed assessment of risk of an immune response to FVIII towards antibodies with direct function/inhibitor capacity. The prediction of risk of antibody formation to FVIII and the response to treatment is based on a triad of genetic, environmental and diagnostic factors. An incomplete understanding at all three of these levels impairs the ability to accurately predict antibody occurrence or outcomes. Greater understanding of factors underlying FVIII formation will allow development of biomarkers of risk offering more personalised (stratified) approaches to the management of FVIII antibodies.

In the first part of this thesis, I will evaluate clinical and laboratory factors in settings where FVIII antibody formation is a rare event (i.e. non-severe haemophilia and acquired haemophilia). In both settings current clinical and laboratory practices surrounding testing for FVIII antibodies and their management is poorly defined. I hypothesise that heterogeneity relating to both clinical and laboratory approaches directly impacts on the outcomes reported within recent observational studies. In non-severe haemophilia A, I hypothesise that a lack of testing for FVIII antibodies by clinicians leads to underestimation of the occurrence of an allo-immune response and subsequently biases towards detection of cross-reacting antibodies to endogenous FVIII. In acquired haemophilia A, I hypothesise that heterogeneity in the management of immunosuppression and testing methodology affects prediction of response or relapse in patients.

In the second part of this thesis, I will evaluate laboratory methodologies for the detection of FVIII antibodies. This follows on from evaluation of clinical factors, as both non-severe haemophilia and acquired haemophilia are settings where there are limitations to using a functional inhibitor assays due to the kinetics of FVIII antibodies and the presence of residual FVIII prior to testing. There have been no previous diagnostic accuracy studies in haemophilia evaluating a new assay in comparison to the gold standard methodology (Nijmegen-Bethesda Assay, NBA) following the international recommendations of the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) initiative. I hypothesise, that immunological testing (ELISA) for FVIII antibodies will provide a screening test capable of detecting both inhibitory

and non-neutralising antibodies improving detection of FVIII antibodies where FVIII:C is present. Following on from this, I will evaluate a modification to inhibitor testing (pre-analytical heat treatment), to assess whether this may improve the sensitivity of testing for FVIII antibodies, where there is significant FVIII:C present in samples (i.e. acquired haemophilia A). There is no data describing the optimal incubation conditions for this modification. I hypothesise that different incubation conditions will have a differential effects on inactivation of FVIII:C and FVIII antibodies. I will systematically evaluate different incubation conditions in a controlled setting with an aim of providing guidance for the optimal conditions for routine usage of this modification in clinical laboratory practice.

Within the third section of this thesis, I will explore the usage of novel high-throughput techniques with the aim of developing biomarkers for risk prediction in severe haemophilia A. In the first part, developing my prior work in improving detection of FVIII antibodies using immunological methodology, I will evaluate a peptide microarray for B-cell epitope mapping (amino acid sequence level) of FVIII antibodies. I hypothesise that FVIII antibodies in patients with severe haemophilia A fall within immunodominant epitopes on the surface of FVIII. Given that a functional assay (NBA) is currently used for detection of inhibitors in clinical practice, I hypothesise these antibodies will display epitopes in antigenic regions that have direct functional roles in haemostasis. Evaluation of this platform will allow high-throughput characterisation of inhibitors in severe haemophilia A to provide biomarkers to predict response to treatment such as immune tolerance induction. In the second part of this section, I will evaluate the practicalities of sample collection for Next Generation Sequencing (NGS) of the transcriptome (RNA-Seq) across first exposure to FVIII concentrate in boys with severe haemophilia A. I hypothesise that use of a modified RNA low-volume sample tube (prepared within our laboratory) will provide RNA of sufficient quantity and quality to allow downstream transcriptome analysis in healthy volunteers and previously untreated patients with severe haemophilia A, which will be acceptable to clinicians with an international study. Application of NGS techniques, such as RNA-Seq will open up new strategies to contribute to further dissecting the immune response to FVIII at a molecular level.

Chapter 3: Materials and Methods

Within this section, detail will be provided of the materials and methods that are used in multiple parts of this thesis or within one of the core facilities. Within each chapter, specific methodology which is salient to the interpretation of the data provided will be described.

3.1 Factor VIII Antibody Testing

Materials

The following sourced reagents were used for testing of FVIII:C, FVIII antibody testing and optimisation of the pre-analytical heat treatment conditions: Lyophilised FVIII deficient plasma, Dade Standard human plasma, Dade Citrol 1, Owren Veronal Buffer, Control N and Control P (Siemens, Germany). Lyophilised Technoclone Factor VIII Inhibitor Plasma (Pathway Diagnostics, Dorking, UK). FVIII deficient plasma with inhibitor (George King Bio-Medical, Inc, Kansas, USA). Distilled Water (Baxter Healthcare, Deerfield, USA). VWR® Traceable® Water-Resistant Thermometer (VWR International Ltd, Lutterworth, UK). Solid phase indirect FVIII ELISA kit (Lifecodes/Immucor, Norcross, USA). Advate® 500IU (Baxter Healthcare, Deerfield, USA) and Wilate® 500IU (Octapharma AG, Lachen, Switzerland) which were kind gifts from The Royal London Hospital Haemophilia Centre (London, UK).

Sample Collection and Preparation

Blood samples were collected following local venepuncture policy. Following skin cleaning, samples were collected directly using a closed vacutainer system into sample tubes containing buffered 0.105M sodium citrate, (1 part anticoagulant to 9 parts blood) and mixed by gentle inversion. Platelet poor plasma (platelet count <10,000 per μL) was prepared by centrifugation at 2000g for 5 minutes. For samples in which batch testing was to be performed at a later date a double spin was performed (2000g for 10 minutes) to give platelet poor plasma with a platelet count <5,000 per μL and the plasma was transferred to a plastic tube and frozen directly at <-70°C. Prior to testing all frozen samples were thawed rapidly at 37°C for 5 minutes and testing was performed within 2 hours.

Automated Factor VIII Activity Measurement (One-Stage)

Lyophilised FVIII deficient plasma (F8DP), Dade Standard human plasma (SHP), Control N and Control P, were reconstituted each using 1mL of distilled water and these were allowed to equilibrate at room temperature for 15 minutes. Automated calibration was performed prior

to batch testing of samples using the Holder Calibration Curve Order. The target value (FVIII:C) for the SHP was entered in the assay sheet value box and the calibration curve was generated automatically and validated. Internal quality control was performed using Control N (normal control) and Control P (abnormal control) plasmas, which following reconstitution were transferred into labelled 2mL cups and loaded onto the analyser. Once the internal quality control was acceptable, assays were batch tested using an automated work list. Within clinical samples, found to have FVIII:C <10IU/dL or >200IU/dL samples were repeated using the MDA Low and MDA High settings, respectively. The results of the one stage FVIII:C was reported to one decimal point (1DP) between 1-800IU/dL FVIII:C. For FVIII:C outside of this range results were reported as either <1IU/dL or >800IU/dL respectively. Within testing of routine samples an in-house determined level of imprecision of 6% (normal control: Siemens Control N) and 7% (abnormal control: Siemens Control P) samples has previously been calculated.

An automated one stage FVIII:C assay, was performed for clinical samples tested at The Royal London Hospital, using a Sysmex CS2100i analyser. Within routine laboratory testing, multiple dilutions of platelet poor plasma were performed by the aPTT methodology with comparison of the clotting times to a standard curve generated by five dilutions of Standard Plasma (SHP) with known FVIII:C in Owren's Buffer added to an equal volume of FVIII deficient plasma. Patient samples were performed using three different dilutions (multi dilution analysis, MDA) with an average FVIII:C, obtained from these to give a final result. A line is plotted with the clotting times for the MDA and compared to the standard curve, to check for parallelism.

Internal and External Quality Control

Internal quality control (IQA) was performed prior to inhibitor testing. The negative control consisted of Factor VIII deficient plasma was reconstituted in 1mL of distilled water. This was mixed gently and left to equilibrate at room temperature for 15 minutes and then used within 8 hours of reconstitution. A positive control of lyophilised Technoclone Factor VIII Inhibitor Plasma was reconstituted in 1 mL of distilled water and left to equilibrate at room temperature for 15 minutes prior to use. The laboratory within The Royal London Hospital (Barts Health NHS Trust) participates in national and international external quality assessment (EQA) schemes. These include the UK NEQAS for Blood Coagulation, European Committee for Action on Thrombosis [ECAT] Foundation Quality Assurance Scheme and the

World Federation for Hemophilia International External Quality Assurance Scheme. Within the ECAT scheme, sample testing is performed twice per year for FVIII inhibitor assays. Within the UK NEQAS scheme, FVIII assay evaluation is performed 4 times per year with ad-hoc distribution of FVIII inhibitor samples for quantification.

Nijmegen-Bethesda Assay

Inhibitor testing following the principles of the NBA was performed as previously described³⁰⁸. Briefly, FVIII deficient (control) and patient plasma were incubated at 37°C for 2 hour in equal volumes (160µL) of buffered control plasma (lyophilised Control N or Dade Ci-Trol 1, Siemens, Germany). In the routine testing of clinical samples with low suspicion for inhibitor presence, this assay is performed as an inhibitor screen using neat plasma. For inhibitor quantification, serial dilutions (1 in 2, 4, 8, 16 and 32) were performed, with samples diluted in FVIII deficient plasma. Residual FVIII:C was measured by a one stage FVIII:C assay, using a Sysmex CS2100iv (Sysmex, Milton Keynes, UK) analyser. The percentage residual FVIII:C was calculated based on the difference between the FVIII:C within each sample relative to the negative control. Quantification of inhibitory activity was based on the principle that 1BU/mL represents an inhibitory activity that results in a 50% reduction in FVIII:C. The inhibitor titre was then calculated using linear regression equation; $INH = [(2 - \log_{10}R)/0.30103] \times D$; where INH=Inhibitor titre (BU/mL); R=Percentage Residual FVIII and D=Dilution Factor. Samples with inhibitor titres ≥ 0.6 BU/mL were defined as positive. In-house testing for variability in inhibitor testing has previously demonstrated a group imprecision (co-efficient of variability) of 38.8% and 28.0%, for testing of a high (8.5BU/mL) and low (0.9BU/mL) titre FVIII inhibitor (Platton S., Principal Biomedical Scientist, The Royal London Hospital, London, UK, personal communication).

Factor VIII ELISA Kit

A commercially available, solid phase indirect FVIII ELISA (Immucor, Norcross, Georgia, USA) was used as per the manufacturer's instruction. This kit contains 96 microwells pre-coated with H1 haplotype rFL-FVIII (Kogenate[®], Bayer Corp., Pittsburg, PA, USA)^{179;307}. Samples and controls were initially diluted 1:4 with diluent buffer and added to the microwells. The wells were sealed with a plate sealer and incubated at 37°C for 30 minutes in a water-bath. Following incubation, the contents of each well were decanted and blotted on absorbent towelling. Wells were washed 3 times with 150µL of working wash solution, with this being decanted and discarded in-between washes and inverted onto absorbent towelling (washing

step). Next, 15µL conjugate (alkaline phosphatase conjugated goat antibody to human IgG, 0.1% sodium azide diluted 1:100 in specimen diluent) was added to all wells, these were sealed with a plate sealer and incubated at 37°C for 30 minutes in a water-bath. The microwells washed three times as per the previous washing step. Next, 50µL substrate (P-nitrophenyl phosphate, reconstituted in de-ionized water and diluted 1:100) was added to all wells and the ELISA plate was incubated in the dark at room temperature (22-25°C) for 30 minutes. The reaction was stopped using 50µL Stopping Solution (3M Sodium Hydroxide) to each of the sample's wells and the absorbance was read at 405nm using a GEN5 plate reader (BioTek, Winooski, USA). All samples were tested in duplicate and defined as being positive if they displayed an OD greater than that of the kit control (KC). The ELISA kit has three controls: negative, positive and kit controls, which are run in parallel with the tested samples. Within each ELISA the first two wells are left blank allowing. The negative control is derived from a normal (non-haemophilia) human donor, with no demonstrable anti-FVIII antibody presence. The positive and kit controls are derived from human serum containing antibodies to human FVIII. The KC is lot specific and is tested by the manufacturer to ensure that its use results in the expected reportable results (positive/negative) in over 90 test samples. Testing of this KC previously within our laboratory has given an inhibitor titre of 0.4BU/mL (Platton S., Principal Biomedical Scientist, The Royal London Hospital, London, UK, personal communication).

3.2 Collection and Extraction of Total and miRNA

Materials

The following sourced reagents were used for production of the low volume modified PAXgene (PG) tubes and for RNA extraction: PAXgene Blood RNA tubes (2.5mL blood, 6.9mL PG reagent) (Beckton Dickinson, BD, Franklin Lakes, USA). Ultimate Security 4.5mL cryovials and cryoboxes (Alpha Laboratories, Eastleigh, UK); RNaseZap® wipes and UltraPure DNase/RNase free distilled water (Invitrogen, Carlsbad, USA); Pipetman Classic P1000 pipette and Diamond® Tips STERILPACK™ (Gilson Scientific, Luton, UK); PAXgene Blood mRNA kit and PAXgene Blood miRNA kit (PreAnalytiX, Hombrechtikon, Switzerland); RNeasy mini kit (Qiagen, Hilden, Germany)

Modified Sample Tube Production Protocol

Modified PG blood tubes were prepared in RNase free conditions within a positive pressure tissue culture hood. Prior to sample tube production the hood and pipette were cleaned with RNaseZAP® followed by RNase/DNase free distilled water. The production of modified PG cryovials was performed by two operators using sterile gloves to further reduce risk of RNase contamination in sample tubes. The ratio of PG media to blood for the modified tubes was maintained as per that in the commercially available tubes (ratio 1mL blood to 2.76mL PAXgene reagent). Pipette tips were changed regularly and cryovials were re-sealed inside the tissue culture hood. The integrity of the seal for each cryovial was re-tested and sample tubes were labelled with the expiry date of source PAXgene media. Any cryovial in which the seal was inadequate was disposed of. Sample tubes were stored at ambient temperature in a sealed cryobox prior to blood sampling.

Extraction of Total RNA and miRNA

Manual purification of RNA was performed within the Genome Centre Core Facility at Queen Mary, University of London following the manufacturer's protocols for whole blood RNA/miRNA extraction. This included three extraction protocols for extraction of either, total RNA (Protocol A) or total RNA and miRNA using either a single (Protocol B) or two tubes (Protocol C) methodology. All extractions were performed in RNase free conditions within a dedicated laboratory for RNA handling, with surfaces cleaned with RNaseZAP® prior to commencement of the extraction. Prior to extraction for all 3 protocols, frozen samples were allowed to thaw at room temperature (15-25°C) and incubated for a further 2 hours prior to extraction. Following completion of all protocols, samples were frozen at -80°C.

Total RNA Extraction Protocol (Protocol A)

PAXgene blood tubes were first centrifuged at 4,000g for 10 minutes in a swing out rotor. The supernatant was removed by decanting and the pellet was re-suspended in 4mL RNase-free water by vortexing and centrifuged at 4,000g for 10 minutes. The supernatant was discarded and 350µL of Buffer BR1 was added to the pellet, which was re-suspended by vortexing. This was transferred by pipetting into a 1.5mL micro-centrifuge tube and 300 µL Buffer BR2 and 40 µL Proteinase K were added, with this being mixed by vortexing. Samples were incubated for 10 minutes at 55°C for in a shaker-incubator. The lysate was pipetted into a PAXgene shredder spin column in a 2ml processing tubes and centrifuged at maximum

speed for 3 minutes. The supernatant from the flow-through fraction was transferred into a fresh 1.5ml micro-centrifuge tube and 350 μ L of 100% ethanol was added, which was mixed by vortexing for 1-2 seconds at 1,000g. 700 μ L of each sample was transferred into a new spin column within a 2mL processing tube and centrifuged at 14,000g for 1 minute. Following centrifuging, the processing tube containing the flow-through was discarded and the spin column was transferred to a new 2mL processing tube. The remaining sample was applied to this and centrifuged at 14,000g for 1 minute and the flow-through discarded. 350 μ L Buffer BR3 was added to the PAXgene spin column within a new 2mL processing tube and centrifuged for 1 minute at 14,000g. The PAXgene spin column was then transferred to another new 2mL processing tube and the flow-through discarded. A DNase treatment step was performed by adding 10 μ L of DNase1 diluted in 70 μ L DNA digestion buffer to the spin column of each sample and left to incubate at room temperature for 15 minutes. 350 μ L Buffer BR3 was then added to the spin column and this was centrifuged for 1 minute at 14,000g and the flow-through discarded. 500 μ L of Buffer BR4 (diluted 1 in 4 in 100% ethanol) was added to the spin column and centrifuged at 14,000g for 1 minute. This spin column was transferred to a new 2mL processing tube and the flow-through discarded and a further 500 μ L of Buffer BR4 (diluted 1 in 4 in 100% ethanol) was added and the spin column was centrifuged at 14,000g for 3 minutes. The flow-through was discarded and the spin column was transferred to a new 2mL processing tube and centrifuged for 1 minute at 14,000g. The follow-through was discarded and the PAXgene spin column transferred to a new 1.5mL micro-centrifuge tube. Finally an elution step was performed applying 40 μ L of Buffer BR5 to spin column membrane and this was centrifuged at 14,000g for 1 minute. The eluate was incubated for 5 minutes at 65°C and the samples were transferred onto ice prior to analysis.

miRNA Extraction Protocol : Protocol B (Single Tube)

PAXgene blood tubes were first centrifuged at 4,000g for 10 minutes in a swing-out rotor. The supernatant was removed by decanting and the pellet was re-suspended in 4mL RNase-free water and the tube was closed using a fresh secondary Hemogard closure. The tube was vortexed until the pellet had dissolved and this was centrifuged for a further 10 minutes at 4,000g using a swing-out rotor. The supernatant was discarded by decanting and the pellet was re-suspended in 350 μ L Buffer BM1 and vortexed until this had visibly dissolved. This was pipetted into a 1.5mL micro-centrifuge tube and 300 μ L Buffer BM2 and 40 μ L proteinase K were added. This was vortexed for 5 seconds and then incubated at 55°C for 10 minutes in a shaker incubator. The sample was then pipetted into a PAXgene shredder spin column placed

in a 2mL processing tube and centrifuged for 3 minutes at maximum speed. The entire supernatant was transferred into a new 1.5mL micro-centrifuge without disturbing the pellet in the processing tube. 700µL of isopropanol (100% purity grade p.a) was added and mixed by vortexing. 700 µL of the sample was pipetted into a PAXgene RNA spin column placed in a 2mL processing tube. The lid was closed gently and this was centrifuged at 14,000g and the flow through was discarded. The spin column was then placed in a new 2mL processing tube and the remaining sample was pipetted into this PAXgene spin column. The lid was closed and this was centrifuged for 1 minute at 14,000g and the flow-through discarded. The spin column was placed in a new 2mL processing tube and 350µL of Buffer BM3 was added. The lid was gently closed and this was centrifuged for 15 seconds at 14,000g and the flow-through discarded. The spin column was then placed in a new 2mL processing tube and 80µL of DNase I incubation mix (10µL DNase I stock solution diluted in 70µL Buffer RD) was added and this was mixed by gentle flicking and incubated at room temperature (20-30°C) for 15 minutes. 350µL of Buffer BM3 was added to the PAXgene RNA spin column. The lid was closed gently and this was centrifuged for 15 seconds at 14,000g and the flow-through was discarded. The spin column was then placed in a new 2mL processing tube and 500µL Buffer BM4 was added to the spin column. The lid was closed and this was centrifuged for 15 seconds at 14,000g and the flow through discarded. The spin column was then placed in a new 2mL processing tube and a further 500µL Buffer BM4 was added to the PAXgene RNA spin column, the lid was closed and this was centrifuged for 2 minutes at 14,000g. The spin column was then placed in a new 2mL processing tube and the old processing tube and flow through were discarded. This was then centrifuged at 14,000g for 1 minute. The processing tube containing flow-through was discarded and the PAXgene RNA spin column was placed in a new 1.5mL micro-centrifuge tube. Finally, 40µL Buffer BR5 was pipetted directly onto the spin column membrane. The lid was closed gently and this was centrifuged for 1 minute at 14,000g. The eluate was incubated for 5 minutes at 65°C and the samples were transferred onto ice prior to analysis.

miRNA Extraction Protocol : Protocol C (Two Tubes)

PAXgene blood tubes were first centrifuged at 4,000g for 10 minutes. The supernatant was removed by decanting and the pellet was re-suspended in 4 ml of RNase-free water and the tube was closed using a fresh secondary Hemogard closure. The tube was vortexed until the pellet had dissolved and then centrifuged for 10 minutes at 4000g. The supernatant was removed and discarded by pipetting. The pellet was then re-suspended in 350µL

resuspension buffer (BR1) and vortexed until the pellet had visibly dissolved. This sample was then pipetted into a 1.5mL micro-centrifuge tube and 300µL binding buffer (BR2) and 40µL proteinase K (PK) were added. This was mixed by vortexing for 5 seconds and incubated for 10 minutes at 55°C using a shaker-incubator. The lysate (maximum 700µL) was pipetted directly into a PAXgene shredder spin column in a 2 mL processing tube, and centrifuged for 3 minutes at maximum speed (but not to exceed 20,000g). The entire supernatant of the flow-through fraction was transferred to a fresh 1.5mL micro-centrifuge tube without disturbing the pellet in the processing tube. 350µL ethanol (100%, purity grade p.a.) was added and this was mixed by vortexing. 700µL sample of the sample was pipetted into the spin column in a 2mL processing tube and centrifuged for 1 minute at 14,000g. The spin column was placed in a new 2 mL processing tube and the flow through was kept for the later stages for miRNA purification. The remaining sample was pipetted into the PAXgene RNA spin column and centrifuged for 1 minute at 14,000g. The spin column was placed into a new 2 mL processing tube (PT). The flow through from each of these steps was kept and added to the flow through from the previous step. 350µL BR3 buffer was pipetted into the PAXgene RNA spin column and these samples were left to one side whilst the flow-through for microRNA purification is prepared. miRNA was then prepared using the RNeasy mini kit following the manufacturer's instructions. 700µL ethanol (98%) was added per 500µL flow-through from the PAXgene Blood RNA binding step and mixed carefully by pipetting. This was applied in 700µL portions to RNeasy mini spin column placed in a 2mL collection tube and centrifuged for 1 minute at 14,000g with the flow-through being discarded following each step. 350µL Buffer RW1 was then added to the RNeasy spin column and the lid closed. For the remainder of the protocol sample preparation for total RNA and miRNA were performed in parallel using the PAXgene RNA spin columns and RNeasy spin columns, respectively. The spin columns were centrifuged for 1 minute at 14,000g and the flow-through discarded. 80µL of DNase incubation mixture (10µL DNase I stock solution diluted in 70µL DNA digestion buffer) was added to the spin columns and these were incubated at room temperature (20-30°C) for 15 minutes. 350µL wash buffer BR3 was pipetted into the PAXgene RNA spin column and centrifuge for 1 minute at 14,000g, with the flow-through being discarded. The spin columns were placed into new 2mL processing tube. 350µL Buffer RW1 was added to the RNeasy spin column and 500µL wash BR4 buffer into the PAXgene spin column and these were centrifuged for 1 minute at 14,000g. The flow-through was discarded and the spin columns were placed into new 2mL processing tubes. 500µL Buffer RPE was added to the RNeasy spin column and a further 500µL wash buffer BR4 to the PAXgene RNA spin column

and these were centrifuged at 14,000g for 3 minutes. The flow through was discarded and the spin columns were transferred to new 2mL processing tubes. 500µL Buffer RPE was added to the RNeasy spin column this was centrifuged at 14,000g for 1 minute. The flow-through was discarded and the spin columns were transferred to new 2mL processing tubes and centrifuged at 14,000g for 1 minute and the flow through discarded. The PAXgene spin column was transferred into a 1.5mL micro-centrifuge tube and 40µL elution buffer BR5 was added directly onto spin column membrane. The RNeasy spin column was transferred into a new 1.5mL collection tube and 40µL RNase-free water was added to the spin column membrane. These were both centrifuged for 1 minute at 14,000g to elute the RNA. The eluate was incubated for 5 minutes at 65°C and the samples were transferred onto ice prior to analysis.

3.3 Nomenclature and Statistical Analyses

All numbering of amino acid positions within this work are stated in “legacy” format for the mature FVIII protein (total length 2332aa), lacking in the 19 amino acid signal peptide to allow comparability with previously published similar work.

Specific statistical methodology, is presented prior to the analysis of results within each chapter. Evaluation of data distribution was performed, through histograms, Q-Q plots or the Kolmogov-Smirnov test. All statistical tests performed were two sided, with a p value of <0.05 taken as being significant. Statistical analyses were performed and figures presented were created using either IBM SPSS version 21 (IBM Corp., Armonk, New York, USA), Stata (StataCorp. 2011. Stata Statistical Software: Release 12.1 College Station, TX: StataCorp LP) or GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

Chapter 4: Compliance with Inhibitor Testing in Non-Severe Haemophilia A: An Evaluation of Practice in London

4.1 Introduction

The cumulative incidence of inhibitory antibodies in patients with non-severe haemophilia A within the recently published INSIGHT study was been reported at 5.3% (54/1112) ¹⁶⁶, which is lower than that seen in severe haemophilia A (32%) ¹⁴⁹. Inhibitors in non-severe haemophilia A patients however, constitute 20% (109/534) of all previously reported inhibitors in the UK within the most recent UKHCDO report (2013-14) ⁶¹. These antibodies are of concern clinically due to reported cross-reactivity against endogenous FVIII ^{64;166}, change in bleeding phenotype ⁶⁴ and increased mortality ¹⁶⁸. In one small study a change in bleeding phenotype and fall in baseline FVIII:C (bFVIII:C) was seen in 22/26 and 24/26 of patients, respectively ⁶⁴. More recently in the INSIGHT study a fall in bFVIII:C and a change in bleeding phenotype was reported in 34/54 (58%) and 30/54 (51%) of patients ¹⁶⁶. In both of these reports, inhibitor screening was only performed in the context of clinical suspicion of a FVIII inhibitor (change in bleeding phenotype, bFVIII:C or impaired treatment efficacy or FVIII recovery) ^{64;166}. Only 11 (19%) asymptomatic patients were diagnosed with an inhibitor on routine screening within the INSIGHT study ¹⁶⁶. There is also a lack of clarity regarding the optimal management of inhibitors in this population ^{64;169;214}, with selection of immunosuppression (or observation) being influenced by clinical and laboratory features ²³³, which complicates interpretation of observation study data ¹⁶⁹.

Patients with non-severe haemophilia A receive treatment with FVIII concentrates in a different clinical and potentially immunological contexts to those with severe haemophilia A. In severe haemophilia A, treatment begins early in life, initially with on-demand treatment for bleeding (spontaneous or following minimal trauma) and a transition to prophylaxis. Understanding of the timing of inhibitor formation (median 14ED) ^{100;149}, from trial data has allowed the development of national guidance for inhibitor monitoring in patients with severe haemophilia A ²¹⁴. These guidelines have focussed on the necessity for close monitoring for inhibitors in patients with severe haemophilia A, in the first 50 ED, which has resulted in an increase in inhibitor screening in the last two decades ²¹⁵. Treatment in non-severe haemophilia A is given less frequently and often in the context of trauma or surgery. Although the age at commencement of treatment is variable, this occurs later than in patients with severe haemophilia A and many patients will not (or never) receive significant

FVIII exposure until later in life. Data from the INSIGHT study has suggested that there is a lifelong risk of inhibitor formation in this group of patients ¹⁶⁶.

The optimal approach to when (and how) to test for FVIII antibodies in non-severe haemophilia A is not clear and there is a paucity of data to guide these practices. Guidance in the UK (published prior to the INSIGHT study) recommends inhibitor screening in situations perceived as being of high immunological risk ²¹⁴. Within these guidelines, the evidence basis (grade 1C) for recommendations was made from pooled data from small retrospective observational studies or extrapolated from data in severe haemophilia A. Further research is required to define optimal inhibitor screening practices in non-severe haemophilia A. There is no data describing current inhibitor testing practices in patients with non-severe haemophilia A. Despite the publication of consensus guidance for inhibitor testing from organisations, such as the UKHCDO, it is unknown whether these impact on clinical practice. Within this chapter, a retrospective audit and evaluation of screening practices (inhibitor and genetic) from a large cohort of patients with non-severe haemophilia A treated at all haemophilia centres in the London region was performed.

4.2 Hypothesis and Aims

The hypothesis for this chapter is that the true allo-immune response to FVIII infusions in non-severe haemophilia A is underestimated due to inconsistent inhibitor testing following FVIII treatment. Despite published national guidance for inhibitor screening, it is hypothesised that there is poor compliance with testing for inhibitors in patients treated with FVIII. This in turn may result in a bias in the detection of cross-reactive FVIII antibodies. The aim of this evaluation was to gain a greater understanding of current practices of inhibitor screening, treatment and testing of *F8* genotype in patients with non-severe haemophilia A. The specific aims of this work were as follows:

- 1) To assess treatment patterns of patients with non-severe haemophilia A managed at the London haemophilia treatment and comprehensive care centres
- 2) To describe occurrence of bleeding and evaluate association between baseline FVIII:C and bleeding phenotype
- 3) To assess compliance with national (UKHCDO) guidance of genetic screening in patients with non-severe haemophilia A
- 4) To assess compliance with national (UKHCDO) guidance for inhibitor testing following FVIII exposure in patients with “high” and “standard-risk” *F8* mutations.

4.3 Materials and Methods

Case and Centre Selection

A retrospective review was performed of the management and follow-up of all patients with non-severe haemophilia A treated at seven active London haemophilia centres (4 comprehensive care centres, CCCs and 3 haemophilia treatment centres, HTCs). These centres included, The Royal London (CCC), Royal Free (CCC), St Thomas' (CCC), Great Ormond Street (CCC), St George's (HTC), Hammersmith (HTC) and Lewisham (HTC) Hospitals. Of these CCCs, Great Ormond Street Hospital purely manages children and The Royal London Hospital and St Thomas' Hospital manage adults and children. The Royal Free Hospital primarily manages adult patients, with a small number of registered adolescent patients (>12 years). All sequentially treated patients with non-severe haemophilia A receiving treatment over a two year period (1/1/11-31/12/12) were included.

The primary objective was to audit compliance with inhibitor testing against published UKHCDO guidelines for the diagnosis of coagulation factor inhibitors²¹⁴. These guidelines recommend that patients with non-severe haemophilia A receive inhibitor screening in the following circumstances²¹⁴:

- "Standard-risk" *F8* mutation: Annual inhibitor testing in all patients who have received exposure to FVIII. Follow-up testing after intensive exposure (≥ 5 EDs) or surgery.
- "High-risk" *F8* mutation: Follow-up inhibitor testing is advised after all episodes of exposure to FVIII.

No guidance however, is provided for the optimal time period in which testing should be performed following FVIII exposure (so called "convalescent inhibitor screening") which is perceived as being "high-risk", whether by *F8* genotype or treatment exposure. For the purpose of this study, convalescent screening was defined as testing performed within six weeks (≤ 42 days) of the first treatment day.

Secondary objectives were to assess compliance with *F8* genotype testing, evaluate treatment patterns, bleeding patterns, timing of inhibitor screening and the incidence of new inhibitors. This study was developed as the first in a series of pan-London initiatives to audit and evaluate clinical and laboratory practices in the management of bleeding disorders. The study design and data to be collected were discussed with the Clinical Effectiveness Unit

(CEU) at The Royal London Hospital and Research Approvals Office (Joint Research Management Office). The study was registered locally at each centre as part of clinical audit and service evaluation by the lead treating physician.

Diagnosis and Definitions of Non-Severe Haemophilia A

All patients were diagnosed locally with baseline FVIII:C (bFVIII:C) levels based on those recorded within the centre's patient registration records. Non-severe haemophilia A was defined as a FVIII:C of 1-50 IU/dL⁶⁴. Patients were then further sub-categorised by the investigator as having either, moderate (FVIII:C >1 to ≤5 IU/dL) or mild (FVIII:C >5 to ≤50 IU/dL) haemophilia A^{63;64;70}.

F8 Genotype Testing and Definitions of "High-Risk" F8 Genotypes

Testing of personal *F8* genotype was determined by any previous testing of a patient's *F8* genotyping. In those, without previous personal *F8* genotype testing, centres were asked if there was knowledge of the familial *F8* mutation. In those in whom *F8* genotyping had been performed, or was known these were categorised as having either "high" or "standard-risk" *F8* mutations. Within recent national guidance, no clear summary of "high-risk" mutations is provided and this advises referring to published data²¹⁴. In this evaluation, *F8* missense mutations published within the HIGS cohort were used to define "high-risk" *F8* mutations¹⁹⁴. These were derived from mutations with an increased incidence of inhibitor formation within the HADB database^{194;210}. This included the following mutations, which are stated in legacy format, with the HGVS assignment given in parentheses: Arg593Cys (Arg612Cys), Tyr2105Cys (Ty2124Cys), Arg2150His (Arg2169His), Arg2163His (Arg2182His), Tyr2229Cys (Trp2248Cys), Asn2286Lys (Asn2305Lys) and Pro23000Leu (Pro2319Leu)^{71;194;210}.

Data Collection

The aim for data collection was to allow the majority of data to be directly retrieved from electronic patient records (EPR) of clinical and laboratory data. A standardised data-reporting tool was distributed to each of the haemophilia centres, following piloting of this tool at The Royal London Hospital. Examples (4 treatment episodes for 3 patients) of how to complete data entries were provided within this tool to help standardise data collected. Data collection took place by centres, between 4/4/13 and 18/6/13. All data provided to the lead investigator was anonymised, using sequential coding and returned using a secure email server (nhs.net). All data entries were checked manually for discrepancies or missing data, with these being

followed up by the lead investigator with the audit co-ordinator for each centre. Recoding and pooling of data was performed, with this being checked both manually and electronically with the source data on three occasions.

Baseline demographic data, including age, bFVIII:C, inhibitor history, *F8* genotype testing (“high-risk” or “standard-risk”) were collected for all patients. Information was collected on treatment modalities used by patients, including usage of DDAVP, FVIII concentrate or bypassing agents. In episodes treated with a FVIII concentrate centres were asked to provide information on the treatment indication and the timing and results (positive or negative) of all inhibitor tests performed following that episode. Treatment indications were categorised as home-treatment or on-demand (hospital based treatment). On-demand treatment was further sub-categorised as bleed/trauma, surgery, other or not-stated. Surgery was classified as any surgical or dental intervention/procedure requiring FVIII replacement therapy. No sub-classification of bleed data (location or spontaneous/traumatic) was performed. An exposure day (ED) was defined as a calendar date in which one or more FVIII infusions was used⁹⁹. In episodes treated with DDAVP or a bypassing agent, no additional information was retrieved. Analysis of inhibitor testing was performed for on-demand episodes as these episodes had clear documented evidence of FVIII being infused (i.e. bleeding or surgery). Home-treatment, was defined as an issue of FVIII concentrate that was supplied by the centre for the treatment or prevention of bleeding that was not administered within the centre. This could include treatment issues for prophylaxis, immune tolerance induction, on-demand home-treatment for bleeding or issue of concentrates prior to travel. Given the heterogeneity and lack of data on the indication and whether these issues had been administered no further evaluation was possible.

Assessment of Timing of Inhibitor Testing

All inhibitor testing was performed by local haemophilia centre laboratories. These laboratories are all accredited by the Clinical Pathological Accreditation (CPA) and triennial UKHCDO inspections. Centres were asked to provide the date and result of all inhibitor tests performed within the study period and following treatment episodes. Data from inhibitor testing was then manually paired by the investigator to the nearest chronological on-demand treatment episode, providing this occurred within one calendar year of the treatment episode.

For evaluation of annual inhibitor screening, treatment episodes were paired to the nearest inhibitor test, providing that it occurred within one year of the first treatment day. For patients who received multiple treatment episodes within a year period, a single inhibitor test could represent an annual inhibitor screen for multiple treatment episodes. For evaluation of paired inhibitor screening, tests were paired to the nearest treatment episode provided that this test occurred within one year of the first day of treatment and no treatment episode occurred in the intervening time. If no test was sent within that year or if another treatment episode occurred before an inhibitor screen within the same year, the treatment episode was judged as not having paired inhibitor screening. If more than one inhibitor test was sent, the treatment episode was paired to the first inhibitor screen sent with subsequent assays being excluded from the analysis. Consequently, for this analysis an inhibitor screen could only be paired with a single treatment episode. An assessment was also made to evaluate whether inhibitor testing occurred due to the patient receiving further treatment. Screening was assessed as having being sent due to treatment if the test was performed within (\pm) one day of the following treatment episode. Finally assessment of whether a paired inhibitor screen was performed as a true “convalescent screen”, was based on if this was performed within six weeks (≤ 42 days) of the first day of treatment.

Statistical Analyses

Analysis was performed of the pooled anonymised data of patients. Descriptive statistics, including mean, median, standard deviation, inter-quartile range (IQR) and frequency were performed. The annualised bleed rate (ABR) was calculated from the total number of bleeding episodes recorded in the 2 year data collection window, divided by two. Comparative statistics were performed using the Mann-Whitney U and Kruskal-Wallis H tests for continuous and the Chi-squared, Chi-squared goodness of fit or Fisher’s exact test for categorical variables. Adjustment for missing data, where stated was performed using multiple imputation (5 imputations). Assessment of association was performed using Spearman’s rank correlation. Graphical representation of annualised bleed rates was performed by local smooth polynomials as previously described ⁶⁶.

4.4 Results

There were 853 patients with non-severe haemophilia A, registered at the 7 haemophilia centres within the London region. Of these patients, a treatment episode was recorded in 377 (44%) over the two year study period. The mean age of patients treated was 35.7 years

(range 0.2-89.0 years). The mean bFVIII:C was 12.9IU/dL (range 1-44) with 102 (27.1%) patients having moderate and 275 (72.9%) mild haemophilia A. Seven patients had a bFVIII:C \geq 40IU/dL (mean bFVIII:C 41.7IU/dL, range 40.0-44.0).

4.4.1 Uptake of Testing of *F8* Genotype

There was a documented history of personal testing (and knowledge) of the *F8* mutation in 79% (297/377) of patients who received any form of treatment in the study period. Evaluating personal *F8* testing by centre, all of the centres except one small HTC (19 patients treated) had performed personal *F8* genotype testing in \geq 70% of patients. The haemophilia causative *F8* mutation for the family was known in a further 34 patients (9%), who had not had their personal *F8* genotype tested. As a result, there was knowledge of the causative *F8* mutation in 88% (331/377) patients treated in the study period. In patients in whom the *F8* mutation was known, 18% (58/331) had a “high-risk” *F8* mutation.

4.4.2 Treatment Characteristics

A large proportion (79%, 296/377) of patients were treated with a FVIII concentrate during the study period, with 259 patients only receiving treatment with FVIII (Figure 4.1). DDAVP was used as part of treatment for 110 patients (29%), with 76 (20%) only receiving DDAVP, 33 (9%) FVIII & DDAVP and 1 (<1%) DDAVP and bypassing agents. DDAVP was used in the management of 10% (10/102) patients with moderate haemophilia A (DDAVP alone=4, FVIII & DDAVP=6) in comparison to 36% (100/275) patients with mild haemophilia A (DDAVP alone=72, FVIII & DDAVP=27 and DDAVP and bypassing agents=1). Three patients in whom DDAVP was used had an inhibitor history.

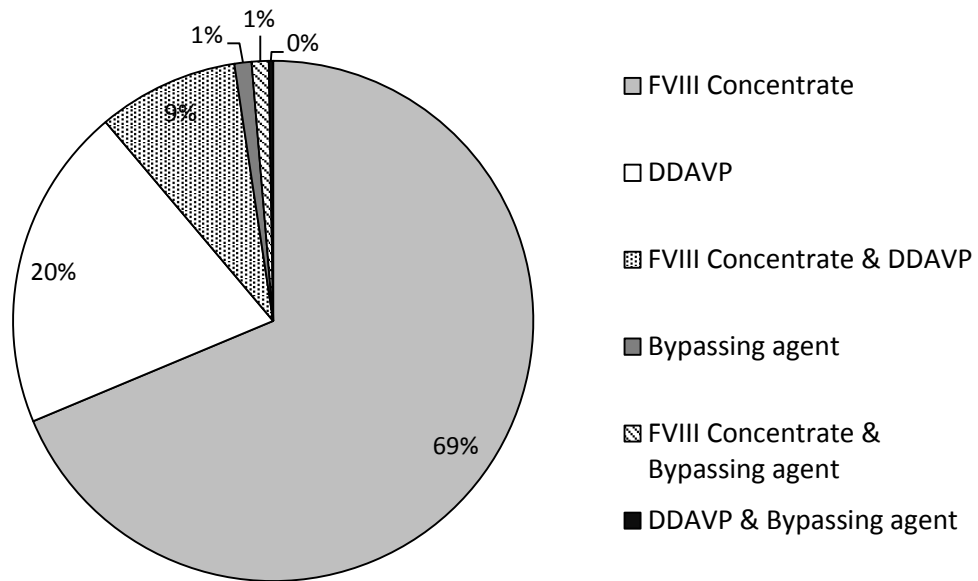


Figure 4.1: Treatment modalities used in the management of non-severe haemophilia A, representing the percentage of patients (total: 377) treated within the observation period.

An evaluation of the demographics by treatment choices was performed for categories in which there were more than five individuals (FVIII alone, DDAVP alone and FVIII & DDAVP). Patients treated with FVIII alone were significantly older (35.8 years, IQR 16.6-57.2) than those treated with DDAVP alone (29.2 years, IQR 14.6-44.5, $p=0.024$). Although, a difference in age was seen between those treated with FVIII alone (35.8 years, IQR 16.6-57.2) and those treated with FVIII & DDAVP (25.3 years, IQR 12.6-48.2), this did not reach statistical significance ($p=0.063$). No significant difference in age was seen between patients treated with DDAVP alone (29.2 years, IQR 14.6-44.5) compared to those treated with FVIII & DDAVP (25.3 years, IQR 12.6-48.2, $p=0.813$). Patients treated with DDAVP alone had significantly higher median bFVIII:C (20.5IU/dL, IQR 11.3-29.0) than those treated with FVIII alone (8.0IU/dL, IQR 4.0-14.0, $p<0.0005$) or FVIII & DDAVP (10.0IU/dL, IQR 7.0-19.0, $p=0.001$). No difference in bFVIII:C was seen in those treated with FVIII alone (8.0IU/dL IQR 4.0-14.0) compared to those treated with FVIII & DDAVP (10.0IU/dL, IQR 7.0-19.0, $p=0.0760$). A whole case analysis of the frequency of “high-risk” *F8* mutations in patients treated with FVIII alone (40/226, 17.7%), DDAVP alone (9/64, 14.1%), FVIII & DDAVP (4/32, 12.5%) did not differ from that expected by chance ($p=0.642$). Repeating this analysis using multiple imputation for the missing data points ($n=46$) also demonstrated no significant difference in the frequency of “high-risk” *F8* mutations between these treatment categories ($p=0.075$).

4.4.3 On-Demand (Bleeding and Surgery) Treatment with FVIII Concentrate

Over the study period, 236 patients received FVIII concentrate alone to cover 562 on-demand treatment episodes (surgery=211, bleeding=351). Patients who received treatment on-demand with FVIII received a median of 4ED (range 1-159) of FVIII in the two year study period (Table 4.1). These on-demand episodes resulted in a cumulative of 2041ED (bleeding=887 and surgery=1154).

In the treatment of bleed presentations, 157 patients received treatment with a FVIII concentrate to treat a median of 1 bleeding episode (range 1-15). Of these patients, 51 had moderate haemophilia A and 106 mild haemophilia A. A total of 130 patients received treatment with a FVIII concentrate to cover a median of 1 surgical episode (range 1-11). A high proportion of patient (n=96, 32.4%) were issued FVIII for home-treatment, with 50 patients only having home-treatment episodes. A significant difference was seen in age and bFVIII:C, comparing patients treated on-demand with those on home-treatment alone or a combination of both. Patients receiving on-demand treatment alone were significantly older (39.9 years, IQR 17.6-59.4) than those treated with home-treatment alone (23.0 years, IQR 13.3-39.2, p=0.011). No significant difference in age was seen comparing patients treated with both on-demand and home-treatment (38.7 years, IQR 18.8-54.0) compared to those treated on home-treatment alone (23.0 years, IQR 13.3-39.2, p=0.164) or on-demand treatment alone (39.9 years, IQR 17.6-59.4, p=0.488). Patients treated with on-demand treatment alone had significantly higher bFVIII:C (10.0IU/dL, IQR 6.0-15.8) than those treated on home-treatment alone (5.0IU/dL, IQR 3.0-9.3, p<0.0005) or on both home-treatment and on-demand treatment (6.6IU/dL, IQR 3.3-11.0, p=0.009). No significant difference for bFVIII:C, was observed comparing patients treated with home-treatment alone (5.0IU/dL, IQR 3.0-9.3) with those treated with both home-treatment and on-demand treatment (6.6IU/dL, IQR 3.3-11.0, p=0.223). Treatment indication were described as either being for other indications or not-stated in 56 episodes (37 patients). No further characterisation of treatment within these categories (home-treatment, not-stated and other) was possible.

	Bleeding	Surgery	Total
Patients (episodes)	157 (351)	130 (211)	236 (562)
Age (years)			
Mean±SD	34.9±22.8	46.6±23.0	39.1±24.1
Median (range)	32.7 (0.2-89.0)	48.5 (2.1-89.0)	39.9 (0.2-89.0)
bFVIII:C			
Mean±SD	10.0±7.6	13.0±9.5	12.2±9.1
Median (range)	8.0 (1.0-42.0)	10.0 (1.5-43.8)	10.0 (1.0-43.8)
Severity			
Moderate	56	28	67
Mild	101	102	169
F8 Mutation			
“High-risk”	27 (17.2%)	24 (18.5%)	42 (17.8%)
“Standard-risk”	109 (69.4%)	88 (67.7%)	162 (69.1%)
Not known	21 (13.4%)	18 (13.8%)	31 (13.1%)
Treatment Episode			
Mean±SD	2.2±2.2	1.6±1.4	2.38±2.2
Median (range)	1 (1-15)	1 (1-11)	1 (1-15)
Total ED / Patient			
Mean±SD	7.4±10.2	6.8±15.5	8.7±14.6
Median (range)	4 (1-82)	2 (1-159)	4 (1-159)

Table 4.1: Baseline demographics of patient with non-severe haemophilia A receiving on-demand treatment with a FVIII concentrate

4.4.4 Assessment of Bleeding in Non-Severe Haemophilia A

An assessment of bleeding was performed in the subgroup of 193 patients (45 moderate and 148 mild HA) without a history of inhibitor who only received on-demand FVIII treatment (bleeding or surgery). The median annualised bleed rate was 0.5 episodes/year (range 0-4.5) with the majority of patients having an ABR ≤ 1 (right/positive skewed distribution, Figure 4.2). A weak, but statistically significant non-parametric correlation ($r=-0.25$, $p=0.0005$) was seen between bFVIII:C and ABR (Figure 4.3). No significant correlation ($r=-0.15$, $p=0.105$) was seen when this analysis was repeated in patients ($n=120$), treated for 1 or more bleeding events within the study period (i.e. excluding those who were treated only for surgery).

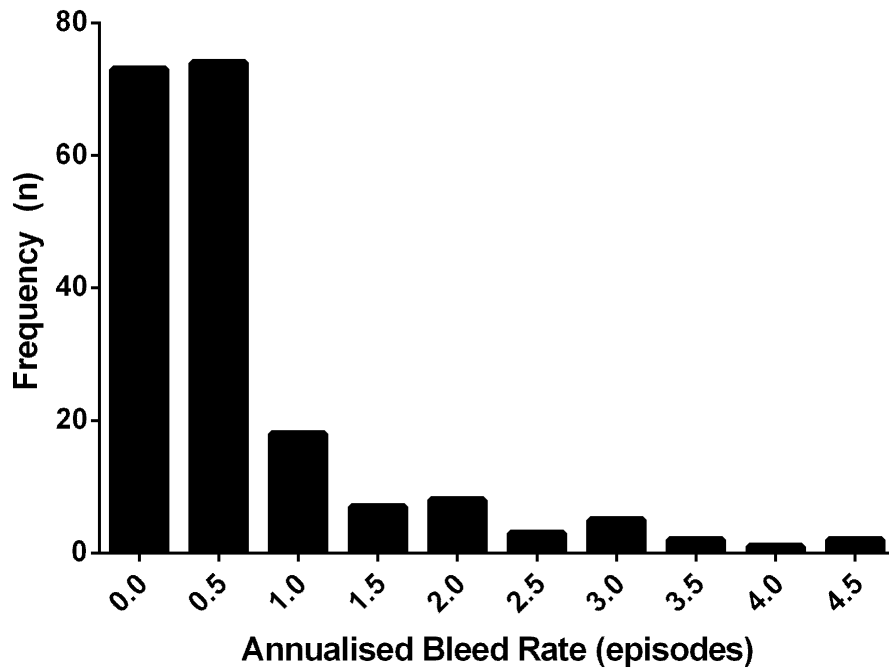


Figure 4.2: Frequency of bleeding in patients with non-severe haemophilia A. A right (positive) skew in distribution of bleeding episodes (annualised bleed rate) was seen for patients treated with on-demand FVIII (median 0.5 bleeds/year)

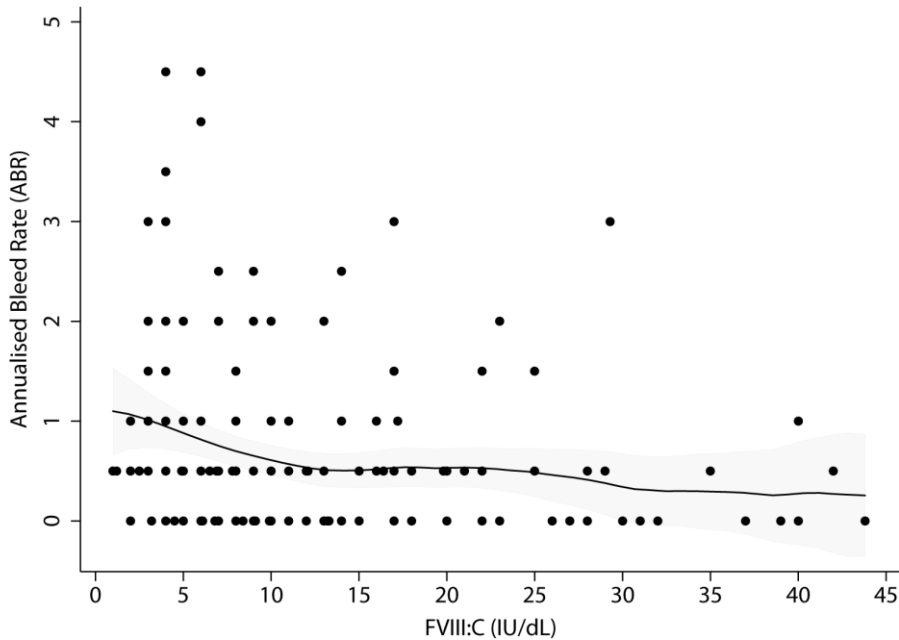


Figure 4.3: Bleeding patterns in non-severe haemophilia A. A weak significant correlation ($r=-0.25$, $p=0.0005$) was seen between the baseline FVIII:C (bFVIII:C) and annualised bleeding rate (ABR) in patients treated with on-demand FVIII.

4.4.5 Inhibitor Screening: “Standard-Risk” *F8* Mutation

In patients with “standard-risk” *F8* mutations, national guidance ²¹⁴ advises inhibitor screening following exposure to FVIII for “high-risk” treatment episodes (bleed \geq 5ED or surgery) and annually following any less intensive treatment with a FVIII concentrate.

There were 194 patients with a “standard-risk” or unknown *F8* mutations, treated with a FVIII concentrate for one or more on-demand treatment episode (n=474) over a cumulative of 1753ED. The mean age was 37.7 years (range 0.2-89.1) and the mean baseline FVIII:C was 11.6IU/dL (range 1.0-42.0), with 142 having mild and 52 moderate haemophilia A. Within this group of patients with a “standard-risk” or unknown *F8* mutations, 58.7% (114/194) had an inhibitor screen sent at some point within the 2 year study period. Of these on-demand treatment episodes, 51.3% (243/474) were followed by an inhibitor screen within one year of treatment, at a median of 106 days (range 2-365). Of all on-demand treatment episodes, 33.3% (158/474) received a directly paired annual inhibitor screen (i.e. inhibitor screen performed prior to subsequent FVIII exposure), with only 12.0% (57/474) having inhibitor screening within 6 weeks of treatment (Table 4.2). Therefore, 66.7% of treatment episodes were not screened prior to subsequent FVIII exposure. At a patient level this represented 101/194 (52%) patients who received annual screening and 93/194 (48%) patients who were not screened annually following treatment with a FVIII concentrate.

FVIII was used to provide haemostatic cover for 175 surgical episodes (106 patients), treated with a median of 1 day (range 1-157) of FVIII over a total of 773ED. Inhibitor screening was performed within one year of treatment for 40.6% (71/175) surgical episodes (Table 4.2). Paired inhibitor testing (i.e. screen before subsequent FVIII exposure) was recorded following 27.4% (48/175) surgical episodes at a median of 67.0 days (range 2-329). Only 9.7% (17/175) surgical episodes had a true “convalescent inhibitor screen” performed within 6 weeks of treatment. Of the episodes that received inhibitor screening within 6 weeks, 41.2% (7/17) tests coincided with a subsequent treatment episode

There were 299 bleeding episodes (130 patients) treated with a median of 2 days (range 1-75) of FVIII over a cumulative of 980ED. Following usage of a FVIII concentrate for treatment of bleeding for \geq 5 days (n=39), 53.8% (28/52) episodes had an inhibitor screen performed within one year of treatment (Table 4.2). Paired inhibitor testing was recorded in 40.3% (21/52) episodes at a median of 40.0 days (range 4-316). True “convalescent inhibitor screening” within 6 weeks of treatment was performed following 21.2% (11/52) of episodes.

Of the episodes that received inhibitor screening within 6 weeks, 21.2% (2/11) tests coincided with a subsequent treatment episode

Taken together, the combined data for bleeding (≥ 5 ED) and surgery demonstrated that although 30.4% (69/227) of episodes had paired inhibitor testing, only 12.3% (28/227) of these received true “convalescent inhibitor screening” within 6 weeks of treatment as per national guidance (Table 4.2 and Figure 4.4).

4.4.6 Inhibitor Screening: “High-Risk” F8 Mutation

For “high-risk” F8 mutations, current national guidance ²¹⁴ advises inhibitor screening following any exposure to a FVIII concentrate.

There were 58 patients with a “high-risk” F8 mutation treated in the study period, with a mean age of 40.9 years (range 0.9-84.4). The mean bFVIII:C was 11.2IU/dL (range 1.9-43.8), with 19 (32%) having moderate and 39 (67%) mild haemophilia A. Of these patients, 46 (79%) received one or more treatment with a FVIII concentrate. This group of patients were mainly treated using on-demand treatment only (38/46). Two patients received home-treatment only, four on-demand and home-treatment and two treatment for other indications. This group of patients underwent treatment with a FVIII concentrate for 36 surgical episodes (114 ED) and 52 bleeding episodes (174 ED), resulting in 88 on-demand episodes with a cumulative exposure of 288ED. Surgical episodes were treated with a median of 1 day (range 1-20) and bleeding episodes with a median of 2 days (range 1-19) of FVIII treatment.

Of the 42 patients with “high-risk” F8 mutations treated within the study period, 69.0% (29/42) had an inhibitor screen performed at some point within the 2 year study period (Table 4.2). Of the 88 on-demand (surgery and bleeding) treatment episodes, 47.8% (42/88) had an inhibitor screen performed within one year of treatment at a median of 87.0 days (range 2-359). A paired inhibitor screen was performed following 33.0% (29/88) of episodes at a median of 93.5 days (range 2-365) after the first treatment day. Of these episodes, a true “convalescent inhibitor screen” was performed in only 13.6% (12/88) within six weeks of treatment (Table 4.2 and Figure 4.4). Of the episodes that received inhibitor screening within 6 weeks, 16.7% (2/12) tests coincided with a subsequent treatment episode

		Episodes	Tested		Not Tested	
			Tested (%)	Treatment screen (%)	Not Tested (%)	Episode <42 days (%)
“High-Risk” F8 Mutation	Annual	88	42 (47.7%)	9 (21.4%)	46 (52.3%)	-
	Pair	88	29 (33.0%)	9 (31.0%)	59 (67.0%)	-
	<42 days	88	12 (13.6%)	2 (16.7%)	76 (86.4%)	15 (19.7%)
“Standard-Risk” F8 Mutation	All Annual	474	243 (51.3%)	42 (17.3%)	231 (48.7%)	-
	All Pair	474	158 (33.3%)	42 (26.6%)	316 (66.7%)	-
	All <42 days	474	57 (12.0%)	21 (36.8%)	417 (88.0%)	75 (18.0%)
	Surgery Annual	175	71 (40.6%)	14 (19.7%)	104 (59.4%)	-
	Surgery Pair	175	48 (27.4%)	14 (29.2%)	127 (72.6%)	-
	Surgery <42 days	175	17 (9.7%)	7 (41.2%)	158 (90.3%)	22 (13.9%)
	≥5ED Bleed Annual	52	28 (53.8%)	2 (7.1%)	24 (46.2%)	-
	≥5ED Bleed Pair	52	21 (40.4%)	2 (9.5%)	31 (59.6%)	-
	≥5ED Bleed <42 days	52	11 (21.2%)	2 (18.2%)	41 (78.8%)	13 (31.7%)
All Episodes	Annual	562	285 (50.7%)	51 (17.9%)	277 (49.3%)	-
	Pair	562	187 (33.3%)	51 (27.3%)	375 (66.7%)	-
	<42 days	562	69 (12.3%)	23 (33.3%)	493 (87.7%)	90 (18.3%)

Table 4.2: Frequency of inhibitor screening in non-severe haemophilia A. Treatment screen=Inhibitor screen performed within (±) one day of the following treatment episode. Episode <42 days=Patient received another treatment episode in <42 days.

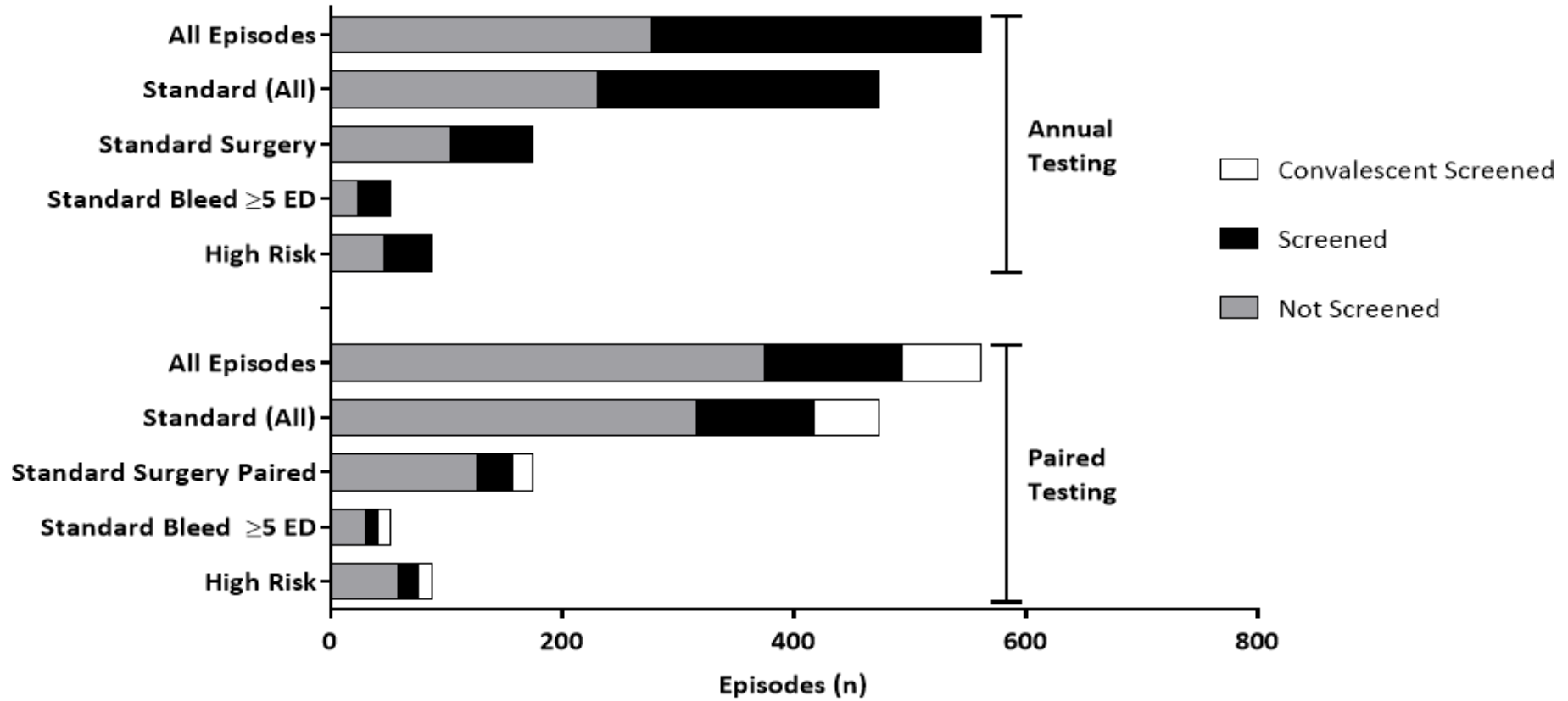


Figure 4.4: Inhibitor screening in non-severe haemophilia A. Standard="standard-risk" or *F8* mutation not recorded. High Risk="high-risk" *F8* mutation.

4.4.7 Factor VIII Inhibitor Formation

Thirteen patients had an inhibitor history, with a mean age of 53.6 years (7.5-80.5) and bFVIII:C of 7.6IU/dL (1.9-16.0). These patients were treated with FVIII alone (n=5, 38.5%), DDAVP alone (n=2, 15.4%), bypassing agents alone (n=4, 30.8%), FVIII and bypassing agents (n=1, 7.7%) and DDAVP and bypassing agents (n=1, 7.7%). Home-treatment was used for 3/5 patients treated with FVIII alone.

Of the patients treated with FVIII (n=290) at risk of inhibitor formation, three developed a new FVIII inhibitor within the study period. All of these cases were diagnosed in one centre, with these inhibitors being detected during treatment for bleeding (n=2) or on inhibitor screening (n=1). A change in the bleeding phenotype was seen in all three patients and fall in bFVIII:C<1% in two patients. One of these inhibitors was initially only detectable by the inhibitor assay (NBA) following pre-analytical heat treatment, discussed in more detail in Chapter 7. Two patients had “high-risk” and one a “standard-risk” *F8* mutation.

The inhibitor prevalence within patients treated within the study period was 4.2% (16/377). Patients with an inhibitor were significantly older (51.9 years, IQR 37.2-70.2) than those without an inhibitor at the start of the study (32.0 years, IQR 16.1-52.5, p=0.008). Patients with an inhibitor also had significantly lower bFVIII:C (6.5IU/dL, IQR 3.3-10.0) than those without an inhibitor (10.0IU/dL, IQR 5.0-18.0, p=0.014). *F8* genotype testing was performed in all patients (16/16) with an inhibitor, compared to 281/361 patients without an inhibitor (77.8%, p=0.034). In patients in whom the *F8* genotype was known (n=331), there was an increased frequency of patients with a “high-risk” *F8* mutation (50.0%, 8/16) in those with an inhibitor compared to those without an inhibitor (15.9%, 50/315, p<0.0005). Although all three new inhibitors were detected in a single centre, the total inhibitor frequency for each centre did not differ significantly than that expected by chance (p=0.654).

4.5 Discussion

Within the London region, 44% (377/853) of patients with non-severe haemophilia A received haemostatic treatment over the two year study period. A large proportion (n=296, 79%) of these patients received exposure to a FVIII concentrate to cover on-demand treatment episodes. Despite national guidance for inhibitor testing in patients with non-severe haemophilia A, compliance with convalescent inhibitor screening was poor. In patients with a “standard-risk” *F8* mutations, although around half (n=243, 51.3%) of the

treatment episodes were followed with an annual inhibitor screen, only 12.3% (28/227) were screened within six weeks of treatment when indicated for by higher intensity exposure or surgical intervention. Similarly in patients with “high-risk” *F8* genotypes only 13.6% (12/88) of episodes were screened with six weeks of treatment. All three inhibitors detected in this study were associated with a change in bleeding phenotype and no inhibitors were detected purely on inhibitor screening.

4.5.1 Testing of *F8* Genotype and “High-Risk” *F8* mutations in Non-Severe Haemophilia A

For unbiased assessment of genetic risk of inhibitor formation in non-severe haemophilia A, there is a need for high levels of uptake of *F8* genotype testing. Within the London haemophilia centres, good compliance (88% *F8* genotype known) with *F8* genotype testing was observed, within all except one small HTC testing >70% of treated patients. Marked variation in *F8* genotype testing practice have been described in an international survey (13 centres)³⁰⁹. In this survey, for sites outside of the USA, genetic testing had been performed in >75% of cases in 7/8 centres. In the USA, 4/5 had performed genetic testing in <50% of patients. The authors of this survey speculate that these differences in genetic screening practices in the USA, may relate to test funding and the need to demonstrate how these alter clinical management, for which there is no strong clinical data to support this at present. Generalisation of these findings is however limited due to the small number of centres surveyed. Similarly, in the INSIGHT study evaluation of inhibitor risk associated with *F8* genotype was performed only in centres that had genotyped ≥70% of patients, resulting in inclusion of 41% (14/34) of centres¹⁶⁶. Given the high compliance with *F8* testing and the authorship of the INSIGHT cohort, patients from 3-4 of the London haemophilia centres may have contributed substantially to this analysis¹⁶⁶. Risk evaluation of inhibitor formation and the response to inhibitor treatment in large observational studies such as the INSIGHT study is dependent on adequate laboratory detection of the primary endpoint. With poor compliance in “convalescent” inhibitor testing as seen in the London cohort, this will significantly impact on data quality within observational studies. This makes interpreting data on inhibitor risk associated with mutations¹⁶⁶ and whether patients with inhibitors respond to immunosuppression¹⁶⁹ difficult.

When designing and piloting this evaluation, there was a paucity of data on inhibitor risk relating to specific *F8* mutations. Within the UKHCDO guidance, no specific *F8* mutations were identified, rather advising referral to the HADB and the awaited publication of the

INSIGHT study to identify “high-risk” *F8* mutations²¹⁴. The “high-risk” *F8* mutations selected, therefore were based on information available within a recently published international study¹⁹⁴ to represent mutations that clinicians may be aware of and could influence inhibitor screening practices. Of these “high-risk” *F8* mutations, with reported inhibitor association assessed within the London cohort, 4/7: Arg593Cys, Tyr2105Cys, Arg2150His and Trp2229Cys, have subsequently been described as being associated with increased risk of inhibitor formation within the INSIGHT study¹⁶⁶. These four *F8* mutations accounted for 16% (179/1112) patients within the INSIGHT study, with 57% (29/51) of patients who had an inhibitors having one of these *F8* mutations. No inhibitors were seen in the remaining three “high-risk” *F8* mutations in the INSIGHT study although the number of patients with these mutations was low, accounting for only 9 patients of the whole cohort¹⁶⁶. All three of these mutations (Arg2163His^{64;310}, Asn2286Lys³¹¹ and Pro2300Leu³¹²) have been described to have been associated with inhibitor formation within the HADB⁷¹. Data within the London cohort, appears to further support the idea of “high-risk” *F8* mutations with these “high-risk” *F8* mutations being over-represented within the group of patient with an inhibitor.

Further study into genetic risk factors of inhibitor formation in non-severe haemophilia A is required. Evaluation of risk is limited by difficulty in case-control matching, given some controls may develop inhibitors later in life with adequate FVIII exposure. Given the number of patients and length of follow-up required, construction of such prospective study would prove difficult even within an international study. *In-silico* approaches to prediction of inhibitor risk offers an interesting approach to address some of these difficulties. Work performed by investigators at the Institut national de la santé et de la recherche médicale (INSERM)³¹³ and by our research group³¹⁴ have attempted to identify prediction models based around peptide presentation for *F8* mutations at the MHC Class II / TCR interface. These data suggest that risk of inhibitor formation in non-severe haemophilia A is more complex than simple knowledge of the *F8* mutation. Addition of MHC Class II into these algorithms may improve risk prediction, but further work is required to establish risk based on context of FVIII exposure within these models.

4.5.2 Timing and Methodology of Inhibitor Testing in Non-Severe Haemophilia A

There is currently no available data to guide the optimal timing for performing inhibitor screening in non-severe haemophilia. A cut-off of ≤ 6 weeks was selected as being representative of when a primary immunological response would be detectable and to

provide some certainty that testing was performed to screen for inhibitor formation following treatment. The FVIII subcommittee of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) has proposed guidance suggesting that all patients with mild haemophilia A should have “convalescent” inhibitor screening performed by the Bethesda assay six weeks after exposure to FVIII ³¹⁵. This provides a pragmatic approach for inhibitor screening in mild haemophilia A in which there are still many unanswered questions as to what constitutes a high risk exposure. More widespread inhibitor screening will facilitate study of both genetic and environmental causes of antibody formation as well as allowing complete identification of the immune response to FVIII. Current inhibitor screening based on a subset of *F8* genotypes selected as being “high-risk” may skew toward detection of inhibitors in these groups, particularly where a founder effect is seen in some countries. For other *F8* mutations where there is little data, this may lead to false reassurance that these patient are not at risk of antibody formation and could result in lack of detection if these are not screened following treatment. Despite earlier reports of what constitutes environmental risk within non-severe haemophilia, a preliminary report of data from the case-control study of the INSIGHT study suggest no strong predictors of risk ¹⁶⁷. More widespread screening such as the approach suggested by the SSC may help in the detection of inhibitors in this group. Whether this approach will lead to increased detection of low-titre (or transient) inhibitors, as has been recently described in patients with severe haemophilia A²¹⁵ is not clear. Finally, although increased screening from a scientific point of view allows clearer understanding of the immune response, this will be associated with substantial increases in cost. Of the whole London cohort, paired testing was performed in 33.3% (187/562) and 12.3% (69/562) of episodes were screened within six weeks of treatment (Table 4.2 and Figure 4.4). Meeting this screening target, however would have required an additional 375 tests being performed within this study period. Piloting of more intensive screening practice in a prospective study may help provide a health economic assessment of this approach to inhibitor screening.

4.5.3 Laboratory Testing of FVIII Antibodies in Non-Severe Haemophilia A

Within this cohort inhibitor testing was based on the usage of a functional inhibitor assay. This methodology has limitations in the detection of FVIII antibodies in non-severe haemophilia A due to many patients with mild haemophilia A having residual endogenous FVIII:C prior to testing. In this cohort over half of the patients (53%, 199/377) had a FVIII:C ≥ 10 IU/dL and 22.5% (85/377) a FVIII:C ≥ 20 IU/dL. Some large haemophilia centre laboratories,

including one of the large CCCs included within this study have previously reported not testing samples in which FVIII:C ≥ 20 IU/dL²⁹⁰ when using a functional inhibitor assay. This approach would have resulted in 1 in 5 patients not being screened based on their bFVIII:C alone. In this setting a screening test with high specificity (i.e. correctly identifies negative results), which is insensitive to residual FVIII:C may facilitate inhibitor detection and result in increased understanding of the immune process in non-severe haemophilia A. Usage of an ELISA or modifications to the NBA, such as pre-analytical heat treatment could assist in the detection of inhibitors in this scenario which is evaluated further in Chapters 6 and 7.

4.5.4 Classification of Mild Haemophilia A

There is ongoing discussion about classification of patients with mild haemophilia A with a FVIII:C >40 IU/dL³¹⁵. Within this cohort seven patients (4/7 centres) were registered as having FVIII:C ≥ 40 IU/dL and 4 received treatment with a FVIII concentrate (bleeding=2, surgery=2). The mean age was 33.6 years (range 8.6-88.4) with three <18 years (8.6, 9.5 and 16.0 years). *F8* genotype testing had been performed in 6/7 of these patients and 1 had a “high-risk” *F8* mutation. Although, representing a small proportion of patients in clinical practice, this subset represents a clinical and laboratory challenge. Current international guidance classifies mild haemophilia A as FVIII:C 5-40 IU/dL⁷⁰, although previous reports from the UK have used a higher threshold of <50 IU/dL⁶⁴. The FVIII Subcommittee of the SSC of the ISTH has proposed individuals with FVIII:C >40 IU/dL, on repeat testing could be classified as having haemophilia if: the *F8* mutation is associated with haemophilia within one of the databases; a family member with the same *F8* mutation has a FVIII:C <40 IU/dL; or in the presence of the same mutation or a database record listing the *F8* mutation is associated with FVIII:C <40 IU/dL in other individuals³¹⁵. This group of patients offer an interesting area for further study through application of *in-silico* and *in-vitro* assessment of effect of these missense mutations on FVIII structure and function.

4.5.5 Annualised Bleed Rates in Non-Severe Haemophilia A

A negative correlation between FVIII:C and ABR was seen, similar to that described within other reports⁶⁶. A Dutch survey of 433 patients with non-severe haemophilia A (119 mild, 314 moderate) of self-reported ABR (preceding year), demonstrated a mean of 1 bleed/year with right (positive) skew, similar to the data described within this chapter (Figure 4.2). Within this cohort patients with moderate haemophilia A (bFVIII:C $<5\%$) had the highest risk for joint bleeds with no expected joint bleeds seen in patients with a bFVIII:C $\geq 15\%$. Statistical

modelling, using a multivariate regression analysis (negative binomial distribution) demonstrated an 18% reduction in bleed frequency for every 1% increase in FVIII:C³¹⁶. The correlation between ABR and bFVIII:C in patients treated in London was however weak, which may relate in part to the methodology of patient selection, analysis (post-hoc) and reliance on registration data for bFVIII:C.

4.5.6 Treatment of Non-Severe Haemophilia A with DDAVP

The number of patients managed with DDAVP within this cohort was lower than expected. DDAVP offers a cheap and safe (no inhibitor risk) method of treating responsive patients for minor bleeding or surgery⁶⁷. There has been varying reports in the literature on factors affecting response to DDAVP which include age, bFVIII:C and *F8* mutation³¹⁷. Within the London cohort, patients treated with DDAVP had a higher bFVIII:C than those patients treated with FVIII alone. This is in keeping, with data from small studies suggesting increased DDAVP response associated with bFVIII:C, with lower responses in patients with moderate haemophilia A. The frequency of “high-risk” *F8* mutations was similar in patients treated with FVIII alone, DDAVP and DDAVP and FVIII within the London cohort. Usage of DDAVP could minimise or avoid exposure to treatment with FVIII in responsive patients with “high-risk” *F8* mutations. Of the “high-risk” *F8* mutations within the London cohort three have previously published data on DDAVP responsiveness (Arg593Cys, Tyr2015Cys and Arg2169His) albeit in small numbers of patients³¹⁷. Further research is ongoing in this area within the RISE (Response to DDAVP In mild hemophilia A patients, in Search for dEterminants) study, a satellite study of the INSIGHT study.

4.6 Limitations

The main limitation from this study stems from the retrospective design which provided limited information on patient demographics and the treatment episodes. As such, it is not possible to confirm whether patients received treatment or were registered at more than one centre, which may affect estimations of proportions of patients receiving FVIII and calculation of inhibitor prevalence. Usage of FVIII:C without additional genetic information to define severity could have resulted in some patients with severe haemophilia A being included. This likely represents a small number of patients as only 6/377 (1.6%) patients of the whole cohort had a FVIII:C <2IU/dL. With this methodology, further assessment of the nature of bleeding (spontaneous or traumatic) or type of surgery was also not possible, both which will constitute heterogeneous groups of treatment indications. This approach however

allowed unbiased assessment of screening associated with FVIII treatment, without making judgement of severity/complexity in subsequent post-hoc analyses. Another difficulties in this and similar data-sets is in the assessment of usage of home-treatment. A high proportion of patients (n=96, 32.4%) were issued FVIII for home-treatment, which could have included a number of treatment indications, including, regular/targeted prophylaxis, on-demand home-treatment, ITI as well as issues prior to travel (that may not have been administered). No further analysis was performed within this group, although it is recognised that the true rate of bleeding within the cohort may be higher than the figure reported.

Within these data sets it is not possible to assess the reason for outlying data-points with high numbers of EDs, which could represent inpatient commencement of prophylaxis. Similarly, in patients treated with DDAVP it is not possible to make further assessment of treatment episodes or whether these usages were linked to subsequent FVIII usage within the same treatment episode (i.e. DDAVP treatment failure or pre-planned combined use to minimise FVIII exposure). It would be interesting to assess the uptake of assessment of DDAVP responsiveness in this cohort. DDAVP responsiveness should be evaluated where there is no contraindication, in any patient with mild haemophilia A. In those with moderate haemophilia A consideration of assessment of DDAVP response would seem appropriate especially in the context of family history of inhibitor formation, but the likelihood of developing adequate response may be lower. Use of DDAVP in such high risk individuals for minor incidental events that may be amenable to DDAVP and tranexamic acid, incrementally through a life time may however save a meaningful number of FVIII concentrate exposures. It is possible that in selection of FVIII over DDAVP, for treatment episodes that clinicians may not have been aware of the *F8* genotype at the time of treatment, or baseline (or target) FVIII:C was such that DDAVP was not sufficient to allow DDAVP alone. Although, there is significant heterogeneity described with the haemophilia databases for bFVIII:C for different *F8* mutations, groups of mutations may have lower FVIII:C (or lack of response) making these not amenable to DDAVP treatment. In the screening and treatment practices described, it is of note that these appear similar in those with “high-risk” and “standard-risk” *F8* mutations, which could imply that at present this does not impact on clinical management.

Another limitation in this approach to assessment of inhibitor screening practices, is follow-up of events that occurred within the last six months of the study, which although, providing sufficient data to allow assessment of testing for “convalescent” screening, did not have a full year of follow-up for assessment of annual screening. This could mean that there is an

underestimation in the uptake of annual screening in those with “standard-risk” *F8* mutations. Interestingly, the frequency of annual inhibitor screening was similar for all four six month (Q) periods (Q1: 44.1%, Q2: 51.7%, Q3: 57.7% and Q4: 50.4%). Patients in whom the *F8* genotype was unknown, were grouped within the assessment of inhibitor screening with patients with “standard-risk” *F8* mutations. This group of patients may well have contained a number who had a “high-risk” *F8* mutation if genotype testing had been performed, which may have a small effect on the proportion of episodes included within this assessment. Assessment of all episodes regardless of *F8* genotype evaluating “convalescent inhibitor screening”, however allowed unbiased assessment of episodes based on this status. One of the strengths of this data set however, relates to this unbiased approach to the assessment of uptake of inhibitor screening, which is not possible within a prospective setting. This study provides an informative snapshot of current inhibitor screening practices in non-severe haemophilia A, which underpin the data provided within observational studies examining risk of inhibitor formation.

4.7 Conclusions

Within a large cohort of patient with non-severe haemophilia A, although there was good compliance with genetic testing, poor compliance with “convalescent” inhibitor screening following FVIII treatment was observed. Re-auditing of inhibitor testing practices presented is required to evaluate the impact of these data on clinical practices. Further study is required to assess optimal practice and methodology of inhibitor screening in non-severe haemophilia A. Improvement in laboratory detection and planning for routine screening, through more sensitive and/or batched screening tests may help in earlier identification of FVIII antibodies, which is discussed in Chapters 6 and 7.

Chapter Five: Immunosuppression for Acquired Haemophilia A: A National Survey of Clinical and Laboratory Practice

5.1 Introduction

Factor VIII antibodies occurring in haemostatically normal individuals although a very rare phenomenon result in significant morbidity and mortality^{107;318}. Previous survey data has suggested that even within the haemophilia CCC clinicians may manage only a small number of cases per year^{110;114;115}. As such, clinicians may have limited personal experience of treating AHA, which has been highlighted in previous studies. In an international survey of ISTH members and CCC clinicians from the early 1980s, 47% (55/118 respondents) reported having not seen a case of AHA in the previous 10 years. In those who had treated AHA, 75% (47/63) had seen ≤ 4 cases, 16% (10/63) 5-10 cases, 8% (5/63) 11-20 cases and only one respondent reported treating >20 cases¹¹⁰. In a more recent European survey (Germany, Austria and Switzerland), 44% of respondents, reported personal treatment experience of 1-4 cases, 24% 5-10 cases and only 18% 11-20 cases. It is difficult however to compare these survey data due to changes in clinical and laboratory practices over time and within both survey methodologies it is not clear which types of clinicians were surveyed or responded. Nevertheless as a rare disorder management decisions will be likely be influenced by recent publication, guidance (local, national or international) or anecdotal experience.

In contrast to severe haemophilia A, treatment aimed at auto-antibody eradication hinges around the use of immunosuppressive agents^{119;319}. Despite recent increased understanding of the natural history of this disorder (discussed in Chapter 1), the optimal approach to first line immunosuppression remains to be defined and there is a lack of consensus as to the optimal duration and approach to withdrawal of immunosuppressive therapy^{119;319}. As a result differences in the management of immunosuppression in observational studies could affect reported clinical outcomes and predictors of risk. One of the greatest current challenge in the management of AHA is the high rates of morbidity and mortality that relate to the treatment of this disorder^{117;118;318}. Historically, the major concern in the management of AHA was mortality resulting from haemorrhage which historically occurred in 15-22% of cases^{110;320}. With improvements in early recognition and haemostatic management, mortality directly attributable to bleeding has reduced to around 3%¹⁰⁷. Within the EACH2 study however, the same number of patients died as a result of immunosuppression (3%, 16/331) as from bleeding (3%, 16/331)¹⁰⁷. Alongside this, one or more adverse event was

associated with immunosuppression in 25% and 44% of patients treated with steroids or steroids and cyclophosphamide respectively³¹⁸. Given these high rates of toxicities it would be desirable to risk stratify patients at commencement or early into immunosuppressive treatment to minimise the risk of toxicity in those more likely to achieve remission. Despite the existence of national guidance for the management of AHA in the UK³¹⁹ there has been no previous national study looking into how haematologists report their implementation of immunosuppression in AHA.

5.2 Hypothesis and Aims

The hypothesis for this chapter is that there is significant heterogeneity in the management of immunosuppression and its withdrawal in the treatment of AHA. It is hypothesised that differences in approaches to management may affect responses to treatment and occurrence of treatment related morbidity in clinical trials. The specific aims are as follows:

- 1) To evaluate national treatment practices in the first line management (immunosuppressive and haemostatic agent) of AHA.
- 2) To evaluate laboratory approaches to weaning and monitoring of weaning of immunosuppression.
- 3) To evaluate clinical approaches to weaning of immunosuppression assessing: timing, speed and variables affecting speed of immunosuppressive weaning.
- 4) To assess clinical case volumes managed by haemophilia treaters within the UK and provide pilot data to inform study design and protocol acceptability for a national, prospective, interventional study in AHA.

5.3 Materials and Methods

Survey Design and Distribution

An assessment of clinical and laboratory practices of haematologists treating AHA in the UK was performed using a national, anonymised, electronic short survey (10 questions). This survey was constructed using the online SurveyMonkey® (SurveyMonkey Inc., Palo Alto, California, USA; <https://www.surveymonkey.com/>) survey tool on behalf of the UKHCDO Inhibitor Working Party. The members of which reviewed and approved the survey topics and questions prior to distribution.

This process of survey distribution was centrally co-ordinated by the UKHCDO, allowing additional blinding/anonymity of potential respondents to the investigator. E-mail invitations were sent to all haemophilia centre directors/co-directors (n=82), at the 78 haemophilia centres (CCC=28 and 50 HTC=50) in the UK. This invitation email contained a hyperlink to the survey (<https://www.surveymonkey.com/s/QDFCFVV>), which allowed direct access for online survey completion viewable as a single webpage. Questions were presented sequentially with no question randomisation. This survey platform was chosen as this was identified as a platform that physicians had previous experience of completion of web surveys. The survey was an “open” survey allowing access and completion of the online questionnaire without registration and no financial incentive was offered. Each survey response was assigned a unique sequential response number by the web-server, with no identifiable respondent or centre data collected. Survey responses could be edited by respondents up until the last section of the survey had been completed, with no completeness check performed prior to survey submission. A maximum of one response per computer was allowed, through cookie placement following survey completion to prevent repeated access from the same web-browser from the same device. The survey was conducted via a secure encrypted SSL (Secure Sockets Layer) connection with responses stored on the SurveyMonkey® servers located in Luxembourg and the United States. The date and time taken to complete the survey were recorded for each participant. At the time of answering, the SurveyMonkey® web-server logs the internet provider (IP) address as part of standard survey recording. No demographic (personal or centre) or contact information was requested and no geo-positional data was electronically obtained. No paper or e-mail response options were provided to standardise the survey format and to help ensure anonymity for respondents. A reminder email was sent one month later, to all centre directors/co-directors, with the survey being open for four months (9/12-12/12).

Data Entry and Statistical Analyses

Individual survey responses were collated and coded electronically to allow more detailed analysis of respondent data. These processes were checked manually and electronically using the summary data provided by the SurveyMonkey® website. Categorical data were used to calculate response rates (n) and frequencies (%), with statistical analyses performed using the chi-squared, chi-squared goodness-of-fit and binomial probability tests. Analysis of survey data was performed within each question, based on the number of complete responses. No mathematical correction (imputation) was made for missing data, as it was

felt that these data points may be missing not at random. Inter-rater agreement was assessed using the Fleiss kappa. Agreement was rated as being either poor (<0), slight (0.0-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80) or almost perfect (0.81-1.00).

5.4 Results

The online survey was completed by 36 respondents, representing haemophilia centre and centre director/co-director response rates of 46.2% (36/78) and 43.9% (36/82), respectively. There were 26 survey responses received following the first survey email with an additional 10 responses obtained following a reminder email being sent one month later. 44.4% (16/36) of responses were completed on the same day that the first survey email was distributed. The median time taken to complete the survey was 3 minutes and 4 seconds (range 20 seconds-30 minutes and 30 seconds).

The majority of clinicians (n=30, 83.3%) responding to the survey reported treating less than five (0-5) new cases of AHA per year. All of the remaining respondents (n=6, 16.7%) responding reported treating between five and ten new cases per year. No respondent reported treating more than ten new cases of AHA per year.

5.4.1 Selection of First Line Haemostatic Agent

Assessment of first line haemostatic agents used in the treatment of bleeding demonstrated that 61.8% (n=21) of respondents use Factor Eight Inhibitor Bypassing Activity (FEIBA®), 35.3% (n=12) recombinant activated factor VII (rFVIIa, NovoSeven®) and 2.9% (n=1) high dose factor VIII. Despite a higher number of treaters opting to use FEIBA® in comparison to NovoSeven®, this did not reach statistical significance (p=0.117).

5.4.2 Selection of First Line Immunosuppression

An assessment of favoured first line immunosuppressive agent(s) was evaluated in three representative clinical groups (Figure 5.1). These consisted of: (A) patients <65 years or with minimal medical co-morbidity; (B) patients >65 years or with significant medical co-morbidity and (C) pregnancy associated AHA. The majority of respondents chose steroids alone or steroids and a cytotoxic agent (Group A 91.1%, B 82.4%, C 96.9%) as first line immunosuppression regardless of the clinical group (Figure 5.1). The remaining respondents mainly used rituximab based regimens either alone, or in combination with steroids. No respondent reported using a ciclosporin or a FVIII (ITI) based regimen as first line treatment.

A comparison was then made of usage of steroids alone versus steroids in combination with a cytotoxic agent for these three clinical groups. Data for other immunosuppressive choices, which contained ≤ 3 respondents were excluded to allow comparison with consensus guidance^{119,319}. In the treatment of pregnancy associated AHA (Group C), significantly more respondents reported using steroids in isolation (83.9%, 26/31) compared with steroids and cyclophosphamide (16.1%, 5/31; $p < 0.0005$). In patients < 65 years (Group A), although the percentage of respondents using steroids alone (64.5%, 20/31) was higher than for steroids plus cyclophosphamide (35.5%, 11/31), this did not reach statistical significance ($p = 0.106$). Similarly, in patients > 65 years (Group B), although a higher proportion of respondents reported using steroids alone (64.3%, 18/28) compared to steroids and cyclophosphamide (35.7%, 10/28), this did not reach statistically significant ($p = 0.131$).

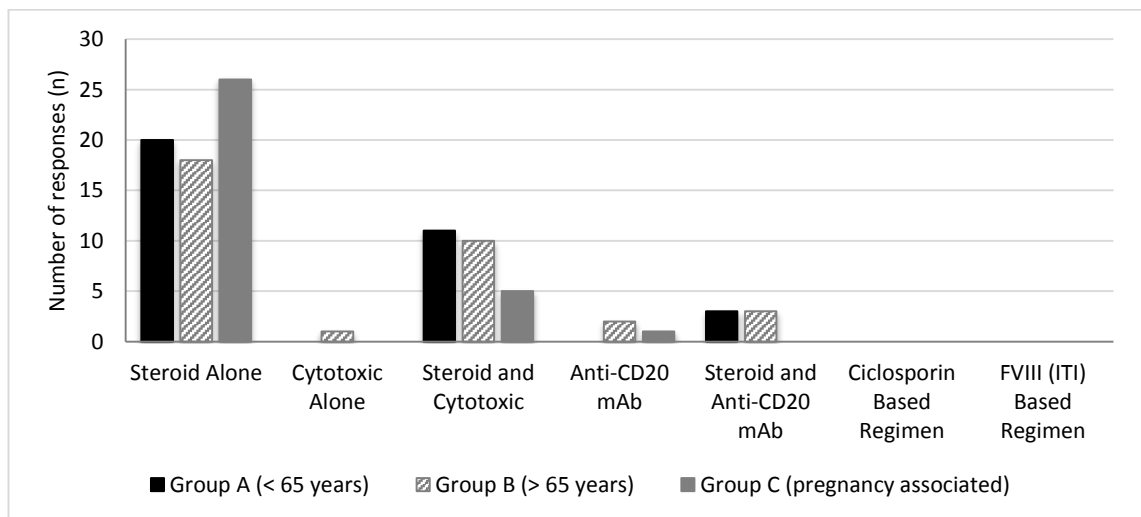


Figure 5.1: First line immunosuppressive agent choices for the management of acquired haemophilia A, in three clinical scenarios. (Group A) < 65 years or with minimal medical co-morbidity; (Group B) > 65 years or with significant medical co-morbidity and (Group C) pregnancy related AHA. Based on a figure in Batty et al³²¹, reproduced with the permission of the publisher.

5.4.3 Laboratory Monitoring of Treatment and Immunosuppressive Weaning

Next an evaluation of the laboratory tests performed to monitor treatment response and immunosuppressive weaning was performed. All participants reported using a FVIII:C assay in combination with an inhibitor assay (Bethesda assay or equivalent) to monitor treatment response. For guiding of weaning of immunosuppression the majority (85.3%) used the FVIII:C in combination with an inhibitor assay and 14.7% used the FVIII:C alone. Only one respondent reported using pre-analytical heat treatment to denature residual FVIII:C prior to

inhibitor testing. No respondents reported using either an ELISA (immunological testing) or global haemostatic assays to monitor treatment or guide weaning.

5.4.4 Factors Affecting Timing of Commencement of Weaning of Immunosuppression

Next an assessment of timing of commencement of weaning of immunosuppression was performed based on the results of laboratory results (6 potential options). There was marked variation in the reported timing of the weaning of immunosuppression (Table 5.1). The commonest strategies seen were to start weaning after the FVIII:C had normalised and the inhibitor assay had become negative (n=11, 33%), a time-point, equivalent to complete remission in recent studies^{117;118}. The second commonest timing to commence weaning was after the FVIII:C, had started to rise, but had not yet normalised (n=8, 24%). Analysis of the distribution of responses for timing of weaning, did not differ significantly from that expected by chance (p=0.073). A secondary analysis was performed grouping responses into two categories to compare commencement of weaning prior to (responses 1-4) or following (responses 5-6) obtaining complete remission (Table 5.1). This demonstrated no significant difference in the proportion of respondents who commence weaning prior to (51.5%, 17/33), or upon achieving (48.5%, 16/33) complete remission (p=0.862).

	Laboratory Variables	Response % (n)	Response
1	As soon as inhibitor assay* negative	9.1% (3)	NR
2	Once FVIII:C starts to rise but not yet normal	24.2% (8)	NR
3	After a period of maintained inhibitor assay* negativity	9.1% (3)	NR
4	As soon as FVIII:C within the normal range, regardless of low level inhibitor assay* positivity	9.1% (3)	PR
5	Once FVIII:C is within the normal range and inhibitor assay* is negative	33.3% (11)	CR
6	After a period of FVIII:C being within the normal range and inhibitor assay* negativity	15.2% (5)	CR

Table 5.1: Timing of commencement of weaning of immunosuppression based on laboratory variables in acquired haemophilia A.*Bethesda assay (or equivalent). NR=No response, PR=Partial remission, CR=Complete remission. Categories 1-4 indicate scenarios of patients not in complete remission, categories 5-6 represent scenarios of patients within complete remission. Based on a table in Batty et al³²¹, reproduced with the permission of the publisher.

5.4.5 Factors Affecting Speed of Weaning of Immunosuppression

Next approaches to speed of weaning of immunosuppression, were evaluated in a scenario of an uncomplicated patient in complete remission following treatment with steroids alone. Respondents could choose one of three speed of weaning options: gradual (e.g. 60mg-50-40-35-30-25-20-15-10-5-2.5-stop); intermediate (e.g. 60mg-40-30-20-10-5-stop) or rapid (e.g. 60mg-30-15-7.5-5-stop). Fifty percent (n=17) opted for a gradual, 44% (n=15) an intermediate and 6% (n=2) a rapid approach to weaning of immunosuppression

	Speed Up	No Change	Slow Down	p
Bleeding at diagnosis (requiring bypassing agents)	2.9% (1)	58.8% (20)	38.2% (13)	0.392
Supra-normal FVIII:C (e.g. >200IU/dL)	55.9% (19)	41.2% (14)	2.9% (1)	0.392
Steroid side effects	82.4% (28)	14.7% (5)	2.9% (1)	<0.0005
Cytotoxic side effects	90.9% (30)	3.0% (1)	6.1% (2)	<0.0005
Only achieving low normal FVIII:C (e.g. 50-60IU/dL)	2.9% (1)	70.6% (24)	26.5% (9)	0.0243
Pregnancy associated AHA	29.0% (9)	71.0% (22)	0.0% (0)	0.0294

Table 5.2: Factors affecting weaning of immunosuppression in acquired haemophilia A. Numbers in brackets represent number of respondents. P refers to a two-tailed binomial probability test, with the null hypothesis that p=0.5 for the selection of no change. Based on a table in Batty et al ³²¹, reproduced with the permission of the publisher.

Finally, respondents were asked to rate whether a number of clinical and laboratory factors might influence their speed of weaning of immunosuppression (Table 5.2). For each scenario, an option of speed up, no change or slow down weaning of immunosuppression was provided. There was fair overall inter-rater agreement ($\kappa=0.335$) for factors influencing weaning of immunosuppression. Looking at each category, there was moderate agreement for factors relating to shortening of the weaning period ($\kappa=0.490$), with only fair and slight agreement to factors leading to no change ($\kappa=0.261$) or prolongation ($\kappa=0.158$) of the weaning period. Toxicity, either from steroids or cytotoxic agents was significantly associated with a change in weaning speed, with 82.4% (n=28, $p<0.0005$) and 90.9% (n=30, $p<0.0005$) of respondents reporting shortening of the weaning period. Achievement of only a low normal FVIII:C e.g. 50-60IU/dL (n=24, 70.6%, $p=0.0243$) and pregnancy associated AHA (n=22, 71.0%, $p=0.0294$), were significantly associated with no change in weaning strategies. Although, a number of respondents reported shortening weaning for supra-normal FVIII:C

e.g. >200IU/dL (n=19, 55.9%, p=0.392) and prolonging for bleeding at diagnosis (n=13, 38.2%, p=0.392), these changes were not statistically significant.

5.5 Discussion

This is the first nationally co-ordinated survey in the UK of treatment and immunosuppressive weaning practices amongst treaters of AHA. These survey data, demonstrate that within a group of haematologists from a country with published consensus guidelines for the management of AHA, there is heterogeneity at all stages of treatment choices. This starts from the initial selection of immunosuppression, but is even more notable in the approaches seen to weaning of immunosuppression.

5.5.1 Choice of Haemostatic Agents to Treat Bleeding

In the treatment of bleeding in AHA, there are a number of options including, aPCC, rFVIIa and high dose FVIII. Of these, bleeding control has been demonstrated to be significantly higher for the usage of bypassing agents over FVIII (93.3% v 68.3%, p=0.003)²²⁰. In keeping with these data, all except for one respondent selected using a bypassing agent for first line management of bleeding. In the selection of bypassing agents, although there has been no direct comparison of efficacy for these agents in AHA, a retrospective analysis of the data from the EACH2 cohort has suggested these to have similar efficacy and safety profiles²²⁰. In this survey there was a trend (non-significant) towards selection of an APCC over rFVIIa (61.8 v 35.3%) for treatment of bleeding. This finding, is similar to data observed within the UKHCDO annual reports over many years (Hay C.R., Professor in Haematology, Manchester Royal Infirmary, personal communication)³²¹. Similar data has also recently been reported in a cohort of patients (n=65) treated within the London CCCs and HTC (1/2009-12/2012)³²². In this, aPCC alone were used to treat 33/65 patients in comparison to 5/65 patients who were treated with rFVIIa alone. These findings however, are in contrast to bypassing agent use previously reported in a national UK AHA surveillance study¹⁰⁶, the EACH2²²⁰ and Surveillance des Auto anti-Corps au cours de L'Hémophilie Acquisée (SACHA) studies¹¹⁷. Within the reported national surveillance study, similar proportions of patients were treated with both agent (APCC alone=25%; rFVIIa alone=21%) and in the EACH2 study, rFVIIa was used in twice as many patients as aPCC (56.7 v 20.5%)³²³. Similarly in the SACHA study, rFVIIa alone, was used to treat 28/37 patients and aPCC alone 6/37 patients¹¹⁷. These recent data may represent a change in practice in the UK, since the previously published two year (1/5/01 and 30/4/03) surveillance study¹⁰⁶.

5.5.2 Choice of First Line Immunosuppression

In the treatment of AHA unrelated to pregnancy, there was a divide in respondents favouring steroids in isolation to steroids in combination with a cytotoxic agent. Forty one percent of respondents (n=13) reported only using steroids in isolation regardless of the clinical scenario (Group A, B or C). This variability, reflects the lack of clear data to provide treatment guidance for first line immunosuppression within the published literature ^{119;319}. Within the EACH2 study, although the combination of steroids and cyclophosphamide was associated with higher rates of stable complete remission (70% v 48%) compared to steroids alone, this did not translate into a survival benefit at final assessment and was associated with a higher rate of treatment related toxicity (25% v 41%) ³¹⁸. To date, there has been only one small prospective interventional study (n=31) investigating first line immunosuppression ³²⁴. This study showed no difference with the addition of an alkylating agent to steroid treatment in patients with residual FVIII auto-antibody following treatment with steroids alone for 3 weeks. The study was underpowered to identify differences between the treatment arms and only managed to recruit 31 patients (of a target of 100) over 5 years.

A small proportion of respondents (Group A, n=3; Group B, n=5 and Group C, n=1), opt to use an anti-CD20 monoclonal antibody (rituximab) as part of first line therapy. Although, a number of recent reports have described high response rates for the use of first line rituximab ^{325;326}, there are no data yet to suggest this approach is superior to conventional first-line immunosuppression ³¹⁸. No respondent reported using ciclosporin or a FVIII (ITI) based regimen as first line treatment. There has been one case series describing the use of calcineurin inhibitors in 11 patients (8 ciclosporin and 3 tacrolimus) in combination with steroids (pulsed intravenous methylprednisolone day 1-3, followed by oral prednisolone), which demonstrated sustained response in 10 patients, at a median of 3 weeks (2-8 weeks), with no relapse at 14 months of follow-up. In this series, there was one serious adverse event (hypertensive posterior progressive encephalopathy) and the 5 year survival rate was 54.5%, with the cause of mortality being the underlying disorder or senescence in all 5 cases ³²⁷. Within the EACH2 study, although 5 patients received treatment that included cyclosporin no response data was presented due to the small number of cases included. There have also been two single centre descriptions of the use of ITI for the treatment of AHA ³²⁸⁻³³⁰. The first, used a three week protocol of intravenous methylprednisolone (100mg; one week tapering for week 2-3), cyclophosphamide (200mg; 10-15 days) and daily FVIII (30IU/kg week 1, 20IU/kg week 2 and 15IU/kg week 3) ³²⁸. Inhibitor eradication was seen in 13/14 patients

treated with ITI at a mean of 4.6 weeks, compared to 4/6 patients at 28.3 weeks in a historical control group. There was no bleed related mortality in the ITI group, with 2 deaths (33%) related to bleeding in the control group. Interpretation of this study is limited by poor selection of historical controls, which are unmatched in all baseline characteristics. It is also not clear, how much of this response was a result of more intensive immunosuppression in the ITI group, especially in view of relatively low FVIII dosing in patients who will have normal endogenous FVIII production. Finally usage of the Bonn protocol (immunoabsorption days 1-5; IVIg days 5-7; cyclophosphamide and prednisolone from day 1 until remission; high dose FVIII (100IU/kg) every 6 hours), has been described over a period of 19 years^{329;330}. This group treated 65 “high-risk” patients, which were defined as one acute bleeding episode, with a fall in haemoglobin to <8.0 mg/dl and the presence of multiple hematomas. Using this protocol, 63 patients completed treatment, with complete remission seen in 54/63 and partial remission in 5/63 (overall response rate 93%). The median time until the inhibitor became undetectable was 3 days, with a median of 17 days FVIII treatment and 16 days immunoabsorption. Although both studies demonstrated fast responses to treatment with no bleed related mortality, lack of adequate control groups make interpretation of these response rates difficult and require confirmation in larger studies.

In the management of AHA associated with pregnancy, the majority of respondents (n=26, 81%), chose steroids alone as their preferred first line immunosuppressive agent(s). This is in keeping with the treatment options seen in the EACH2 study, in which data is presented on 39 cases received immunosuppression (total 42 cases, 3 cases no data available). Of these the majority (n=27) were treated with steroids alone, with 6 patients receiving steroids and cytotoxic, 4 steroids and IVIg and 2 steroids and rituximab¹⁰⁹. A total of 29 patients (79%), went in to complete remission with first line treatment and at final follow-up, 86% of the total cohort (36/42) were in complete remission. All 40 of the patients, in which data was available were alive at final follow-up.

5.5.3 Weaning of Immunosuppression and Treatment Related Toxicity

Toxicity relating to treatment is currently one of the main difficulties in the management of AHA as highlighted in recent studies^{106;117;118;318}. In keeping with these trial data toxicity from immunosuppression (steroids or cytotoxic agents) was reported in this survey to significantly impact on weaning of immunosuppression, with the majority of respondents shortening the weaning period on this basis. One potential way to limit the toxicity of immunosuppression

would be to wean earlier. The GTH-AH 01/2010 study is the only multicentre studied published to date in which there was clear guidance on weaning of immunosuppression. Tapering of immunosuppression was commenced at achievement of partial remission (FVIII:C >50IU/dL; no active bleeding after stopping haemostatic drugs for >24 hours), with cessation of cyclophosphamide/rituximab and then tapering of steroids over 6 weeks (descending doses of 50, 25, 20, 15, 10, 5mg, for 1 week each). With this rapid weaning schedule, of those achieving partial remission 19 (22%) had a fall in FVIII:C <50% requiring an increase in steroid dose and a more prolonged tapering, with 11 (95%) of these patients going on to obtain a complete remission. Data from this UK survey, demonstrated a more cautious approach to weaning immunosuppression, with only 9.1% (3/33) commencing weaning of immunosuppression at a timing which would be similar to partial remission in the GTH-AH 01/2010 study (Category 4). In looking at approaches to weaning speed, 44% (n=15) respondents, took an intermediate approach to weaning, similar to the weaning protocol in the GTH-AH 01/2010 study, although without a proscriptive timing over which weaning is performed within this survey this is difficult to compare directly. This weaning schedule in the GTH-AH 01/2010 study may be appropriate for some patients with a lower risk of relapse to prevent additional toxicity, although requires further assessment in prospective study.

5.5.4 Laboratory Monitoring of Treatment Response in Acquired Haemophilia A

Monitoring of response and weaning was almost entirely based on the results of the FVIII:C and inhibitor assay. This is in keeping with published international consensus recommendations for the management of AHA ¹¹⁹. It is interesting to note that only one respondent reported using pre-analytical heat treatment to denature residual FVIII:C prior to testing. At the time this survey was conducted, this was routine practice in our centre (discussed in more detail in Chapter 7), but appears not to have been as widely used in the rest of the UK. One year after this survey was conducted, following the presentation of a number of reports by our group and others on usage of pre-analytical heat treatment, there appears to have been a change in practice within the UK with increased usage in a NEQAS exercise from November 2013 ³³¹. Usage of pre-analytical heat treatment as part of FVIII antibody testing in AHA is discussed in more detail in Chapter 7.

5.5.5 Limitations and Use of Web Based Questionnaires in Healthcare Research

With the increased routine use of the internet in healthcare environments and user friendly interface of online survey platforms, online surveys provides an attractive platform for

researchers in rare disorders. These provides a rapid, cheap and standardised methodology for collecting information on current practice, attitudes of physicians and the impact of recent research on their clinical practice^{332;333}. Understanding of these areas can help define healthcare policy, identify areas of required research and inform potential future study design. However, when using this approach it is important that the group that are sampled form a representative population of clinicians in whom findings will be generalised. The response rate (44-46%) seen in this survey is comparable to other online (single media) surveys conducted without provision of financial incentive³³⁴⁻³³⁶. Within the centres surveyed there were paediatric centres (n=7) and centres which subsequently became inactive (n=10) in the year following the survey. If only active adult haemophilia centres were assumed to have participated, it is possible that the response rates could have been in the region of 59.0% (36/61). It is however not possible to confirm the exact proportions of individual and centre response or the split between haemophilia centres and comprehensive care centres consequent on maintaining anonymity for respondents. Previous international and European surveys, carried out in AHA demonstrated response rates of 59% (118/200) and 16% (81/522) respectively^{110;115}. Despite technological advances to facilitate conducting surveys there are concerns that there may be a decline in the response to surveys by physicians. A review of response rates to physician surveys, demonstrated a small, but significant difference in response rates in surveys conducted between 1989 and 1995 (61.2%, 257 studies) and those between 1996 and 2005 (57.5%, 237 studies, p=0.02)^{337;338}. A Canadian study carried out in general practitioners and gynaecologist investigating reasons for lack of survey response, found reasons included inaccurate central listings (17.3%), physicians being away (2.6%), ineligibility due to practice type (18.5%) and office policy not to participate in surveys (36.3%)³³⁹. There are conflicting data from previous research as to factors that may affect response rates to surveys based on their methodology and design. Specific methods applied within this survey to maximise response rates^{336;340} were usage of a short survey format (median response time 3 minutes and 4 seconds), UKHCDO centralised study, follow-up contact (repeat email after 1 month) and an interesting questionnaire topic (following recent publication or the EACH2 study). Given the design of the study it was not possible to allow personalised contact of non-responders or to provide incentives to survey. Methods that may have provided greater response rate would have been to allow respondents to reply via different modalities (e.g. post or internet) and through additional reminder emails, being sent to the target population.

One of the main advantages of using this methodology, was that it ensured anonymity for survey respondents, which could potentially reduce selection and response biases. It is likely that responses may be influenced by respondents' anecdotal experience of managing this rare condition in addition to awareness of recent data ^{318;323;341-344}. The usage of anonymisation may have limitations as it is not possible to confirm that the survey was answered by the intended audience of the original email. For example, the email invitation could have been forwarded on to other interested members of the treatment team in one centre. An internet option was chosen to allow ease/speed of response and anonymity, despite recognition that this may potentially result in lower response rates. Although usage of a centralised method of disseminating the email link for this survey, allowed complete blinding to the clinicians in whom the survey was directed this has a number of potential problems, including difficulty in calculation of the precise number recipients for of the denominator. It is also not possible to assess how many individuals either did not open original emails or accessed the survey site and did not proceed with survey.

5.5.6 Aging Population in the United Kingdom

Similar to other areas of medicine, we are faced with an aging population. In 2010, the percentage of the population aged >65 years was 17%, with this projected to rise to 23% by 2035 ³⁴⁵. In the management of AHA this potentially poses two difficulties. Although, the overall incidence of AHA is reported at 1.48 million/year, this has been reported as increasing with age. In patients aged 16-64, there is an incidence of 0.3 million/year, which increases from 9 million/year to 15 million/year in patients aged 65-84 and ≥85 years old, respectively ^{106;113}. Given the projected rise in patients over 65 in the next decade, one would expect to see an increased incidence of AHA, assuming that the primary immunological mechanism is loss of tolerance to FVIII, resulting from the aging immune system (immunosenescence). Secondly, there is a well reported increase in multi-morbidity (presence of two or more co-existing medical conditions) associated with age. In a large cross sectional study of the Scottish population (1.75 million individuals), 64.9% of patients aged 64-84 had multi-morbidity, with this number rising to 81.5% in those aged ≥85 years ³⁴⁶. With the prevalence of multi-morbidity in the elderly, it is perhaps unsurprising there have been a large number of adverse events reported in previous studies of AHA relating to steroids and myelosuppressive drugs. Although there is requirement for more intensive immunosuppression in some patients with AHA, there is a need for further study of risk stratification based on clinical data and biomarkers. Development of such prognostic models,

given the rarity, heterogeneous aetiologies, limitations of diagnostic testing, and incomplete understandings of the underlying immunological mechanism may be difficult even in the context of an international study.

5.6 Conclusions

Conducting prospective interventional studies in very rare diseases such as AHA is challenging on account of the acute nature of presentation, significant multi-morbidity and statistical power considerations. Understanding trends in treatment practices, which have been obtained through these survey data are helpful for the design of protocols and gauging acceptable to physicians and patients. There are many unanswered questions in the management of AHA, which limit the development of clinical prediction tools to stratify risk. Although this survey is underpowered to make conclusions to guide clinical practice, I feel it offers a representative impression of treaters' preferences within one country, confirming the clinical equipoise that existing in the management of AHA. Despite commonly cited, peer-reviewed guidelines in the UK, immunosuppression choice remains at the physician's discretion and there is no proscriptive guidance on immunosuppression weaning³⁴⁷. One of the aims of this survey was to describe current treatment practice in the United Kingdom in AHA, in advance of the development of a prospective interventional study in the management of AHA (Collins P., Professor in Haematology, Arthur Bloom Haemophilia Centre, Cardiff, personal communication). The heterogeneity highlighted in this survey, reflects the lack of clarity in published guidance, itself a function of a paucity of data and need for an appropriately designed prospective randomised study.

Chapter 6: Diagnostic Accuracy Study of a Factor VIII ELISA Kit for Detection of Inhibitory Antibodies in Congenital and Acquired Haemophilia A

6.1 Introduction

Antibodies to FVIII may arise at any stage of life in a person with any severity of haemophilia A ^{166;174;199}. The urgency of testing for these antibodies will vary depending on the clinical context. In severe haemophilia A, laboratories are required to provide urgent inhibitor surveillance assay results during the early treatment of previously untreated patients (PUPs) ^{149;214} or where there is impaired treatment efficacy or pharmacokinetic parameters (recovery or half-life) following an infusion of a FVIII concentrate. Urgent testing is important in these circumstances to allow provision of adequate haemostatic therapy for treatment of acute bleeding or prevention in the peri-operative period. Early detection may be associated with an improved outcomes through early commencement of ITI ³⁴⁸. There is similar urgency to confirm the presence of auto-antibodies to FVIII in AHA ^{119;319}, to allow early commencement of haemostatic and immunosuppressive therapy. Aside from these two scenarios, comprising a minority of tests in most centres, most FVIII antibody assays performed within the CCC haemostasis laboratory are for the purpose of routine surveillance or screening. This may however, differ between centres depending on the age range of patients treated within that centre.

The optimal test for FVIII antibody screening would be one that could be performed simply with both high specificity and negative predictive value. This would also ideally provide information to the clinician about the functional capacity of the FVIII antibody to influence treatment decisions. The most widely used assay for the detection of antibodies to FVIII is the NBA ^{273;276}, which is associated with well documented inter-laboratory variability ^{278;298;299} and affected by numerous pre-analytical variables ^{288;289} as discussed earlier in Chapter 1. Despite these limitations the NBA is considered the “gold standard” assay for detection of FVIII antibodies ^{214;270}.

A number of “immunological” assays, have been described for the detection of FVIII antibodies in research settings (discussed in Chapter 1), which include enzyme linked immunosorbent assays (ELISA) ^{262;300-303;349}, fluorescence based immunoassays ²⁵⁹⁻²⁶¹ and immunoprecipitation ^{265;266}. A FVIII ELISA offers a simple approach to detect FVIII antibodies and is potentially less susceptible to the pre-analytical variables affecting functional inhibitor assays. Despite descriptions of usage of both commercial and in-house ELISAs in research

settings, this approach is not currently part of routine laboratory practice. A FVIII ELISA kit was introduced into routine laboratory practice in 2009-2010 in two London haemophilia CCCs. In this chapter the performance of this FVIII ELISA kit will objectively reviewed following implementation into routine laboratory practice.

6.2 Hypothesis and Aims

The hypothesis for this chapter is that a FVIII ELISA kit offers a simple approach for batch screening for FVIII antibodies in congenital and acquired haemophilia A in routine laboratory practice. The role of this assay as a screening test in routine haemostasis laboratory practice test has not previously been described. This is also the first formal comparison of the performance of a new assay compared to the NBA following the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) criteria ²⁷¹ for the reporting of diagnostic accuracy studies. This ELISA kit could allow early detection of FVIII antibodies that may provide a biomarker to predict the development of an immune response. It is hypothesised that the semi-quantitative results of this assay is directly associated with inhibitory capacity when measured using a functional inhibitor assay. The specific aims for this chapter are as follows:

- 1) Compare the diagnostic accuracy of a FVIII ELISA kit to the NBA for detection of FVIII antibodies in patients with congenital and acquired haemophilia A.
- 2) To model the effect of inter-individual clustering of data on the primary estimates within the diagnostic accuracy assessment.
- 3) To describe the utility of the FVIII ELISA in testing of clinical samples for FVIII antibodies based on the results of the diagnostic accuracy assessment.
- 4) To evaluate association between the results of the NBA and FVIII ELISA and to assess whether there is correlation between the results of these two assays.

6.3 Materials and Methods

Sample Selection and Inclusion/Exclusion Criteria

All samples sent for routine FVIII inhibitor testing from patients with congenital and acquired haemophilia A were tested in parallel using the Nijmegen-Bethesda assay and a FVIII ELISA as part of routine clinical care. At centre 1 (The Royal London Hospital, London, UK), samples were tested between 9/9/2010-23/4/2012 and at centre 2 (St Thomas' Hospital, London, UK) between 11/12/2009-28/09/2011. Testing was performed within the specialist haemostasis laboratories of both centres. The results of both assays were made available on the electronic

reporting systems to the treating haematologist, with neither laboratory nor clinical staff being blinded to results of these tests. Samples were tested only once in the laboratory of the haemophilia centre in which the patient was being managed and no repeat testing was performed.

A retrospective assessment of the performance of the ELISA was made based on the results of anonymised samples (discussed below), with a sub-group analysis performed using diagnostic labels provided at the time of venepuncture. No additional demographic or clinical data were retrieved to maintain patient anonymity. Samples were included, if they had a diagnostic label of either congenital (severe or non-severe) or acquired haemophilia A and where results from both assays were available. Samples were excluded, if the diagnostic label indicated a non-haemophilia A (congenital or acquired) diagnosis or if one (or more) of the assays had not been performed. All diagnoses were made by the local laboratory, with severe haemophilia A defined as a baseline FVIII:C <1IU/dL and non-severe haemophilia A 1-40 IU/dL^{63;70}. Acquired haemophilia A, was defined as the presence of bleeding (acute or recent onset) with an unexplained prolongation of the activated partial thromboplastin time (aPTT) that does not correct on appropriately incubated mixing studies, associated with a low FVIII:C^{119;319}. Local approval was granted at both centres for the evaluation of the efficacy (service evaluation) of the introduction of the ELISA in routine laboratory practice.

Nijmegen-Bethesda Assay

Inhibitor testing was performed following local protocols using the Nijmegen-Bethesda assay as described earlier (Chapter 3). At centre 1, assessment of residual FVIII:C was performed using a one stage FVIII:C assay with a cut-off for positivity ≥ 0.6 BU/mL based on consensus recommendations²⁷⁰. At centre 2, 4%BSA was used in the place of FVIII deficient plasma in the control sample and for test dilutions, with measurement of residual FVIII:C using a chromogenic FVIII assay. The cut-off for centre 2 was ≥ 0.7 BU/mL, defined by in-house by dilution studies of a commercially available lyophilised FVIII inhibitor plasma³⁵⁰. Inhibitors at both centres were defined as being of low-titre if the NBA was ≤ 5 BU/mL and high-titre if the NBA was >5 BU/mL^{63;70}.

Factor VIII Indirect ELISA Kit

A FVIII ELISA was performed as part of routine testing of samples sent for inhibitor screening at both centres as described in Chapter 3. Samples were defined as being positive if the ELISA optical density (OD) was greater than that of the kit control (KC).

Statistical Analyses

A diagnostic accuracy evaluation was performed for the results of samples tested in parallel by the FVIII ELISA (index test) and NBA (reference test) as a post-hoc analysis on an anonymised grouped database. Sub-group analyses, were performed based on diagnostic headers provided at the time of venepuncture. A whole case analysis was performed, as it is likely that missing data was missing not at random (MNAR); for example a test may not have been performed due to high or low clinical suspicion on the basis of previous clinical/laboratory information. The performance of the index test (ELISA) was summarised using sensitivity, specificity and predictive values (negative and positive). Confidence intervals (binomial exact) were calculated from the standard error taking these values as proportions ³⁵¹. In view of multiple assays being performed in some patients, adjustments were made to these calculations to account for inter-individual clustering. This was modelled using logistic regression with sandwich estimator, logistic random-effects model (random effect across clusters) and generalised estimating equations (exchangeable correlation matrix) as described previously ³⁵².

An additional post-hoc analysis of the unadjusted data is presented in the form of likelihood ratios (negative and positive) and diagnostic odds ratios ³⁵³. Confidence intervals were calculated from the standard error (SE) for the likelihood ratio as previously described ³⁵⁴ and for the diagnostic odds ratio using the formula $\log \text{DOR} \pm 1.96\text{SE}(\log \text{DOR})$ ³⁵³. Assessments of association of the results of both assays was performed utilising correlation (Pearson) and linear regression using the log-adjusted results of the NBA in comparison to the ELISA OD. To account for inter-individual clustering of data within these analyses the first recorded positive database entry was used. Statistical analysis of continuous variables was performed using a one-way ANOVA, with post-hoc comparison of groups using the post-hoc Turkey test ³⁵⁵.

The findings of this study are presented, following the recommendations of the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) Statement ²⁷¹. The STARD initiative

reported in 2002, with an aim of improving the quality of reporting of diagnostic accuracy studies, which includes a 25 item checklist and study flow chart template. The aim of this was to increase transparency of reporting and ensure all relevant study details are present which facilitates inclusion of data within meta-analysis and interpretation of potential bias. The STARD flow chart is presented in Figure 6.1 and the checklist in Appendix 1, respectively.

6.4 Results

6.4.1 Cohort Demographics

During the observation period 569 samples (273 patients) were sent for inhibitor testing, with a mean number of 2.8 samples tested per week. Of these samples, 17 (14 patients) were excluded due to a non-haemophilia A diagnosis and 55 (45 patients) due to incomplete testing (Figure 6.1). In samples excluded due to incomplete testing, the reasons for these exclusion were the absence of NBA (n=51), lack of ELISA testing (n=2) or neither test being performed (n=2). Following exclusions, data were evaluable for 497 samples (239 patients). Of these samples, 59% (n=291, 140 patients) were from patients with severe haemophilia A, 26% (n=129, 86 patients) from patients with non-severe haemophilia A and 15% (n=77, 14 patients) from patients with acquired haemophilia A (Figure 6.1). Although, similar numbers of samples were tested at each centre; 113 (47.3%) for centre 1 and 126 (52.7%) for centre 2, there was a difference in the clinical case load. At centre 1, more samples were tested from patients with non-severe haemophilia (54 v 31 patients) or AHA (10 v 4 patients) and at centre 2 more samples were tested from patients with severe haemophilia A (91 v 49 patients). The mean number of samples tested per patient was 2.1 (range 1-20), with a single sample tested in 145 patients (60.7%) and more than one sample in 94 (39.3%). Patients with AHA, had significantly more samples (mean $5.5 \pm \text{SD } 6.0$) tested during the observation period than those with severe (mean $2.1 \pm \text{SD } 2.0$, $p < 0.005$) or non-severe HA (mean $1.5 \pm \text{SD } 1.9$, $p < 0.005$).

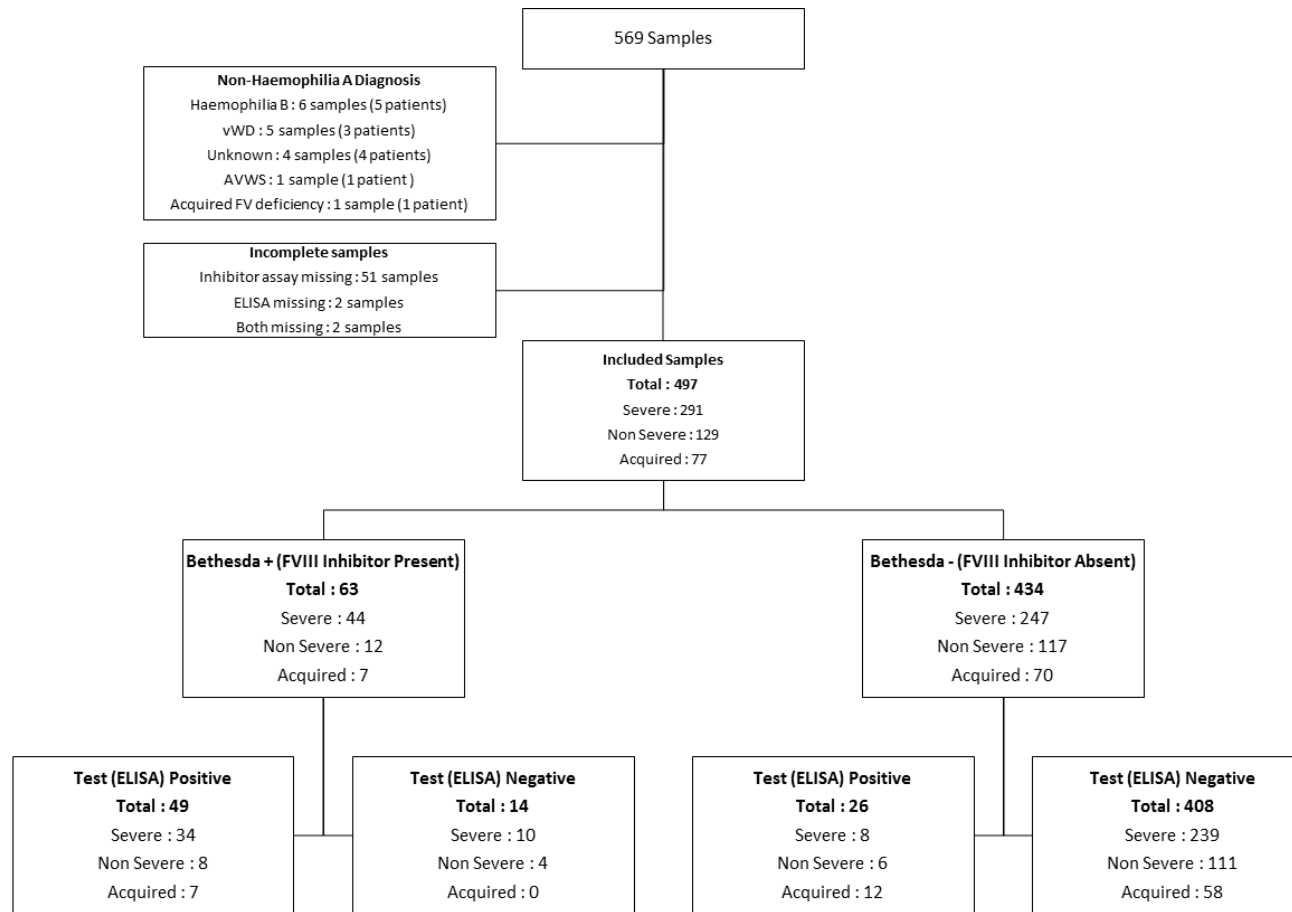


Figure 6.1: STARD diagnostic accuracy study flow chart. Flow chart shows samples included and excluded within this analysis, with classification by test result and diagnostic header.

6.4.2 Samples Positive by the Nijmegen-Bethesda Assay (Reference Test)

There were 63 samples, that tested positive by the NBA from 28 patients (severe=17, non-severe=6, acquired=5), resulting in an inhibitor sample and patient prevalence of 12.7% and 11.8%, respectively. The median inhibitor titre of NBA positive samples was 1.2BU/mL (range 0.7-978.0) with 49 being low-titre (median 1.1BU/mL, range 0.7-4.8) and 14 high-titre (median 63.7BU/mL, range 11.8-978.0). Of these 63 NBA positive samples, 49 (18 patients) were also positive by ELISA and 14 (13 patients) negative by ELISA (Figure 6.1). In samples classified as low-titre by the NBA and ELISA positive (n=35), the mean ELISA OD was 0.82 (range 0.25-1.98). The ELISA OD was ≤ 1.0 , in 29/35 (82.9%) of these samples. An ELISA OD of >1 and <2 was seen in 5/35 samples and an OD of 2.0 in a single sample. For samples classified as high-titre (n=14), the mean ELISA OD was 2.03 (range 0.59-3.36). Within this group, the majority (85.7%, 12/14) of these samples, had an OD ≥ 1.0 and 9/14 (64.3%) had an OD ≥ 2.0 . Two samples, from one patient with high-titre antibodies by the NBA, were recorded as having an OD ≤ 1.0 .

All samples that tested negative by the ELISA and positive by NBA (n=14) were of low-inhibitory titre with a mean inhibitor titre of 0.8BU/mL (range 0.7-1.0). The mean ELISA OD for these samples was 0.23 (range 0.04-0.58), with the mean ELISA KC for these samples being similar (mean=0.44, range 0.22-0.62) to the cut-off seen for all samples. All of these patients had congenital haemophilia A (severe=9, non-severe=4) and the majority (13/14) were tested in centre 2. A further review of all samples sent in this group of patients was then performed. An additional sample was tested at some point within the observation period in 84.6% (11/13). All other tests recorded were negative by both assays in 8/11 (73%) patients, with only 3/11 (27%) patients demonstrating positivity by either the ELISA and/or the NBA in an additional sample (Table 6.1; Groups X and Y).

6.4.3 Samples Negative by the Nijmegen-Bethesda Assay (Reference Test)

There were 434 samples (226 patients) that tested negative by the NBA, of which 26 (15 patients) tested positive by the ELISA and 408 (218 patients) negative by the ELISA. For samples that tested negative (n=408) by both assays the mean ELISA OD was 0.12 (range 0.03-0.59) with a mean KC OD of 0.36 (range 0.16-0.67). The diagnostic header on these samples was severe HA=130, non-severe HA=78, AHA=10.

Of the samples that tested negative by the NBA and positive by the ELISA (n=26) the mean ELISA OD was 0.63 (range 0.20-1.59) with a mean KC OD of 0.34 (range 0.16-0.67). The mean sample FVIII:C was 62.4IU/dL (range <1-362.4), with 18 samples (69%) having a FVIII:C \geq 5IU/dL. Of these 26 samples (15 patients) positive by the ELISA, but negative by the NBA, 14 (5 patients) were tested by centre 1 (non-severe HA=3, AHA=1) and 12 (10 patients) by centre 2 (severe HA=6, non-severe HA=3 and AHA=1). Additional samples were tested in 8/15 patients at another point within the observation period. A positive result was seen on another sample by the NBA and or the ELISA in 75% (6/8) of these cases (Table 6.1, Groups X and Z), in contrast to patients with samples that were NBA positive and ELISA negative (Group Y). In the remaining two patients all other samples tested were negative by both assays.

Patient	Samples	B+E-	B-E+	B+E+	B-E-	Group	
1	14	1	1	6	6	X	
2	10	1	2	1	6		
3	8	2	0	3	3	Y	
4	5	1	0	0	4		
5	4	1	0	0	3		
6	3	1	0	0	2		
7	3	1	0	0	2		
8	3	1	0	0	2		
9	2	1	0	0	1		
10	2	1	0	0	1		
11	2	1	0	0	1		
12	1	1	0	0	0		
13	1	1	0	0	0		
14	16	0	1	6	9		Z
15	12	0	1	0	11		
16	10	0	7	0	3		
17	7	0	4	1	2		
18	2	0	2	0	0		
19	2	0	1	0	1		
20	1	0	1	0	0		
21	1	0	1	0	0		
22	1	0	1	0	0		
23	1	0	1	0	0		
24	1	0	1	0	0		
25	1	0	1	0	0		
26	1	0	1	0	0		
Total	114	14	26	17	57		

Table 6.1: Assessment of sample discrepancy of the Nijmegen-Bethesda assay and FVIII ELISA at a patient level. B=Nijmegen-Bethesda assay. E=ELISA. Grouping of patients; X=Samples discrepant by both ELISA (E+B-) and Bethesda status (B+E-); Y=Samples discrepant by the Bethesda status only (B+E-); Z=Samples discrepant by the ELISA status only (E+B-).

6.4.4 Diagnostic Accuracy Assessment

An assessment of the performance of the FVIII ELISA (index test) compared to the NBA (reference test) was made for all samples. This demonstrated a specificity 94.0% (95%CI 91.3-96.0), sensitivity 77.8% (95%CI 65.5-87.3), negative predictive value (NPV) 96.7% (95%CI 94.5-98.2) and positive predictive value (PPV) 65.3% (95%CI 53.5-76.0). Further post-hoc assessment based on these primary calculations (unaffected by prevalence) demonstrated a positive likelihood ratio (LR+) 13.0 (95%CI 8.7-19.3) and negative likelihood ratio (LR-) 0.2 (95%CI 0.1-0.4). Global evaluation of the performance of the ELISA, demonstrated a diagnostic odds ratio (DOR) of 54.9 (95%CI 27.0-112.0) (Table 6.2).

	Severe HA	Non-Severe HA	Acquired HA	Total	Centre 1	Centre 2
Samples	291	129	77	497	237	260
Patients	140	85	14	239	113	126
Prevalence	15.1% (11.0-19.8)	9.3% (4.9-15.7)	9.1% (3.7-17.8)	12.7% (9.9-15.9)	7.2% (4.2-11.2%)	17.7% (13.3-22.9)
Sensitivity	77.3% (62.2-88.5)	66.7% (34.9-90.1)	100.0% (59.0-100.0)	77.8% (65.5-87.3)	94.1% (71.3-99.9)	71.7% (56.5-84.0)
Specificity	96.8% (93.7-98.6)	94.9% (89.2-98.1)	82.9% (72.0-90.8)	94.0% (91.3-96.0)	93.6% (89.6-96.5)	94.4% (90.4-97.1)
PPV	81.0% (65.9-91.4)	57.1% (28.9-82.3)	36.8% (16.3-61.6)	65.3% (53.5-76.0)	53.3% (34.3-71.7)	73.3% (58.1-89.9)
NPV	96.0% (92.7-98.1)	96.5% (91.3-99.0)	100.0% (93.8-100.0)	96.7% (94.5-98.2)	99.5% (97.3-100.0)	94.0% (89.9-96.7)
LR+	23.9 (11.8-48.1)	13.0 (5.4-31.2)	5.8 (3.5-9.8)	13.0 (8.7-19.3)	14.8 (8.8-24.9)	12.8 (7.17-22.8)
LR-	0.2 (0.1-0.4)	0.4 (0.2-0.8)	0.0*	0.2 (0.1-0.4)	0.1 (0.0-0.4)	0.3 (0.2-0.5)
Diagnostic OR	102.0 (37.9-272.0)	37.0 (9.1-152.0)	*	54.9 (27.0-112.0)	235.4 (29-#)	42.7 (18.0-101.7)

Table 6.2: Diagnostic Accuracy Evaluation of the FVIII ELISA (index test) in comparison to the Nijmegen Bethesda assay (reference test). Figures in parenthesis, represent 95% confidence intervals (95%CI). PPV=Positive predictive value, NPV=Negative predictive value, LR= Likelihood ratio. OR=Odds ratio. # >1000. *Calculation not possible due to no false negative results.

A subgroup analysis was performed based on diagnostic headers at the time of venepuncture. Similar findings were seen (Table 6.2), with high specificity, negative predictive values and positive likelihood ratios in patients with congenital (severe and non-severe) HA. In patients with AHA, a high sensitivity (100.0%, 95%CI 59.0-100.0%) was also noted. This estimate may however, be skewed by the smaller sample size (n=77, 14 patients) and no “false-negative” results seen for the ELISA in this group (Table 6.2). Of the samples from patients with AHA, 12 (56%) tested positive by ELISA, but were negative by the NBA, accounting for 46% (12/26) of the E+B- discrepant samples. This resulted in a lower PPV (36.8%, 95% CI 16.3-61.6%) than was seen for samples from patients with congenital HA.

The diagnostic accuracy assessment was then repeated to compare testing at both centres (Table 6.2). Although, similar findings were seen in both centres for specificity and NPV, a higher sensitivity was seen for centre 1 (94.1% v 71.7%) and higher PPV for centre 2 (73.3 v 53.3%). Differences in these estimates may relate to a lower number of “false negative” results seen at centre 1 and lower number of “false positive” results seen at centre 2. An increased inhibitor prevalence for samples tested at centre 2 (18.0%) compared to centre 1 (7.2%) may have skewed these primary estimates, which is supported by similar likelihood ratios (unaffected by prevalence) being seen for both centres.

6.4.5 Adjustment for Clustering within the Diagnostic Accuracy

In view of multiple samples from some patients being tested within the diagnostic accuracy assessment, the primary estimates (sensitivity, specificity, NPV and PPV) were re-calculated using modelling to account for inter-individual clustering. This was performed using three different methodologies (Table 6.3) as previously described ³⁵².

Ignoring Clustering	Sensitivity	Specificity	PPV	NPV
Binomial proportion (exact CI)	77.8% (65.5-87.3)	94.0% (91.3-96.0)	65.3% (53.5-76.0)	96.7% (94.5-98.2)
Variance Adjusted	Sensitivity	Specificity	PPV	NPV
Logistic regression with sandwich estimator	77.8% (63.0-87.8)	94.0% (88.8-96.9)	65.3% (44.6-81.5)	96.7% (94.4-98.1)
Logistic random effects model	80.5% (44.8-95.4)	100.0% (96.0-100.0)	71.3% (28.8-93.8)	97.5% (92.3-99.2)
GEE (exchangeable correlation matrix)	69.4% (52.8-82.2)	95.3% (92.0-97.3)	58.4% (41.0-74.0)	96.7% (94.4-98.0)

Table 6.3: Modelled effects of inter-individual clustering on the estimates of diagnostic accuracy. Figures in parenthesis, represent 95%CI. GEE=Generalised estimating equations, PPV=positive predictive value, NPV=negative predictive value, CI=confidence interval.

All three methodologies, demonstrated similar results to the non-adjusted diagnostic accuracy assessment for the specificity and negative predictive values (Table 6.2 and 6.3, binomial proportion). Greatest variation was however, seen for the adjusted sensitivity (69.4-80.5%) and PPV (58.4-71.3%). Taking the most conservative model (generalised estimating equations), this gave a specificity of 95.3%, sensitivity 69.4%, NPV 96.7% and a PPV 65.3% (Table 6.3). Due to the small number of patients with AHA (n=14) further sub-group analysis was not possible using these approaches.

6.4.6 Assessment of Association of ELISA Optical Density with Nijmegen-Bethesda Assay

An assessment of association was performed for all samples (63 samples, 27 patients) positive by the NBA (regardless of ELISA status), to further evaluate the observation of higher ELISA ODs being seen in patients with high-titre inhibitors. Assessment of the Q-Q plot and histograms for the results of the NBA titres (data not shown) demonstrated a non-parametric distribution with multiple outliers. The results of the NBA titre were normalised using log transformation. There was strong positive correlation ($r=0.790$, $p<0.0005$) between the results of the NBA (log-transformed) and ELISA OD.

To account for inter-individual clustering this analysis was then repeated using the first recorded database entry for each patient, resulting in 28 samples (severe=17, non-severe=6 and acquired=5). The median NBA titre was 1.1BU/mL (range 0.7-488.2) and ELISA OD 0.71 (range 0.04-2.59). There was again strong correlation ($r=0.774$, $p<0.0005$) between the log-transformed result of the NBA and the ELISA OD. The effect of variability in methodology between centres on measures of association was also assessed, repeating this analysis by centre (centre 1=7 samples, centre 2=21 samples). Comparison of correlation by centre demonstrated r values of 0.670 ($p=0.10$) and 0.894 ($p<0.0005$) for centres 1 and 2 respectively.

Finally assessment of association (linear regression) was performed to test the hypothesis that the result obtained by the ELISA OD could predict the inhibitor titre by the NBA (Figure 6.2). This was performed on the subset of samples in the correlation analysis, positive by both assays (n=18). There was a significant association ($R^2=0.475$, $p=0.002$) with data fitting to the regression equation, $\log\text{BU} = (0.9512 \times \text{OD}) - 0.4590$; where OD=ELISA OD. The inverse equation for calculating the ELISA OD is, $\text{OD} = (\log\text{BU} + 0.495)/0.9512$. Using this equation a high-titre inhibitor $\geq 5.0\text{BU/mL}$ would be expected to have an ELISA OD ≥ 1.217 .

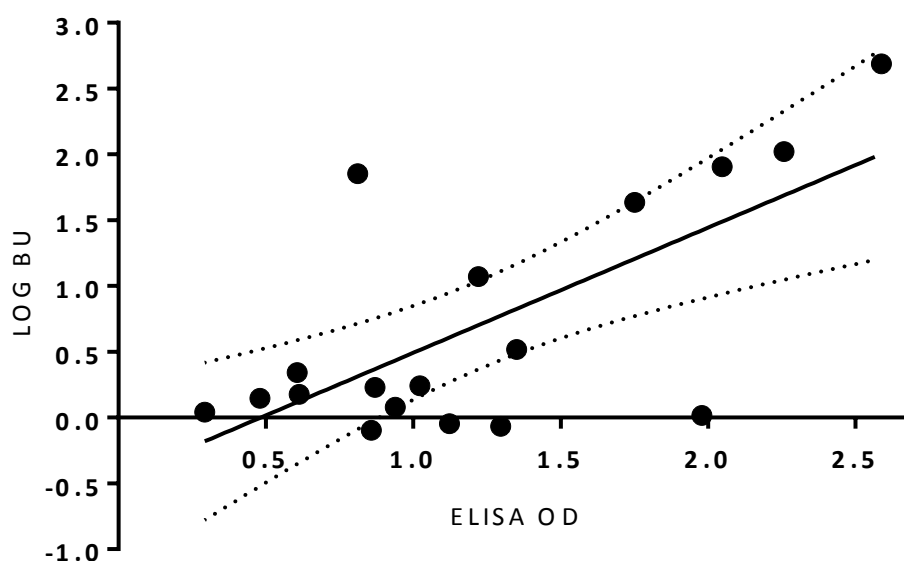


Figure 6.2: Association between the Nijmegen-Bethesda assay and ELISA OD. Plotted line indicates the linear regression line: $\log BU = (0.9512 \times OD) - 0.4590$, with dotted lines representing the 95% confidence interval.

6.5 Discussion

This is the largest retrospective diagnostic accuracy study comparing the performance of a commercially available FVIII ELISA with the NBA in routine laboratory practice in patients with congenital and acquired haemophilia A. This is also the first study reported in the haemophilia setting, following the recommendations of the STARD initiative for reporting of these findings ²⁷¹. This methodology of reporting standardises presentation and interpretation, facilitating comparison of these assay in future meta-analysis. The FVIII ELISA kit demonstrated high specificity, negative predictive value and diagnostic odds ratio in both laboratories, suggesting a role for the FVIII ELISA as a screening test for FVIII antibody detection. The ELISA OD demonstrated a semi-quantitate measure for assessment of inhibitory capacity in keeping with the findings of other studies ^{300;303}.

6.5.1 Application of ELISA in FVIII Antibody Testing

Two previously published studies ^{300;303} and six conference abstracts ³⁵⁶⁻³⁶¹ have investigated usage of this commercially available FVIII ELISA kit in comparison to a functional inhibitor assay, from which diagnostic accuracy estimates are calculated or can be derived from presented data (Table 6.4). Within these studies, estimates of sensitivity and specificity range from 77.8-100.0% and 60.0-100.0% respectively. A High NPV, as seen in this study is similar

to that seen in previous studies (90.6-100.0%)^{300;358;360;361}. Data reported in this study however, demonstrated a greater specificity (94.0% v 78.4%)³⁰⁰, albeit with a lower sensitivity (77.8% v 97.7% v 95.5%)^{300;303} for the ELISA kit. This may relate to differences in sample selection methodology in previously published studies, which used samples either known to be positive³⁰³ or “at risk of inhibitor development”³⁰⁰, rather than as a screening tool in routine laboratory practice. These differences will affect the prevalence and pre-test probability of sample positivity, which could in turn influence the primary diagnostic accuracy estimates.

Samples (Patients)		Sens	Spec	PPV	NPV	R	Cal	Reference
131 (93)	CHA AHA	97.7%	78.4%	68.9%	98.6%	0.68	Y	Martin et al. A ³⁰⁰
246 (176)	CHA AHA	95.5%	-	-	-	0.82	N	Sahud et al. A ³⁰³
497 (239)	CHA AHA	77.8%	94.0%	65.3%	96.7%	0.77	Y	Batty et al. A ³⁶²
NS (30)	CHA AHA	-	-	-	-	0.97	N	Bowyer et al. B ³⁵⁶
200 (200)	CHA	-	-	74.3%	-	-	N	Srivasta et al. B ³⁵⁷
42 (42)	CHA	100.0%	60.0%	33.3%	100.0%	-	N	Riddell et al. B ³⁵⁸
71 (43)	CHA AHA	93.0%	95.0%	-	-	-	Y	Nougier et al. B ³⁵⁹
28 (28)	CHA	88.9%	100%	100%	94.4%	0.94	Y	Needham et al. B ³⁶⁰
160* NS	CHA AHA	82.5%	93.2%	87.0%	90.6%	-	N	Riddell et al. B ³⁶¹

Table 6.4: Comparison of studies using the FVIII ELISA and NBA in congenital and acquired haemophilia A. HA=Congenital haemophilia A; AHA=Acquired haemophilia A. r=Correlation co-efficient. Cal=Diagnostic accuracy evaluation calculated by the study authors (yes/no). A=Published article, B=Conference abstract (poster/oral communication). *Comparison is between a NBA with pre-analytical heat treatment and the FVIII ELISA.

6.5.2 FVIII ELISA Specificity

Since the previous publications of the usage of the FVIII ELISA by Martin et al³⁰⁰ and Sahud et al.³⁰³ there has been a change in rFVIII plate antigen and methodology used for determining positivity for this ELISA. The data presented in this chapter, is the first study comparing this newer version of the ELISA kit with the NBA. In earlier studies, the plate antigen used was Recombinate[®] (Baxter Healthcare Corp, Deerfield, IL, USA), an H2 haplotype rFL-FVIII^{179;307}, with positivity defined by an absorbance value (OD), twice the mean of the negative control^{300;303}. In this newer version of the ELISA, the plate antigen used is Kogenate[®], an H1 haplotype rFL-FVIII, with positivity defined by an absorbance value (OD) greater than the kit control (human serum containing FVIII antibodies in bovine albumin and 0.1% NaN₃)¹⁷⁹. The difference in primary sequence between H1 and H2 haplotype FVIII, is a single nucleotide polymorphism within the B domain, encoding either aspartic acid or glutamic acid at position 1241, respectively¹⁷⁹. There will also be differences in post-translation modifications, between these two recombinant products, with Recombinate[®], being produced from a baby hamster kidney cell line and Kogenate[®] from a Chinese hamster ovary cell line. Whether this change in plate antigen may affect the results obtained is not known, although differences in the reactivity of inhibitors to different FVIII concentrates has been previously reported (discussed below). Good correlation has however been demonstrated between the results of the two FVIII ELISAs in an evaluation of the effect of antiphospholipid antibodies³⁰⁷.

6.5.3 Discrepant Samples and Residual FVIII:C using the FVIII ELISA

The presence of FVIII:C in samples, when using a functional inhibitor assay such as NBA, may impair detection of potentially clinically relevant FVIII antibodies²⁹¹, which is discussed in more detail in the following chapter. Given the difficulties in the diagnosis and accurate quantification of FVIII inhibitors in patients with type II antibodies (complex kinetics), who often have residual FVIII:C, the ELISA technique offers an attractive platform for inhibitor surveillance. Within this analysis, discrepancy between the results of the two assays was seen in 40 samples. Due to the methodology of this analysis no further evaluation of clinical parameters in these samples, or reproducibility of these results, was possible. The relevance of antibodies with discrepancy between assays requires further study. In congenital haemophilia, non-neutralising antibodies, may constitute one of these discrepant groups, which have been reported in approximately 18% of patients with congenital haemophilia²⁶¹.

The clinical significance of this group of non-neutralising antibodies remains unclear, with conflicting reports in the published literature²⁶². Further study of non-neutralising antibodies using parallel assays to evaluate binding affinity (e.g. surface plasmon resonance, biolayer interferometry or competition ELISA), epitope profiles (discussed in Chapter 8) or global haemostatic effects (e.g. thromboelastography or thrombin generation assays) may provide further insight into this class of antibodies.

In the sub-group analysis of samples from patients with AHA, a substantial proportion (n=12, 15.6%, 3 patients) were positive by the ELISA (mean OD 0.58, range 0.25-1.59) but negative by the NBA. In these the degree of positivity was significant, to a level of ≥ 2 fold the KC in 58.3% (7/12) samples. All of these discrepant samples, had substantial FVIII:C measurable (mean 122.8IU/dL, range 40.8-362.4) prior to testing. Despite anecdotal reports of the significance of FVIII antibody detected using immunological methodology, with a negative functional inhibitor, the relevance of these findings are not clear. The first of these reports, compared the FVIII ELISA, with the Bethesda assay demonstrating prolonged ELISA positivity in 2 patients, with a negative Bethesda assay, although there was insufficient details provided to calculate assay performance, from the data presented within the abstract³⁶³. In a recently published study, evaluating FVIII antibody subclasses and binding affinity, longitudinal follow-up of a patient with AHA (days 10, 31 and 57) demonstrated, persistent IgG1 and IgG4 antibodies throughout the observation period. At day 31, the inhibitor assay became negative and the patient was weaned off immunosuppression by day 42. He then relapsed at day 57 (2.8BU/mL)²⁶⁹. Further prospective study, is required to evaluate if this may represent a biomarker for prediction of response to treatment or relapse in AHA.

6.5.4 Role of New Assays in FVIII Antibody Testing

There are a number of difficulties, in assessing new diagnostic tests for detection of FVIII antibodies. Firstly, there is a lack of consensus, as to what represents an important positive result for clinical practice. Although data supports the clinical relevance of “high-titre” FVIII antibodies, this is less clear for antibodies with weak or no inhibitory capacity. The next problem, relates to the intended role that a new test would play in laboratory practice, which could either be for screening, replacement or as an add-on test³⁶⁴. The ideal test from an immunological perspective would be an assay allowing detection of both inhibitory and non-neutralising antibodies. Clinically however, it would be desirable that this test gave a reproducible quantitative result correlating with inhibitory capacity using simple

methodology and is insensitive to the pre-analytical variables affecting current laboratory methods. It is unclear however, whether there is a clinical desire for a new test to act as a triage/screening test, replace or as an add-on test to the NBA. Each approach would subsequently require different methodology to validate such an assay. The second limitation is where there is an imperfect reference standard or where the true relevance of the spectrum of pathological findings is unknown, which is the current situation in testing for FVIII antibodies. In the evaluation of new tests, given the limitations of the NBA, this assay forms an imperfect reference standard. Methodology for assessment of diagnostic tests is limited in the situation where a new assay may detect potentially relevant findings that are not detected on current assays, as may be the case for apparent “non-neutralising” antibodies. Using the NBA as a reference test in statistical comparison of a new assay with high sensitivity for the detection of non-neutralising antibodies, will result in a low PPV, which is seen in this and other studies (Table 6.4) using immunological assays. Despite recent advances in the understanding of non-neutralising antibodies, the long-term relevance of these remains uncertain and it is not known whether these have prognostic significance in predicting inhibitor formation (epitope spreading) or relapse from immunosuppression or ITI.

6.5.5 Association between NBA Titre and ELISA OD

Despite the limitations of the NBA in the detection of FVIII antibodies, one of the great strengths of this assay is that the quantitative results has been used to guide management of patients with bleeding episodes. For an alternative assay, it would be desirable to give a qualitative read-out that may differentiate, high and low-titre inhibitors. Within this study strong correlation was seen between the results of the ELISA OD when compared to an adjusted (log) Bethesda value, which are similar to the findings of other investigators (Table 6.4).

6.6 Limitations

One of the major limitations of this study relates to the retrospective design, evaluating the performance of the FVIII ELISA with reliance on the diagnosis assigned when samples were collected. Using anonymised samples, could have resulted in patients being mis-categorised and does not take into account other important clinical variables present at the time of testing. To minimise mis-categorisation, data from patients with mild and moderate haemophilia A were grouped into a non-severe haemophilia A category. Retrospective analysis of these data does also not allow assessment of assay reproducibility or to further

investigate discrepancies seen between the results of the two assay. This study similar to other diagnostic accuracy studies contains multiple sample results from the same patient, which results in the formation of clustered data³⁶⁵. Without adjustment, due to lack of statistical independence in these clusters, this could skew the size of one of the diagnostic accuracy estimates or result in inappropriately narrow confidence intervals. For example, a patient may always test positive by one assay and negative by the other assay, which will result in false elevation of the rate of false positives or negatives, directly affecting subsequent calculations. Within my analysis, adjustment for this is presented both for diagnostic accuracy and correlation, although even using the most conservative model (GEE) to adjusting for clustering this clustering only had a minor effect on the estimates of diagnostic accuracy. Although significant correlation was seen between the results of the ELISA OD, this is limited by the number of samples included within this analysis and further study of this is required within a larger cohort.

A further limitation to these data is that there were differences in the NBA testing methodology used at the two centres, although both approaches have been well described within the literature^{279;280;366}. Usage of 4%BSA in place of FVIII deficient plasma has been described in two studies, which has been discussed previously (Chapter 1)^{279;280}, with this modification being recently validated and demonstrating good agreement between the results of the two assays, especially samples with low-inhibitory capacity (<2BU/mL)²⁸⁰. A comparison between the one stage and chromogenic FVIII assay as part of the NBA has also been recently presented in 1005 samples (702 patients)³⁶⁶. In this study, 880/883 samples negative by the NBA, were also negative by Chromogenic Bethesda assay (ChBA). For inhibitor titres <2BU/mL, 43/80 specimens (53.8%) positive by the NBA were also positive by the ChBA. In samples positive by the NBA, with an inhibitor titre ≥ 2.0 BU/mL, 42/42 were positive by ChBA with excellent correlation ($r=0.98$, $p<0.0001$) between the two assay results. Variation in inhibitor testing methodology, is a common limitation in any study using local inhibitor testing to define positivity rather than centralised testing. To assess the impact of these differences, both the diagnostic accuracy analysis and correlation assessment were repeated to compare the results obtained in both centres. A differences in the correlation was seen, which most likely relates to the smaller number of NBA positive samples included in the analysis from centre 1 ($n=7$). An alternative explanation may relate to a higher proportion of samples from centre 1, being from patients with acquired or non-severe

haemophilia (centre 1: 4/7, 57.1%; centre 2: 7/21, 33.3%) which could display more complex inactivation kinetics ²⁵⁰.

Finally, a potential limitation to this commercially available ELISA is the use of a single plate antigen (Kogenate®). Differences in specificity to different recombinant antigens have been described for both functional inhibitor assays ²⁸⁶ and in validated research-laboratory ELISAs ^{258;367;368}. These findings are of interest clinically given such small differences in primary sequence between different rFL-FVIII concentrates. Within samples tested by the ELISA, it is possible that some samples may have tested negative, due to the presence of antibody specificity to a different FVIII antigen or presence of non-IgG FVIII antibodies, although further evaluation of these samples was not possible within this work aimed at assessing the role for this ELISA in routine laboratory practice.

6.7 Conclusions

In summary, a commercially available FVIII ELISA kit in comparison to the NBA demonstrated good specificity and negative predictive value for FVIII antibody detection in samples from patients with congenital and acquired haemophilia A. The ELISA displays a semi-quantitative result which provides good correlation between the log-adjusted inhibitor titre and ELISA OD. With ELISA being a routinely available assay platform in most hospital laboratories, this offers the potential for efficient batching of FVIII antibody surveillance for non-urgent routine clinical samples.

Chapter 7: Pre-Analytical Heat Treatment Improves FVIII Antibody Detection by the Nijmegen-Bethesda Assay and Factor VIII ELISA

7.1 Introduction

The presence of residual FVIII:C in samples limits detection of inhibitors when using a functional inhibitor assay such as the NBA. There are however, multiple situations where inhibitor testing is required despite presence of residual FVIII:C, where it is not practical (or possible) to await a sample where FVIII:C is absent. One approach taken previously by some laboratories was to not test samples where there was substantial residual FVIII:C ²⁹⁰. In the assessment of patients with non-severe haemophilia A treated within London, presented in Chapter 4, 22.5% (85/377) patients had a FVIII:C ≥ 20 IU/dL and in the previous chapter evaluating the application of a FVIII ELISA a FVIII:C ≥ 20 IU/dL was seen in 25% (122/497) of samples tested. In these circumstances it would be desirable to have an assay insensitive to residual FVIII:C (e.g. FVIII ELISA) or to use a standardised modification to adjust for this. Although usage of a FVIII ELISA as a screening test forms one option this does not form part of current routine testing practice in the UK, at least in the setting of AHA as seen in Chapter 5. Given current reliance on functional inhibitor assays, a more feasible option would be to use a modification (*in-vitro*) or correction (mathematical) to these assays to account for pre-analytical FVIII:C. Perhaps, the simplest of these modification for the busy CCC laboratory is to use pre-analytical heat treatment (PHT) to denature FVIII:C, allowing standardisation of sample preparation prior to inhibitor testing ²⁹¹. Although PHT has received a degree of interest within the recent literature ^{283;291;294;297;369}, there are a number of questions surrounding usage of this modification that should be addressed prior to use in routine clinical practice. This modification is based on observations of differences in thermostability between FVIII:C and FVIII inhibitors from small studies performed in the 1960s ³⁷⁰ and 1970s ^{292;371}. In the study that is likely to have resulted in the recent use of this modification, sample incubation at 56°C for 30 minutes resulted in 95±3% of FVIII:C being inactivated with no effect on FVIII inhibitor neutralising activity ²⁹². PHT resulted also in loss of reactivity of denatured FVIII in a neutralisation assay, suggested structural changes disrupting the B-cell epitopes on the FVIII surface, a finding which been confirmed in more recent experiments ¹⁴⁷. These earlier experiments used a functional inhibitor assay similar to, although not the same as the CBA ²⁹², with a limited range of incubation conditions. There is a lack of controlled data to supporting optimal PHT incubation conditions (temperature and time) for use with current inhibitor assays (NBA and FVIII ELISA). Although the most commonly used incubation

conditions described in clinical studies range from 56°C to 58°C^{283;294;296;297}, transition in FVIII:C stability has been seen between 46 and 66°C¹⁴⁷. The effect of using either higher or lower PHT temperatures or incubation times is not known. Another consideration for using this modification is that different sources of FVIII:C found in samples (exogenous or endogenous) could exhibit differences in thermostability. Finally, all studies using this modification have investigated usage as part of testing with a functional inhibitor assay and the effects of PHT on FVIII antibody detection using a FVIII ELISA is not known.

7.2 Hypothesis and Aims

In the first part of this chapter, the effect of the introduction of PHT in patients with AHA as part of routine clinical laboratory practice will be evaluated and discussed. Within the haemostasis laboratory of our CCC (The Royal London Hospital), a switch was made to using PHT for all samples requiring inhibitor testing from 2012 onwards. Following this change in practice, I evaluated the effect of using PHT during routine practice for samples from patient with AHA, where a high residual FVIII:C would be expected either at diagnosis or following response to treatment. The hypothesis for this first part, is that PHT improves the detection of FVIII antibodies through denaturation of FVIII:C in samples.

In the second part of this chapter the effect of different PHT incubation conditions (time/temperature) will be systematically evaluated to assess the effect on residual FVIII:C and inhibitory activity seen in samples. PHT has not been evaluated using current laboratory methodologies, to assess FVIII:C and inhibitor presence using a functional (NBA) and an immunological (FVIII ELISA) assay. The hypothesis for this second part, is that different PHT incubation conditions will have different effects on the proportion of FVIII and inhibitor denatured. The specific aims of this chapter are as follows:

- 1) To describe the effect of PHT in a pilot study at the introduction of this methodology as part of routine laboratory practice in patients with acquired haemophilia A.
- 2) To assess the effect of different PHT incubation conditions on FVIII:C from endogenous and exogenous sources.
- 3) To assess the effect of different PHT incubation conditions on FVIII inhibitor detection using the NBA and a FVIII ELISA.
- 4) To provide guidance of the optimal conditions for the usage of PHT for inhibitor testing within the laboratory of The Royal London Hospital Haemophilia Centre.

7.3 Materials and Methods

Study Samples and Approvals

An assessment of the effect of PHT on inhibitor detection was made in routine inhibitor samples tested from patients with AHA (8/11-2/12). De-identified samples were tested in parallel using both the NBA and FVIII ELISA assays, before and after heat treatment at 58°C for 90 minutes²⁹¹. Study approval was granted by the Clinical Effectiveness Unit (CEU) Barts Health, as part of assay development and service evaluation at The Royal London Hospital (part 1 and 2). The assessment and optimisation of the PHT conditions, presented in part 2 were discussed with the research approvals department at Queen Mary University London (QMUL) and these experiments did not represent material ethics.

One Stage FVIII:C and Nijmegen Bethesda Assay

These assays were performed as described previously in the materials and methods section (Chapter 3). Samples within part 1, were tested as part of routine care within the central laboratory of The Royal London Hospital. In the second part evaluating optimal PHT incubation conditions I performed all assays although due to limitations in available sample volumes and inhibitor titres the FVIII:C and NBA assays were performed undiluted.

Factor VIII ELISA

A commercially available, solid phase indirect FVIII ELISA (Lifecodes/Immucor, Norcross, USA) was performed as described within Chapter 3.

Part 1: Pre-Analytical Heat Treatment: Evaluation of the Effects of Switching to Usage of PHT in Testing for FVIII antibodies in Acquired Haemophilia A

Samples were tested in the presence or absence of heat treatment (58°C for 90 minutes), with samples left to cool to room temperature (RT) for 20 minutes following PHT. No precipitant was seen following heat treatment. FVIII:C was measured prior to and following heat treatment.

Part 2: Effects of Pre-Analytical Heat Treatment on FVIII:C and FVIII Inhibitor Detection

Preparation of Pooled Platelet Poor Plasma (Endogenous FVIII Source)

Discarded platelet-poor plasma, collected in 3.2% sodium citrate from 43 anonymous consecutive, routinely tested, non-haemophilia samples was collected and pooled. Following pool preparation, 200µL samples were aliquoted into cryovials and frozen at -80°C prior to testing.

Preparation of FVIII Concentrates (Exogenous FVIII Source)

The effect of pre-analytical heat treatment on FVIII from an exogenous source was evaluated using a rFVIII (Advate®, Baxter, Deerfield, USA) and a pdFVIII concentrate (Wilate®, Octapharma AG, Lachen, Switzerland). This was evaluated for the most commonly described temperatures used in previous studies (56 and 58°C). Pools were prepared for each concentrate in Factor VIII deficient plasma (F8DP). Lyophilised Advate® (500 IU) and Wilate® (500 IU) were reconstituted as per the manufacturers' recommendations using sterile water (2mL) or sterile water/0.1% polysorbate-80 (5mL) respectively. These were then both diluted separately in 250mL of sterile water aiming to give a similar FVIII:C as seen for the pooled FVIII plasma (c. 200IU/dL). The diluted FVIII concentrates were allowed to sit on the bench for 10-15 minutes and mixed gently. Vials of lyophilised FVIII deficient plasma were then reconstituted using 1mL of the FVIII concentrate in sterile saline. These were allowed to stand on the laboratory bench for 15 minutes, prior to gentle mixing as per the manufacturer's recommendations. The vials of FVIII concentrate diluted in FVIII deficient plasma were then combined to give two separate pools and mixed by gentle inversion. These were then aliquoted into 200µL samples and directly frozen at -80°C prior to testing.

Evaluation of the Effect of Pre-Analytical Heat Treatment on FVIII:C

A sequential evaluation was performed of the effect of PHT on endogenous and exogenous FVIII:C. Assessment of the effect of PHT on FVIII:C activity was made across six temperature points (37, 47, 52, 56, 58 and 64°C), until FVIII:C was completely denatured within five minutes of PHT. The effect of PHT was evaluated over seven incubation times (control, 5, 15, 30, 60, 90 and 120 minutes). All incubation temperatures and times were assessed in triplicate. This evaluation of PHT was made using methodology as available in a routine haemostasis laboratory using a water-bath for sample incubation. Prior to assessment, the water-bath being used for PHT was heated to the selected temperature for 30-60 minutes. The water-bath temperature was then monitored prior to commencement of sample incubation to ensure that the desired temperature had been obtained and this was not fluctuant for 10-15 minutes. Throughout each experiment, the water-bath temperature was

monitored, using a traceable digital thermometer (accuracy $\pm 0.5^{\circ}\text{C}$) with temperature recordings performed every second, with a record of minimum and maximum temperatures. Frozen samples were rapidly thawed in a water-bath at 37°C for 5 minutes and all samples (except the control samples) were then immediately transferred for pre-analytical heat treatment into the second water-bath. Following PHT, samples were removed from the water-bath, the external surface was dried and allowed to cool at RT for 20 minutes and then directly tested using a one stage FVIII:C assay.

Evaluation of the Effect of Pre-Analytical Heat Treatment on Inhibitor Plasma

An assessment of the effect of PHT on inhibitor activity and detection was performed using a commercially available FVIII inhibitor plasma (George King Bio-Medical Inc., Kansas, USA). This inhibitor plasma was a commercially available FVIII inhibitor (48 BU/mL) sample obtained from a single donor by apheresis frozen directly at -80°C without undergoing lyophilisation. This was diluted 1 in 21 in FVIII deficient plasma, then directly aliquoted into 250 μL samples and frozen at -80°C prior to testing. Due to small sample volumes being used two test sample were thawed and evaluated using the Nijmegen-Bethesda assay to assess the dilutions required within the Nijmegen-Bethesda assay and to compare the predicted to actual inhibitor titre. On testing these thawed sample gave an inhibitor titre of 1.1 BU/mL which was only just detectable on the 1 in 2 dilution (67% residual FVIII:C), as such all subsequent sample were tested undiluted. Inhibitor samples were incubated using PHT in a similar manner to the previous experiments over four temperature points (56°C , 58°C , 64°C and 72°C), with all samples being tested in duplicate. This temperature range was chosen based on temperatures at which Ig disruption would be expected to occur. Following sample incubation, the external surface was dried and samples were allowed to cool for 20 minutes at RT. Each sample was divided into a sample for use in the Nijmegen-Bethesda assay (160 μL) and the remainder for the FVIII ELISA. These were then refrozen at -80°C prior to batch testing by the NBA and ELISA.

Data Interpretation and Statistical Analyses

Descriptive data, including raw data, mean, SD and co-efficient of variation (CV) of these experiments are presented. Co-efficient of variation, was calculated by dividing the standard deviation by the mean. Given lack of independence, between the datasets no further comparative statistics were performed. For the experiments looking at FVIII:C, these data were reported as mean raw FVIII:C and a normalised mean percentage FVIII:C. Normalisation

was performed relative to the mean FVIII:C of control samples to allow inter-experimental comparison of residual FVIII:C following PHT.

7.4 Results

7.4.1 Pre-Analytical Heat Treatment Improves Detection of FVIII Antibody Formation in Acquired Haemophilia A

Thirty nine samples (8 patients) were tested using the FVIII ELISA and NBA before and after heat treatment. Prior to PHT all samples had detectable FVIII:C (median 75IU/dL, range 24.1-362.4) which became undetectable in all samples following PHT.

7.4.1.1 Nijmegen-Bethesda Assay

Prior to PHT, only 2/39 samples (1 patient) were positive by NBA which increased to 20 samples (6 patients) positive by the NBA following PHT (Table 7.1). All inhibitors, detected by NBA following heat treatment were “low-titre” (median 0.6BU/mL, range 0.6-2.0). Both samples that were positive by the NBA prior to PHT remained positive following PHT.

	NBA	ELISA	Parallel Testing
Untreated	2 (5%)	11 (28%)	12 (31%)
Pre-Analytical Heat Treatment	20 (51%)	16 (41%)	24 (62%)

Table 7.1: Factor VIII antibody detection by NBA and FVIII ELISA pre and post heat treatment. Figures shown represent the number of positive samples (%). Parallel testing, corresponds to positivity by one or more assay. Table reproduced from Batty et al. ²⁹⁶, with the permission of the publisher.

7.4.1.2 FVIII ELISA

Using the FVIII ELISA, 11/39 samples (3 patients) were antibody positive prior to PHT, which increased to 16/39 positive samples (5 patients) following PHT (Table 7.1). In samples positive by ELISA prior to PHT, parallel PHT NBA assays were positive in 7, equivocally positive (0.5 BU/mL) in 2 and negative in 2. Interestingly, 5 samples (2 patients) positive by the ELISA prior

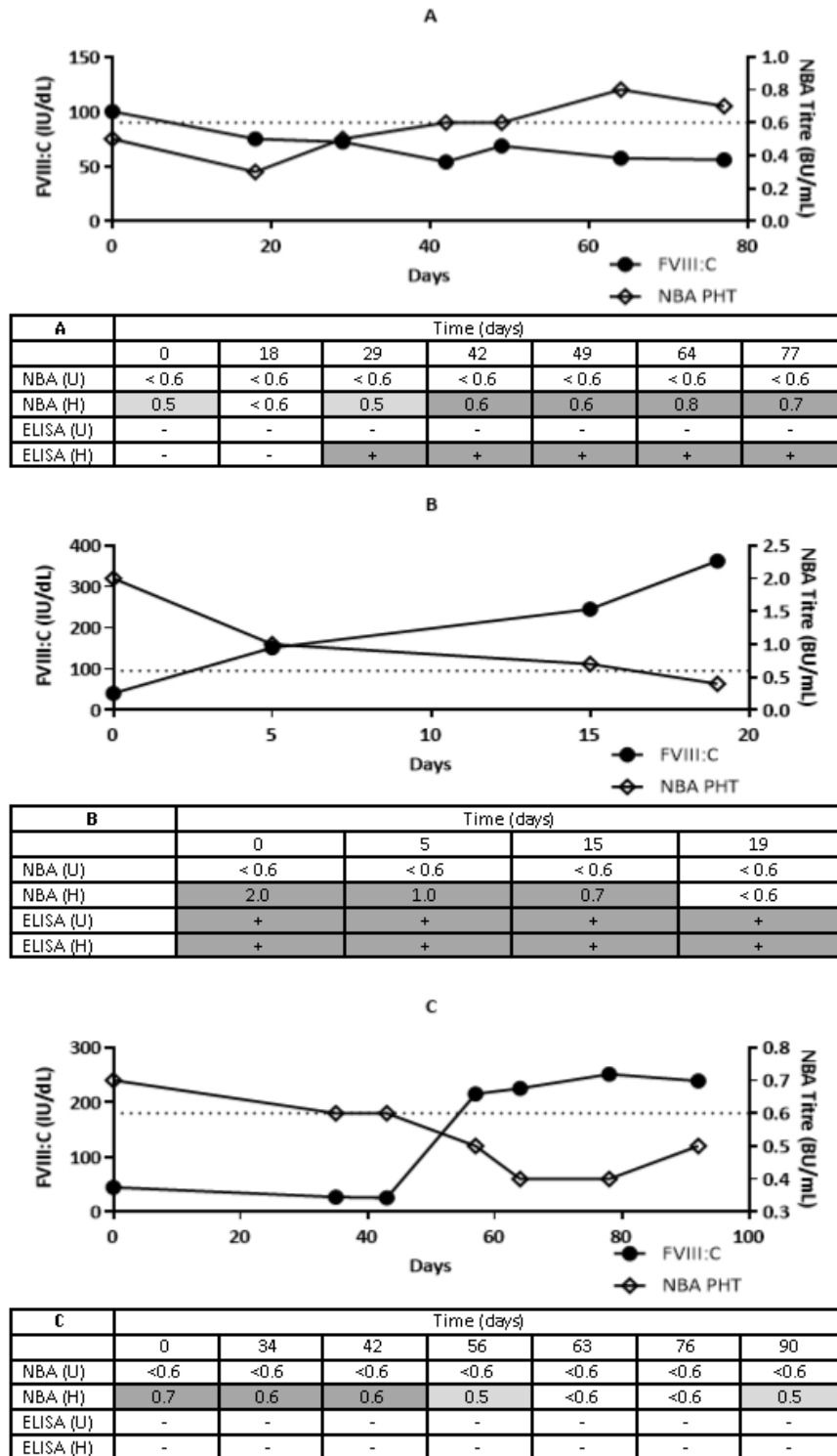


Figure 7.1: Effect of PHT and ELISA on FVIII antibody detection in acquired haemophilia A. Plotted curves show FVIII:C and PHT NBA titre. U=Untreated; H=Pre-analytical heat treatment. A: Undetectable FVIII antibody with NBA(U), transition to positive by ELISA (H) and NBA (H), with fall in FVIII:C. B: Undetectable FVIII antibody by the NBA (U), detectable by ELISA (U & H) and NBA (H); transition to negative on NBA (H) with rising FVIII:C. C: Undetectable FVIII antibody by NBA (U) and ELISA (U & H); Transition from low-titre positive by NBA (H) to negative with rise in FVIII:C. Based on a Figure in Batty et al ²⁹⁶, reproduced with the permission of the publisher.

to PHT, became negative following heat treatment. Of these samples 2 were positive, 2 equivocally positive and 1 negative by the NBA following PHT.

7.4.1.3 Parallel testing (NBA and FVIII ELISA)

The number of samples with detectable antibody increased from 5% (2/39) identified using a conventional NBA without heat treatment to 62% (24/39) identified using parallel testing by heat-treated NBA *and* ELISA. Six patients (75%) had ≥ 1 sample negative using a conventional NBA without heat-treatment that became positive following PHT by the NBA and/or ELISA. In the remaining 2 patients, all 5 samples tested were negative by both assays, before and after PHT. In a longitudinal assessment of samples tested, PHT appears to improve detection of low-titre FVIII antibodies by NBA. The results for samples tested in three patients, are shown in Figures 7.1A-C.

7.4.2 Optimisation of Pre-Analytical Heat Treatment Conditions for the Detection of FVIII Antibodies

Within the clinical data, a tenfold change from negative to positive was seen comparing the results of samples tested by the NBA prior to and following PHT. Within these observations an unexpected finding was that 5 samples tested by the ELISA became negative following PHT. This raises the possibility that the incubation using PHT may have a detrimental effect on antibody activity. To investigate the optimal conditions for routine laboratory usage the effect of varying PHT incubation conditions were tested to assess effects on FVIII:C and inhibitor detection (NBA and ELISA).

7.4.2.1 Assessment of the Effect of Pre-Analytical Heat Treatment on Endogenous FVIII:C

Pooled, non-haemophilia plasma samples were sequentially incubated in triplicate over a range of six temperatures for up to 120 minutes. Within all experiments some denaturation of endogenous FVIII:C was seen following PHT for 120 minutes. PHT of endogenous FVIII at 36, 47, and 52°, resulted in an incremental decline in FVIII:C up to 120 minutes, with adjusted residual FVIII percentages of 68.9%, 57.4% and 13.5% respectively. Of the commonly used temperatures for PHT, differences were seen in the time taken to reach FVIII:C ≤ 1 IU/dL. PHT at 56, 58 and 64°C resulted in a fall in mean FVIII:C to ≤ 1 IU/dL (>99% reduction in adjusted residual FVIII%) after incubation for 60, 30 and 5 minutes respectively (Figure 7.2 and Table

7.2). Finally the experiments using commonly cited (56°C and 58°C) temperatures for PHT were repeated, the results of which are plotted as a control in the following set of experiments (Table 7.2 and Figure 7.3).

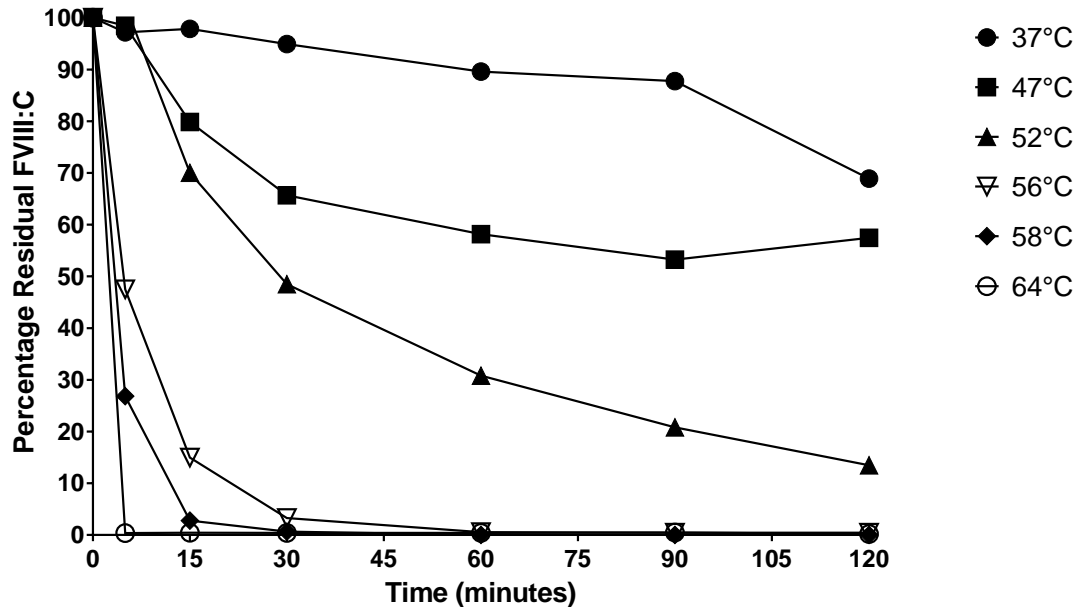


Figure 7.2: Effect of Pre-Analytical Heat Treatment on Endogenous (pooled plasma) FVIII:C. Data-points represent the mean inter-experiment normalised values (%), with samples tested in triplicate. Measures of error within these samples are shown for raw values within Table 7.2

7.4.2.2 Assessment of the Effect of Pre-Analytical Heat Treatment on Exogenous FVIII:C

The effect of PHT was compared between FVIII of endogenous (pooled, non-haemophilia plasma) and exogenous sources (rFVIII and pdFVIII). The effect of PHT using commonly cited incubation conditions (56°C and 58°C) was evaluated with samples incubated for up to two hours, in triplicate as per the previous experiments. Within these experiments a further set of endogenous FVIII:C samples were incubated to act as a control and to confirm the findings within the first dataset. With these experiments differences were seen both in the rate of decline of FVIII:C and time to reach a normalised residual FVIII:C $\leq 1\%$, comparing FVIII from endogenous and exogenous sources (Figure 7.3). With incubation at 56°C, FVIII:C became undetectable (FVIII:C $\leq 1\text{IU/dL}$) after 60 minutes for endogenous FVIII and after 120 minutes for rFVIII. Even after PHT at 56°C for 120 minutes, FVIII:C remained detectable (3.1IU/dL, normalised residual FVIII 2.6%) for the pdFVIII concentrate. For incubation at 58°C, a reduction in FVIII:C to $\leq 1\text{IU/dL}$ was seen at between 15-30 minutes PHT for endogenous FVIII and following 60 minutes of PHT for both the rFVIII and pdFVIII concentrates.

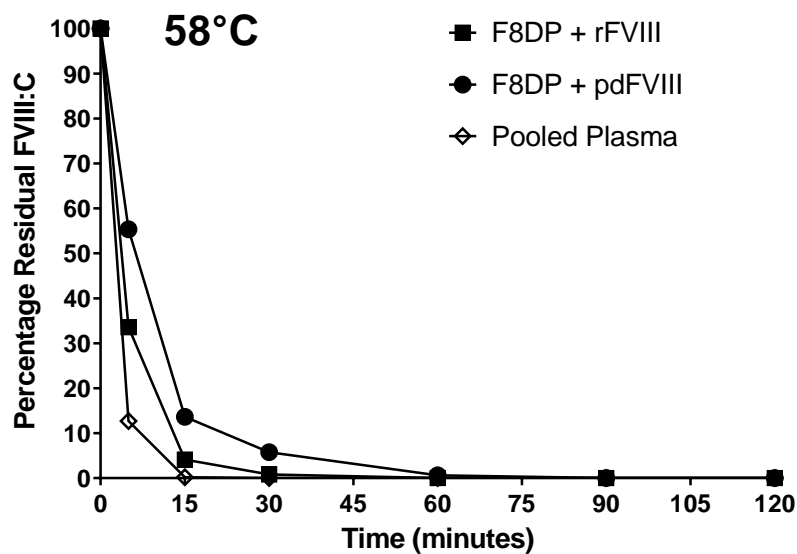
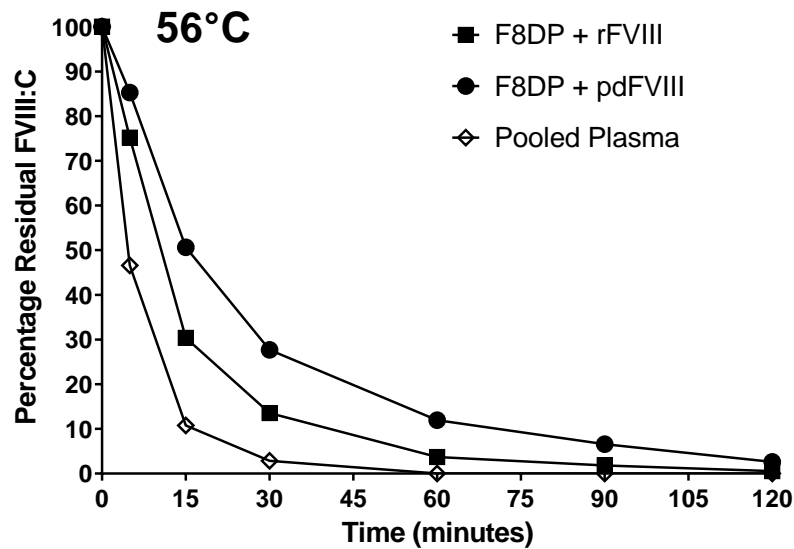


Figure 7.3: Comparison of the effect of Pre-Analytical Heat Treatment on Endogenous (pooled plasma) and Exogenous (FVIII concentrates) FVIII:C. PHT was performed at 56 and 58°C. Data-points represent the mean inter-experiment normalised values (%), with samples tested in triplicate. Measures of error within these samples are shown for raw data within Table 7.2. F8DP=Factor VIII deficient plasma. rFVIII=recombinant FVIII concentrate (Advate®). pdFVIII=plasma derived FVIII concentrate (Wilate®).

Temperature	Range	Control	5 min	15 min	30 min	60 min	90 min	120 min
37°C	37.5-37.7	233±11 (100)	226±6 (97)	228±20 (98)	221±4 (95)	209±12 (90)	204±5 (88)	160±20 (69)
47°C	46.6-47.2	261±2 (100)	257±6 (98)	208±11 (80)	172±12 (66)	152±4 (58)	139±3 (53)	150±12 (57)
52°C	51.9-52.5	197±24 (100)	202±4 (103)	138±22 (70)	95±8 (48)	61±6 (31)	41±6 (21)	27±3 (13)
56°C A	55.9-56.7	232±24 (100)	110±14 (48)	35±1 (15)	8±2 (3)	1±0 (1)	1±0 (0)	1±0 (0)
56°C B	55.9-56.8	188±13 (100)	88±4 (47)	20±3 (11)	5±1 (3)	<1±0 (0)	<1±0 (0)	<1±0 (0)
56°C rFVIII	55.9-56.8	173±3 (100)	130±4 (75)	52±5 (30)	23±2 (14)	6±0 (4)	3±0 (2)	<1±1 (1)
56°C pdFVIII	55.9-56.5	118±1 (100)	100±4 (85)	60±1 (51)	33±3 (28)	14±1 (12)	8±1 (7)	3±0 (3)
58°C A	57.4-58.2	196±6 (100)	53±3 (27)	6±2 (3)	1±0 (1)	<1±0 (0)	<1±0 (0)	<1±0 (0)
58°C B	57.8-58.7	192±7 (100)	24±4 (13)	<1±1 (0)	<1±0 (0)	<1±0 (0)	<1±0 (0)	<1±0 (0)
58°C rFVIII	57.8-58.7	170±8 (100)	57±3 (34)	7±0 (4)	1±0 (1)	<1±0 (0)	<1±0 (0)	<1±0 (0)
58°C pdFVIII	57.7-58.3	123±5 (100)	68±2 (55)	17±5 (14)	7±0 (6)	<1±1 (1)	<1±0 (0)	<1±0 (0)
64°C	63.7-64.6	207±9 (100)	<1±0 (0)	<1±0 (0)	<1±0 (0)	<1±0 (0)	<1±0 (0)	<1±1 (0)

Table 7.2: Effect of Pre-Analytical Heat Treatment of Endogenous and Exogenous FVIII:C. Figures represent mean raw FVIII:C±SD (IU/dL). Figures in parenthesis represent the normalised FVIII percentage. A=1st experiment; B=2nd experiment. rFVIII=recombinant FVIII concentrate. pdFVIII=plasma derived FVIII concentrate.

Temperature	Cut-Off	Control	5 min	15 min	30 min	60 min	90 min	120 min
56°C (56.0-56.6) NBA (BU/mL)	≥0.6	1.4±0.0	1.3±0.0	1.5±0.1	1.5±0.0	1.5±0.0	1.5±0.0	1.5±0.0
ELISA OD (1)	≥0.217	0.44±0.14*	0.60±0.18*	0.67±0.06	0.46±0.20*	0.62±0.05	0.68±0.33*	0.67±0.33*
ELISA OD (2)	≥0.414	0.54±0.25*	0.66±0.00	0.66±0.06	0.63±0.01	0.50±0.08	0.54±0.04	0.61±0.01
58°C (57.8-59.1) NBA	≥0.6	1.3±0.0	1.4±0.0	1.5±0.1	1.4±0.1	1.4±0.0	1.7±0.2	1.6±0.0
ELISA OD (1)	≥0.22	0.50±0.26*	0.70±0.26*	0.70±0.28*	0.79±0.16*	0.67±0.44*	0.67±0.25*	0.63±0.16*
ELISA OD (2)	≥0.41	0.44±0.04	0.63±0.03	0.42±0.15*	0.81±0.37*	0.52±0.01	0.57±0.00	0.53±0.04
64°C (63.8-65.0) NBA	≥0.6	1.4±0.1	1.5±0.1	1.5±0.1	1.7±0.3	1.3±0.2	1.1±0.3	0.4±0.2
ELISA OD (1)	≥0.22	0.61±0.21*	0.77±0.47*	0.75±0.20*	0.67±0.14*	0.66±0.15*	0.62±0.13*	0.72±0.02
ELISA OD (2)	≥0.25	0.27±0.01	0.34±0.02	0.42±0.05	0.38±0.08*	0.29±0.01	0.41±0.00	0.45±0.01

Table 7.3: Effect of Pre-Analytical Heat Treatment on Inhibitor Detection. Cut-Off=Assay cut-off. Values shown represent mean values for samples tested in duplicate.* CV≥20%.

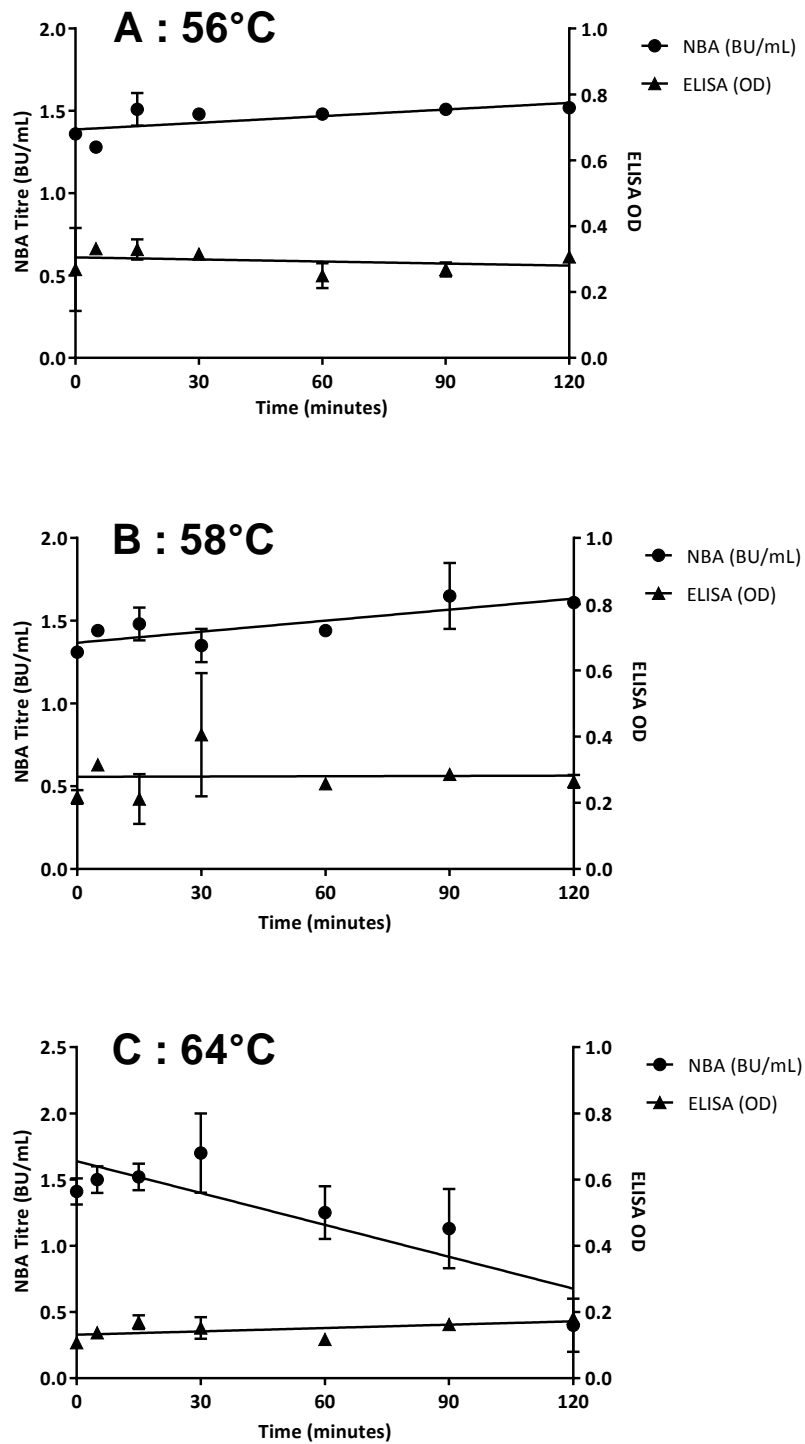


Figure 7.4: Effect of Pre-Analytical Heat Treatment on FVIII inhibitor detection at 56°C (A), 58°C (B) and 64°C (C) evaluated using a FVIII ELISA and the Nijmegen Bethesda Assay (NBA). Data plotted for the NBA is the mean±SD for sample evaluation performed in duplicate and the data from the second ELISA experiment with OD±SD.

7.4.2.3 Assessment of the Effect of Pre-Analytical Heat Treatment on FVIII Inhibitor Detection

Finally, an assessment of the effect of PHT was performed on pooled FVIII deficient plasma spiked with a low-titre FVIII inhibitor (1.4BU/mL) from a single donor. The effect of PHT on inhibitor detection by the FVIII ELISA and NBA was evaluated over four temperatures (56, 58, 64 and 72°C) to assess whether this resulted in loss of inhibitor detection. A higher temperature range was used in these experiments to the previous experiments to assess a temperature range which resulted in impaired antibody activity. PHT at 56 and 58°C did not result in any change in inhibitor detection by the NBA and FVIII ELISA for up to 120 minutes of sample incubation (Table 7.3 and Figure 7.4). PHT at 64°C resulted in a loss of antibody detection by the NBA after 120 minutes of sample incubation, with the suggestion of a decline in inhibitor titre with increasing incubation time (Figure 7.4). PHT at 64°C, did not affect inhibitor detection by the FVIII ELISA even after 120 minutes of incubation. PHT at 72°C resulted in plasma gelation within 15 minutes of heating. This process was irreversible and it was not possible to obtain sufficient sample volumes for inhibitor testing by the NBA or FVIII ELISA.

7.5 Discussion

The data presented for clinical usage of PHT, demonstrated improvement (10 fold) in the detection of FVIII auto-antibodies, using a functional inhibitor assay (NBA) in parallel with the FVIII ELISA. A change from negative to positive was seen in samples from 6/8 patients, which could have had implications for clinical management. Evaluating the longitudinal time lines (Figure 7.1 A-C), PHT using a functional inhibitor assay (NBA) and/or a FVIII ELISA, allowed earlier detection of FVIII auto-antibodies, allowing unmasking of otherwise undetectable low-titre FVIII antibodies ²⁹². Improving the sensitivity of antibody detection using PHT, described in this chapter and by other investigators (Table 7.4) could have application as a biomarker for early detection of relapse or as a more sensitive marker of remission in AHA. Evaluation of commonly used PHT incubation conditions (56 and 58°C), demonstrated differences in denaturation of FVIII dependent on the incubation time, without affecting inhibitor detection by the NBA or FVIII ELISA.

7.5.1 Pre-Analytical Heat Treatment in Congenital and Acquired Haemophilia A

There has been increased research interest in the usage of PHT, as part of inhibitor testing in both congenital and acquired haemophilia A. The key features of these previously described studies are summarised in Table 7.4. All of these used PHT incubation temperatures between 56 and 58°C, although there were some difference in incubation times and use of centrifugation. The largest of these studies (1353 samples, 710 patients), was carried out as part of a national inhibitor surveillance study from the US²⁸³. In samples from patients with severe haemophilia A, 55% (126/228) contained measurable FVIII:C. A total of 202 samples (159 negative inhibitor history, 43 positive inhibitor history and 30 controls) were tested using the NBA before and after PHT. Of these, six became positive following heat treatment with only one from a patient with no inhibitor history (pre: 0BU/mL to post: 0.7BU/mL) and the remainder from patients with a previous inhibitor history²⁸³. A more recent publication has systematically evaluated the effect of PHT in patients with severe haemophilia A, with no inhibitor history (group I, n=20); inhibitor history with no recent FVIII exposure (group II, n=21) and patients on ITI (group III, n=5, 68 samples). Although no samples from patients without an inhibitor history became positive post PHT, samples in both other groups with an inhibitor history had a change in inhibitor status post PHT (group II=6 and group III=5). It is interesting to note that all of these samples that became positive had very low amounts of FVIII:C (range 0.1-1.8IU/dL) prior to PHT, although there was demonstrable FVIII:Ag in all samples. This change in positivity in these samples could support previous findings²⁹² that an additional mechanism of PHT in increasing inhibitor testing sensitivity is through disassociation of bound FVIII antibodies.

Data for usage of PHT in AHA is more limited with three small unpublished studies (not including the data presented in this chapter) being presented to date in abstract format. The first of these, presented in 2005, represents one of the first recent descriptions of routine use of this modification²⁹³ (Sahud M., Senior Academic Associate, Quest Diagnostics, personal communication). This study looked at 120 samples from patients with FVIII antibodies; auto-antibodies (n=84) and allo-antibodies (n=9)²⁹³. Following PHT (56°C for 60 minutes), FVIII:C became undetectable in all samples. There was an increase in auto-antibody inhibitor titre in 86% (66/77) samples (mean increase 28.3±71BU/mL), although all of these samples appear to have detectable antibody present prior to PHT (i.e. no negative samples). Relatively little change was seen in inhibitor titre following PHT in a small number (n=9) of samples containing allo-antibodies. Similar findings to the data presented in this chapter

PHT	Cent	Samples (pt)	Diag	Findings	Ref
56°C 60mins	No	120 (NS)	AHA CHA	+PHT at 56°C for 1 hour, FVIII:C <1%, no effect on inhibitor activity +66/77 positive AHA samples mean increase in Bethesda titre 28.3±71	Sahud et al B ²⁹³
58°C 90mins	Yes Dual	20 (7)	CHA	+Low-titre assay cut-off >0.03BU/mL +6/7 patients post ITI, low-titre NBA+, with impaired FVIII pharmacokinetics (PK) +3 patients, normalisation of PK with negative low-titre NBA. +2 patients continued impaired PK with ongoing low-titre NBA+	Dardikh et al A ²⁹⁴
56°C 30mins	Yes 5000rpm 5mins	202 (NS)	CHA	+ PHT resulted in undetectable FVIII:C & FVIII:Ag in severe (n=15) and non-severe (n=7) haemophilia A + No inhibitor history: 1/159 became NBA+ + Inhibitor history: 5/30 became NBA+	Miller et al A ²⁸³
58°C 90mins	Yes Dual	38 (15)	AHA	+ 7 patients (FVIII <70IU/dL and BU ≤5BU/mL). 5/7 patients had ≥1 BA+ sample following PHT, +8/14 samples BA- with FVIII <70IU/dL pre-PHT were positive post PHT. +All BA+ samples remained positive post PHT. +No samples BA+ post PHT if FVIII >70IU/dL (n=24)	Bodo et al B ²⁹⁵
58°C 90mins	No	39 (8)	AHA	+ Pre PHT: 2/39 samples NBA+ and 11/39 ELISA+. + Post PHT: 20/39 NBA+ and 16/39 ELISA+ + 6/8 patients, ≥1 sample NBA+ post PHT	Batty et al A ^{296;372}
56°C 60mins	Yes 2700g 5mins	1092 (NS)	CHA AHA	+Increased sample testing, post PHT (95v72%) + 0/31 samples with severe HA NBA+ with FVIII:C>20IU/dL. +3/14 samples NBA+ with non-severe HA with FVIII:C >20 IU/dL. + 91/140 samples NBA+ with AHA, were NBA+ with FVIII:C >20 IU/dL	Gandhi et al B ²⁹⁰
56°C 30mins	Yes 2500g 5mins	109 (46)	CHA	+ No inhibitor history (n=20): PHT no change + Inhibitor history, no recent FVIII (n=21): 6 samples became positive with PHT. Increased mean inhibitor titre (p<0.05) + ITI patients (n=5, 68 samples): 5 samples became positive with PHT. 3 fold mean increase in inhibitor titre in positive samples.	de Lima Montalvão et al A ²⁹⁷

Table 7.4: Clinical usage of Pre-Analytical Heat Treatment in Congenital and Acquired Haemophilia A. NS=Not-stated. Cent=Centrifugation. Dual=Two step centrifugation, with initial short centrifugation at 18,000g for 5 minutes, followed by centrifugation of supernatant plasma at 4200g for 45 minutes at room temperature in a Ultra-Free-4 centrifugal filter with a nominal molecular weight limit of 100kD. Concentrates were collected from the filter unit and mixed in a ratio of 3:1 with 0.1mol/L imidazole buffered normal plasma (pH 7.4). AHA=acquired haemophilia A, CHA=congenital haemophilia A. A=Published article, B=Poster or conference presentation.

have been described in a cohort of patients (n=15), which was presented in abstract form in 2013²⁹⁵, alongside my data^{296;372}.

7.5.2 Laboratory Monitoring of Treatment Response in Acquired Haemophilia A

Interestingly, five clinical samples positive by ELISA prior to PHT became negative by ELISA following PHT. Although, one could suspect, these represented false positives on the initial ELISA, this seems unlikely given that there was some detectable inhibitory capacity using the NBA in four of these following PHT. An alternative explanation could be that PHT has a greater effect on the immunoglobulin structure than previously suggested^{292;370;373}. Experimental data, in another setting has shown heating results in alterations in immunoglobulin secondary structure affecting the Fab region at 61°C, followed by the Fc region at 71°C³⁷⁴. One could hypothesise that some antibodies' binding kinetics, for example those with low binding affinity may be more sensitive to temperature induced disruption of Ig structure. This would in turn lead to a lack of detection when using a FVIII ELISA. In contrast, immunoglobulins might still be able to exert inhibitory effect through steric hindrance resulting in detectable inhibitory capacity even with lower binding affinity. In my subsequent experiments following on from these observations, PHT at 56°C and 58°C did not affect antibody detection by either the NBA or ELISA, even when incubated at 64°C for 120 minutes, despite loss of detection by the NBA.

Finally, the clinical and function relevance of antibodies detectable following PHT remains unknown. Whether these represent truly pathogenic antibodies, or are similar to "natural" FVIII antibodies seen following heat treatment of samples from normal individuals and patients with congenital haemophilia A is not clear³⁷⁵. In my view, introduction of PHT in the absence of prospective clinical trial data of clinical significance may adversely influence clinical treatment decisions in AHA. For example, in the weaning of immunosuppression this could result in patients with persistently detectable antibody following PHT, being exposed to protracted immunosuppression, with associated risk of morbidity. The approaches described in this and the previous chapter (PHT and ELISA) could provide biomarkers to allow more personalised management of immunosuppression in AHA. To date there has been no description of the usage of ELISA or similar technology to provide long term monitoring in AHA. As such, it is not known if some patients develop a long term (chronic) immune response to FVIII, even following normalisation of FVIII:C, that remains detectable using a more sensitive assays. On the other hand, some patients who achieve antibody negativity by

all assays/modifications, as seen for two patients in this study, might form a distinct subgroup of patients in whom immunosuppression could be de-escalated more rapidly and toxicity minimised. Whether the first of these scenarios represents an analogous status to the concept of minimal residual disease, used for in leukaemia risk stratification in haemato-oncology studies of treatment response, is not clear. Further prospective study would be required to determine whether differences exist in relapse rates in patients with persistent detectable low level antibody, compared to those whose antibodies become undetectable by all assays. This would optimally be performed in a multicentre study with centralised laboratory testing and would be a difficult study to conduct even within the setting of a national or international cohort study.

7.5.3 Usage of Pre-Analytical Heat Treatment in the United Kingdom

Within the UK there appears to have been an increased uptake in usage of PHT as part of inhibitor testing. Survey data presented in Chapter 5, demonstrated that only one respondent used PHT as part of their inhibitor testing in AHA³²¹. Since this survey, a NEQAS exercise (November 2013), reported 21% (23/110) of centres surveyed reported using PHT to inactivate residual FVIII:C as part of their inhibitor testing³³¹. There was variation in incubation temperature (56-58°C), incubation time (15-120 minutes) and centrifugation use (none, or 3000-15,000 x g), reflecting a lack of previous data comparing these conditions. This change in laboratory practice may have resulted from recently presented data on usage of PHT (Table 7.4). An alternative explanation may be that clinicians surveyed regarding management of AHA were unaware of the specifics surrounding inhibitor testing within their centre's laboratory.

The data from my experiments assessing different PHT conditions has demonstrated differences between the commonly cited PHT (temperature and incubation time) conditions. Although, incubation at 56°C resulted in a FVIII:C <1IU/dL for endogenous pooled plasma and rFVIII after 60 and 120 minutes respectively, there was detectable FVIII:C for the pdFVIII concentrate following 120 minutes of incubation. PHT at 58°C, as used in our laboratory practice, had a clearer pattern of denaturation for both endogenous and exogenous FVIII:C, which resulted in FVIII:C <1IU/dL after 60 minutes. Within these temperatures there was no significant denaturation of FVIII antibodies seen. Data from the usage of a higher temperature (64°C) for heat treatment appear to suggest a modification that may be associated with significant time saving for laboratory staff, in that endogenous FVIII:C was

completely denatured within 5 minutes of PHT, although there was a trend for loss of inhibitory potential with increased incubation time. The findings of incomplete FVIII:C inactivation with usage of an incubation of 56°C, although in keeping with the earlier experimental data^{292;371}, differ from recent studies that found undetectable FVIII:C following 30²⁹³ and 60²⁸³ minutes of PHT. Whether these differences relate to the high levels of FVIII:C present in my experiments, relative to those seen in these recent studies, is not clear and requires further study.

7.6 Limitations

Within the sample testing used within this work, there are a number of limitations due to the protocols used. Within testing of the FVIII:C for the endogenous and exogenous plasma, with these samples having baseline FVIII:C >100IU/dL, the precision in testing at this point will be lower, which is seen through the wide SD for these data-points (Table 7.2). In reporting of the effect of PHT on FVIII:C, both the normalised and raw data were reported to provide transparency of reporting, with the absolute cut-off of a FVIII:C of ≤1IU/dL being used. In this it is recognised that the level of effect of PHT might be affected by the starting proportion of FVIII:C. Additional limitations exist in the precision of reporting of the inhibitor titres and FVIII:C, in which only testing of undiluted samples were performed due to cost limitations and the low-titre of inhibitor present. Although this may have resulted in lower precision being insensitive to subtle changes following PHT, the primary outcome being evaluated was loss of FVIII:C (<1IU/dL) or loss of inhibitor detection, which demonstrated consistent results on repeat testing.

Finally it should be noted that as there is increased usage of PHT in routine laboratory practice, greater care is required in the calibration and validation of laboratory equipment used for these purposes. Even within these controlled experiments, variability in the water-bath temperatures were seen for each temperature point. In the usage of water-baths for sample incubation, centres should not assume temperature settings of the water-bath will provide accurate measures of water temperature, highlighting the importance of usage of calibrated devices (as required by ISO 15189:2012) and temperature monitoring during the process of PHT. Secondly in the usage of the ELISA, within these experiments, although no detectable change in signal was seen, even with duplicate testing there was a greater than desired CV (>20%) for some samples tested, although this was improved with the addition of a gentle vortexing step between stages within the ELISA protocol. As no further ELISA plates

were available for usage in these experiments, it was unfortunately not possible to repeat the tests in which the CV was >20%.

Finally, a significant limitation in the review of the clinical data was that the ELISA results were only available for analysis in a binary format (positive or negative). This did not allow comparison of the ELISA OD pre and post PHT, or evaluation of the testing QC (KC and CV). A variation in CV seen in the ELISA could potentially impact on the results of this assay, although it was not possible to repeat testing on these samples due to the study design. Measures of imprecision should ideally be reported alongside results if they are to be used in clinical decision making processes.

7.7 Conclusions

PHT improves detection of FVIII antibodies in congenital and acquired haemophilia A. The proposed mechanism for this is through denaturation of residual FVIII:C within samples prior to analysis and/or release of bound FVIII antibodies. Commonly cited PHT protocols could yield different results in their effect of denaturing FVIII:C, especially when exogenous FVIII:C is present in samples. PHT at 58°C for 60 minutes yielded consistent results for denaturation of both endogenous and exogenous FVIII without affecting inhibitory capacity. The rapid denaturation of FVIII:C, seen using PHT at 64°C without impacting on inhibitor detection, could result in time and cost savings for use of this modification, requires further study.

Chapter 8: Amino Acid Sequence Epitope Mapping of Factor VIII Antibodies in Severe Haemophilia A

8.1 Introduction

Polyclonal FVIII antibodies have been reported to interact with multiple domains on both the heavy and light chain of FVIII in congenital and acquired haemophilia A. Although modifications to FVIII antibody testing described in the previous chapters improved detection of FVIII antibodies, these do not provide a mechanism of inhibitory action for FVIII antibodies. Understanding where these antibodies interact (B-cell epitope) with FVIII may provide further information for understanding of the immune response to FVIII. Improved characterisation of antibody interaction sites may identify regions of increased immunogenicity (immunodominant epitopes) and allow greater understanding of differences in antibody classes (inhibitory/non-neutralising) and kinetics. This could in turn allow development of modified FVIII products with reduced immunogenicity as demonstrated in a murine haemophilia A model ³⁷⁶ by mutagenesis at residues within an immunodominant epitope in the A2 domain (R484-I508) ³⁷⁷. In this model, an R484A/R489A/P492A mutated B domain deleted (BDD) FVIII had reduced immunogenicity compared to a control wild type BDD FVIII ³⁷⁶. Certain epitope profiles may be predictive of ITI response (or failure) which could provide a biomarker for further stratification of patients towards different treatment approaches. In keeping with this, small studies of patients undergoing ITI have suggested that the FVIII antibody domain epitope may predict likelihood of ITI success ³⁷⁸⁻³⁸⁰. In the most recent of these studies (15 patients), the presence of FVIII antibodies to the A1 and A2 domain prior to ITI initiation demonstrated significant discriminatory ability (AUC >0.875) for prediction of ITI failure ³⁸⁰. Similar findings of poor response to ITI in patients with A2 domain binding antibodies has been reported ^{378;379}. These data support a hypothesis that differences in B-cell epitopes of FVIII antibodies may influence outcomes, although this requires further study in larger cohorts. There has been substantial research interest over the last three decades attempting to characterise the B-cell epitopes of FVIII antibodies ^{239;247;381}, via a number of different approaches at high (amino acid sequence) and low (domain or sequence region) resolutions, which are summarised in Table 8.1 ³⁸².

	Domain	Seq	Linear	Conformational	Time	Ref
Luminex®	+ / -	-	+	+	+	246;247;261;380
IB / IP	+	LRes	+	-	++	135;383-385
MS	+	LRes/HRes	+	+	++	386;387
Recombinant hybrid porcine/human FVIII	+	LRes/HRes	+	+	++	133;377;388;389
Alanine scanning mutagenesis	+	HRes	+	+	+++	390
Phage display	+	HRes	+	+	+++	391-393
Peptide array	+	HRes	+	+ / -	+	394-398
SPR	+	HRes	+	+	++	399
X-Ray crystallography	+	HRes	+	+	++++	400

Table 8.1: Comparison of assays used in detection and description of FVIII antibodies in congenital and acquired haemophilia A. Time=Time taken to perform epitope mapping. LRes=Low-resolution (region of amino acids), HRes=High-resolution (amino acid sequence). IB=Immunoblotting. IP=Immunoprecipitation. MS=Mass spectrometry. SPR=Surface plasmon resonance.

Prior to consideration of the optimal approach for epitope mapping in the setting of haemophilia, it is useful to consider some of the core determinants of B-cell epitopes in a broader context⁴⁰¹⁻⁴⁰⁵. Although the majority of epitopes described form conformational or discontinuous epitopes⁴⁰¹, 85% of epitopes contain linear stretches of 5 or more residues^{403;405}. The positioning of epitopes protrude from the antigen surface⁴⁰⁶ and are more exposed than the remaining antigen⁴⁰⁵. Within these regions, the secondary structure is depleted of strands/helices and enriched in loops^{401;407}, with paired amino acids (Y:Y, C:P, N:Y, G:Y, D:P, T:Y, R:Y, H:Y and H:M)^{135;383;384} represented more frequently in the epitope than the remaining antigenic surface^{401;403}. As such, the optimal epitope mapping platform should allow characterisation of continuous and discontinuous epitopes at an amino acid level. This would allow high-throughput assessment within an integrated bioinformatics algorithm to provide epitope data for clinicians and translational researchers. This would also ideally allow evaluation using low sample volumes without complex sample preparation. Previous techniques used for epitope mapping in haemophilia (Table 8.1), all have specific advantages and limitations, but none have provided an approach amenable for high-throughput, high resolution epitope profiling of continuous and discontinuous B-cell epitopes. Of these multiple methodologies, peptide arrays consisting of synthesised peptides of variable lengths on a solid phase medium provide an attractive approach due to relatively low costs and potential to allow high-throughput testing⁴⁰⁴. A number of groups have used linear peptide

arrays to provide a low-throughput approach to epitope profiling of FVIII inhibitors³⁹⁴⁻³⁹⁸. These studies have applied different sequence lengths of synthesised peptides and have only provided a mimic of the primary FVIII structure (linear sequence). As a result, this could lead to conformational differences between a region represented in the array and the corresponding region within the intact protein^{408;409} and bias towards the detection of linear epitope sequences⁴⁰⁴.

A novel, commercially available peptide microarray (Pepscan), offers a high-throughput reusable approach for high-resolution mapping of antibody-protein interactions to an amino acid sequence level⁴¹⁰. This uses customised chemically synthesised overlapping peptide sequences, in a 455 well micro-card format, for any protein in which the amino acid sequence is known. This platform offers a variety of different arrays providing *in-vitro* representation of linear (primary sequence) or a conformation (primary and secondary structure) through constrained scaffolded peptide sequences. This approach has been used in a number of other settings describing the B-cell epitopes for a number of monoclonal (e.g. FSH- β , CD20, CC-chemokine receptor CCR5)⁴¹⁰ and polyclonal (e.g. wheat allergy: α -, γ , ω 2, and ω 5-gliadin)^{411;412} antibodies. This technique has also notably been used to describe the binding epitope of two anti-CD20 monoclonal antibodies (rituximab and obinutuzumab)^{413;414}, delineating discrete binding profiles for each with potential mechanistic implications. A reusable high-throughput peptide microarray, providing a conformational mimic, has not previously been described for epitope mapping of FVIII antibodies, which potentially offers a novel approach for characterising the binding of FVIII antibodies within clinical studies. Within this chapter I have evaluated the application of this platform in the characterisation of B-cell epitopes of monoclonal and polyclonal FVIII antibodies.

8.2 Hypothesis and Aims

The hypothesis is that a novel, high-throughput peptide microarray provides a feasible platform for epitope mapping of FVIII antibodies, which will allow high-throughput analysis of oligo-/poly-clonal B-cell epitopes. This technique will allow high-resolution epitope mapping of clinical samples containing FVIII antibodies and identify recurrent immunodominant sequence epitopes. The specific aims from these experiments are as follows:

- 1) To evaluate a novel, high-throughput microarray for the description of B-cell epitopes recognised by a panel of FVIII monoclonal antibodies (mAbs).

- 2) To define the array cut-off for positivity and validate the re-usability to allow interpretation of clinical samples.
- 3) To describe the B-cell epitopes of inhibitory antibodies in a cohort of patients with severe haemophilia A.
- 4) To assess whether inhibitory antibodies have common FVIII (immunodominant) B-cell epitopes and to correlate these with FVIII structure and function.

8.3 Materials and Methods

Monoclonal Antibodies

GMA-012 (R8B12) and GMA-8011 (2A9) were purchased from Green Mountain Antibodies (Burlington, Vermont, USA). The remaining monoclonal antibodies were kind gifts from the following: C5, Prof. Z.M. Ruggeri (The Scripps Research Institute, La Jolla, CA, USA), 58.12 & 2D2, Dr L. Regan (Bayer HealthCare, Berkeley, CA, USA). The known characteristics of these monoclonal antibodies are summarised in Table 8.2.

Monoclonal Antibody	Domain	Sequence	Epitope	Technique	Reference
C5	A1	338-362	L	Phage	Ware et al. ⁴¹⁵
		351-365	L	C.ELISA	Foster et al. ³⁸³
58-12	A1	N/A	N/A	N/A	Nil
GMA-8012 (R8B12)	A2	563-740	L	IB	Fay et al. ⁴¹⁶
		497-510; 584-593	D	MS	Ansong et al. ⁴¹⁷
2D2	A3	N/A	N/A	N/A	Nil
GMA-8011 (2A9)	C1	N/A	C	ELISA	Summers et al. ⁴¹⁸

Table 8.2: Factor VIII murine monoclonal antibodies previously characterised epitopes. L=Linear, D=Discontinuous, C=Conformational. C.ELISA=Competition ELISA. IB=Immunoblotting. MS=Mass spectrometry.

Pepscan Epitope Mapping Platform

Epitope mapping of FVIII monoclonal and polyclonal antibodies was performed using peptide micro-arrays produced by a commercially available precision epitope mapping service (Pepscan, Lelystad, Netherlands). These microarrays allow high-throughput epitope mapping of any protein in which the sequence is known, using low volumes of antibody (100µg) or untreated plasma (100µL). These micro-arrays consist of a proprietary re-usable 'mini-card' with overlapping solid-bound peptide sequence of up to 40-mer in length, offering varying

degrees of protein scaffolding, from “un-scaffolded” (linear T1) to “scaffolded” (looped, T2). Plate design was based on FASTA sequence for the mature FVIII protein (P00451). Selection of peptide sequence lengths for the array wells was made based on previous data of epitopes’ characteristics as discussed earlier (majority of epitopes containing 15 residues with linear stretches of 5 or more residues within these regions)^{404;405}. Two arrays were created to mimic linear and conformation structures of FVIII. The linear (T1) array consisted of 20-mer peptide sequences within each well, with a 5-mer sequence overlap between adjacent wells (Figure 8.1). The looped (T2) array consisted of 15-mer sequences with a 5-mer overlap between each microwell, with a conformational mimic created through the addition of cysteine residues at the 5’ and 3’ ends of the peptide sequence resulting in a 17-mer amino acid in each microwell (Figure 8.1). Peptides were robotically synthesised using a syro-synthesiser onto 455 well micro-cards for both the linear (T1) and looped (T2) arrays, by Pepscan (Lelystad, Netherlands). Solid-phase synthesis was performed using standard Fluorenylmethyloxycarbonyl (Fmoc) chemistry onto polypropylene supports containing carboxylic acid groups, with peptides sequences being covalently bonded at the C-terminal peptide to each well^{410;419}. Residues were then de-protected using trifluoric acid (TFA) with scavengers. For the creation of peptide sequences within the looped array (T2), each peptide sequence was capped with a cysteine residue at the C- and N-terminus. Within the T2 array, where a cysteine occurred within the primary protein sequence, an alanine substitution was made. There are 23 cysteine (22 represented in assay) residues in FVIII, which occur in the following domains: A1=5; a1=0; A2=4; a2=1; B=4; a3=0; A3=5; C1=2; C2=2 (1 C2 domain cysteine represented in the array). An alanine substitution was made in 61/455 wells on the looped array to allow provision of conformational mimics for these regions. These plates were designed within our group prior to the commencement of these experiments and analyses. The sequence coverage for the T1 and T2 arrays, was 98.2% (1-2290): A1/a1 (100%), A2/a2 (100%), B (100%), a3/A3 (100%), C1 (100%), C2 (73.8%). Formation of scaffolds was performed using Chemical Linkage of Peptides onto Scaffolds (CLIPS™) technology in 100% H₂O at room temperature at pH 7.8-8.0⁴¹⁰. This process involves the chemical reaction between the cysteine residues and a template (T) containing a benzyl bromide group, a reaction which is selective to the nucleophilic (SH) group of cysteine^{410;419}. Following micro-card construction and between each experiment, plates were “cleaned” by sonication in “disrupt buffer” at 70°C for 30 minutes followed by sonication in millipore H₂O for 45 minutes.

Samples were transported to Pepscan using the recommended sample transportation conditions and frozen at -80°C prior to testing. Samples were prepared for analysis by Pepscan staff and robotically applied to the array within the Pepscan laboratories. Samples were incubated (diluted 1 in 1000 in buffer) overnight at 4°C. After washing, the wells were incubated with either an anti-human or murine peroxidase conjugated secondary antibody for 1 hour at 25°C. After washing, the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate and 3% H₂O₂ were added for 1 hour and colour development was measured using a charge-coupled device camera^{420;421}. The results of all experiments were reported to the investigator as raw optical densities (ODs) for each well of the microarray. No cut-off or bioinformatics interpretation was performed at the point of testing within Pepscan.

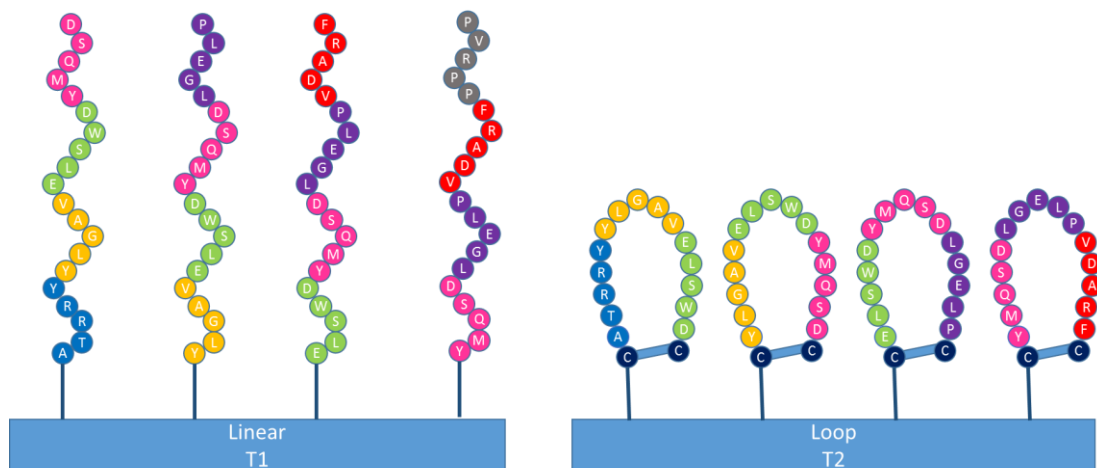


Figure 8.1: Pepscan Linear (T1) and Loop (T2) arrays demonstrating first four wells on these plates. These arrays consist of 20 (T1) and 15 (T2) mer peptide sequences, overlapping by 5 amino acids between each wells.

Clinical Samples

Samples were obtained (non-sequentially) from patients with severe haemophilia A patients with a current inhibitor at the Angelo Bianchi Bonomi, Hemophilia and Thrombosis Center, IRCCS Maggiore Hospital, University of Milan, Italy. Local institutional approval was obtained and informed written consent was obtained from all patients with the study being performed in line with the Declaration of Helsinki.

Demonstration of Pepscan Assay Cut-Off

To evaluate feasibility of assay use for characterising the B-cell epitope of FVIII antibodies, five FVIII mAbs with known domain or sequence epitopes (Table 8.2) (positive controls) and

eight negative controls (buffer media) were tested. These samples were tested sequentially with a negative controls tested prior to and following each mAb to evaluate assay reusability by identifying potential signal carry-over between experiments. Assessment of the data from all experiments, including the mAbs (n=5), negative controls (n=8) and clinical samples (n=13) was performed to establish a cut-off for positivity.

On examination of the raw OD data for these experiments, clear binding peaks were seen for 4/5 mAbs and 12/13 clinical samples. The raw assay OD data from the three sets of samples (negative controls, FVIII mAbs and clinical samples) were initially examined to determine whether this could provide an absolute cut-off for positivity for both arrays. For the linear (T1) array, the mean (range) OD for the negative controls, mAbs and clinical samples were 37.9 (0-126), 112.2 (0-2860) and 239.0 (2-2552) respectively. Similarly, for the looped (T2) array the mean (range) OD for the negative controls, mAbs and clinical samples were 37.3 (0-140), 94.0 (0-2857) and 265.6 (41-2661). Given the range of OD seen for the negative controls compared to the clinical samples (Figure 8.2), it was not possible to define a cut-off based on OD alone. In the clinical samples 72.7% (4303/5915) and 71.0% (4200/5915) of wells on the linear and looped arrays had optical density values greater than the maximum values seen using buffer media.

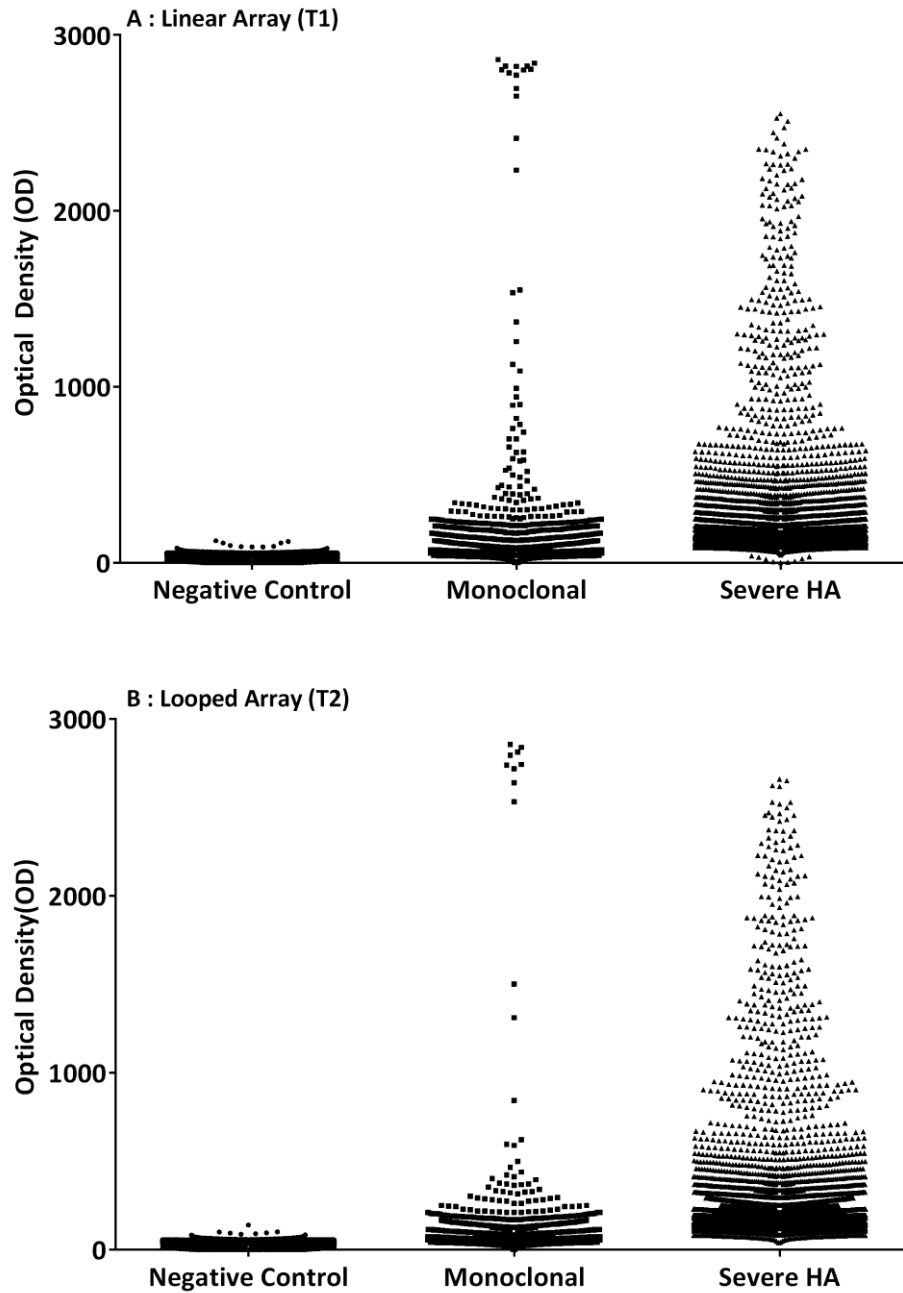


Figure 8.2: Scatterplot of optical density data for monoclonal antibody, negative controls and inhibitor samples from patients with severe haemophilia A.

Examination of the distribution of pooled data points for each group of experiments demonstrated a normal distribution for the negative controls and the appearances of a bimodal distribution curve for the mAbs and clinical samples. Further assessment of assay cut-off was then performed based on variance of results from the experimental mean. For the FVIII mAbs, the second peak was seen at approximately $\geq 5SD$ from the experimental mean (Figure 8.3) on both the linear and looped arrays. Taking this cut-off of $\geq 5SD$, the

binding peaks in 17 (linear) and 12 (loop) array wells for the mAbs all coincided with clear binding peaks seen on examination of the experimental raw data. Using a cut-off of $\geq 5SD$, only 8 (linear) and 1 (looped) array wells for the negative controls were positive (Figure 8.3). This threshold was used as an absolute cut-off to describe antibody binding similar to that seen for the mAbs. Applying this threshold alone to clinical samples was too restrictive giving positivity in only 32/5915 (linear) and 26/5915 (loop) of the array wells, despite additional peaks being evident on examination of the raw data. An evaluation of a “relative cut-off”, was performed based around the repetition of 5-mer sequences in subsequent wells on the linear (n=4) and looped (n=3) arrays. Given most epitopes contain linear stretches of ≥ 5 residues ^{403;405} this could allow identification of these regions. An assessment of a relative threshold based around binding in the sequential well containing the same 5-mers sequence was performed. Manual filtering was performed for all array wells in which the OD was $\geq 2SD$ from the experimental mean. Positive wells with a $\geq 2SD$ were then grouped into “epitopes” falling within sequential or semi-sequential wells of the array (see Epitope characterisation and description). With the repetition of 5-mer sequences in subsequent wells, it would be expected to see binding in two or more continuous or discontinuous wells on the array if this sequence formed part of the B-cell epitope. Evaluation of the characteristics of all potential epitopes, based on binding data (SD and binding in subsequent wells), suggested that using such a filtering strategy with a relative cut-off of $\geq 2SD$ and binding in ≥ 2 sequential/semi-sequential wells would allow inclusion of more of the binding peaks seen in clinical samples and a reduction in non-specific binding seen in the negative controls (Appendix 2). An absolute cut-off of $\geq 5SD$ and relative cut-off of $\geq 2SD$ was therefore set for these experiments. This approximates to a raw well specificity of 98.9% and 99.3% for the negative controls on the linear and looped arrays respectively.

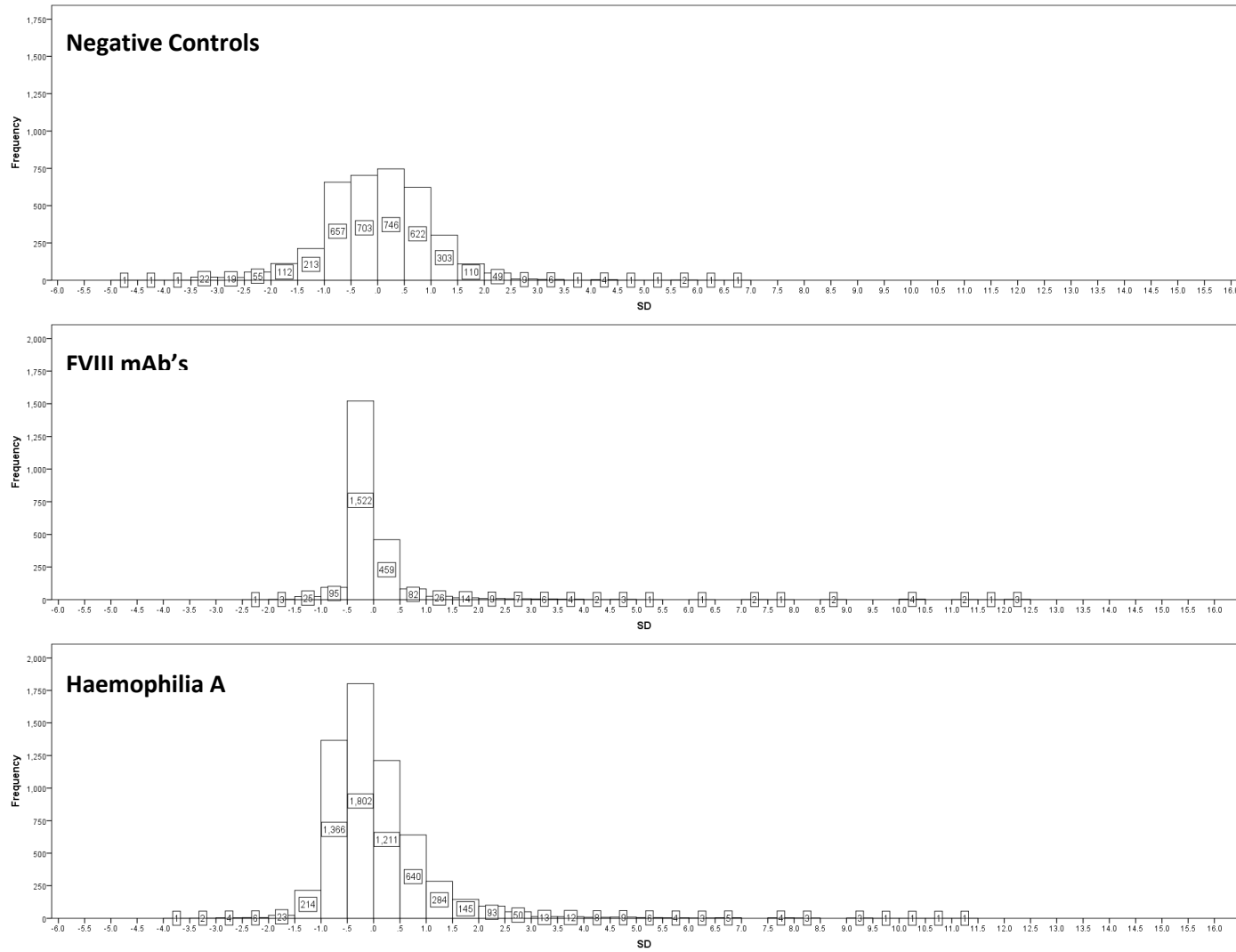


Figure 8.3A: Linear (T1) array, distribution (SD) of epitope binding data, from the experimental mean. Data shown represents the combined normalised results for all array wells for each group of experiments. Distribution of binding data has a peak occurring around the mean ($\pm 0.5SD$, normal distribution), with bimodal distribution of monoclonal antibody and clinical (haemophilia A) data. mAb's=monoclonal antibodies. SD=standard deviations.

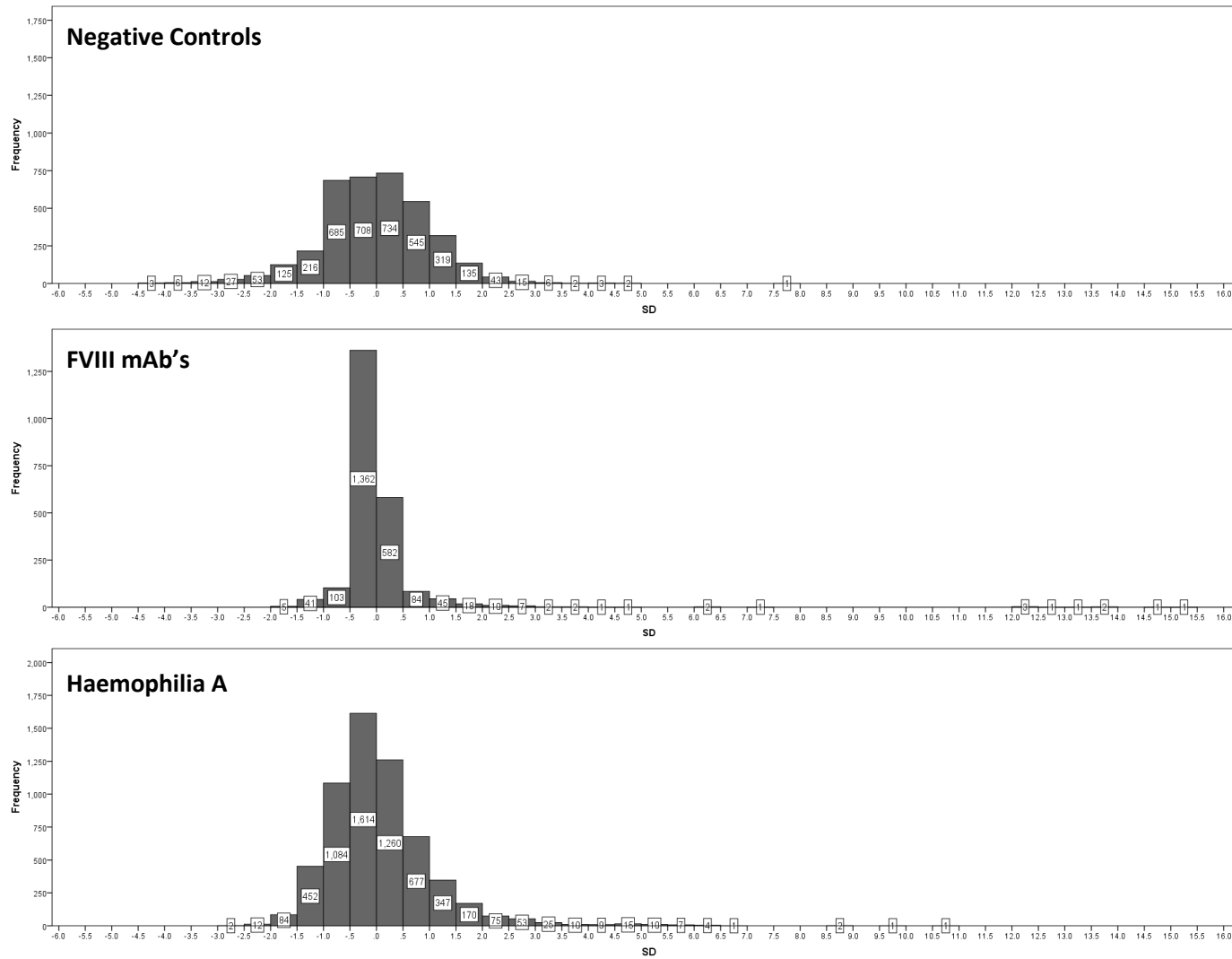


Figure 8.3B: Looped (T2) array, distribution (SD) of epitope binding data, from the experimental mean. Data shown represents the combined normalised results for all array wells for each group of experiments. Distribution of binding data has a peak occurring around the mean ($\pm 0.5SD$, normal distribution), with bimodal distribution of monoclonal antibody and clinical (haemophilia A) data. mAb's=monoclonal antibodies. SD=standard deviations.

Demonstration of Re-usability of the Pepscan epitope mapping array

Evaluation for carryover of binding between experiments was performed, comparing the OD seen for the mAb peaks, with the OD within the same well prior to and following that experiment. This was performed for experiments in which all three data points (pre, mAb peak and post) were available (R8B12, GMA-8011, 2D2 and 58-12). No significant difference was seen for the mean negative control OD before and after mAb binding peaks for both the linear (pre 37.7 ± 14.4 v post 37.2 ± 19.8 , $p=1.0$) and looped arrays (pre 39.6 ± 11.7 v post 34.6 ± 15.8 , $p=1.0$). The mean peak mAb OD, was significantly higher than the preceding negative control OD, for both the linear (pre 37.7 ± 14.4 , post 1483.9 ± 1086.5 , $p < 0.0005$) and looped arrays (pre 39.6 ± 11.7 v post 1327.3 ± 1231.7 , $p=0.001$). When positivity for the negative controls was defined based on the experimental SD (absolute cut-off $SD \geq 5$ and relative cut-off $SD \geq 2$ in ≥ 2 wells containing the same 5-mer sequence) none of the wells in the negative controls following the mAb peaks were positive.

Epitope Characterisation and Description

Low-resolution (LRes) epitopes described represent the total sequence length in which positivity was seen. For example, if binding was seen in first three array wells of the linear plate, well 1 (1-20), well 2 (well 6-25), well 3 (11-30), the low-resolution epitope would represent the first aa to last aa in which positive binding seen, which in this example would be 1-30. The high-resolution epitopes (HRes) presented represent a putative epitope based on the recurrence of 5-mer sequences in subsequent wells on the array. Using the same example as above, the sequence that is represented in all three of these wells on the linear array is the region 11-20. A similar approach was also taken if a discontinuous signal was seen, for example if binding was seen in wells 1, 3 and 4. Using this approach, the low-resolution epitope would correspond to positions 1-35 and the high-resolution epitope positions 16-20, based on this 5-mer sequence being represented in all three of these wells. Within the summary of epitope data, these are also described based on the array in which positivity was demonstrated, with a “shared epitope” being positivity seen within the same sequence region on both the linear and looped arrays.

Bioinformatic Resources, Structural Analysis and Epitope Representation

The FVIII amino acid sequences was obtained from the Universal Protein Resource (www.uniprot.org) for human coagulation factor VIII (P00451-FA8_HUMAN)⁴¹⁹. The 3.7Å B

domain deleted FVIII crystalline structure (PDB ID: 2R7E) ⁷ was obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Databank (www.rcsb.org)⁴²⁰. As discussed in Chapter 1, this crystalline structure has disordered regions not represented within the crystalline structure. For the purpose of visual presentation of the sequence epitopes, prediction for these regions were performed using pGenTHREADER ⁴²¹ (accessed 9/6/15) part of the PSIPRED v3.3 protein sequence analysis workbench (www.bioinf.cs.ucl.ac.uk/psipred) ⁴²². Predictions were made for the heavy chain of Factor VIII based on the primary sequence (P0451) for the mature FVIII heavy chain (legacy positions 1-740). This resulted in 31 statistically significant ($p < 0.05$) predictions for the heavy chain, with 3 predictions with a net GenTHREADER score > 200 . The 2R7EA0 prediction was used based on the basis of sequence length coverage (735aa) and visual similarity of the heavy chain to that modelled in the original crystalline structure (2R7E). The heavy chain prediction was aligned electronically to the structure of 2R7E within PYMOL. This resulted in additional representation of the following regions within the heavy chain: 215-221, 335-359, 726-735 within the heavy chain for demonstration of epitope positions.

Assessment of solvent accessibility (accessible surface area, ASA) was performed for areas with positive binding (epitopes) to evaluate whether these regions were likely antibody interaction sites. ASA assesses the surface area of a protein that is accessible to a solvent giving a representation of surface exposed residues. Within an antigenic region, residues involved within the B-cell epitope are more surface exposed than the remaining antigen ⁴⁰¹. The absolute ASA represents the ratio between the solvent ASA of a residue within a three-dimensional structure and that in an extended tripeptide (A-X-A) conformation. Absolute solvent accessible surface area for all residues represented (i.e. not predicted) within the FVIII crystalline structure were obtained using the Dictionary of protein secondary structure (DSSP) ⁴²³. Relative accessible surface area was then calculated using previously published scaling values (AA_{SF}) ⁴²⁴ using the following formula: $Relative\ ASA = (Absolute\ ASA / AA_{SF}) \times 100$. These AA_{SF} values are : A (110.2), D (144.1), C (140.4), E (174.7), F (200.7), G (78.7), H (181.9), I (185.0), K (205.7), L (183.1), M (200.1), N (146.4), P (141.9), Q (178.6), R (229.0), S (117.2), T (138.7), V (153.7), W (240.5) and Y (213.7) ^{424;425}. This gives estimates of relative ASA ranging from 0% (completely buried) to 100% (completely accessible). A relative ASA $\geq 20\%$ was used to classify residues as being exposed and $< 20\%$ as being buried ⁴²⁶. Secondary structure assignments were obtained using DSSP ^{423;427} from the RCSB Protein Databank, for the 2R7E FVIII crystal structure (last accessed 7/7/15) ⁴²⁰. Secondary structure assignments

are: H= α -helix, B=residue in isolated β -bridge, E=extended strand, participates in β ladder, G=310 helix, I=5-helix (π -helix), T=hydrogen bonded turn and S=bend. Blank entries, represent either a loop, irregular or no reported output ⁴²³. Assessment of potential discontinuous epitopes was made through calculation of pythagorean distance between the co-ordinates (X,Y,Z) of the α -carbons of residues obtained using DSSP ^{423;428}. A cut-off of $\leq 10\text{\AA}$ was used to assess potential proximity of residues likely to form part of a discontinuous epitope ⁴²⁹. Analysis of sequence similarity was performed using the local sequence alignment server Emboss Matcher based on the LALIGN application prediction server ⁴³⁰. Representation of epitopes was performed using PYMOL (The PYMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC).

Data Analysis and Statistical Analysis

Sequence epitopes described are based on the numerical sequence position within the mature protein, i.e. legacy format. Data is presented as mean, range and standard deviation. Assessment of the data distribution was performed using scatterplots and histograms of the grouped data. Comparison of the mean optical density was performed using a one-way repeated measures ANOVA (pre, peak, post) for positively defined peaks from the monoclonal antibody experiments. Within the one-way repeated measures ANOVA sphericity was assessed using Mauchly's test⁴³¹ and where significant (i.e. sphericity violated) corrections to degrees of freedom were made using the Greenhouse-Geisser correction ⁴³² with post-hoc comparison being made using the Bonferroni test.

8.4 Results

8.4.1 Amino Acid Sequence Epitope Mapping of Factor VIII Monoclonal Antibodies

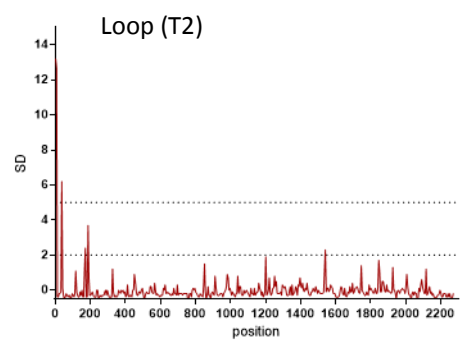
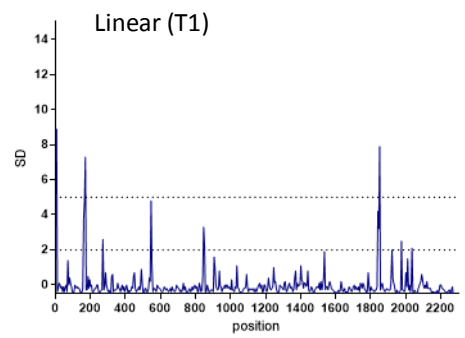
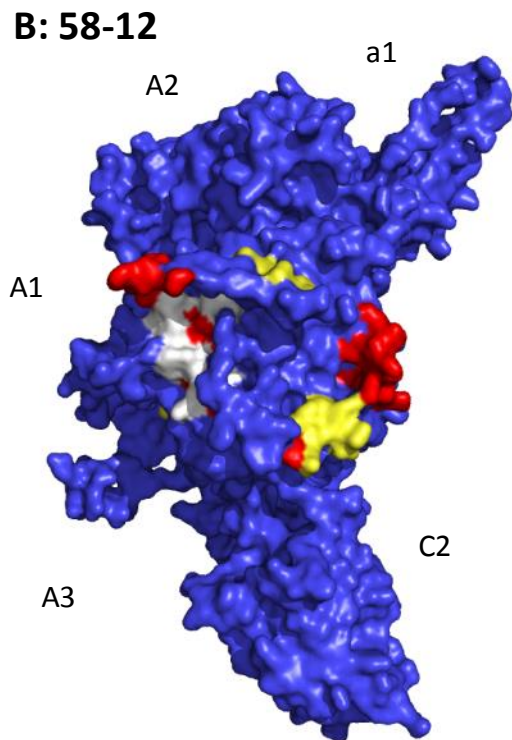
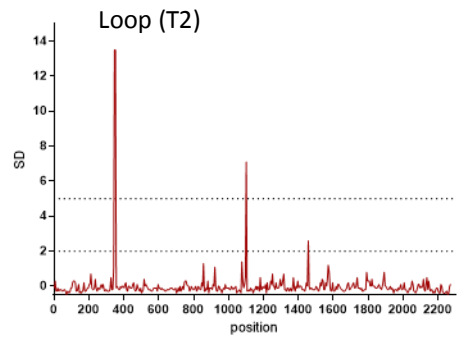
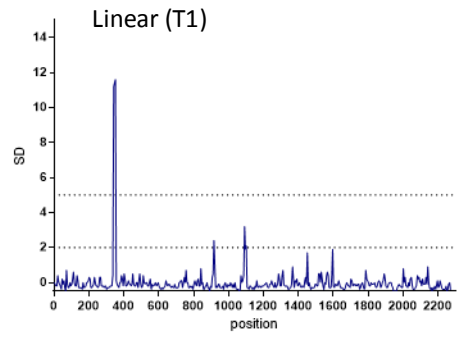
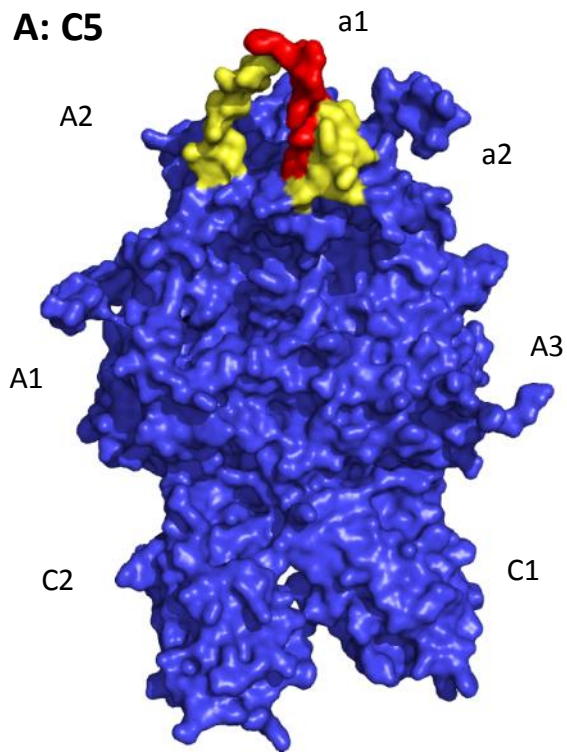
Five anti-FVIII monoclonal antibodies with known sequence or domain epitopes (Table 8.2) to the heavy (n=3) and light (n=2) chain were tested on both the linear and looped peptide microarrays. Clear binding signals (Figure 8.4) were seen for 4 of these monoclonal antibodies (C5, R8B12, 2D2 and 58-12).

A shared epitope for the a1 domain binding mAb (C5) is characterised between positions 341-370 (HRes 351-360, TDSEMDVVRV), as shown in Figure 8.4A. This acidic terminal region of the A1 domain (a1), which includes the HRes epitope, lies within a disordered region of the FVIII crystal structure ⁷, although all amino acids represented (360-370) within the remainder of the LRes epitope are highly solvent accessible (Figure 8.5). Importantly, the HRes epitope

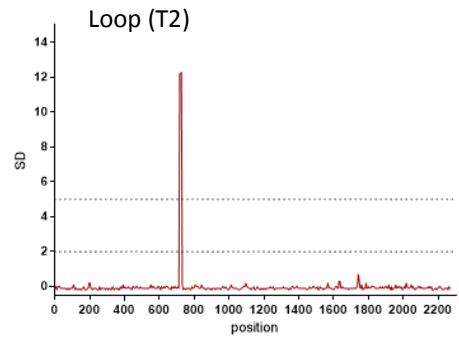
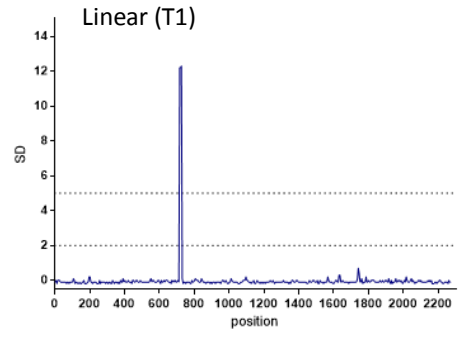
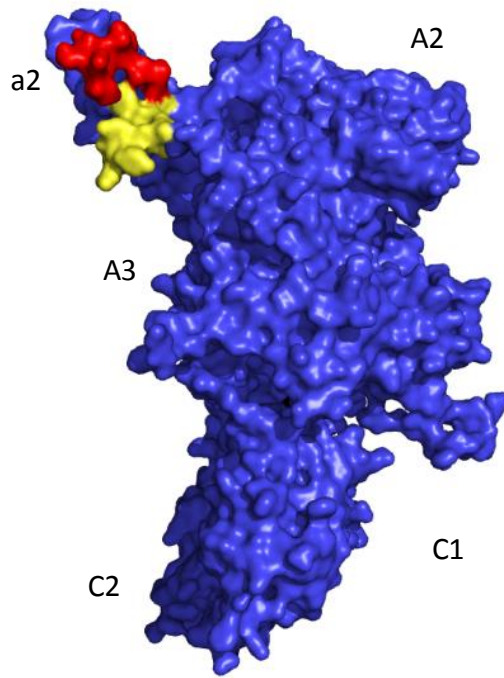
seen in these experiments is identical to the previous reported linear epitope for this mAb^{383;415}. Significant binding was also seen within the B domain, with a shared epitope at positions 1091-1120 (HRes 1101-1110, FLPESARWIQ). These two regions showing significant similarity (underlined) at 7/16 (43.8%) and are identical (bold and underlined) at 5/16 (31.2%) residues (**LTDSEMDVVRFDDNS** and **LPESARWIQRTHGKNS**), including three residues within the C5 HRes epitope (D352, S353, V357 and R359).

A second A1 domain mAb (58-12), demonstrates a discontinuous epitope (Figure 8.4B). This has a shared epitope at positions 1-25 (HRes 6-15, YLGAVELSWD), with further binding at positions 36-50 (HRes 36-50, KSPFNTSVVYKKTLL) and 161-190 (HRes 171-190, GLIGALLVCR) on the looped and linear arrays respectively. The high-resolution linear epitope of the third region (171-190) is solvent inaccessible within the first 9 amino acids of this sequence (GLIGALLVC), with the terminal 10 amino acids being located distant from the putative discontinuous epitope described above. Analysis of relative spatial positioning of the alpha carbons of residues for these three regions demonstrates the following regions lie within proximity ($\leq 10\text{\AA}$): 7-18 and 43-50; 170-172 and 9, 11, 14; 46-50 and 167-174. Taking into account the relative solvent accessibility ($>20\%$) of these regions, this results in a putative 11 residue discontinuous epitope (shown in white in Figure 8.4B) with the co-ordinates: 11-13(ELS), 17-18 (MQ), 43-47 (SVVYK) and 50 (L). Further binding was seen on the linear array within the B domain (841-870, HRes 851-865, PESGLQLRLNEKLGTT) and A3 domain (1841-1870, HRes 1851-1860, LIGPLLVCHT). The first of these regions demonstrates sequence similarity (similarity 60%, identical 50%) over a stretch of 10 amino acids (**DLVKDLNSGL** and **DMVFTPESGL**) in regions 163-172 and 846-855, respectively. The second of these also demonstrates a high degree of sequence similarity (similarity 78.3%, identical 65.2%) over a stretch of 23aa (**VDLVKDLNSGLIGALLVCREGSL** and **VDLEKDVHSGLIGPLLVCHTNTLL**), in regions 162-184 and 1841-1863, respectively. These regions share a common 5 amino acid motif of SGL and D-X-V where X represents any amino acid.

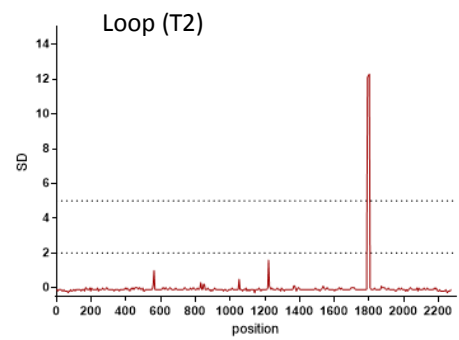
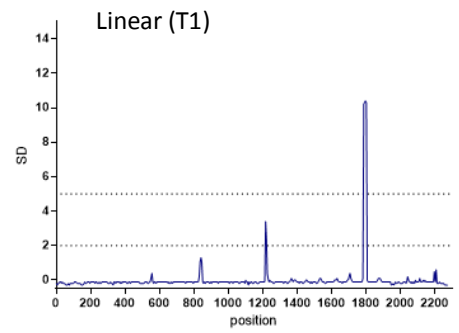
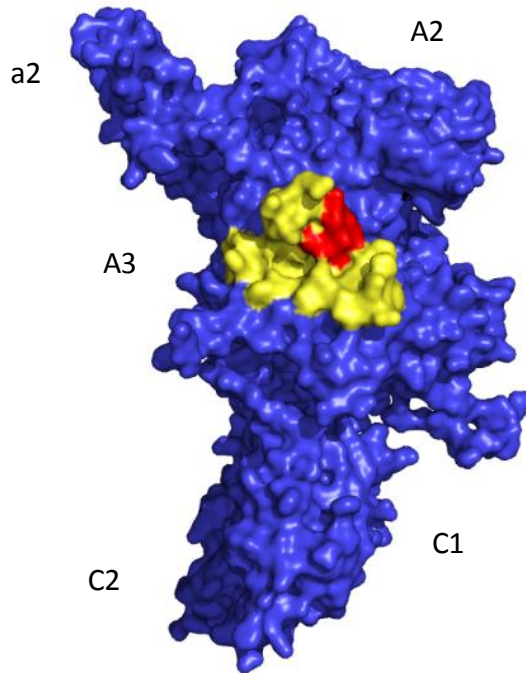
The third monoclonal antibody (R8B12) demonstrates a shared epitope in the $\alpha 2$ domain between positions 716-745 (HRes 726-735, ISAYLLSKNN) (Figure 8.4C). This peak was reproducible on both linear and looped arrays on repeat testing. The high-resolution epitope falls within a disordered region of the crystalline structure, although the peptides within the low-resolution epitope (719-725) demonstrates solvent accessibility (Figure 8.5). This epitope falls within the region described previously (Table 8.2), but differs from a more recently reported discontinuous epitope for this monoclonal antibody between positions



C: R8B12



D: 2D2



E: GMA-8011

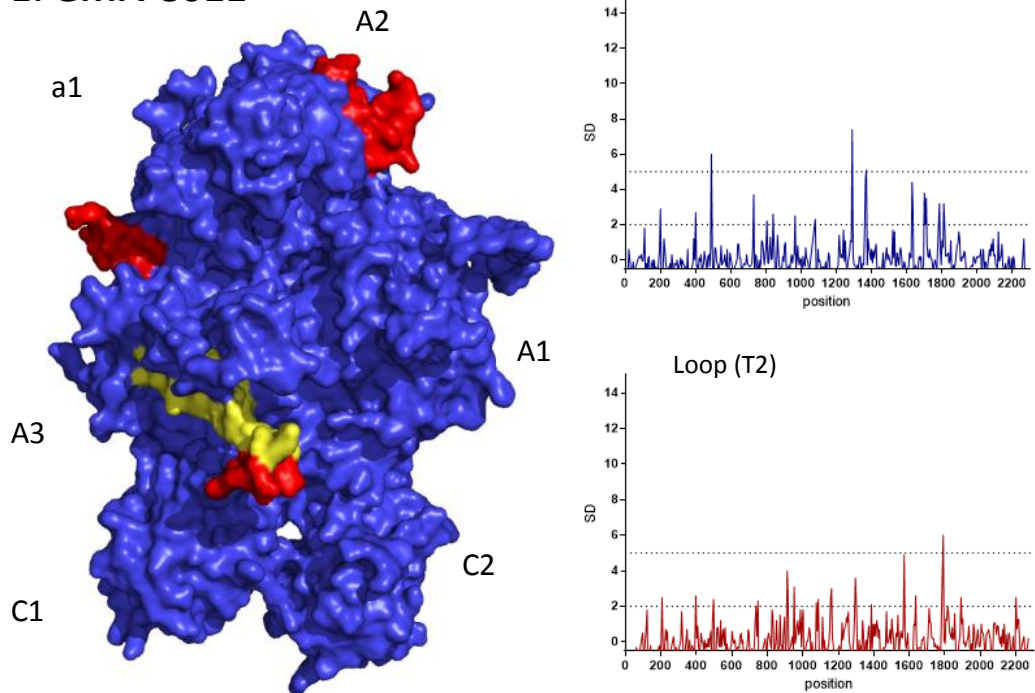


Figure 8.4: Sequence Epitope Mapping of FVIII Monoclonal Antibodies. Low-resolution (LRes) and high-resolution (HRes) epitopes are represented in yellow and red respectively. Normalised data for binding is shown on the curves with the linear and looped arrays plotted in blue and red, respectively. A: C5 mAb (a1 domain): 341-370 (HRes 351-360, TDSEMDVVRV). B: 58-12 mAb (A1 domain): 1-25 (HRes 6-15, YLGAVELSWD), 36-50 (HRes 36-50, KSFPFNTSVVYKCTL) and 161-190 (HRes 171-190, GLIGALLVCR). 58-12 dis-continuous epitope (shown in white): 7-18 and 43-50; 170-172 and 9, 11, 14; 46-50 and 167-174. C: R8B12 mAb (A2 domain) 716-745 (HRes 726-735, ISAYLLSKNN). D: 2D2 mAb (A3 domain): 1786-1820, (HRes 1801-1805, EPRKN). E: GMA-8011 mAb (C1 domain): LRes: 491-510, 736-760, 1701-1730 (HRes: MSSSPHVLRN, 1711-1720) and 1791-1805.

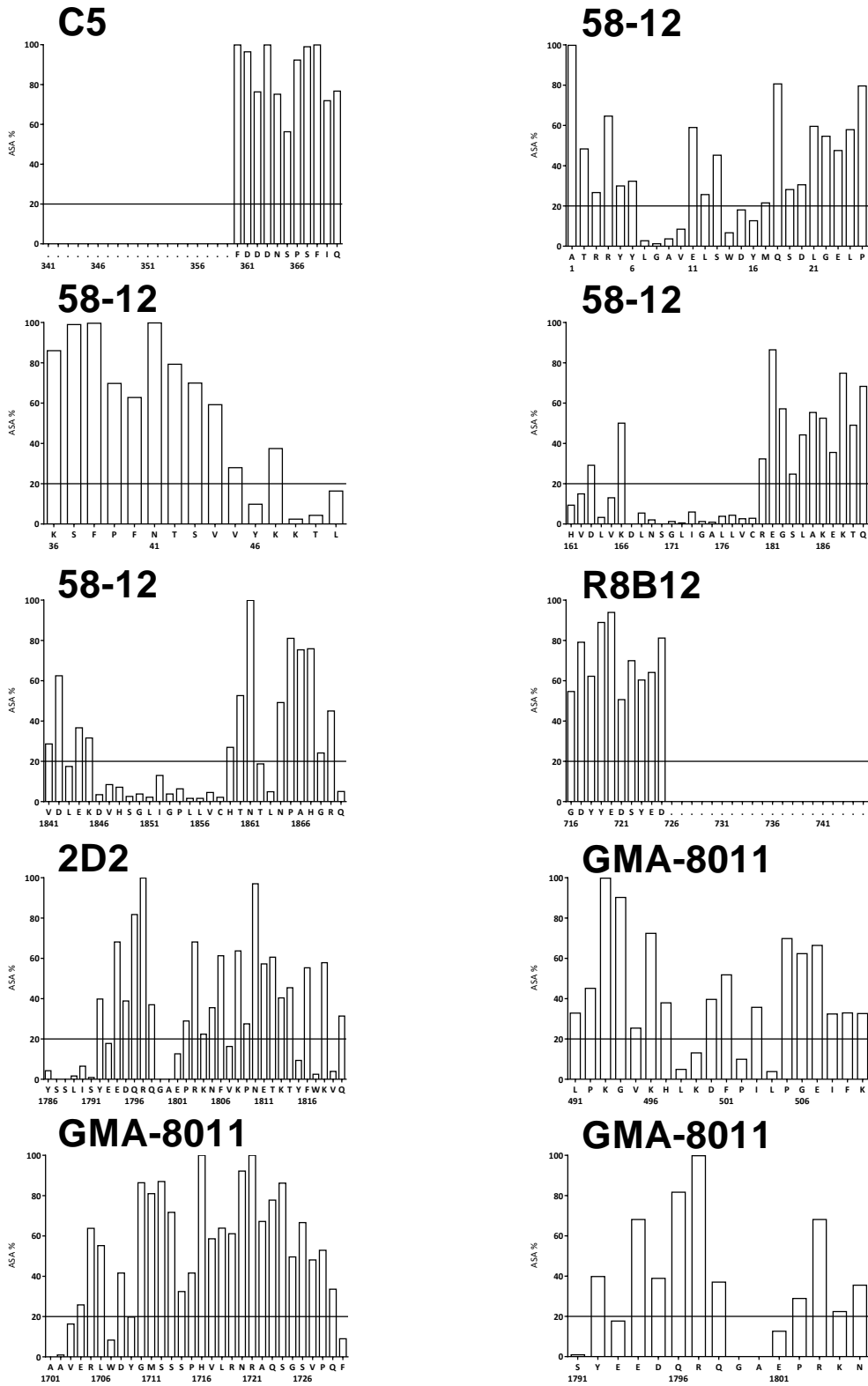


Figure 8.5: Solvent Accessibility of FVIII Monoclonal Antibody Epitopes. Regions denoted (.) represent areas not represented within the FVIII crystalline structure for which estimation of ASA was not possible.

497-510 and 584-593. No significant binding was observed within these two regions on either the linear or looped arrays. The epitope characterised in my data and the previously characterised discontinuous epitope share sequence similarity (75% identical) over a region of four amino acids: SAYL (727-730) and SWYL (584-587).

The fourth monoclonal antibody (2D2) demonstrates a shared epitope within the A3 domain at positions 1786-1820, (HRes 1801-1805, EPRKN) which is solvent accessibility in the residues 1802-1805 (Figure 8.4D and 8.5) Binding for the mAb falls within the domain previously characterised (Regan L., Bayer Healthcare, Berkley, USA) although there is no published experimental data of the sequence epitope. Additional binding is seen on the linear array within the B domain at positions 1216-1240 (HRes 1221-1235, KNFMKNLFLSTRQN). These two sequence regions on pairwise comparison, have 100% similarity (80% identical) for a linear section of five amino acid residues KNFVK (1804-1809) and KNFMK (1221-1225), which includes two of the residues of the high-resolutions epitope (K1804 and N1805).

No clear dominant epitopes were demonstrable for GMA-8011, a commercially available mAb previously described to bind to the C1 domain by ELISA ⁴¹⁸. There were 10 regions (Figure 8.4E) in which positive binding was seen, with no “shared” epitopes seen. Within this experiment the raw optical density for these peaks was lower than seen for the other monoclonal antibodies. Outside the B domain, binding was seen in the following regions 491-510 (A2), 736-760 (a2), 1701-1730 (A3) and 1791-1805 (A3). Although no significant binding was seen within the C1 domain, the binding seen within the A3 domain (1701-1730) is in a region directly adjacent to the C1 domain.

8.4.2 Amino Acid Sequence Epitope Mapping of Factor VIII Antibodies in Severe Haemophilia A

Samples from 13 patients with severe haemophilia A with a current inhibitor were analysed on both the linear and looped arrays. The median inhibitor titre for these samples was 26.0 BU/mL (range 0.8-750.0), with 10 having high-titre and 3 low-titre inhibitors (Table 8.3). The *F8* genotype was known for 9/13 patients which included intron 22 inversion (n=4), nonsense mutations (n=3), large deletions (n=1) and a small deletion (n=1). The *F8* genotype was not known for four patients, with no mutation being found in three and the result not being available for the fourth patient.

	Titre (BU/mL)	F8 mutation	Domain epitopes	Low resolution sequence epitopes	High resolution sequence epitopes
1	22	Not found	A1, a1, A2, a2, B, a3	11-30; 96-125; 321-365; 381-400; 706-725; 801-860; 896-920; 1226-1250; 1646-1670	11-30; 106-115; 336-360; 386-395; 711-720; 806-820; 841-845; 901-915; 1231-1245; 1651-1665
2	19	Not found	A1, a1, B, a3	106-130; 316-370; 931-950; 1261-1280; 1651-1665	111-125; 331-355; 936-945; 1266-1275; 1651-1665
3	193	i22 inv	A1, a1, A2, a2, B, a3	96-125; 316-365; 426-450; 586-610; 701- 725; 896-945; 1646-1670	106-115; 331-335; 341-360; 431-445; 591- 605; 705-720; 901-915; 926-940; 1651-1665
4	0.8	i22 inv	A1, a1, A3	231-255; 331-355; 1916-1940	236-250; 336-350; 1921-1935
5	80	Nonsense (Ex15)	A1, a1, B, a3	96-125; 326-355; 1261-1280; 1651-1665	106-115; 336-345; 1266-1275; 1651-1665
6	26	i22 inv	A1, A2, a2, B, A3	11-35; 231-255; 706-730; 841-870; 896-920; 1231-1255; 1911-1945	16-30; 236-250; 711-725; 851-860; 901-915; 1236-1250; 1916-1935
7	96	i22 inv	A1, a1, A2, a2, B	331-355; 701-725; 1151-1175	336-350; 706-720; 1156-1170
8	4	Nonsense (Ex14)	B	861-880; 1001-1020; 1056-1085; 1101-1130; 1211-1235; 1421-1435; 1466-1505; 1556- 1575; 1581-1605	866-875; 1001-1020; 1066-1075; 1111-1120; 1216-1230; 1421-1435; 1476-1500; 1556- 1575; 1586-1600
9	75	Large Del	A1, a1, B, A3	231-255; 326-350; 1051-1070; 1281-1305; 1921-1945	236-250; 331-345; 1056-1065; 1286-1300; 1926-1940
10	80	Not available	A1, a1, A2, a2, A3	191-205; 231-255; 326-355; 381-400; 701- 725; 1916-1940	191-205; 236-250; 336-345; 386-395; 706- 720; 1921-1935
11	13	Small Del (Ex18)	A1, a1, A2, B, A3	186-205; 231-255; 326-355; 451-465; 1001- 1025; 1871-1885	186-205; 236-250; 331-345; 451-465; 1006- 1020; 1871-1885
12	750	Not found	A1, a1, B, A3, C2	231-255; 341-365; 1001-1025 1916-1940; 2266-2285	236-250; 346-360; 1006-1020; 1921-1935; 2271-2280
14	4	Nonsense (Ex10)	A1, a1, A2, a2 [#] , A3	191-205; 231-255; 331-355; 381-400; 451- 465; 711-730; 1851-1885; 1916-1945	191-205; 236-250; 336-350; 386-395; 451- 465; 716-725; 1866-1880; 1926-1935

Table 8.3: Summary of domain and sequence epitope of patients with severe haemophilia A, with inhibitory antibodies using linear and looped peptide arrays. Colour coding refers to recurrent sequence epitopes and is the same as represented within Figure 8.6, demonstrating position of these sequences. Del=deletion. Ex=exon. # = A single amino acid of the a2 domain at position 730 is covered within this epitope.

Code	Array	Epitope (HRes)	Domain	Frequency	Functional regions	Previously Described
A	LIN	11-30 (16-30)	A1	2	-	Sugihara et al. ³⁹⁵
B	SHARED	96-125 (106-125)	A1	4	Calcium K107, E110, D116, E122, D125, D126 A1/A3C1C2 interaction site	Palmer et al. ³⁹⁴ , Sugihara et al. ³⁹⁵
C	LOOP	191-205 (191-205)	A1	3	-	Griffiths et al. ³⁸⁶
D	LIN	231-255 (236-250)	A1	6	Disulphide bond C248-C329 N-linked glycosylation N239	Sugihara et al. ³⁹⁵
E	SHARED	316-365 (331-360)	A1/a1	11	FX interaction site M337-R372 A1-A2 interaction site FVIIIa inactivation (APC/Xa) R336 Sulphated Y346 Disulphide bond C248-C329	Foster et al. ³⁸³ , Ware et al. ⁴¹⁵ , Huang et al. ⁴³³ , Griffiths et al. ³⁸⁶ , Sugihara et al. ³⁹⁵
F	LOOP	381-400 (386-395)	A2	3	A2-A1/A3C1C2 interaction Ser373-Trp393	Kopecky et al. ³⁹⁶ , Gharagozlou et al. ³⁹⁸ , Ware et al. ⁴³⁴
G	LOOP	451-465 (451-465)	A2	2	-	Palmer et al. ³⁹⁴ Gharagozlou et al. ³⁹⁸
H	SHARED	701-730 (706-725)	A2/a2	6	FIXa interaction site K707-N714 Sulphated Y718, Y718 and Y723	van den Brink et al. ¹³³ , Huang et al. ⁴³³ , Gharagozlou et al. ³⁹⁸
I	LIN	841-860 (N/A)	B	2	-	-
J	LIN	896-920 (901-915)	B	4	N-linked glycosylation N900	Palmer et al. ³⁹⁴
K	LIN/LOOP	931-945 (936-940)	B	2	N-linked glycosylation N943	Palmer et al. ³⁹⁴
L	LIN	1001-1025 (1006-1020)	B	3	N-linked glycosylation N1001 and N1005	Huang et al. ⁴³³
M	LOOP	1061-1070 (1066-1070)	B	3	N-linked glycosylation N1066	Huang et al. ⁴³³
N	LIN	1216-1250 (1236-1245)	B	3	FVIII haplotype SNP D1241	-
O	LOOP	1261-1280 (1266-1275)	B	2	-	-
P	SHARED	1646-1670 (1651-1665)	B/a3	4	VWF interaction E1649-R1689 Sulphated Y1664	Tiarks et al. ²⁴⁴ Huang et al. ⁴³³
Q	LOOP	1871-1885 (1871-1880)	A3	2	VWF interaction E1875	Griffiths et al. ³⁸⁶
R	SHARED	1916-1945 (1921-1935)	A3	5	-	Sugihara et al. ³⁹⁵

Table 8.4: Summary of recurrent inhibitor epitopes seen in clinical samples, with functional correlation to binding regions. LIN=Linear array, LOOP=Looped array. HRes=High-resolution.

Clear binding peaks were seen for 12/13 patients, although there was, predictably, more background signal with samples being polyclonal than for the mAb samples. There were 77 regions of positive binding (linear and looped), with a median of 6 epitopes per patient (range 3-9). Binding was seen to both the heavy and light chain in 11/13 patients and to the heavy chain alone in 2/13 patients. At a domain level, binding was seen to the A1 (12/13), a1 (11/13), A2 (7/13), a2 (6/13), B (10/13), a3 (4/13), A3 (7/13) and C2 (1/13) domains. No patients demonstrated positive binding within the C1 domain and one patient with a low-titre inhibitor (4BU/mL) demonstrated binding to the B domain alone. The sequence and domain epitopes are summarised in Table 8.3.

On analysis of the epitopes from each patient, it became apparent that some epitopes recurred in more than one patient (immunodominant). Summarising all of the positive binding epitopes (linear and looped) by 5-mer sequence demonstrated 18 candidate immunodominant epitopes which were seen in ≥ 2 patients (Table 8.4 and Figure 8.6). There were ≥ 3 solvent accessible residues for antibody interaction in all of these epitopes which are represented within the FVIII crystalline structure ($n=10$) (Figure 8.7). Binding within these characterised epitopes has previously been described in one of more patient in previous studies in 83% (15/18) of these regions. Of the epitopes which were seen outside of the B domain ($n=11$), binding was seen in 7 regions with structural or functional importance (Table 8.4).

The most frequently seen epitope (11/13) was a recurrent shared epitope between positions 316-365 (HRes 331-360) in the A1/a1 domains (Table 8.3 and Figure 8.6). The majority of the high-resolution epitope falls within a disordered region which is only partially represented within the FVIII crystalline structure (disordered 335-359). Residues surrounding this disordered region demonstrate substantial solvent accessibility (Figure 8.7). This epitope contains a paired C:P residues (C329:P330). This region of FVIII contains the FX interaction site (337-372)^{45,435}, a sulphated tyrosine residue (Y346) important for FVIII activation³⁹ and the APC inactivation site (R336)⁴⁸. This region also contains one of the interaction site between the A1 and A2 domains involved in maintaining FVIII stability. Epitopes within this region have previously been described by a number of different methodologies in clinical samples^{386;395;433}. A second shared epitope was seen in 5/13 patients in the a2 domain 701-730 (HRes 706-725, Figure 8.6). This region demonstrates solvent accessibility and paired tyrosine residue (Y718:Y719). This region contains one of the FIXa interaction sites (707-714)⁴³, 3 key sulphated tyrosine residues (Y718, Y719 and Y723)³⁹ and is in a region of previously

characterised inhibitor binding (Table 8.4). Binding to either/both of these two regions involved in tenase complex formation was observed in 12/13 patient.

A third shared epitope was seen at positions 96-125 (HRes 106-125) in the A1 domain in 5/13 patients. The region formed by the HRes epitope predominantly consists of bends within the secondary structure. Functionally, this region contains a predicted calcium ion site^{6,7} and specific residues (R121, E122) are involved in maintenance of FVIII structure through interaction with the A1 and C2 domain (residues Q2266, L2302, K2239)^{6,436} (Figure 8.6). A looped epitope was also seen in 3 patients in the A2 domain at positions 381-400 (HRes 386-395), a region containing paired H:Y residues (H384:Y385) forming a continuous beta-strand within the secondary structure. This region is involved in interaction between the A2 domain and A1/A3C1C2 dimer at positions 373-393⁴³⁷ (Figure 8.6).

Analysis of binding to the FVIII light chain identified recurrent epitopes in areas involved in the interaction between FVIII and VWF. The first of these was a shared epitope seen in 4/13 patients regions within the a3 domain between positions 1646-1670 (HRes 1651-1665). This region falls within a disordered region of the FVIII crystalline structure. The region between positions 1649-1689 has been described as being involved in maintenance of optimal conformation (for maximal binding) of FVIII to the VWF high-affinity binding site (1673-1689)²⁹. The second of these epitopes was seen in 2/15 patients on the looped (T2) array between positions 1871-1885 (1871-1880). Interestingly, this region demonstrates solvent accessibility at only three residues, which likely form the key determinants of antibody interaction at positions V1871, T1872 and D1884. This region contains both bends (T1872, V1873) and a region consisting of a beta strand Q1874-A1877 within which the VWF interaction residue (E1875) is observed.

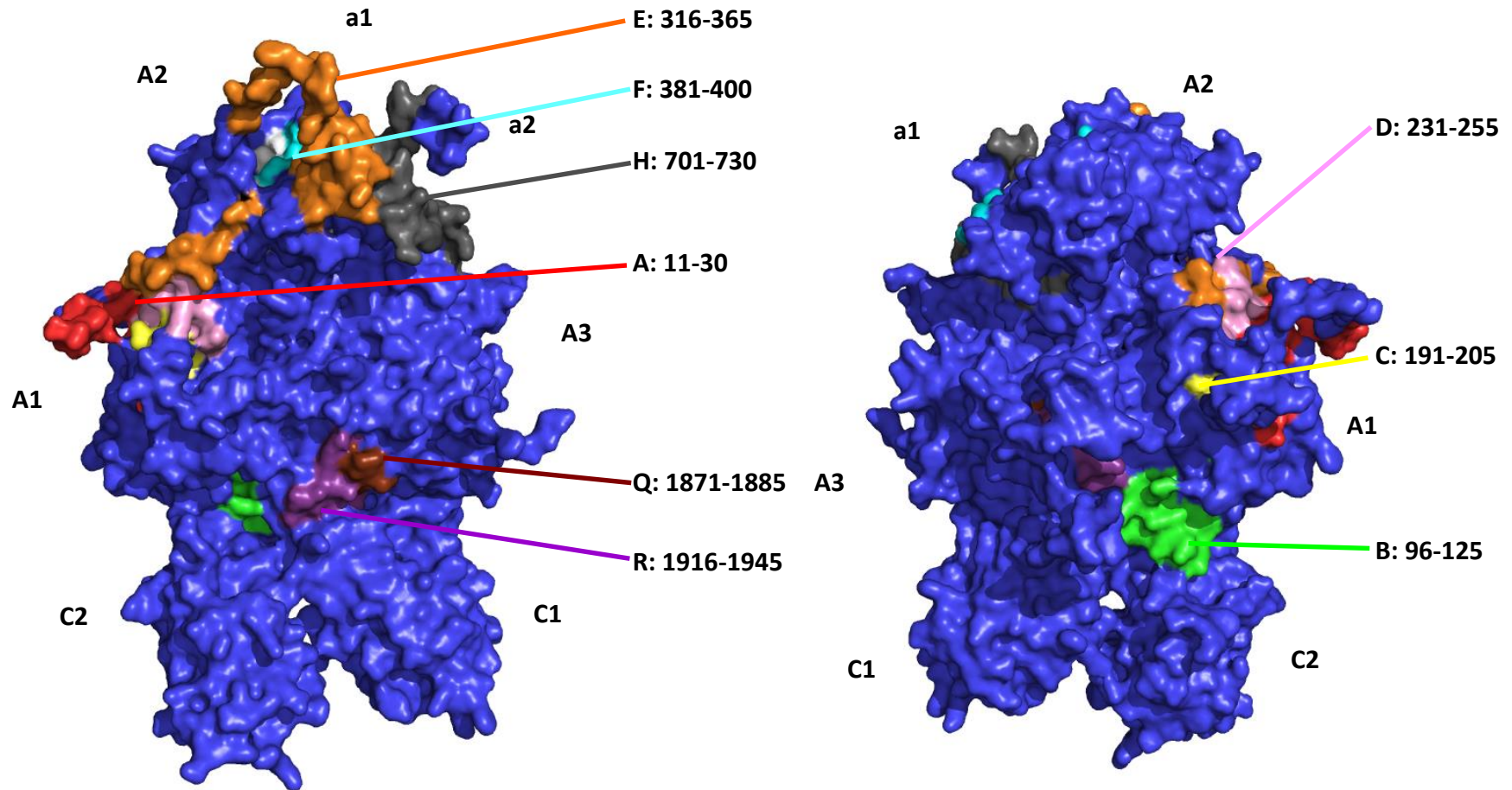


Figure 8.6: Sequence Epitope Mapping of FVIII Inhibitory Antibodies in Severe Haemophilia A. Epitopes shown represent the low resolution epitopes.

8.5 Discussion

These high-throughput peptide microarray experiments have demonstrated amino acid sequence epitopes for both monoclonal and polyclonal FVIII antibodies. Assays were possible using small volumes of patient plasma or monoclonal antibody without requiring purification and demonstrated re-usability without antibody carryover. In patients with severe haemophilia A with inhibitors, recurrent regions (clusters) of binding on the FVIII surface were identified, consistent with immunodominant B-cell epitopes, which were located in regions of functional (tenase and VWF interaction sites) or structural importance (e.g. inter-domain interaction sites). Where structural information was available, all of these regions contained solvent accessible residues (≥ 3 aa) for antibody interaction. In keeping with previous studies, seven of the epitopes contained paired aa, previously shown to occur with increased frequency within B-cell epitopes. Functional and structural correlation between the epitope sequences and previously described *in-vitro* data has provided putative *in-silico* mechanism of FVIII inhibition in these samples.

8.5.1 Epitope Characterisation of Factor VIII Monoclonal Antibodies

Within my experiments characterising the B-cell epitopes of FVIII mAbs, binding was seen within the previously described domains for 4/5 antibodies. Of these, the C5 mAb demonstrated identical binding to that which has previously been described³⁸³. Within these experiments, application of the raw data in combination with positional information has given a putative discontinuous epitope for a monoclonal antibody within the A1 domain (58-12) that has so far only been characterised at a domain level. Binding for the A2 domain mAb R8B12, although falling within the same region as previously described by immunoblotting (563-740), differed from the discontinuous epitope described by affinity directed mass spectrometry (497-510 and 584-593)⁴¹⁷ with a lack of binding seen in these regions on my experiments performed in duplicate. Whether this represents a limitation of this peptide microarray approach compared to this study using affinity directed mass spectrometry is not clear. Comparison of these data is however confounded by differences in coverage of the A2 domain between my experiments (100%) compared to those using affinity directed mass spectrometry (70%) which did not have complete representation of the C-terminal region of the A2/a2 domain.

8.5.2 Factor VIII Immunogenicity and B-Cell Epitopes

I have detected immunodominant epitope profiles in 12/13 patients in regions associated with tenase complex formation (FX or FIXa interaction sites). The predominant B-cell epitope (11/13 patients) was within the a1 domain between positions 316 and 365, which includes the FX interaction site and sites involved in FVIII activation/ inactivation. A second recurrent epitope was seen in around half (6/13) of these patients within one of the sites of the FIXa interaction sites within the a2 domain (707-714)⁴³. Binding within these regions in the a1 (FX)^{383;386;395;415;433} and a2 (FIXa)^{391;433;438} domains, involved within the tenase complex formation, have been described previously and my data provides further supportive information regarding this being an important mechanism of inhibitor action in severe haemophilia A (Table 8.4). A further recurrent epitope was seen in the A1 domain (96-125), within a calcium ion containing region involved in interaction between the A1 and light chain (A3C1C2)⁴³⁶ which has again been characterised in small numbers of patients previously^{394;395}. Although antibody binding to the A1/a1 domain is seen less frequently than other domains (A2 and C2), binding within the a1 domain between positions 351-365³⁸³ and 338-362⁴¹⁵ has been previously characterised. Although the precise crystal structure of the a1 domain has not been characterised⁷, given the functional roles of this region involving protein-protein interactions, it is likely that this would be solvent accessible. Although binding to the A1 domain is less common, a study by Palmer et al., demonstrated epitopes in 3/6 patients with congenital haemophilia A with binding occurring in one region that was also seen within my experiments (96-125)³⁹⁴. Studies of FVIII antibody epitope profiles towards the heavy chain of FVIII have demonstrated that a large proportion of patients contain epitopes towards the A2 domain. A recently published study investigating the diversity of the immune response to the A2 domain has shown overlapping epitopes covered the majority of the surface of the A2 domain for 29 anti-human A2 domain mAbs produced in a murine haemophilia A model⁴³⁸. Most of these murine antihuman mAbs, recognised a previously described epitope bounded by R484-I508 and resulted in non-competitive inhibition of the tenase complex. Other epitope regions identified involved in inhibition of FVIII activation by thrombin or were non-neutralising. Similar to these experiments, one of the first epitopes characterised in the A2 domain as being immunodominant by homolog scanning mutagenesis with a hybrid human/porcine FVIII molecules was found between positions 484-508 using the murine monoclonal antibody (mAb 413)³⁷⁷. This interfered with FX binding and non-competitively inhibit the tenase complex. More recent work investigating epitope profiles in patients with

severe haemophilia A has not demonstrated consistent binding within this region ³⁹⁸, in keeping with the results seen in my experiments. A second immuno-dominant epitope has been described in the terminal acidic region of the A2 domain (a2) between positions 712-736 ³⁹¹, a region in which binding was seen in more than half of the inhibitor samples tested within my experiments.

Within my experiments, the predominant area of binding to the light chain of FVIII (3 recurrent epitopes) was in the a3/A3 domain. Two of these epitopes fall within regions that are involved with VWF interaction (Table 8.4). Significant binding in the C2 domain was only seen for one patient (2266-2285), which is in contrast to previous studies ³⁸⁴. The C2 domain has previously been described to contain immunodominant epitopes at the N (2181-2243) and C (2248-2312) terminal regions of this domain. FVIII antibodies binding to the C2 domain have been previously described as either classical (inhibit tenase complex formation on phospholipid membranes) or non-classical (inhibit FVIII activation by thrombin and/or FXa). Whether this lack of binding to the C2 domain in my experiments represents an assay limitation or differences in the characteristics of these C2 domain binding antibodies is not clear, which is discussed in more detail in the following section. It is interesting to note that other groups using peptide arrays have also only identified small numbers of epitopes within the C2 domain relative to those seen in the A2 domain ³⁹⁸.

It was interesting to note that five of the epitopes seen in the B domain occurred in regions containing N-linked glycosylation sites. It is possible these represent areas of increased immunogenicity in patients treated with rFVIII concentrates due to differences in glycosylation seen at these sites from non-human cells lines (CHO and BHK) ¹⁰⁴. Within previously described epitope mapping platforms, only two studies have included representation of the B domain either in large fragments ⁴³³ or partial coverage (760-960 and 1411-1667) ³⁹⁴, with binding being characterised within all of these regions. Unfortunately, no details of treatments received by the patients included in my analysis were available to further assess this hypothesis and due to lack of N-linked glycosylation as part of this assay the relevance of these findings is not clear.

8.6 Limitations

There are a number of limitations with the epitope mapping platform used within these experiments. Although providing clear descriptions of epitopes within the heavy chain and N-terminal region of the light chain (a3/A3 domains), there was a lack of binding in other light chain regions. In the clinical samples tested, only one patient demonstrated significant binding within the C2 domain and no clear binding epitopes were seen for a C1 domain mAb (GMA8011). These findings are in contrast to previously characterised B-cell epitopes in patients with severe haemophilia A. Due to an error which occurred in the final design and manufacture stage at Pepscan, prior to my commencement of these experiments, the arrays utilised gave incomplete coverage of the C-terminal region of C2 domain (2291-2332). This error, unfortunately, only became apparent during data analysis after the maximum number of samples that could be tested had been reached. Given the costs and time associated with plate redesign to provide full FVIII coverage, it was not possible to repeat testing to further investigate this lack of C2 domain binding. Nevertheless, in view of clear epitope peaks being seen, an analysis was performed acknowledging that this limitation is present which may have excluded B-cell epitopes within the C2 domain. An alternative explanation for lack of binding seen within the light chain could relate to structural (secondary/tertiary) differences between the heavy and light chain. Antibodies binding to the light chain could potentially display more complex epitopes, as has been previously described from the crystal structure of a human monoclonal antibody (Bo2C11) in complex with the C2 domain ⁴⁰⁰. Although this represents the epitope for a single mAb which may not be representative for other C2 domain antibodies' binding, *in-silico* predictions of epitopes in the C2 domain support the complexity of epitope profiles seen within this domain of the FVIII structure ⁴³⁹. Similar difficulties in characterisation of light chain antibody epitopes have also been reported by other investigators (Shannon Meeks, Associate Professor, Emory University, Atlanta, personal communication).

There are limitations to all currently available epitope mapping platforms in the evaluation of a polyclonal immune response. In the use of synthesised peptide sequences, this may favour towards identification of linear epitopes and subsequently not allow detection of conformational or discontinuous B-cell epitopes. Using this type of peptide array, with conformational mimics, although helping providing closer representation of native protein structures could in some parts, artificially disrupt relevant epitopes. Other approaches such as mass spectroscopy may be limited in their representation of regions of interest, due to

relying on FVIII cleavage (e.g. thrombin), which could result in difference in secondary/tertiary structure once antigenic regions are not constrained within the full protein conformation. A further limitation of this (and similar) approaches, is that it is more likely to identify antibodies with higher binding affinities and cut-offs assume similar binding kinetics across the surface of FVIII. One difficulty in defining thresholds for epitope mapping studies at the time these experiments were conducted was a lack of positive controls (mAb) with known amino acid sequence epitopes. The absolute and relative cut-offs applied were pragmatic cut-offs based around evaluation of binding across the whole dataset. Usage of these thresholds, however, allowed systematic assessment of data between experiments, which were likely to represent significant FVIII antibody binding. This assay and similar peptide arrays lack in-assay positive and negative controls, which would be of use in discriminating between lack of binding or assay failure. Inclusion of such modifications within future plate design may allow more precise definition of cut-off, standardised for each array plate. This could include negative (e.g. poly-A tails or a lack of plate antigen) and a positive control (e.g. sequence epitope for testing a strongly positive mAb) wells being included within the plate design. In plate redesign, this would include all regions of the FVIII to allow coverage of the C-terminal region of the C2 domain, although changes in sequence length or regions represented would be required to allow this to fit with the 455 available microwells. Finally, due to the commercial nature of this platform there is limited information available in the public domain regarding methodology to allow comparison with other peptide arrays.

8.7 Conclusions

A high-throughput epitope mapping platform has allowed characterisation of B-cell epitope profiles of monoclonal and polyclonal FVIII antibodies, using low sample volumes and demonstrating re-usability. Within clinical samples tested, immunodominant epitopes were seen in regions of functional and structural significance. Usage of constrained peptides within a looped (T2) array, provided an *in-vitro* mimic of primary and secondary structure. This T2 array demonstrated higher specificity for epitope representation, with a reduction in non-specific binding attributable to regions of sequence similarity that was seen in the linear (T1) array. This approach offers a high throughput methodology for epitope mapping of clinical samples which may provide greater insights into the immune response to FVIII in larger cohort studies.

Chapter 9: Next Generation Sequencing (RNA-Seq) to Identify Prognostic Variables for Inhibitor Formation in Severe Haemophilia A

9.1 Introduction

Previous observational studies in severe haemophilia A have demonstrated a strong genetic component to inhibitor formation. A family history of inhibitor formation gave a three-fold (RR=3.2; 95%CI 2.1-4.9) increased risk of inhibitor formation within the Malmö International Brother Study (MIBS) ¹⁷⁸. Of the genetic factors investigated to date the underlying class of *F8* gene mutation appears to be one of the most significant risk factors. A meta-analysis of 30 published studies (5383 patients, including 1029 inhibitor patients), demonstrated that the risk of high-titre inhibitor formation (comparator intron 22 inversion) was greatest in patients with large deletions (OR=5.18; 95%CI 3.35-7.99), with this risk being greatest when these deletions involved multiple exons (OR=14.79; 95%CI 7.79-28.11) ¹⁷⁷. Alongside the underlying *F8* mutation there is an increased incidence of inhibitor formation associated with black or Hispanic ethnicity, although the biological mechanisms for this are not clear (discussed in Chapter 1). Variable risk has been described in single nucleotide polymorphisms in the genes involved in the immune response (CTLA-4, TNF- α , IL-10) ^{188-191;440-443} and some HLA Class II alleles ¹⁸⁶⁻¹⁸⁸. A more detailed assessment of genetic risk has been presented in a large study of 833 subjects from 3 independent cohorts, investigating 1081 genes involved in the immune response or immune modifier genes (13,331 SNPs). This identified 13 SNPs, associated with either increased (n=5) or reduced risk (n=8) of inhibitor formation risk, including SNPs within the following genes: CD44, CSF1R, DOCK2, MAPK9, and IQGAP2. ¹⁹⁴.

Recently advances in sequencing technology and a reduction of costs has made this technology more accessible for usage within clinical studies. These approaches allow detailed evaluation at a DNA (genome wide or exome mapping), RNA (transcriptome / gene expression), proteomic and epigenetic level. Alongside investigator lead studies, population based genomics projects such as the 100,000 (100k) Genome Project will create vast open-access repositories of genomic/transcriptomic data to increase the understanding of the pathogenesis of disease processes. It is hoped that these studies will herald an era of genomic diagnostics and personalised medicine. Within the haemophilia setting, it is hoped these approaches may allow prediction of patients who will develop inhibitors and allow identification of patients in whom ITI is unlikely to be successful. These approaches may allow personalised management of patients at high (or low) risk of inhibitor formation to modify

replacement therapy and potentially justify novel interventions (e.g. selective or targeted immunomodulation) in particular high risk individuals.

Although studies have described the epidemiology of inhibitor formation in severe haemophilia A in some detail, there is still an incomplete understanding of this process. Clinical studies at the initiation of FVIII treatment offer a unique opportunity to further develop an understanding of the allo-immune response. Analysis of changes occurring in the gene expression (transcriptome) will allow assessment of dynamic change occurring on exposure to a novel stimulus. Usage of Next-Generation Sequencing (NGS), by RNA-Seq allows quantitative assessment of the whole transcriptome including both coding (messenger RNA, mRNA) and non-coding/regulatory (including micro RNA, miRNA). Within such studies, patients are treated with a single protein therapeutic with regular laboratory testing for antibody formation and recording of relevant clinical data occurring at the time of treatment. In the design of such studies in severe haemophilia A there are however, a number of sampling considerations. Due to the early age of commencement of FVIII treatment (median 9.8 months)¹⁴⁹ and the blood sample requirements for evaluation of safety and efficacy, the blood volume available for additional studies is limited. Alongside this when collecting samples for transcriptome analysis, pre-analytical variables may artificially induce changes in mRNA expression without adequate consideration. Following venepuncture blood cells are exposed to a change in environment and new stimuli, which results in changes in the cellular activation status and/or *ex-vivo* change in mRNA expression (induction or downregulation). Speed of blood sampling⁴⁴⁴, sample tube surfaces⁴⁴⁵, activation of coagulation⁴⁴⁶, endotoxins⁴⁴⁷, contents of lysed cells (e.g. haemoglobin)^{448;449} and endogenous nucleases⁴⁵⁰ have all been shown to affect mRNA expression previously.

Routinely available anticoagulated sample tubes, which do not contain RNA stabilisation media, could potentially affect cytokine expression in whole blood samples. Whole blood collected into EDTA sample tubes and incubated at room temperature (7 days), resulted in increases in IL-6 and TNF- α expression and an initial decrease in IL-1- β expression (6 hours) with subsequent increases between 1-3 days. In comparison whole blood in a sample tube containing an RNA stabilisation media (PAXgene, PG) did not demonstrate significant differences in mRNA expression for these cytokines up to 7 days⁴⁵¹. This supports the observation that *ex-vivo* changes in gene expression following blood sampling can be blunted either, by the addition of a transcription factor inhibitor such as actinomycin-D⁴⁵² or usage of RNA stabilising media containing tubes as in the aforementioned PG tube⁴⁵¹⁻⁴⁵³. There are

two commercially available storage solutions for RNA collection from whole blood, the PAXgene and Tempus™ RNA systems, which require 2.5mL and 3mL of blood respectively.

Although in an adult study the blood volume required for RNA storage tubes is of no significant concern, this may not be the case in paediatric studies, especially where there is requirements for multiple blood tests for efficacy and safety. For investigators looking to reduce blood volume requirements, this either means allowing under (and variable) filling of commercially available sample tubes or the development of modifications to currently available sample tubes. Usage of under-filled Tempus™ RNA tubes has resulted in suboptimal values for RNA yield, integrity and purity (260/280 and 260/230 ratios) ⁴⁵⁴. Usage of a modification allowing maintenance of the ratio of storage reagent media to whole blood has been described previously. The first study collected small blood volumes (50-300µL) from mice, using maintained ratios of blood to PG media ⁴⁵⁵. Even using sample volumes of 50µL the mRNA obtained was of sufficient quantity (average 2.3µg) and quality to allow multiple gene expression determinations. The second study was carried out to investigate invasive pneumococcal disease in children in Malawi (n=87, median age 3.25 years, range 0.17-13 years) ⁴⁵⁶. This study firstly, evaluated using smaller blood volumes (maintaining the blood to PG media ratio) of 2.5ml, 1ml and 0.3ml, demonstrated total RNA yields of 4.5-11.6µg, 5.1-8.3µg and 1.6-5.0µg respectively, with high RNA integrity (RIN 8.2-9.6) and purity (260/280 ratios 1.97-2.14). Although the lowest blood volume (0.3mL) gave sufficient mRNA for multiple RT-PCR experiments this did not consistently produce sufficient mRNA for application on a microarray ⁴⁵⁶.

Changes in gene expression have been described at first exposure to a novel stimulus and have provided novel biomarkers for the prediction of risk in another clinical setting, such as response to influenza vaccination ⁴⁵⁷. There is only one case-control small (n=20) study comparing gene expression profiles in severe haemophilia A ¹⁹⁵. This study demonstrated downregulation of CCL3L1, CCL3L3, CXCL7 and IL8, with upregulation of CXCL9 and CXCL11 in inhibitor patients. This study took a cross-sectional (rather than longitudinal) approach and it is not clear from the methodology how samples were collected. This approach may mean that gene expression may be affected either by environmental factors occurring at the time of blood sampling or from sample collection methodology. To allow a more structured dynamic evaluation of changes in transcriptome profiling prospective clinical study is required. To this end I have developed a protocol and initiated sample collection, longitudinally evaluating changes in gene expression profiles at first exposure to FVIII or

during ITI in severe haemophilia A. This forms part of an international collaboration as a satellite study within the trial of a new human cell line rFVIII concentrate (Nuwiq®) in previously untreated patients (PUPs) with severe haemophilia A.

9.2 Hypothesis and Aims

As part of an investigator lead collaboration, I have developed a protocol (Appendix 3) for the prospective sampling of total and miRNA (RNA-Seq) at first exposure to FVIII and during ITI in severe haemophilia A. This is being performed within an international study (GENA-05 / NuProtect study) of previously untreated patients (PUPs) treated with a new human cell line (HEK293) rFVIII concentrate (Nuwiq®). To minimise sample volume requirements for this paediatric study, I have tested the application of a modified PG RNA storage tube, created within our laboratory. Within this chapter, the application of this low volume sampling for application in genomic studies will be evaluated in healthy volunteers and boys with severe haemophilia A. I hypothesise that this modified sample tube will allow blood sampling using a reduced blood volume (1mL) providing RNA (total and miRNA) of sufficient quantity and quality for use in transcriptome analysis by next generation sequencing (NGS). This methodology for sample collection and storage will be acceptable to paediatricians and allow evaluation of changes in gene expression at first exposure to FVIII and during ITI in patients with severe haemophilia A. This will allow high-throughput global assessment of changes occurring in patients who are at risk (or no risk) of inhibitor formation in severe haemophilia and identification of novel biomarkers of inhibitor risk. The specific aims are as follow:

- 1) To validate usage of a modified RNA storage tube for collection of peripheral blood samples for transcriptome (mRNA and miRNA) analysis in healthy volunteers.
- 2) To evaluate the effect of pre-analytical (clinical and laboratory) variables on quantity and quality of RNA collected using this modified tube.
- 3) To demonstrate validity of using this modified RNA storage tube for transcriptome analysis in patients with severe haemophilia A at first exposure to FVIII and to demonstrate acceptability for use in paediatric clinical studies.

9.3 Materials and Methods

Modified Sample Tube Production Protocol

Modified PG blood tubes were prepared in RNase free conditions within a positive pressure tissue culture hood as described earlier (Chapter 3).

9.3.1 NGS Pilot Study Materials and Methods (Part 1)

Recruitment of Study Participants and Blood Sampling (NGS Pilot Study)

Normal healthy volunteers were recruited from staff and students at Barts and The London School of Medicine and Dentistry (Whitechapel Campus) and The Royal London Hospital. The inclusion criteria were: staff or student; age ≥ 18 and ≤ 65 years and the ability to give informed written consent. Participants not meeting these criteria, or who were currently taking immunosuppressive or chemotherapeutic agents were excluded. No NHS patients or vulnerable groups were recruited. Anonymised demographic data on age and gender of participants was collected. The study was approved by proportionate review by the Joint Research Management Office (Barts Health NHS Trust and Queen Mary University of London), ReDA Reference: 008864. All sample tubes were kept at RT (18-25°C) prior to sampling. Blood was collected into the modified PG tube using the same protocol as in the GENA-05 study (Appendix 3). Blood collected into the standard, unmodified PG tube was collected as per the manufacturer's recommendation. Following collection of blood into sample tubes these were gently inverted 8-10 times to allow mixing of blood with PG media and sample tubes were incubated at room temperature and frozen directly to $< -80^{\circ}\text{C}$ until extracted.

Phase 1: Investigation of Pre-Analytical Sampling Variables

Within the first phase of this study blood was collected from 10 healthy volunteers. The aim of this phase was to evaluate the performance of the modified sample tube in comparison to the standard PG tube and the effect of simulated pre-analytical sampling variables. Within this the internal control for comparison were 2.5mL (standard PG) and 1mL (modified PG) samples incubated for 4 hours and frozen at -80°C for 2 months. Pre-analytical variables evaluated were sample freezing duration (no freezing/immediate extraction, 2 months, 4 months), sample incubation time (< 60 minutes, 4 hours and 24 hours) and sample fill volume (0.5, 1 and 1.5mL).

Phase 2: Assessment of Inter-Batch Variability

The second phase of this pilot study was performed to evaluate whether differences in batches of PG media or extraction kits affected RNA quality and quantity. A secondary aim of this phase was to evaluate lower than expected RNA integrity (RIN) values obtained in the first phase of the pilot study. Samples were collected from 5 further healthy volunteers, into 1mL modified PG tubes (total 4) containing PG media from 2 separate batches (i.e. different lot numbers and expiry dates). These samples were incubated at room temperature for 4 hours prior to freezing at -80°C for 2 months. Following thawing of samples these were extracted using one of two extraction kits, consisting of the same kit used in the previous phase and a second kit used by another group within our laboratory.

Phase 3: Investigation of Feasibility of miRNA Extraction and Protocol Comparison

In the third phase of this pilot study, feasibility of usage of the modified (1mL) PG tube for extraction of miRNA was assessed, in comparison to the standard (2.5mL) PG tubes. Blood samples were collected from 5 further healthy volunteers into modified and standard PG tubes. A comparison was also made of two different miRNA extraction protocols, which were based on extraction of all RNA, including miRNA into one tube (extraction protocol B) or a protocol in which miRNA and mRNA were collected into two separate sample tubes (extraction protocol C). Finally a further comparison was made to provide additional assessment of the standard and modified PG tubes for quality and quantity of total RNA, to assess the effect of these extractions over time.

Extraction of Total RNA and miRNA

Manual purification of total RNA and miRNA were performed within the Genome Centre QMUL Core Facility, following the manufacturers' protocols for whole blood RNA extraction as described earlier (Chapter 3).

Measurement of RNA Quality Control

Quantification of total RNA extracted was performed by spectrophotometry using the NanoDrop 8000 spectrophotometer (Fisher Scientific, Wilmington, DE, United States). This gives a report of the total RNA concentration with the eluate (ng/μL), with the total RNA yield in μg being equal to the RNA concentration x 40 x 0.01. For the purpose of downstream analysis, a minimum RNA yield of ≥1μg was targeted. Estimation of RNA quality was

performed through assessment of RNA integrity and purity using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, United States). RNA Integrity numbers (RIN) was calculated using the Agilent 2100 software⁴⁵⁸. This methodology provides a high-throughput standardised approach for the reporting of RNA integrity, which is reported on an ordinal scale ranging from 0 (poor quality) to 10 (highest quality). A RIN value of >5 is thought to be adequate and >8 optimal for downstream analysis⁴⁵⁹. Purity of extracted RNA was evaluated using the 260/280 and 260/230 ratios. These two ratios measure the absorbance by RNA at 260nm and contamination at 280nm (protein) and 230nm (salts, including guanidine thiocyanate and organic contaminants such as phenol or aromatic compounds). For the primary measure of RNA purity (260/280) a ratio of >1.8 is assumed to represent pure RNA suitable for downstream analysis^{460;461}. For the secondary RNA purity measure (260/230) pure RNA should demonstrate a 260/230 ratio of approximately 2.0, although there is no consensus internationally as to what represents the lower limit of acceptability for this ratio.

9.3.2 GENA-05 (NuProtect) Gene Expression Sub-Study (Part 2)

An assessment of changes in gene expression occurring at first exposure to FVIII and during ITI patients is currently underway using this modified PG sample tube, within the GENA-05 (NuProtect) study. This is an international phase 3 study (ClinicalTrials.gov identifier: NCT01712438) of a human cell line (HEK293) rFVIII concentrate (Nuwiq®, Octapharma AG, Lachen, Switzerland). This study aims to assess immunogenicity of this product in the first 100ED, in previously untreated patients with severe haemophilia A. As part of this study, we are co-ordinating an investigator led study of changes in gene expression with an aim of identifying those changes that indicate potential inhibitor formation / failure of ITI and to provide mechanistic insight into these poorly understood process. Samples are being collected in this study for all patients enrolled in this sub-study at the following time-points: at first exposure to FVIII and after every 3-4 ED up until 20ED or inhibitor formation. In patients diagnosed with an inhibitor undergoing immune tolerance induction samples were collected prior to commencement of ITI and then at 2, 4, 8, 12, 16, 20 and 24 weeks. Modified PG sample tubes used for the GENA-05 sub-study were prepared as described in Chapter 3. These were shipped overnight in sealed cryoboxes at ambient temperature to a centralised Clinical Trials management / logistics facility (AdminOps, LabCorp, Marken, Hamburg, Germany) for sample tube pack production for distribution to study sites.

Statistical Analyses

Analysis of the pooled anonymised data for these experiments was made. Descriptive statistics (mean, median, standard deviation and 95% confidence intervals) are displayed for continuous variables. Exploratory parametric statistical comparisons were made for these experimental data. For continuous data, these were performed using the paired T-test or repeated measures ANOVA (one or two way). Assessment of sphericity within these repeated measures ANOVAs was performed using Mauchly's test⁴³¹ and where significant (i.e. sphericity violated) corrections to the degrees of freedom were made using the Greenhouse-Geisser correction⁴³². Where significant difference was found on univariate testing (ANOVA), multiple comparisons were performed using the post-hoc Turkey (one-way ANOVA) and Bonferroni (two way ANOVA) tests.

9.4 Results

9.4.1 Gene Expression (RNA-Seq) Pilot Study

A total of 20 healthy volunteers (8 male and 12 females), with a median age of 35 years (range 26-63) were recruited to the three phases of this pilot study. This resulted in a total of 150 samples being collected into standard (n=25) or modified (n=125) PG blood tubes. RNA extraction was possible in all samples, including one which demonstrated excessive coagulation.

9.4.1.1 Quantitative and Qualitative Effect of Modification to the Standard PAXgene Tube

Assessment of the modification to the PG tube was made in samples from 15 individuals in two phases of recruitment (phase 1 and 3). Significantly lower mean total RNA yields were obtained from the modified PG tube ($2.66 \pm 1.23 \mu\text{g}$) compared to the standard PG tube ($6.12 \pm 3.55 \mu\text{g}$, $p=0.0002$). The mean RNA yield was 2.3 times lower than for the modified RNA tube, closely matching the 2.5 fold reduction in sample volume. A small, but statistically significant difference was seen for the mean RNA integrity (RIN) for samples collected into the modified PG tube (6.8 ± 1.7) compared to the standard PG tube (7.8 ± 0.9 , $p=0.02$). All samples demonstrated high RNA purity (260/280), with values ≥ 2.0 . Samples collected into the modified PG tube (2.24 ± 0.13) demonstrated a small but statistically significant difference in 260/280 ratio in comparison to the standard PG tubes (2.15 ± 0.05 , $p=0.017$). There was no significant difference in 260/230 ratios comparing samples collected into the standard PG (1.18 ± 0.55) and modified PG tube (1.02 ± 0.72 , $p=0.191$)

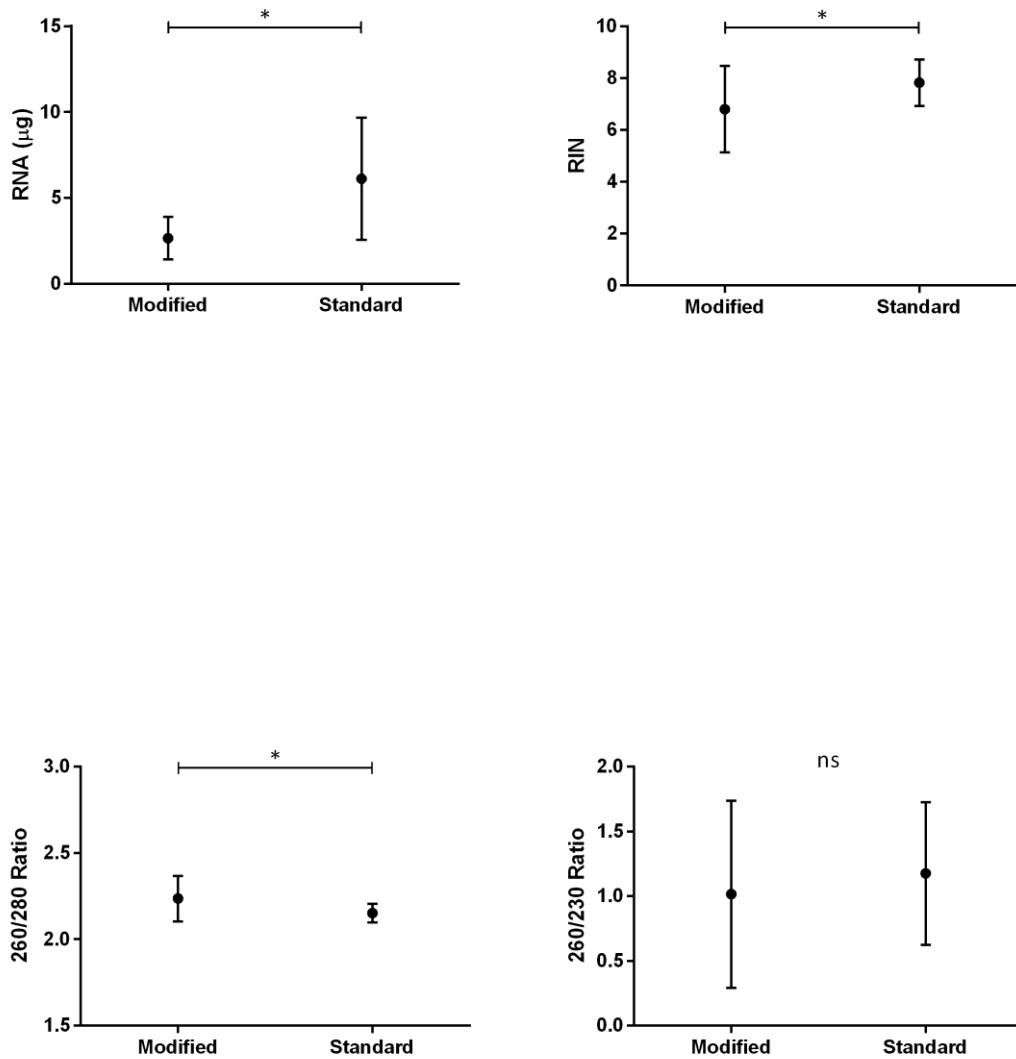


Figure 9.1: Quantitative and Qualitative Effect of Modification to the Standard PAXgene Tube. ns=Not significant. *=Significant p<0.05.

9.4.1.2 Quantitative and Qualitative Effect of Clinical Variable on Whole Blood RNA in Healthy Volunteers (Phase 1)

In the first study phase an assessment was made of pre-analytical variables that might have quantitative or qualitative effects on RNA obtained in 10 healthy volunteers.

9.4.1.3 Quantitative and Qualitative Assessment of Freeze Duration

An assessment of the effect of sample freezing was made comparing samples collected into the modified PG tube with RNA extraction being performed either immediately (control) or after freezing at $<-80^{\circ}\text{C}$ for either 2 or 4 months (Figure 9.2). All samples were made incubated at RT for 4-6 hours following venepuncture. A significant difference was seen for total RNA

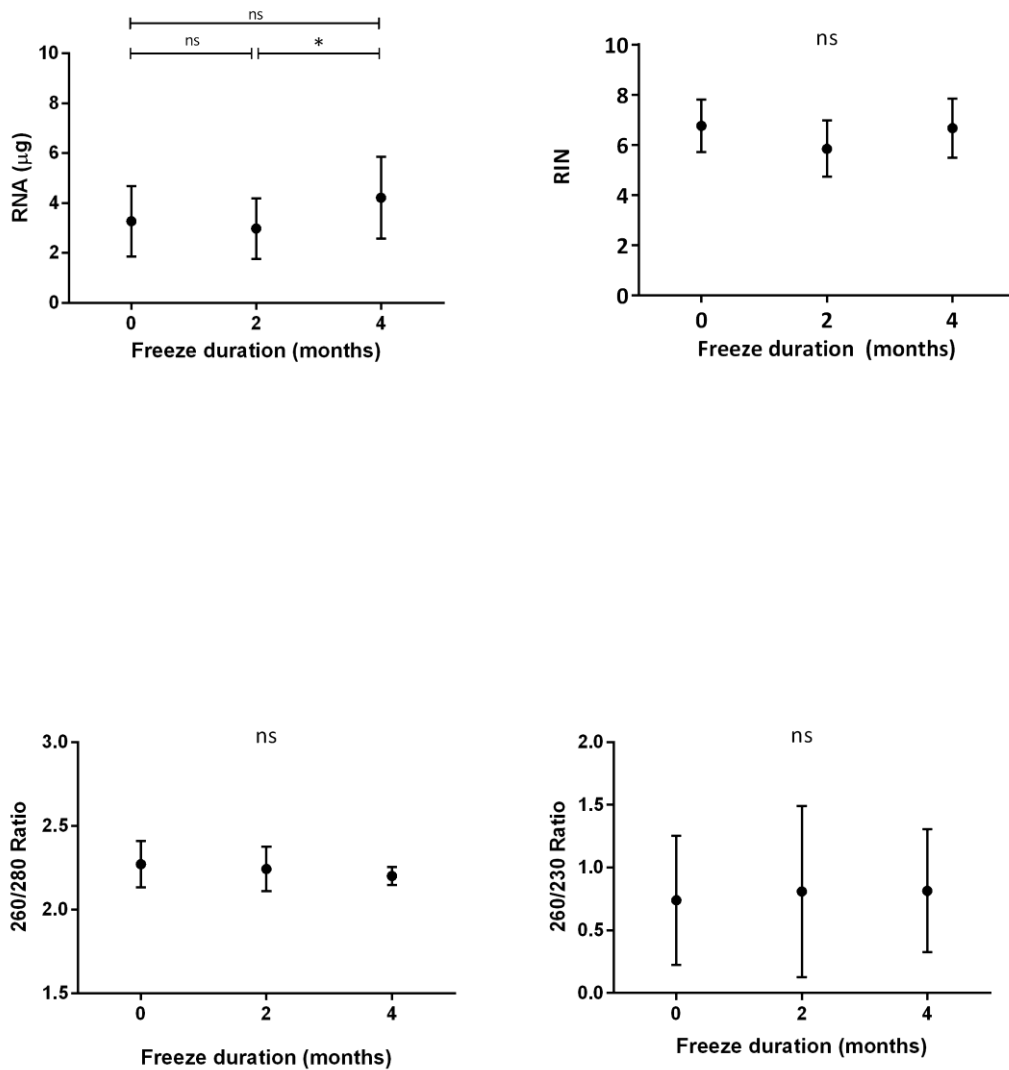


Figure 9.2: Quantitative and Qualitative Assessment of Freeze Duration. ns=Not significant. *=Significant $p < 0.05$.

yield and freeze duration ($p=0.009$). Surprisingly, higher RNA yields were seen for samples frozen for 4 compared to 2 months ($4.21 \pm 1.63 \mu\text{g}$ v $2.98 \pm 1.21 \mu\text{g}$, $p=0.008$), although these difference may represent variance in extraction yields between extraction batches. No significant difference in RNA yield was seen comparing sample extracted immediately

($3.27 \pm 1.42 \mu\text{g}$) or after freezing for either 2 ($2.98 \pm 1.21 \mu\text{g}$, $p=1.000$) or 4 months ($4.21 \pm 1.63 \mu\text{g}$, $p=0.232$). There was no significant difference in the RNA integrity (RIN) of samples extracted immediately (6.8 ± 1.1) or after 2 (5.9 ± 1.1) or 4 months (6.7 ± 1.2) of freezing ($p=0.188$). Similarly, no significant difference was seen in the 260/280 ratio, comparing sample extracted immediately (2.28 ± 0.14) or after 2 (2.24 ± 0.13) or 4 (2.20 ± 0.05) months of freezing ($p=0.416$). There was also no significant difference in the 260/230 ratio comparing sample extracted immediately (0.74 ± 0.51), after 2 (0.80 ± 0.68) or 4 (0.81 ± 0.49) months of freezing ($p=0.886$).

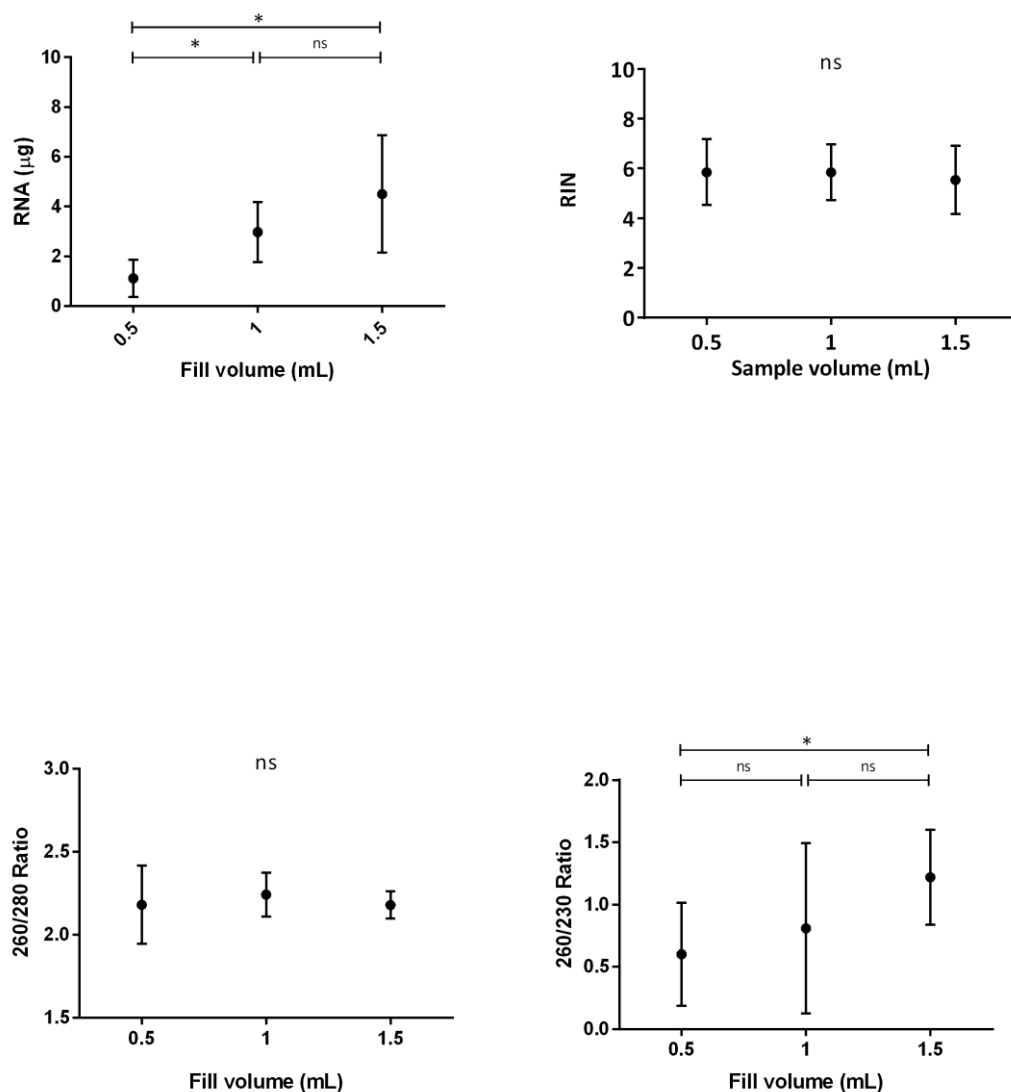


Figure 9.3 Quantitative and Qualitative Effect of Sample Fill Volume. ns=Not significant. *=Significant $p < 0.05$.

9.4.1.4 Quantitative and Qualitative Effect of Sample Fill Volume

An assessment of the effect of sample fill volume was performed comparing under (0.5mL), or over-filling (1.5mL) of the modified PG tube, with a control sample (1mL) (Figure 9.3). Samples were incubated at room temperature for 4-6 hours following venepuncture and subsequently frozen at $<-80^{\circ}\text{C}$ for 2 months. There was a significant difference in mean total RNA yield obtained in relation to fill volume ($p=0.001$). Significantly lower mean total RNA yield were seen in under-filled samples ($1.12\pm 0.75\mu\text{g}$) compared to control ($2.98\pm 1.21\mu\text{g}$, $p=0.002$) and over-filled samples ($4.50\pm 2.36\mu\text{g}$, $p<0.0005$). Although a difference was seen in total RNA yield for over-filled samples ($4.50\pm 2.36\mu\text{g}$) compared to controls ($2.98\pm 1.21\mu\text{g}$), this did not reach statistical significance ($p=0.156$). No significant difference was seen for RNA integrity (RIN) obtained under these conditions (RIN 5.9 ± 1.3 , 5.1 ± 1.1 and 5.5 ± 1.4 , $p=0.775$). Similarly, no significant difference was seen in the 260/280 ratio, comparing these conditions (2.18 ± 0.24 , 2.24 ± 0.13 and 2.18 ± 0.08 , $p=0.496$), under-fill, control and overfill respectively. A significant difference in 260/230 ratio was seen comparing the fill volumes ($p=0.019$), with higher 260/230 ratios seen for over-filled (1.22 ± 0.38) compared to under-filled samples (0.60 ± 0.41 , $p=0.002$). No difference in 260/230 ratio was seen comparing control tubes (0.81 ± 0.68) to either under-filled (0.60 ± 0.41 , $p=1.0$) or over-filled tubes (1.22 ± 0.38 , $p=0.274$).

9.4.1.5 Quantitative and Qualitative Effect of Sample Incubation Time

Assessment was made of the effect of sample incubation time (<1 hour, 4-6 hours (control) or >24 hours) at RT prior to freezing at $<-80^{\circ}\text{C}$ for 2 months (Figure 9.4). There was a significant difference in the mean total RNA yield obtained comparing the different sample incubation conditions ($p=0.008$). Significantly higher total RNA yields were obtained for samples incubated for 4 hours ($2.98\pm 1.21\mu\text{g}$) compared to those frozen within 1 hour ($2.14\pm 1.26\mu\text{g}$) of venepuncture ($p=0.049$). No significant difference was observed for total RNA yields comparing samples incubated for 24 hours ($3.51\pm 1.77\mu\text{g}$) and those incubated for either <1 hour ($2.14\pm 1.26\mu\text{g}$, $p=0.07$) or 4 hours ($2.98\pm 1.21\mu\text{g}$, $p=0.455$). No significant difference in RNA integrity (RIN) in relation to sample incubation time was seen for samples incubated for <1 hour (5.9 ± 1.1), 4 hours (6.6 ± 0.6) or 24 hours (6.0 ± 1.2) ($p=0.263$). A small, but significant difference in the 260/280 ratio was seen comparing the three incubation conditions (<1 hour: 2.33 ± 0.18 , 4 hours: 2.24 ± 0.13 , 24 hours: 2.20 ± 0.078 , $p=0.043$), but this lost statistical significance on pair-wise comparison. Similarly a small, but statistically significant difference was seen for the 260/230 ratio comparing the three incubation

conditions (<1 hour: 0.60 ± 0.53 , 4 hours: 0.81 ± 0.68 , 24 hours: 0.97 ± 0.80 , $p=0.043$), which again lost significance on pair-wise comparison.

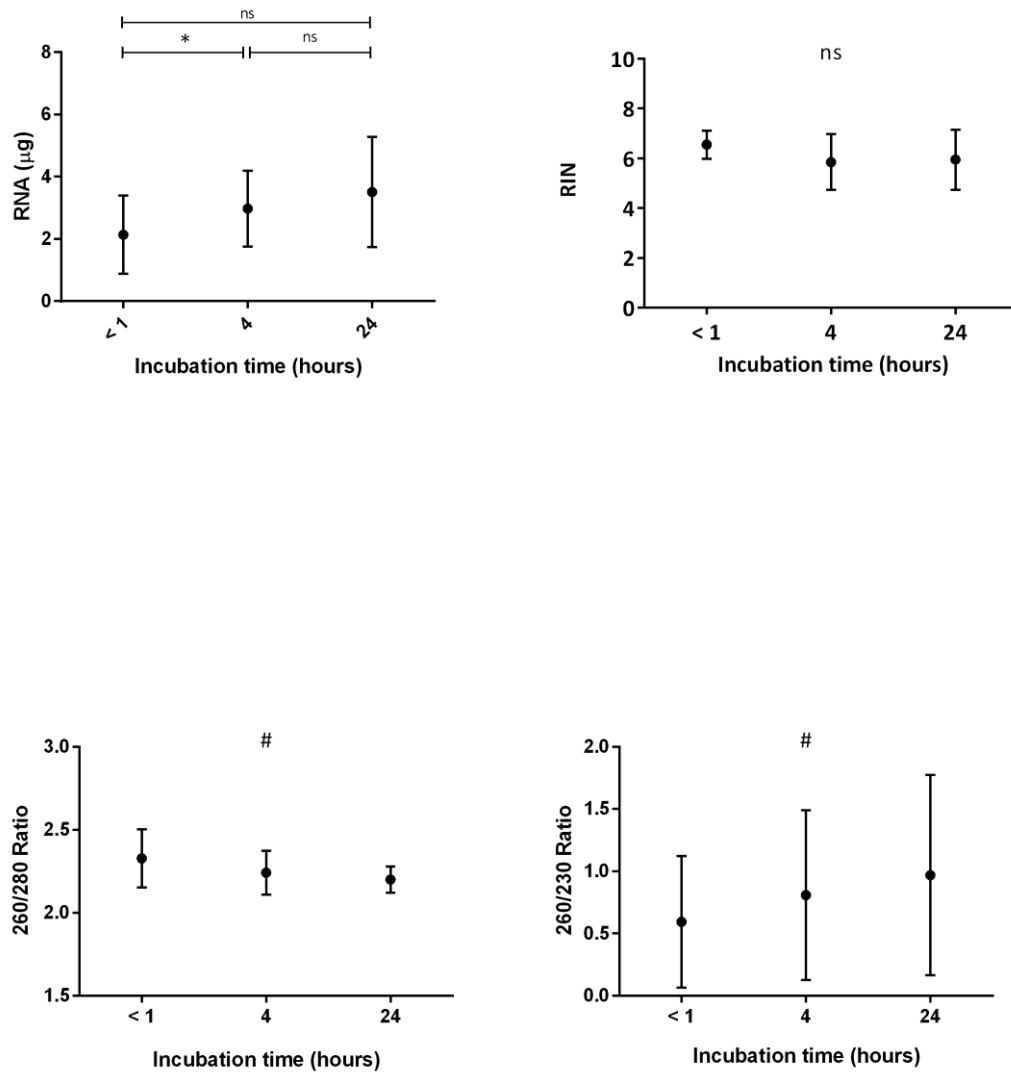


Figure 9.4: Quantitative and Qualitative Effect of Sample Incubation Time. ns=Not significant. *=Significant $p<0.05$. #=Significant on univariate analysis, but not significant on pair-wise comparison.

9.4.1.6 Quantitative and Qualitative Effect of Laboratory Variables on Whole Blood RNA in Healthy Volunteers (Phase 2)

In the second phase of this study, an assessment was made to firstly to investigate, lower than anticipated RNA integrities (RIN) seen within the first study phase and to evaluate variability in RNA yield and quality from different PG media and extraction kits. A cross-over comparison was performed in five additional healthy volunteers, using two separate PG

media and extraction kits. Significantly higher mean RNA integrity (RIN) were seen for samples extracted in this second study phase compared to those extracted in phase 1 (RIN phase 1: 5.9 ± 1.1 v phase 2: 7.5 ± 0.3 , $p=0.007$), with only one sample having a RIN value of <7 (RIN 6.6) in this phase. There was a trend towards higher total RNA yields (phase 1: 2.98 ± 1.21 v phase 2: 5.43 ± 3.72 , $p=0.07$). No significant difference was observed on comparison of different PG media and extraction kits, for total RNA yield (PG media $p=0.478$, extraction kit $p=0.466$), RIN (PG media $p=0.615$, extraction kit $p=0.552$) and 260/280 ratio (PG media $p=0.292$, extraction kit $p=0.958$). A small, but statistically significant difference was observed in the 260/230 ratio, between the two PG medias (media 1 0.45 ± 0.10 v media 2 0.55 ± 0.08 , $p=0.026$).

9.4.1.7 Assessment of Feasibility of miRNA Collection and Optimal Extraction Method (Phase 3)

The aim of the third phase of this study was to evaluate whether the modified PG tube could allow extraction of miRNA for downstream application and to ascertain the optimal laboratory method for extraction. There are two available miRNA extraction which either extract RNA (total and miRNA) into a single (Protocol B) or two tubes (Protocol C). A cross-over comparison was made for samples taken from 5 further healthy volunteers into modified and standard BD PG tubes. to allow comparison of the kits as well as between the standard and modified PG tubes.

Similar to data presented in the previous sections, there was a significant difference in the mean total RNA obtained using the modified PG ($2.47 \mu\text{g}$, 95%CI 0.964-3.966) compared to the standard ($4.12 \mu\text{g}$, 95%CI 2.766-5.482) PG tube ($p=0.007$). There was also a difference observed in comparing the Protocol B ($3.94 \mu\text{g}$, 95%CI 2.559-5.318) to the Protocol C ($2.65 \mu\text{g}$, 95%CI 1.287-4.014) kit for total RNA yield. On cross-over analysis, there was however no significant difference seen for the two variables. No significant difference was seen comparing the RIN, 260/280 and 260/230 ratio between the two PG tubes or extraction kits.

Comparison of the miRNA concentration obtained using the standard ($2266.4\text{pg}/\mu\text{L}$, 95%CI 529.4-4003.4) and modified ($1837.4\text{pg}/\mu\text{L}$, 95%CI 707.6-2967.3) PG tube demonstrated no significant difference ($p=0.640$), Although a slightly lower miRNA concentration was seen for Protocol B ($883.6\text{pg}/\mu\text{L}$, 95%CI -1.628-1768.9) in comparison to Protocol C ($3220\text{pg}/\mu\text{L}$, 95%CI 1128.7-5311.8) this did not reach statistical significance ($p=0.074$).

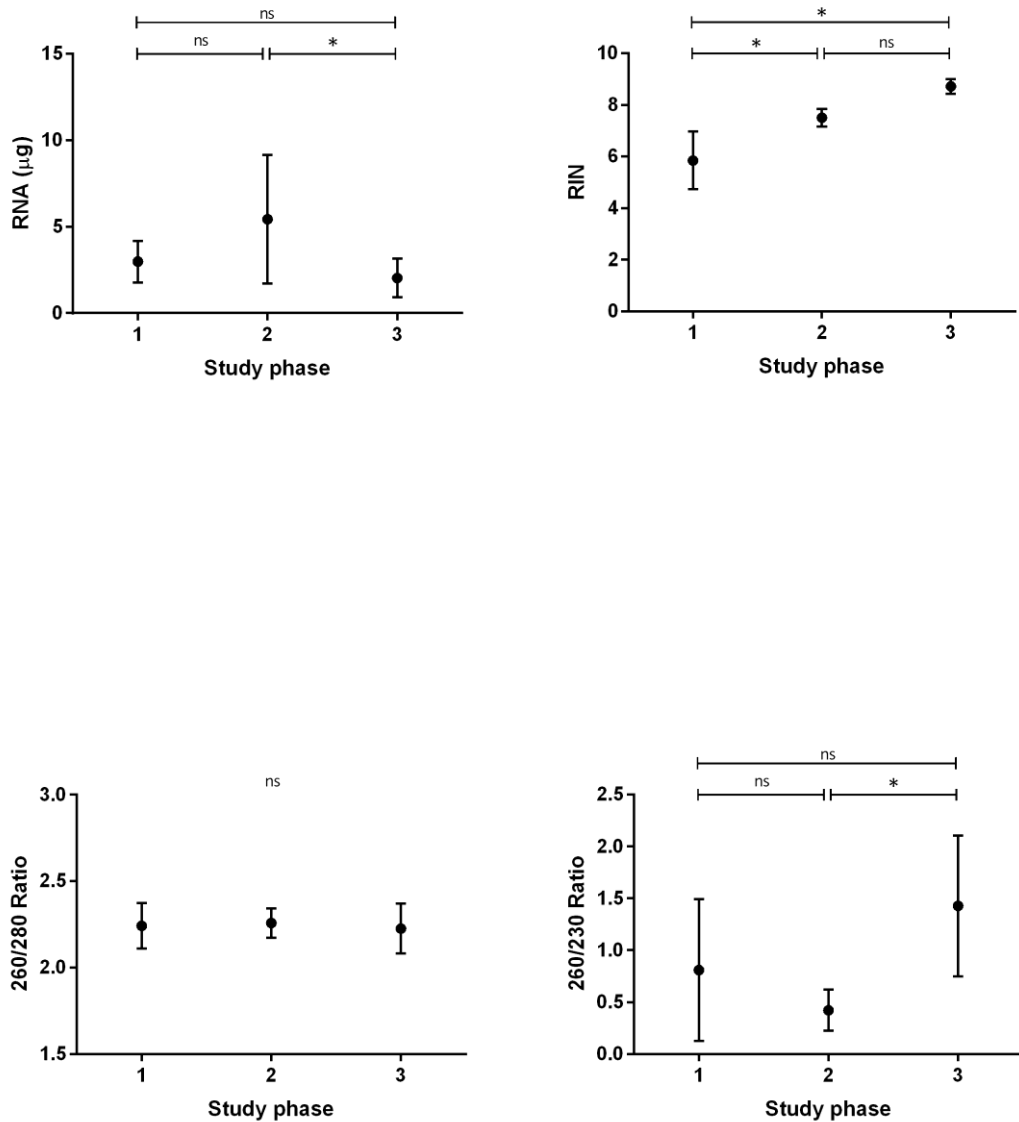


Figure 9.5: Quantitative and Qualitative Evaluation of RNA Obtained Across the Different Study Phases. ns=Not significant. *=Significant $p < 0.05$.

9.4.1.8 Evaluation of Quantity and Quality of RNA Obtained in Different Study Phases

Finally an assessment of the quality and quantity of RNA obtained across the three phases of this pilot study was performed (Figure 9.5). A significant difference was seen for total RNA yields obtained across the different phases of the study ($p = 0.047$). A lower total RNA yields was seen in the third phase ($2.03 \pm 1.11 \mu\text{g}$) compared to the second ($5.43 \pm 3.72 \mu\text{g}$, $p = 0.048$) phase of the study. No significant difference was seen in RNA yields between those obtained in the first ($2.98 \pm 1.21 \mu\text{g}$) compared to the second ($5.43 \pm 3.72 \mu\text{g}$, $p = 0.108$), or third study ($2.03 \pm 1.11 \mu\text{g}$, $p = 0.688$) phases. A significant difference was seen in the mean RNA Integrity

(RIN) across the three phases of the study ($p < 0.0005$). Significantly higher RIN values were seen in the second (7.5 ± 0.3) and third (8.7 ± 0.3 , $p < 0.005$) phases of this pilot study compared to the first phase (5.9 ± 1.1 , $p = 0.006$). No significant difference was seen in the mean 260/280 ratio across all three phases of the study ($p = 0.923$). There was a trend towards difference in the 260/230 ratios obtained across the three study phases ($p = 0.051$), with samples extracted in the third (1.43 ± 0.68) phase of the study having a higher 260/230 ratio compared to those extracted in the second phase (0.42 ± 0.20 , $p = 0.044$).

9.4.2 GENA-05 RNA-Seq Sub-Study

As of June 2015, 79 patients have been screened and enrolled from 11 countries into the GENA-05 (NuProtect) study. There are 28 active study sites internationally and 42 sites initiated. It is anticipated that 45 sites in 17 countries will participate in this study, with an aim to recruit 100 patients. To date, 68 patients have initiated treatment and of these 51 are receiving regular prophylaxis with 36 patients having received treatment for >20 EDs (25 patients ≥ 50 ED). A large proportion of patients (68%, 54/79) have enrolled and have been recruited onto the gene-expression (RNA-Seq) sub-study from 18 centres (3 continents). To date, 195 RNA samples have been received in our laboratory for analysis, with a median of 4 samples per patients (range 1-7). There are ≥ 5 samples available for analysis in 19 patients.

RNA (total) extraction was performed on samples obtained from the GENA-05 study to assess feasibility and proof of principle that the modified RNA sample tube is suitable methodology for RNA collection in a paediatric population. RNA was extracted from study participants with ≥ 5 samples sent for gene expression analysis and in whom a screening sample was recorded at study registration. This included 63 samples from 11 patients, with a median of 6 samples per patient. The mean RNA yield obtained was $7.08 \pm 3.11 \mu\text{g}$, with all samples having a total RNA yield of $>1.00 \mu\text{g}$. The mean RNA Integrity (RIN) obtained was 8.6 ± 1.2 , with 62/63 (96.8%) having a RIN ≥ 5 and 51/63 (81.0%) a RIN ≥ 8 . Two samples had sub-optimal RNA integrity (RIN 2.8 and 5.0) for downstream analysis, although good total RNA quantity was seen for both samples ($4.55 \mu\text{g}$ and $6.41 \mu\text{g}$ respectively). The mean RNA purity (260/280) was 2.12 ± 0.04 , with all samples having a 260/280 ratio ≥ 2.0 . In these samples the mean 260/230 ratio was 1.47 ± 0.47 , which is higher than was seen within the pilot study.

9.5 Discussion

The modified PG RNA sample tube demonstrated a feasible and valid approach for the collection of RNA (total and miRNA) for downstream analysis in healthy adult volunteers and in an international paediatric clinical study. High uptake of recruitment into the GENA-05 sub-study demonstrates that this forms an acceptable methodology for RNA sampling from young children within an international study. Predictable difference in total RNA yields were seen as a result of reduced sample volume being collected into this modified PG tube, but these sample tubes provided RNA of sufficient quantity and quality for downstream sequencing. Within the initial phase of this pilot study lower than expected RNA integrity was seen for samples collected into both the modified and standard PG tubes. With increased experience with sample handling, improvements were seen in the RNA integrity and purity of samples throughout these experiments. It was interesting to note that RNA quantity and integrity obtained in the samples from the NuProtect study were higher than those seen in the pilot study, where sampling may have been performed in a more controlled environment.

9.5.1 Effect of Pre-Analytical Variables on Usage of a Modified PAXgene RNA Blood Tube

Within the first phase of evaluation of this sample tube in healthy volunteers, although good RNA yields and purity were obtained, lower than anticipated RNA integrity was seen in comparison to similar studies in this area⁴⁵⁶. The mean RIN values seen for the standard PG and modified PG tubes in the first phase were 7.4 and 5.9, respectively. Previous reports of usage of the modified PG tube has given RIN values, in the range of 7-8, with very few samples having a RIN <7 (Carrol E, Professor in Clinical Infection, University of Liverpool, personal communication). Although higher RNA integrity was expected using the standard PG tube from previous experience within our laboratory, the mean RIN values seen are in keeping with those seen in previous studies of healthy volunteers (mean RIN 7.4-8.1)^{454,459}. A second phase was performed to further evaluate these finding of sub-optimal RNA integrity demonstrating higher RNA integrity for all samples. Given these findings, the most likely mechanism for suboptimal RNA integrity within the first phase was RNase contamination within one of the extraction kit reagents that was not present in future extractions. Although RNase contamination within the modified PG tubes could provide an alternative explanation, this seems unlikely given higher RIN values seen for samples collected into this same batch of modified PG tubes, in the second study phase and GENA-05 study. Within this

assessment of pre-analytical variables RNA integrity was not effected by sample incubation time, freezing or fill-volume.

9.5.2 Transcriptome Analysis in Haemophilia

The GENA-05 (NuProtect) study, will create a vast database of RNA-Seq data from patients with severe haemophilia A at first treatment and during ITI. One of the problems with such hypothesis generating datasets is the careful construction of subsequent analyses to interpret changes that are seen in gene expression. Within this study, the first analyses that will be constructed will consist of case-control analysis of changes in gene expression in patients with and without inhibitor looking at prior to FVIII exposure and early within treatment with FVIII. The hypothesis of this analysis is that there will be changes in gene expression early in treatment with FVIII that may predict onset of inhibitor formation. Changes in gene expression within these patients would then be validated by quantitative polymerase chain reaction (qPCR) that could provide biomarkers that could be included within prediction algorithms. Alongside this study looking at the usage of whole blood for transcriptome analysis in patients at first exposure to FVIII, there is a similar study the Hemophilia Inhibitor Previously Untreated Patients Study (HIPS, ClinicalTrials.gov Identifier: NCT01652027), which is utilising buffy coat-separated RNA extraction ⁴⁶². Although potentially offering a more difficult approach for sample collection at study sites this will provide a complementary dataset for increasing understanding of the development of an immune response to FVIII.

9.6 Limitations

One of the main limitations of the data described in healthy volunteers relates to the small sample size for describing differences between the pre-analytical samples conditions analysed. This may mean that there is over, or underestimation of the size of effect of one or more of these variable investigated. Although allowing exploratory comparison of the effect of different variables, but due to a lack of power these data will be insensitive to the discovery of small effects and may be more likely to be affected by chance findings. Nevertheless, one of principle aims of these pilot data was to demonstrate validity of usage of this approach in healthy volunteers to allow ethical sample collection within this paediatric study. Finally, although the GENA-05 study will create a vast repository of transcriptome data to help unravel the immune processes, this study is limited by the use of a functional inhibitor assay as the primary outcome measure for assessing FVIII antibody formation. Further study is

required to evaluate changes occur with onset of different classes of antibody (inhibitory and non-neutralising).

9.7 Conclusions

A modified PG sample tube provides a feasible and acceptable methodology for transcriptome (total RNA and miRNA) analysis using whole blood in paediatric studies prior to the availability of commercially manufactured sampling tubes. Samples collected from the GENA-05 (NuProtect) RNA-Seq sub-study will create a vast dataset of RNA-Seq information for the haemophilia community, which will hopefully provide greater insights into the immunological mechanism of antibody formation and provide biomarkers for risk stratification for patients with severe haemophilia A.

Chapter 10: Summary and Conclusions

10.1 Summary

There is a lack of understanding of the total immune response to FVIII, resulting from reliance on functional inhibitor assays for the detection of FVIII antibodies in clinical studies. Although standardisation of these inhibitor assays in the 1970s resulted in improvements in diagnosis and inter-study comparison, there remains questions into the optimal approaches for FVIII antibody testing (laboratory assay and timing). This is particularly the case where FVIII antibody formation is a rare event, such as in non-severe haemophilia A and acquired haemophilia A. Within my work, significant heterogeneity was described in approaches to inhibitor testing and clinical management in both of these settings. Reviewing inhibitor testing practices in a large cohort of patients with non-severe haemophilia A, demonstrated poor compliance with targeted inhibitor testing following “high-risk” treatment. This could result in underestimation of the humoral response to FVIII and skew towards detection of antibodies that result in a change in clinical phenotype (cross reactive antibodies to endogenous FVIII). In acquired haemophilia A, there was significant heterogeneity at all levels of the management of immunosuppression. Selection of immunosuppression in both non-severe haemophilia A and acquired haemophilia A appears to be influenced by clinical and laboratory factors ^{169;233}. These “stratifications” made by clinicians in their approaches to management ^{169;233}, however, lack in an evidence base. This introduces systematic bias in observational studies, limiting interpretation and generalisability of clinical outcomes. There is a need for prospective study in both setting following standardised treatment protocols with centralised testing to improve understanding of the natural history of FVIII antibodies. Usage of a modification (PHT) to current inhibitor assays and/or a FVIII ELISA improves detection of FVIII antibodies. Evaluation of B-cell epitope profiles in patients with severe haemophilia A, using a novel re-usable peptide microarray demonstrated immunodominant epitopes in regions of functional (tenase complex) and structural importance, giving further insight into the mechanism of action of inhibitors. Advances and reduction in costs for high-throughput genomic techniques (NGS), allows evaluation of the immune response to a protein therapeutic, such as FVIII. Modifications to sampling methodology described in this work, demonstrate feasibility of this approach in young boys with severe haemophilia A and samples collected within this study will provide a vast repository of data for unravelling the immune response to FVIII.

10.2 FVIII Antibody Testing in Congenital and Acquired Haemophilia A

The classical Bethesda assay which was first proposed in the 1970s was developed for quantification of type 1 inhibitors in patients with severe haemophilia A. Since introduction of this assay, usage has been extrapolated for detection and quantification of FVIII antibodies in non-severe and acquired haemophilia A; two settings where antibodies may display more complex inactivation kinetics and samples may contain substantial residual FVIII:C. It has not been until more recently that there has been interest in the usage of assay modifications to adjust for residual FVIII:C in samples. Within this thesis, modifications and additional testing approaches have demonstrated improvements in FVIII antibody detection in both congenital and acquired haemophilia A. There appears to be recent changes in laboratory testing practices, with a recent NEQAS exercise reporting increased usage of pre-analytical heat treatment in routine laboratory practice ³³¹. Alongside this, at a local level at The Royal London Hospital, it is interesting to note a recent increase in the number of FVIII inhibitor tests being performed. In the last year (10/14-11/15) a total of 331 inhibitor samples were tested, representing an increase from an average of 2.8 to 6.4 samples per week. Whether this has resulted in increases in the inhibitor detection requires further evaluation.

The usage of a FVIII ELISA either alone or in parallel to a FVIII inhibitor assay may allow improvements in antibody detection, giving further understanding of the immune response to FVIII. From an immunological perspective, there is a need for further prospective studies, using parallel approaches to testing (immunological and functional) to gain further understanding of the natural history of FVIII antibodies. A large monitoring study has recently been presented from the USA, over a 6 year period with prospective central monitoring for FVIII antibodies using three different testing platforms (Nijmegen-Bethesda, Chromogenic Bethesda and a fluorescence immunoassay) with PHT (56°C for 30 minutes) ^{366,463-465}. Within the study protocol there were recommended times for monitoring, including at entry, annually, before product switching and where there was a clinical indication ⁴⁶⁵. Despite the scale of such project, which included 824 patients with all severities of haemophilia A (severe=498, non-severe=326), only 23 inhibitors were detected (9 high-titre and 14 low-titre), highlighting the difficulties in conducting such studies. This study is however an important advance in the conceptualisation of FVIII antibody detection in haemophilia A moving away from simple testing using a functional assay and allows confirmation of FVIII specificity.

Approaches for inhibitor testing using parallel testing will be associated with substantial cost increases for centres if performed within local laboratories. To allow further economic justification for these approaches, collaborative studies are required to provide further data on timing and optimal assays for screening. With increased screening, there is the likelihood that there will be increased detection of FVIII antibodies in which the long-term physiological relevance is not clear, such as low-titre and non-neutralising antibodies. Awareness of this is of importance, in future comparisons of inhibitor incidence both at a local clinical level, but also at a registry level. Reported inhibitor occurrence rates are heavily politicised in haemophilia care due to the commercial interest of pharmaceutical companies. This increases the imperative to understand more fully the basis of inhibitor occurrence in the various scenarios discussed in my thesis. Other approaches such as the usage of thrombin generation assays, offer novel approaches that may allow global assessment of inhibitory capacity⁴⁶⁶. These techniques have been used successfully by laboratories to help guide treatment in patients with inhibitors. Previous lack of standardisation and quality control has mainly limited usage to research settings and does not provide a technique amenable to screening for FVIII antibodies. Thrombin generation in parallel to an immunoassay could provide additional data of the functional capacity of FVIII inhibitors allowing personalised management for bleeding and surgery^{219;467}.

10.3 Future Developments in Haemophilia Treatment

A number of novel approaches are undergoing clinical trials for improvement of treatment for patients with haemophilia A. These include both modified FVIII concentrates and non-FVIII based approaches⁴⁶⁸⁻⁴⁷³. In approaches using modified FVIII concentrates, these have either attempted to improve pharmacokinetic profiles of FVIII or reduce immunogenicity, which include fusion to the immunoglobulin Fc domain, pegylation, modified (covalently bonded heavy-light chain) single chain FVIII and rFVIII produced from a human recombinant cell line (HEK293). Similar approaches in FIX deficiency, using a Fc fusion protein to FIX, in a phase 3 study produced an increase in half-life from 33.8 to 82.1 hours, with low annualised bleeding rates, in patients treated once every 1-2 weeks with this product⁴⁷⁴. There were initial hopes similar strategies for FVIII would result in a significant reduction to the number of infusions required per week on prophylaxis. Unfortunately, similar increases in half-life have not been seen for the extended half-life FVIII concentrates, with an average of a 1.4-1.6 fold half-life extension (13-19 hours). These small extensions in half-life are thought to relate to requirement of molecular chaperoning of FVIII to VWF^{472;473}. However, the potential for

these modifications to reduce the immunogenicity of factor FVIII concentrates are yet to be fully assessed. The studies of usage in haemophilia A PUPs and post marketing surveillance will provide important data to inform decisions of immunogenicity of these new products.

A number of approaches using modified FVIII structures either to reduce immunogenicity, through introduction of mutations within immunodominant epitopes (T or B cell) or to provide increased FVIII hetero-trimer⁴⁷⁵, or domain⁴⁷⁶ stability have been investigated *in-vitro* and in murine haemophilia A models. From the work presented in this thesis, modification of immunodominant B-cell epitopes, may not provide an easy mechanisms for reduction in immunogenicity given the majority that were characterised fell within key functional regions of the FVIII structure. Modifications of residues could result in changes in the functional capacity of FVIII and/or neo-epitope formation. With modification to FVIII sequence there is an importance for pharmacovigilance projects to evaluate increased rates of immunogenicity. This was highlighted firstly through an outbreak of inhibitor formation following usage of a pasteurised FVIII concentrate in the early 1990s⁴⁷⁷⁻⁴⁷⁹ and more recently through concerns of increased immunogenicity of a B domain deleted FVIII concentrate with the presence of a non-native linker sequence⁴⁸⁰. Given the heterogeneity of the human immune system and *F8* causative mutations, one would expect differences in the immunodominant epitopes (T and B cell) seen between individuals. As such, development of “personalised” FVIII concentrates based on risk profiles may prove difficult given regulatory requirements and lack of financial viability for such projects⁴⁸¹.

There are a number of novel non-FVIII approaches to management of patients with congenital and acquired haemophilia A. Agents that are currently undergoing clinical trials are a bispecific monoclonal antibody, which binds FX and FIX (ACE 910)^{482,483}, inhibitors of TFPI (e.g. concizumab)⁴⁸⁴ and a small interfering RNA (siRNA) to anti-thrombin (ALN-AT3)⁴⁸⁵. The results of ongoing trials in these novel agents are encouraging and may provide a non-FVIII based therapy that could personalise management of haemophilia A in patients at high risk of inhibitor formation. There are continued hopes that gene therapy could revolutionise the care of patients with haemophilia A, following the impressive results seen in the first studies of gene therapy in haemophilia B⁴⁸⁶⁻⁴⁸⁸. Further study of gene therapy using an Adenovirus-Associated Viral vector (BMN 270) is ongoing in haemophilia A (ClinicalTrials.gov Identifier: NCT02576795), which may provide an additional option for the long-term management of haemophilia A.

10.4 Further Studies

10.4.1 Laboratory Testing of FVIII Antibodies

Although the evaluation of pre-analytical heat treatment has provided clearer data to guide use of this modification in laboratory practice, there is a need for further study of this modification. This would ideally be performed as part of centralised laboratory testing within a prospective clinical study. Clinical data suggested loss of inhibitor activity by a FVIII ELISA following PHT in some samples from patients with AHA, after incubating for 90 minutes at 58°C, which was not reproducible on more systematic evaluation. Whether some antibodies with weaker affinity or different epitope profiles are more susceptible to the effects of PHT is not clear. Within these experiments, usage of higher temperatures for PHT (58-64°C), could offer time and cost saving approaches to the use of this modification in laboratory practice and further evaluation of this is required. Finally, there is variable reports of usage of centrifugation as part of this modification which warrants further study, given differences seen in this and previous work for FVIII inactivation at 56°C²⁸³.

10.4.2 B-Cell Epitope Mapping of FVIII Antibodies

The approach (Pepscan) described in this thesis, provides a novel high throughput approach for epitope mapping of FVIII antibodies using *in-vitro* scaffolded mimics of secondary structure. This approach given reusability, lack of complex sample preparation is particularly amenable to the evaluation within clinical trials. In the further development of this platform validation of epitope profiles for the clinical samples is planned using an alternative platform³⁹³ as part of a collaboration with the University of Frankfurt (Königs C., Head of Molecular Haemostasis, University Hospital Frankfurt am Main, personal communication). Redesigning of this array is also required to allow completion of coverage of the C2 domain. This approach offers epitope mapping methodology that could be applied within a larger international cohort, to further evaluate the immunodominant epitope profiles seen. This methodology may also allow further investigation of differences between individuals with inhibitory and non-neutralising antibodies to further evaluate differences in epitope profiles and whether epitope spreading occurs in time in both groups. Finally, although this approach offered high-throughput methodology for testing of FVIII antibodies, this lacked high-throughput data analysis, with all data being analysed manually. Interrogation of this dataset has provided the early framework for development of an algorithm for automated interpretation of such datasets, which could allow development of a server for usage with other protein-antibody

interactions. This methodology could allow assessment using variable cut-offs for positivity and give clinicians a summary of B-cell epitopes as well as structural and functional data on these regions.

10.4.3 Transcriptome Analysis in Severe Haemophilia A

Sample collection of RNA within the GENA-05 (NuProtect) study has allowed the development of a vast repository of gene expression data at first exposure to FVIII and during ITI in patients with severe haemophilia A. Work in this thesis demonstrated clinical acceptability and sample viability, but is the first stage in this hypothesis generating project to improve immune response to FVIII and identify biomarkers of risk. The first approach to analysis within this study will be to evaluate changes in gene expression profiles seen at registration compared to an early sample point (3-6ED) in patients developing inhibitors compared to those that remained inhibitor free (case-control study). This approach allows sequencing of both coding and non-coding RNA, with a high sequencing read-depth to identify changes (or lack of change) seen early in treatment in patients with inhibitors. Following on from this, interrogation of the antibody repertoire (Ab-Seq) ⁴⁸⁹ will be performed to evaluate the diversity of the immune response to FVIII to determine whether particular germ lines (clonal diversity) are highly represented and the timing of isotype switching.

10.5 Conclusions

With the ever expanding use of such high-throughput platforms in clinical studies, there is a growing need for increasing collaboration between clinical, genomic and bioinformatic researchers to provide meaningful translational data. With these advances a paradigm shift is required in approaches to FVIII antibody testing as reliance on imperfect laboratory methodology such as a functional inhibitor assays may undermine the relevance of these future findings. Advances in treatment options and understanding of immune processes underlying inhibitor formation will allow more personalised care for persons with haemophilia in the third millennium. Finally, although these advances may have substantial benefits for boys born with haemophilia in developed nations, there is an ongoing need for consideration of the global implications of haemophilia ⁴⁹⁰.

Chapter 11: References

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Appendix 1: STARD checklist for reporting of studies of diagnostic accuracy

Section and Topic	Item		Pages
Title/abstract/ Keywords	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	118
Introduction	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	118-119
Methods			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	119-120
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	119-120
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	119-120
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	121
Test methods	7	The reference standard and its rationale.	60-67 120
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	60-68 72-75 120-121

	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	72-7 120-121
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	119-121
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	119-120
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	121
	13	Methods for calculating test reproducibility, if done.	N/A
Results			
Participants	14	When study was performed, including beginning and end dates of recruitment.	119
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	122 Figure 6.1
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	122 Figure 6.1
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	119-121
	18	Distribution of severity of disease (define criteria) in those with the target condition; other	122 Figure 6.1 Table 6.2

		diagnoses in participants without the target condition.	
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	Figure 6.1 Table 6.2
	20	Any adverse events from performing the index tests or the reference standard.	N/A
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	121 Table 6.2 Table 6.3

Appendix 2: Filtering of B-Cell Epitopes Using FVIII Pepscan Arrays

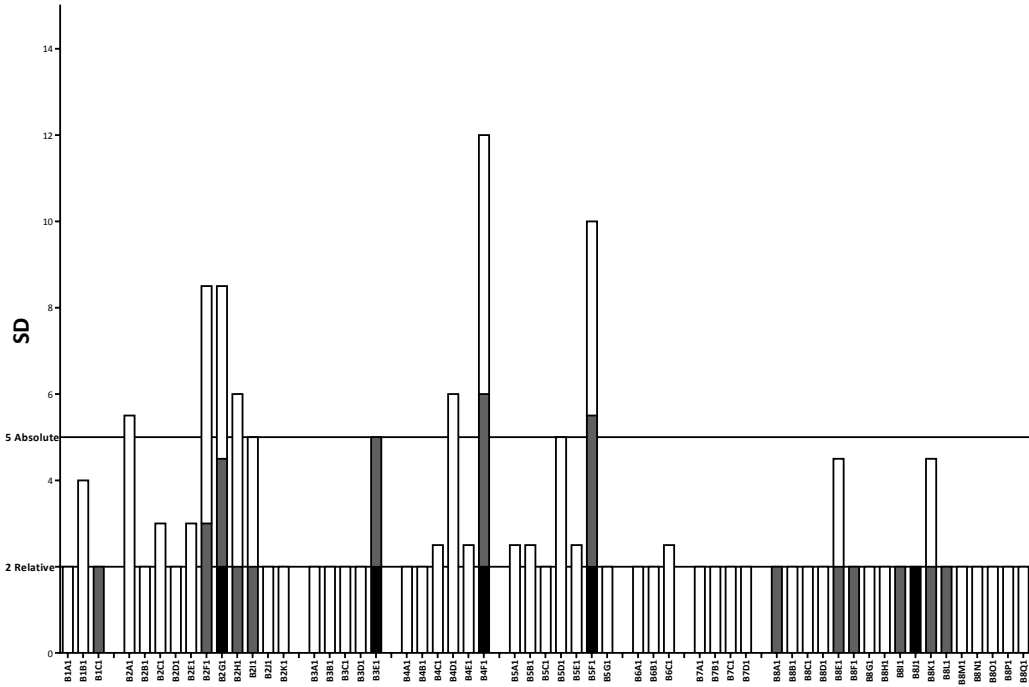
An evaluation of a “relative cut-off”, was performed based around the repetition of 5-mer sequences in subsequent wells on the linear (n=4) and looped (n=3). Manual filtering of positive binding was performed in all array wells for in which the OD was $\geq 2SD$ from the experimental mean. Positive wells with a $\geq 2SD$ were then grouped into “epitopes” falling within sequential or semi-sequential wells of the array. With the repetition of 5-mer sequences in subsequent wells, it would be expected to see binding in two or more continuous or discontinuous wells on the array if this sequence formed part of the B-cell epitope. Figures shown in this section show the effect of application of this strategy and the loss of non-specific binding.

Within these figures each column represents a single epitope for the negative controls, mAbs and polyclonal FVIII antibodies. The shading in these bars as shown in the keys represents the number of sequential or semi-sequential wells in which the result based on the SD would remain positive if this threshold were to be used. This allows assessment of the effect of using a variable cut-off based on either the SD from the experimental mean or number of consecutive wells in which binding was seen. For example, if binding was seen in a single well on the linear array, 3.2SD from the mean, this would result in a column up to level 3SD (i.e. this would not be positive if a cut-off of 3.5SD were used) on the y-axis without shading. If binding is seen in 3 consecutive wells on the linear array with these results being 2.3, 2.4 and 5.1SD from the experimental mean, this would result in the column would being black (n=3) up to a level of 2SD as all 3 results would be positive with this cut-off and then a clear column up to the level of 5SD.

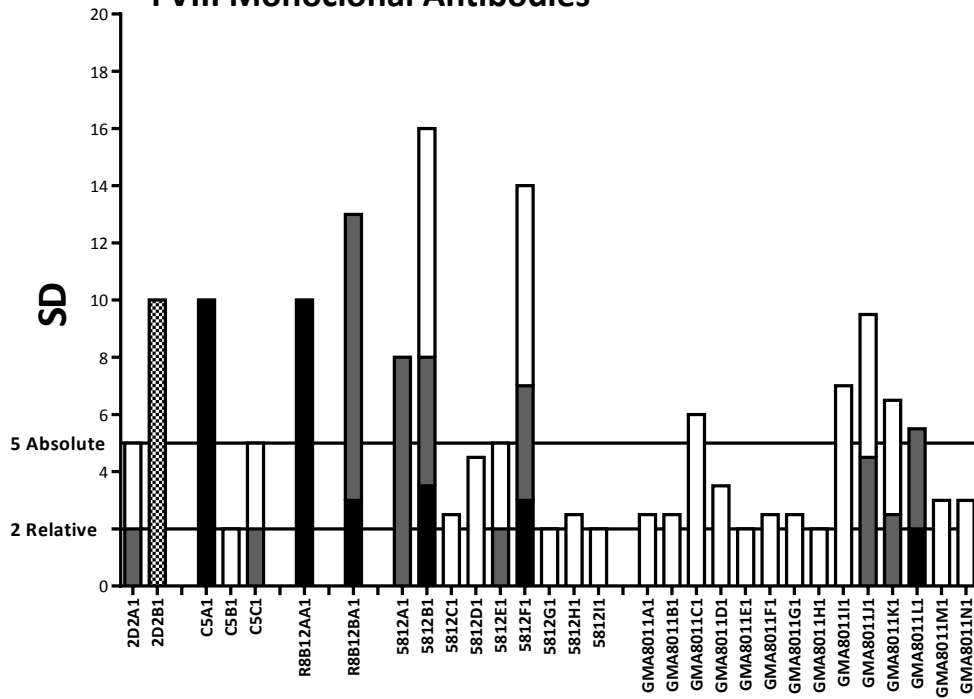
Linear (T1) Arrays

- 1
- 2
- 3
- ▨ 4

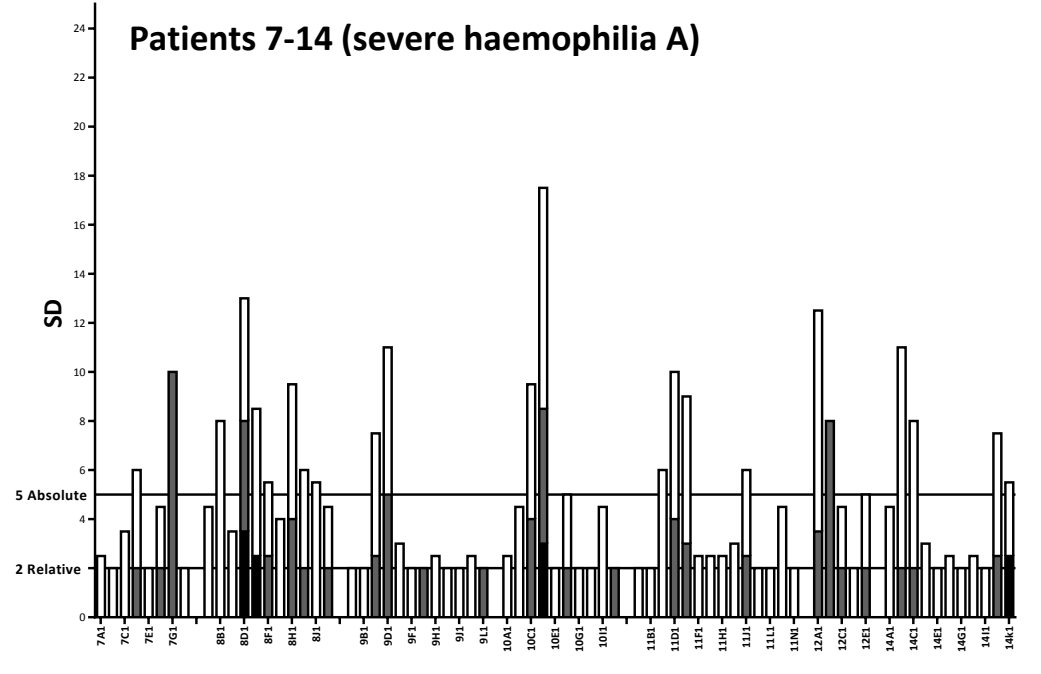
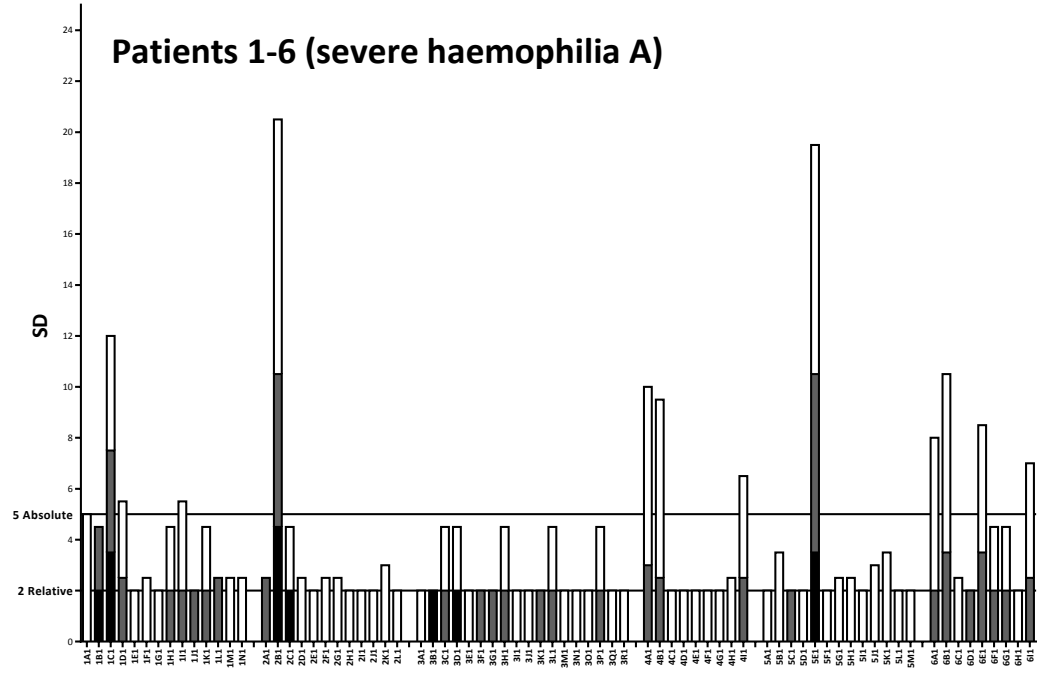
Negative Controls



FVIII Monoclonal Antibodies

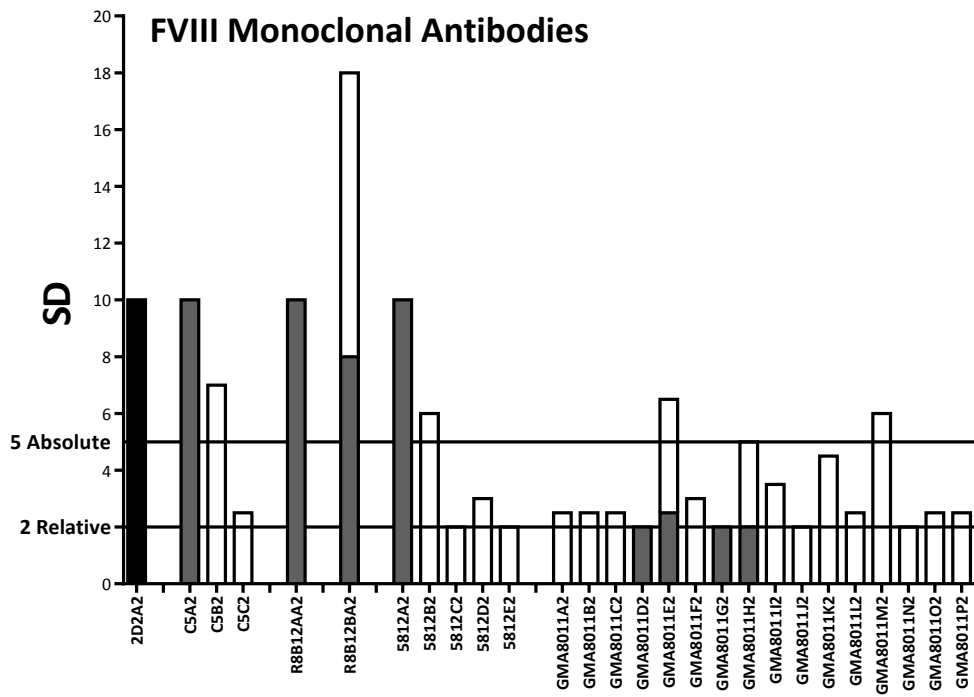
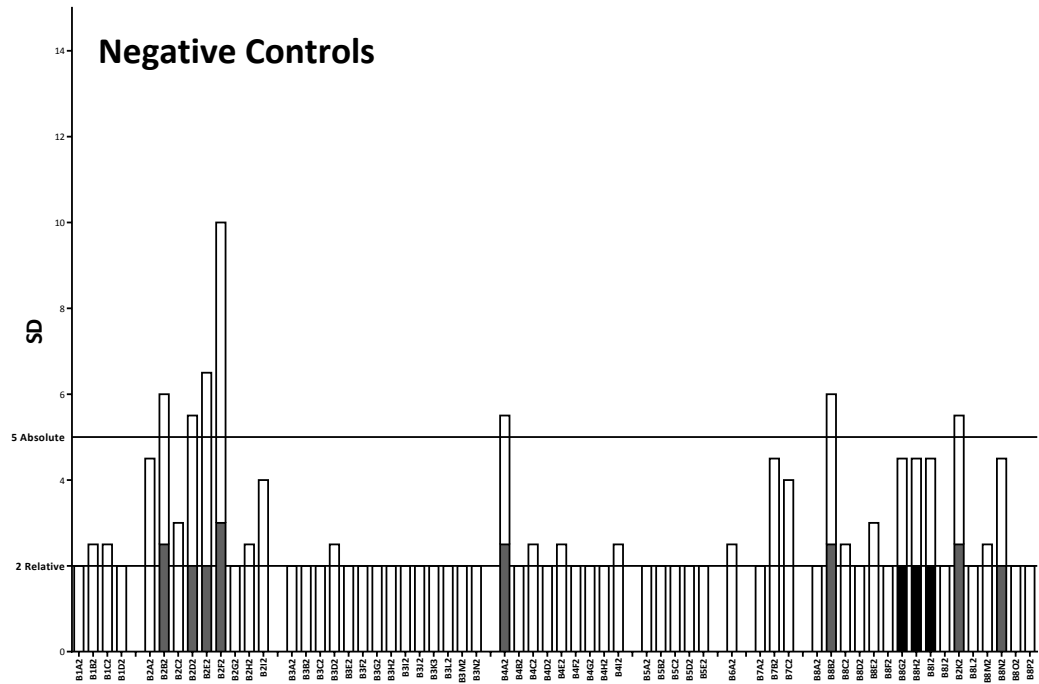


- 1
- 2
- 3
- ▨ 4

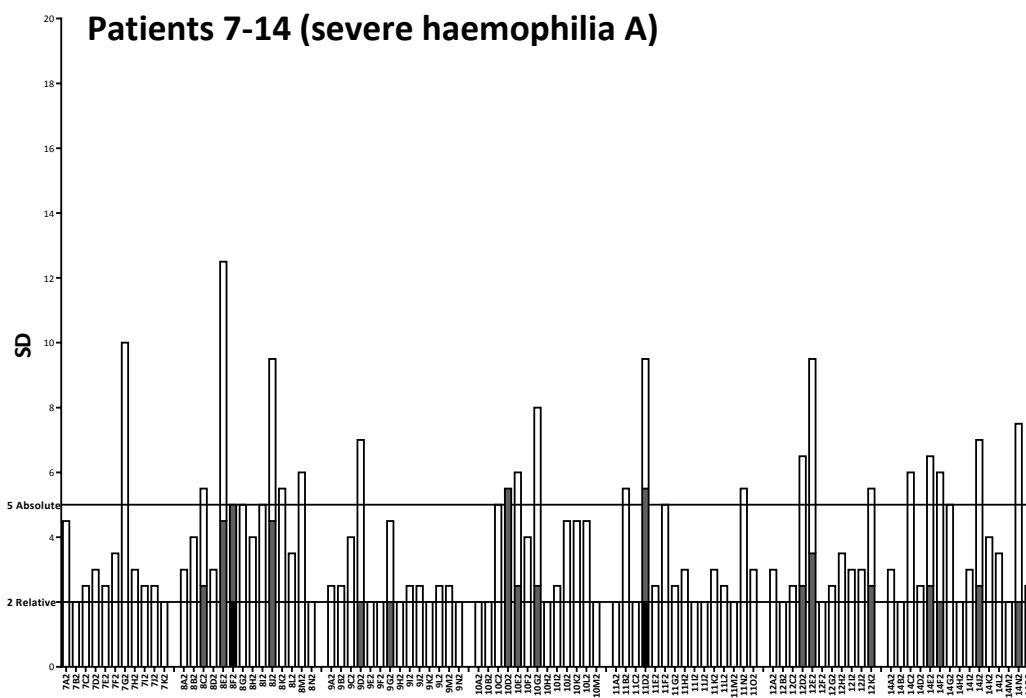
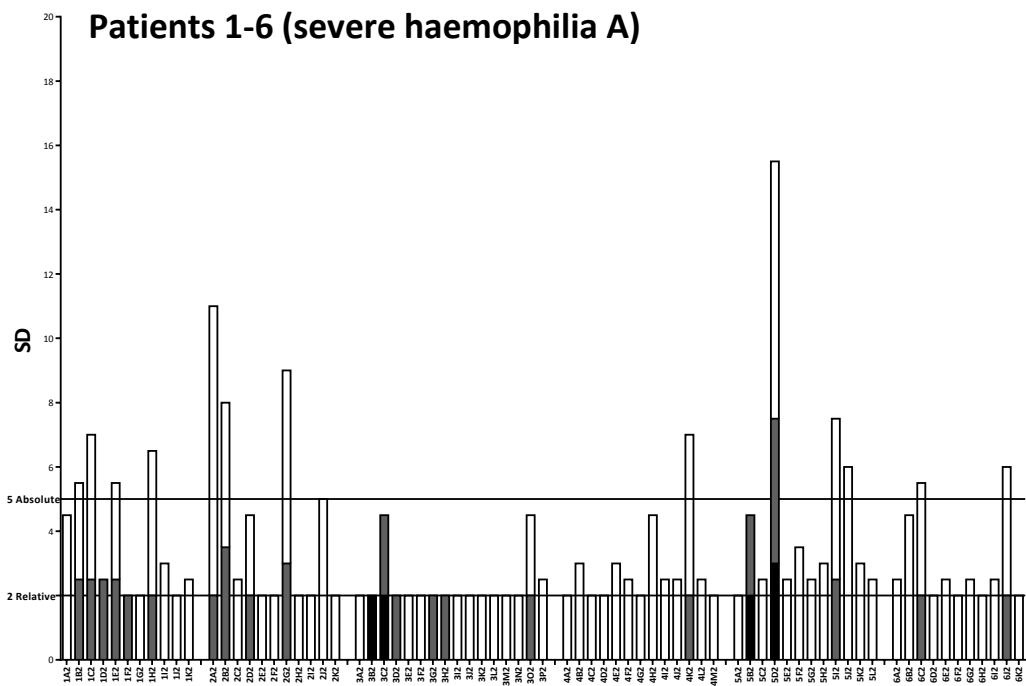


Looped (T2) Arrays

- 1
- 2
- 3



- 1
- 2
- 3



Appendix 3 GENA-05 / NuProtect RNA-Seq Sub-Study Protocol

Inhibitory antibody formation remains the greatest challenge in the management of severe haemophilia A. The focus of genetics studies of inhibitor risk has been on variations / mutations in the inherited DNA sequences of the factor VIII (FVIII) gene (causative haemophilia mutation or FVIII wild type haplotype) and genes involved in the immune response (e.g. IL-10, TNF- α and CTLA-4). However, such DNA based technology only gives the baseline gene profile of the individual without taking into account the dynamic changes that will occur in actual gene expression around the time of exposure to a novel immunogenic stimulus. Quantitative assessment of transcribed messenger Ribonucleic Acid (mRNA) offers a way of measuring differential changes in gene activity in these circumstances. Although technology for assessment of mRNA expression has been available for some years, the arrival of next generation sequencing (RNA-Seq) offers the ability to perform high throughput analysis of gene transcript activity on small volumes of venous blood in the setting of an international clinical study. This technology offers the ability to sequence the gene transcript products of millions of genes from each clinical sample. This enables an unbiased assessment of the genes involved in the immune response as well as other potential novel biological pathways that may prove to be of key importance in the process of inhibitor formation.

This is the first clinical study to prospectively evaluate changes in gene expression around the time of exposure to exogenous FVIII and during immune tolerance induction. Measurement of real-time changes in mRNA expression may give further insight into the complex dynamic processes and pathways involved with the aim of identifying “bio-signatures” that can predict those at greatest risk of inhibitor formation or failure of immune tolerance induction. Sampling of mRNA is feasible in a paediatric study as it only requires small volumes of venous whole blood. Due to instability in mRNA and changes in gene expression following phlebotomy, stabilisation is required at the point of collection (1,2,3). Modifications of commercially available stabilisation systems have been successfully trialled in both paediatric and animal studies demonstrating satisfactory mRNA yields whilst minimising required blood volumes (4,5).

RNA Expression Analysis Sampling Schedule

Formal written consent should be obtained at study entry for storage of whole blood (mRNA) samples for research purposes to investigate the mechanisms of inhibitor formation / immune tolerance.

For a patient enrolled on this optional sub-study, peripheral venous blood samples will be collected on commencement of Human-cl rhFVIII treatment at the time points shown in table 1.

A screening sample (pre-fVIII exposure) is necessary to proceed with follow up samples. If the screening sample is missed, do not collect subsequent follow up samples. However, these patients could still be enrolled in the ITI RNA-Seq study (see Table 2), with a new pre-ITI baseline sample and subsequent follow up samples.

	Pre-1 st exposure (Screening)	Every 3 – 4 ED (with routine inhibitor screen)	20 ED or at inhibitor formation
RNA expression analysis	1mL	1mL	1mL

Table 1: RNA expression analysis sampling schedule: First 20 Exposure Days (20ED)

For patients diagnosed with an inhibitor (commencing on ITI), enrolled on this optional ITI RNA expression analysis sub-study, peripheral venous blood samples will be taken at the time points shown in table 2.

	Pre-ITI Initiation	Week 2	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24
RNA expression analysis	1mL	1mL	1mL	1mL	1mL	1mL	1mL	1mL

Table 2: RNA expression analysis sampling schedule: Immune Tolerance Induction (ITI)

RNA Expression Study Materials

Investigation centres will be provided with centrally prepared customized PAXgene mRNA sampling tubes. The materials required for this study are as follows:

- 1) Customized PAXgene mRNA sampling tube: sterile(RNase / DNase free) cryovial (4.5 mL volume) containing 2.76mL PAXgene reagent (ratio 1mL blood : 2.76mL reagent) (provided)
- 2) Sterile butterfly / winged infusion kit (not provided)
- 3) Sterile 1mL syringe (not provided)
- 4) Sterile gloves (not provided)
- 5) Skin surface wipe (not provided)

RNA Expression Sampling Protocol

- 1) Keep RNA expression sample tube at room temperature (18 – 25°C) prior to sampling. **Do not re-use once opened.**
- 2) **Collect 1mL venous blood** into a 1mL sterile syringe using the local centre's protocol for venepuncture, wearing sterile gloves.
- 3) Open PAXgene sample tube and directly transfer **1mL** of venous blood following venepuncture into the PAXgene tube.
- 4) Close sample tube.
- 5) Gently invert the customized PAXgene tube 8 to 10 times.
- 6) Label sample with patient identifiable information / study number / and study time-point.

RNA Expression Transportation and Storage Protocol

- 1) Keep sample upright for **4-6 hours at room temperature (18 – 25°C)** following blood sampling, prior to freezing. Early freezing of samples will substantially decrease the RNA yield.
- 2) Freeze sample at **-70°C** in a wire rack (not a styrofoam tray as this may lead to sample tubes cracking).
- 3) Transport frozen PAXgene stabilised RNA study samples to LabCorp (LabCorp Clinical Trials, Laboratory Services, 8490 Upland Dr, Ste. 100, Englewood, CO 80112, USA) on dry ice.
- 4) RNA Samples kept at -70°C will be stable for a period of up to 60 months (6).

RNA Extraction and Transcriptosome Analysis

All extraction, purification of RNA and next generation sequencing (RNA-Seq) will be performed at The Genome Centre, John Vane Science Centre, United Kingdom. Interpretation of results will be performed by Dr Paul Batty / Dr Daniel Hart in collaboration with the Bioinformatics Department at The Genome Centre, John Vane Science Centre, United Kingdom.

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