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# Exploring Anti-Factor VIII Antibodies in Haemophilia A

Role in *In Vitro* Haemostasis and Clinical Disease

Jenny Klintman, MD



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Abstract Haemophilia A (HA) is caused by defective synthesis of coagulation factor VIII (FVIII), which has serious effects on haemostasis; joints being the most common site of bleeding. The development of FVIII replacements has improved the situation for patients with haemophilia such that chronic arthropathy can be prevented, and life expectancy and the quality of life have increased. However, approximately 20-30% of patients suffering from severe HA develop neutralizing antibodies (inhibitors) against FVIII. Alternative treatment, using by-passing agents, is available for patients exhibiting inhibitors, but these can only be used for the short-term treatment of acute haemorrhage and as prophylaxis during surgery. Furthermore, the clinical response to by-passing products is unpredictable. Two of the studies included in this thesis evaluated the response to by-passing therapy in plasma from patients with HA. The variation in thrombin production within families was found to be significantly lower than the variation between families, indicating that a familial predisposition may influence thrombin formation in response to by-passing agents (Paper I). Moreover, FVIII clotting factors were found to potentiate the in vitro effect of by-passing agents on thrombin formation in plasma from patients with HA exhibiting inhibitors (Paper II). Not all anti-FVIII antibodies have neutralizing capacity. In the studies presented in Papers III & V, non-neutralizing anti-FVIII antibodies (NNAs) were investigated in two different cohorts, using an enzyme-linked immunosorbent assay (ELISA). NNAs were detected in 18.9% of siblings with HA, and in 12.8% of unrelated HA subjects followed for four years. The NNA response was assayed using three different rFVIII products. The NNA response was found to be heterogeneous, to vary considerably between individuals (Papers III and V), and also over time (Paper V). None of the patients in the cohort with NNAs observed longitudinally developed inhibitors (Paper V). However, in one patient with moderate HA, the detection of NNAs coincided with a change in bleeding phenotype four years prior to FVIII inhibitor development. This finding suggests that immunoassays may be a useful complement in evaluating the immune response to FVIII (Paper IV). The potential clinical impact of NNAs was evaluated in the long term study (Paper V), showing no association between age, F8 mutation, or the influence of immune system challenges on NNA development. Interestingly, patients with NNAs had significantly fewer bleeding episodes than NNA-negative patients ( $p=0.048$ ), raising questions about the possibility of yet undefined types of anti-FVIII antibodies with protective or potentiating effects on FVIII.		
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# Exploring Anti-Factor VIII Antibodies in Haemophilia A

Role in *In Vitro* Haemostasis and Clinical Disease

Jenny Klintman, MD



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Doctoral Thesis  
2013

Department of Clinical Sciences, Malmö, Unit of Clinical Coagulation Research  
Faculty of Medicine, Lund University, Sweden

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# Abstract

Haemophilia A (HA) is caused by defective synthesis of coagulation factor VIII (FVIII), which has serious effects on haemostasis; joints being the most common site of bleeding. The development of FVIII replacements has improved the situation for patients with haemophilia such that chronic arthropathy can be prevented, and life expectancy and the quality of life have increased. However, approximately 20-30% of patients suffering from severe HA develop neutralizing antibodies (inhibitors) against FVIII. Alternative treatment, using by-passing agents, is available for patients exhibiting inhibitors, although these can only be used for the short-term treatment of acute haemorrhage and as prophylaxis during surgery. Furthermore, the clinical response to by-passing products is unpredictable. Two of the studies included in this thesis evaluated the response to by-passing therapy in plasma from patients with HA. The variation in thrombin production within families was found to be significantly lower than the variation between families, indicating that a familial predisposition may influence thrombin formation in response to by-passing agents (Paper I). Moreover, FVIII clotting factors were found to potentiate the *in vitro* effect of by-passing agents on thrombin formation in plasma from patients with HA exhibiting inhibitors, indicating that further assessment of this treatment strategy in a clinical context is warranted (Paper II). Not all anti-FVIII antibodies have neutralizing capacity. In the studies presented in Papers III & V, non-neutralizing anti-FVIII antibodies (NNAs) were investigated in two different cohorts, using an enzyme-linked immunosorbent assay (ELISA). NNAs were detected in 18.9% of siblings with HA, and in 12.8% of unrelated HA subjects followed for four years. The antibody response was assayed using three different rFVIII products. The antibody response was found to be heterogeneous, to vary considerably between individuals (Papers III and V), and also over time (Paper V). None of the patients in the cohort with NNAs observed longitudinally developed inhibitors (Paper V). However, in one patient with moderate HA, the detection of Bethesda-negative anti-FVIII antibodies coincided with a change in bleeding phenotype four years prior to FVIII inhibitor development. This finding suggests that immunoassays may be a useful complement in evaluating the immune response to FVIII (Paper IV). The potential clinical impact of NNAs was evaluated in the long term study (Paper V), showing no association between age, *F8* mutation, or the influence of immune system challenges on NNA development. Interestingly, patients with NNAs had significantly fewer bleeding episodes than NNA-negative patients ( $p=0.048$ ), raising questions about the possibility of yet undefined types of anti-FVIII antibodies with protective or potentiating effects on FVIII.



# Abbreviations

aPC	activated protein C
aPCC	activated prothrombin complex concentrate
APTT	activated partial thromboplastin time
BDD-rFVIII	B domain-deleted recombinant FVIII
CI	confidence interval
CTI	corn trypsin inhibitor
CTLA	cytotoxic T-lymphocyte antigen
ELISA	enzyme-linked immunosorbent assay
ETP	endogenous thrombin potential
FIIa	activated coagulation factor II (e.g. thrombin)
FVII	coagulation factor VII
FVIIa	activated coagulation factor VII
FVIII	coagulation factor VIII
FVIIIa	activated coagulation factor VIII
FVIII:C	FVIII coagulant activity
FL-rFVIII	full-length recombinant factor VIII
FX	coagulation factor X
FXa	activated coagulation factor X
FXIa	activated coagulation factor XI
GPIb	glycoprotein Ib
GPVI	glycoprotein VI
HCV	hepatitis C virus
HIGS	Hemophilia Inhibitor Genetics Study
HIV	human immune-deficiency virus
HLA	human leukocyte antigen
IL	interleukin
ITI	immune tolerance induction
MHC	major histocompatibility complex
MIBS	Malmö International Brother Study
NNAs	non-neutralizing antibodies
pdFVIII	plasma-derived factor VIII
PLs	phospholipids
PPP	platelet-poor plasma
PRP	platelet-rich plasma
rFVIIa	recombinant activated FVII
SD	standard deviation

SNP	single nucleotide polymorphism
$T_{1/2}$	half-life
$T_{\max}$	maximal thrombin production in nM
$T_{\text{regs}}$	regulatory T cells
TCR	T cell receptor
TF	tissue factor (also called thromboplastin or factor III)
TGA	thrombin-generation assay
TGF	transforming growth factor
VWF	von Willebrand factor

# Original Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

- I. Thrombin Generation *In Vitro* in the Presence of By-passing Agents in Siblings with Severe Haemophilia A  
**Klintman J, Berntorp E, Astermark J; On behalf of the MIBS study group**  
Haemophilia, 2010 Jan; 16(1):e210-5
- II. Combination of FVIII and By-passing Agent Potentiates *In Vitro* Thrombin Production in Haemophilia A Inhibitor Plasma  
**Klintman J, Astermark J, Berntorp E**  
British Journal of Haematology, 2010 Nov; 151(4):381-6
- III. Antibody Formation and Specificity in Bethesda-negative Brother Pairs with Haemophilia A  
**Klintman J, Hillarp A, Donfield S, Berntorp E, Astermark J**  
Haemophilia, 2013; 19(1):106-12
- IV. Clinically Relevant Non-neutralizing Anti-FVIII Antibodies in a Patient with Haemophilia A  
**Klintman J, Hillarp A, Berntorp E and Astermark J**  
Submitted to Haemophilia in December 2012
- V. Long-term Anti-FVIII-antibody Response of Bethesda-negative Haemophilia A Patients Receiving Continuous Replacement Therapy  
**Klintman J, Hillarp A, Berntorp E and Astermark J**  
Submitted to Haemophilia in February 2013

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# My contribution to the papers

- Paper I      I performed part of the experimental work (TGA) and analysed the data with support from lab technician Maj Ekman and Jan Astermark. I performed the statistical work with support from the Competence Centre for Clinical Research, at Region Skåne and I wrote the paper.
- Paper II     I performed part of the experimental work (TGA), analysed and prepared the data with support from lab technician Maj Ekman and my supervisor Prof. Berntorp, and I wrote the paper. The statistical work was performed at Rho Inc, Chapel Hill, NC, USA.
- Paper III    I designed the research study together with my supervisor Ass. Prof. Jan Astermark and Ass. Prof. Andreas Hillarp at the Coagulation Unit of the Department of Laboratory Medicine, Skåne University Hospital in Malmö. I performed the ELISA assays in concert with lab technician Kerstin Fridh. I analysed the experimental and clinical data with support from my supervisor Jan Astermark and Ass. Prof. Andreas Hillarp. Andreas Hillarp developed the ELISA. I wrote the paper.
- Paper IV    Case report. I performed the ELISA, collected clinical data from the medical records and the UMAS Hemophilia Database 4.1 and I wrote the paper.
- Paper V     I designed the research study with support from my supervisors Ass. Prof. Jan Astermark and Prof. Erik Berntorp. I collected the clinical data from the medical records and from the UMAS Hemophilia Database 4.1. I performed the majority of the ELISA assays in concert with lab technician Kerstin Fridh. I analysed the clinical and experimental data with support from my supervisors. I wrote the manuscript.



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# Haemostasis

Haemostasis is a dynamic process involving interactions between the procoagulant, anticoagulant and fibrinolytic systems. It maintains blood fluidity and protects the body from extensive blood loss caused by vascular damage. Haemostasis can be divided into three phases: primary haemostasis, secondary haemostasis or coagulation, and fibrinolysis. When the integrity of the vasculature is disrupted, primary haemostasis is activated and platelets are recruited and aggregate at the site of injury. Coagulation (secondary haemostasis) is simultaneously initiated and fibrin is produced, which, together with platelets, forms a platelet plug (1). To avoid the formation intravascular thrombi, haemostasis is highly regulated by blood and vascular inhibitors, and coagulation is localized to the site of injury and on the surfaces of specific cells, such as platelets and endothelial cells (2, 3). The thrombin formed during coagulation activates fibrinolysis, which eventually dissolves the platelet plug (4).

## The Role of Endothelium and Primary Haemostasis

The endothelial lining of the vasculature controls haemostasis in several ways. As long as the integrity of the resting endothelium is maintained, negatively charged heparan sulphate proteoglycans, coagulation inhibitors (e.g. thrombomodulin and tissue factor pathway inhibitor) are expressed on the endothelial cell surface (3, 5). In addition, endothelial cells produce and release nitric oxid and prostacyclin (PGI<sub>1</sub>/PGE<sub>2</sub>), and express ectonucleotidases, preventing the activation of platelets and thus coagulation (5, 6) (Ref. 6, p. 1). Furthermore, when vascular damage occurs, locally produced vasoactive substances are released and vasoconstriction is initiated, making the endothelium part of the first-line defence against blood loss (5).

Rupture of the endothelium exposes the extracellular matrix (subendothelium) to the bloodstream, initiating primary haemostasis, which leads to the slowing down and adherence of circulating platelets to the site of injury. The platelets are anchored mainly through the binding of glycoprotein Ib (GPIb) and glycoprotein (GPVI) receptors on the surface of the platelets to the von Willebrand factor (VWF) and subendothelial collagen, respectively (7, 8). VWF is synthesized in endothelial cells and megakaryocytes, and stored in the endothelial Weibel-

Palade bodies and  $\alpha$ -granules of megakaryocytes/platelets until secreted (9, 10). Upon endothelial activation, VWF is secreted and forms ultralarge VWF multimers that connect to subendothelial collagen and, under the influence of shear stress, the molecule stretches exposing binding sites for the GPIb receptor on the circulating platelets (called adhesion) (8-10). When platelets are bound to collagen by the GPVI receptor, a conformational change is initiated in the platelets, exposing binding sites allowing platelet aggregation. The  $\alpha$ IIB $\beta$ 3 receptor on the platelet surface plays a key role in aggregation by binding the platelets to circulating VWF, and VWF continues to bind to GPIb on circulating platelets. The incoming platelets are activated by signals (e.g. adenosine diphosphate) from the first layer of platelets, and the process of anchoring additional platelets continues, leading to the formation of a platelet plug (8).

## Coagulation

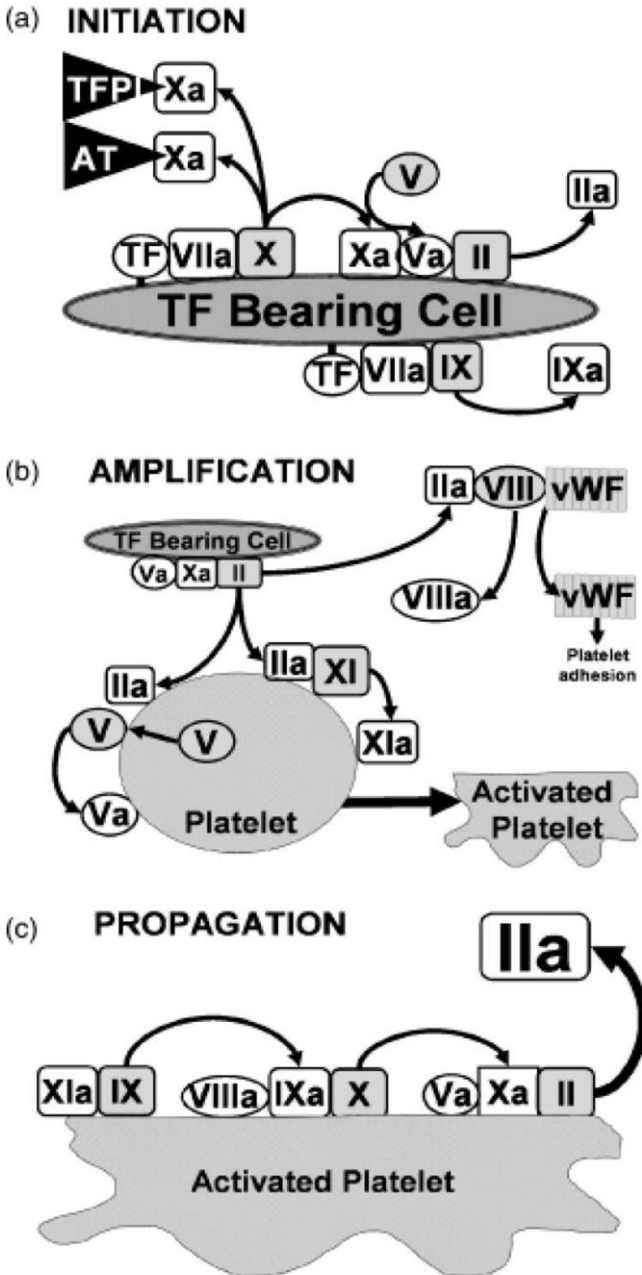
The initiation of *in vivo* coagulation is dependent solely on the plasma-membrane-anchored protein tissue factor (TF) (11), and the process thus takes place at the cell surface (Figure 1.1a). Many different cell types express TF, such as fibroblasts, monocytes, macrophages and endothelial cells (12), and certain organs are particularly rich in TF, including the brain, kidneys, placenta, testis and heart (13). Two kinds of TF are believed to exist, termed “coagulant active” TF and “cryptic” TF: where it has been suggested that cryptic TF is expressed on cells inside the vasculature (e.g. monocytes and endothelial cells), and is not (significantly) involved in initiating coagulation (4, 11). When plasma comes into contact with TF-bearing subendothelial cells the coagulation factor VII (FVII) in plasma binds to TF forming the TF-FVIIa complex (2, 14). Unlike all other coagulation factors found in plasma as zymogens (e.g. inactive enzyme precursors), a very small amount (1-2%) of FVII circulates in the blood in its active form, FVIIa. To become proteolytically active, FVIIa must be bound to TF, and is therefore protected from inhibition by circulating antithrombin III in the blood (15) (pp. 7-8). The subendothelial membrane-bound TF-FVIIa complex activates factor IX (FIX), factor X (FX) and more FVII, leading to the formation of additional TF-FVIIa complexes (3). Activated FX (FXa) forms a complex with its cofactor, activated factor V (FVa), i.e., the prothrombinase complex, which subsequently cleaves prothrombin (FII), producing a small amount of activated thrombin (also denoted FIIa). In the following amplification phase (Figure 1.1b), the thrombin generated/formed during the initiation phase diffuses away from the TF-bearing cell activating the platelets, initiating the release of factor VIII (FVIII) from VWF, and activating FVIII, FV and FXI (4). As a result of conformational changes in the

activated platelets, pro-coagulant phospholipids are exposed on the platelet surface, which are crucial to the assembly of enzymatic complexes during the ensuing propagation phase: i.e., the tenase complex (FIXa-FVIIIa) and the prothrombinase complex (FVa-FXa) (Figure 1.1c). The prothrombinase complex bound to the platelet surface generates a burst of thrombin, which subsequently converts fibrinogen to fibrin, and activates FXIII, producing FXIIIa, which enables cross-linking of the fibrin strands to form a mesh, providing a strong, elastic plug (3).

Coagulation is regulated through various feedback systems. The tissue factor pathway inhibitor expressed on the endothelial cell surface, controls the TF-FVIIa complex on TF-bearing cells and inhibits FXa, which diffuses away from the cell surface in order to prevent FVIIa activation in the circulation (12, 16, 17). Antithrombin III inhibits several activated coagulation factors: the TF-FVIIa complex, thrombin, and FIXa, FXa and FXIa (18). Moreover, the binding of thrombin to the endothelial cell receptor thrombomodulin activates protein C (aPC), an inhibitor of FVa and FVIIIa. This protein C-induced inhibition is greatly increased by complex binding of aPC to its cofactor, circulating protein S (17).

## Fibrinolysis

In order to prevent extensive thrombus formation, fibrinolysis is triggered by the tissue-type plasminogen activator or by the urokinase-type plasminogen activator once the fibrin clot is formed, and plasminogen is then converted to plasmin. Plasmin degrades polymerized fibrin to fibrin degradation products which, when they are cross-linked by FXIII, are released as D-dimers that can be analysed in the blood (19, 20) (Ref p. 463). Under physiological conditions, and in the absence of clot formation, fibrinolysis is kept suppressed by several control systems, and by the fact that fibrin itself is a major cofactor and substrate for plasmin (4, 19).



**Figure 1.1** Cell-based coagulation. Roman numerals indicate inactive and active coagulation factors (see Abbreviations);(reprinted with permission from Wiley-Blackwell) (3).

# Monitoring *In Vitro* Haemostasis

## Screening Assays

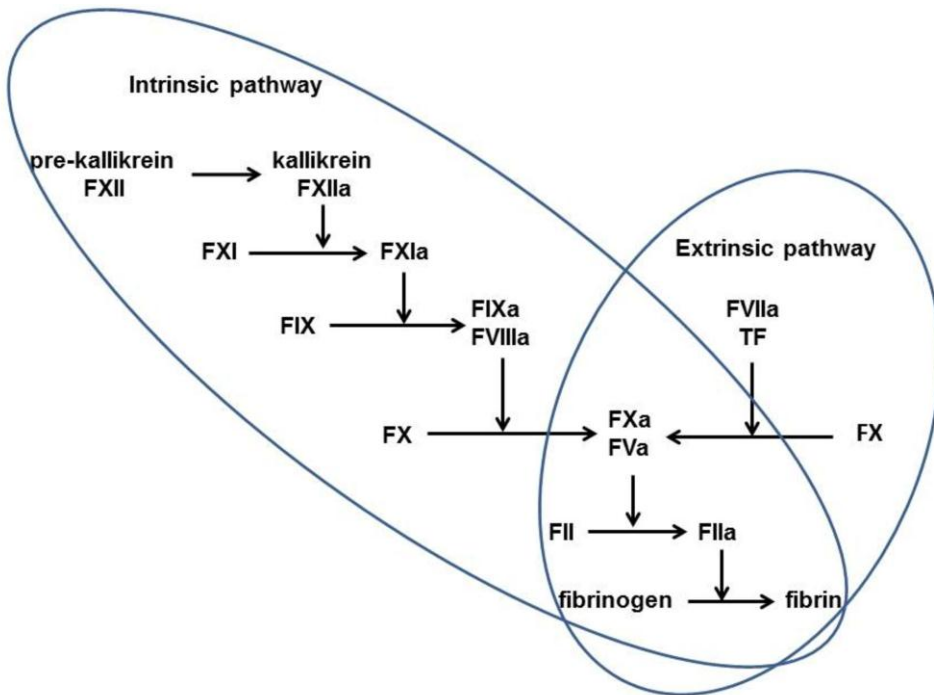
In 2001, Monroe and Hoffman proposed that coagulation, as described in the previous section, is a process involving interactions between clotting factors and specific cell surfaces in the vicinity of the ruptured endothelium. This theory is called the cell-based model of haemostasis, and is today widely accepted as reflecting *in vivo* haemostasis (21). However, the classical cascade model of coagulation (Figure 1.2) is still a useful starting-point to understand the *in vitro* approaches used in the laboratory. Here, two pathways, the intrinsic and the extrinsic, are stimulated in a sequence of enzymatic steps, where one enzyme activates the next zymogen (or pro-enzyme), generating the next enzyme in the cascade. Both pathways converge in the activation of FX to FXa, which results in thrombin activation (3).

The intrinsic pathway, suggested to reflect the coagulation process taking place within the blood vessel, is initiated by contact activation of FXII. *In vitro*, this pathway is activated when blood comes into contact with the test-tube. This is prevented, in both clinical practice and research, by coating the test-tubes with trisodium citrate. This blocks the calcium necessary for the enzymatic reactions that activate FIX and the ensuing reactions. The combined reactions of the intrinsic and common pathway can be monitored using the activated partial thromboplastin time (APTT). An activator is added to the test-tube together with a phospholipid substrate and calcium in order to initiate the activation of FXII and pre-kallikrein to form FXIIa and kallikrein, respectively (6, 21).

The extrinsic pathway reflects the function of factors VII, X, V, II (prothrombin) and fibrinogen, and is activated by tissue factor. In the laboratory, this process is monitored by the prothrombin time assay, in which the extrinsic pathway is activated by TF, together with phospholipids and calcium. Factors X, VII and II are vitamin K-dependent, and the prothrombin time assay is used for monitoring anticoagulation therapy with warfarin<sup>1</sup> (6, 15) (p. 9 & p. 11, respectively).

---

<sup>1</sup> Antagonised with vitamin K epoxide reductase, resulting in decreased carboxylation of coagulation factors in the liver.



**Figure 1.2** Cascade model of haemostasis. Roman numerals indicate inactive and active coagulation factors (see Abbreviations); TF = tissue factor.

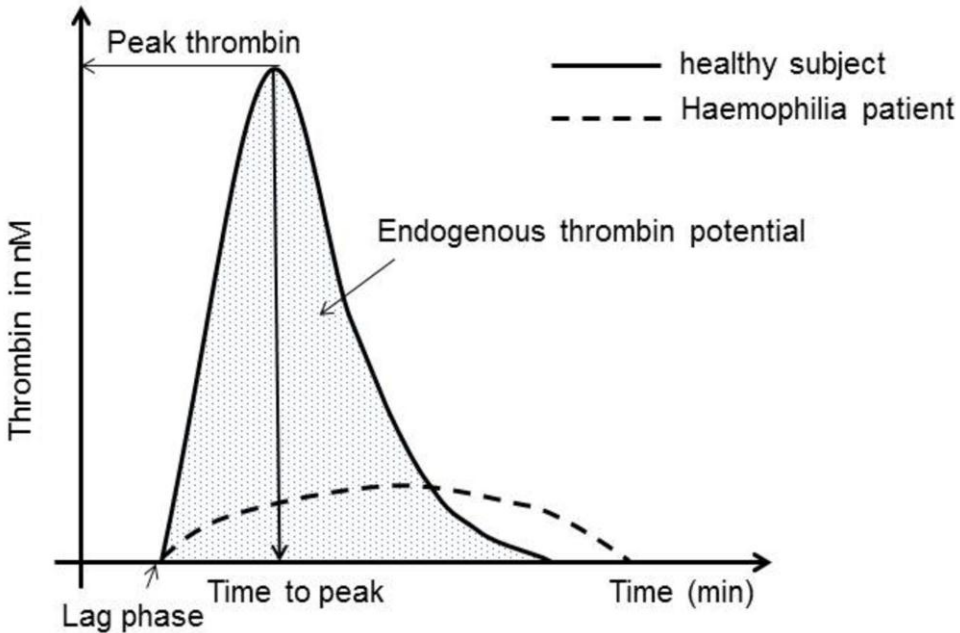
## Assays Monitoring Global Haemostasis

The screening assays described in the previous section are standardized and available at most hospitals, and are usually sufficient to diagnose a clotting factor deficiency. However, these methods are not able to provide complete pathophysiological explanations of the *in vivo* observations made in patients. For example, in patients with FXII deficiency, the APTT will be considerably prolonged, without finding any bleeding tendency of the patient (12). It is well-known among clinicians caring for haemophilia patients that the FVIII coagulant activity (FVIII:C) measured in plasma from an individual patient does not necessarily correspond to the clinical phenotype of the patient (22). This is one reason for the increased interest in developing laboratory methods that provide a more physiological and comprehensive picture of the process of blood coagulation (23). Two principal methodological pathways have been developed, aimed at either the monitoring of whole blood clot formation or the continuous measurement of thrombin formation.

In modern thromboelastography, i.e. testing of the efficiency of the coagulation of blood, the sample is placed in a cup with a pin inserted at the centre. Either the cup (TEG®) or the pin (ROTEM®) is rotated, and a transducer converts the rotation of the pin into an electrical signal recorded in a computer. Methods based on blood clot formation provide information on the continuous changes in the viscoelastic properties that occur during the coagulation of whole blood or plasma (15, 24) (Ref. 15 pp. 266-267), and is assumed to represent the combination of platelet function, coagulation factors and the fibrinolytic system (24). However, thromboelastography mainly reflects the changes in fibrinogen levels, and is not particularly sensitive to the dynamics of thrombin formation (25). Several areas of clinical application have been studied in single-centre settings, including bedside monitoring of haemostasis during surgery, monitoring of blood component therapy during cardiac and liver surgery, and assessment of thrombotic risk. TEG® and ROTEM® have been utilized for monitoring *in vitro*, *ex vivo* and *in vivo* by-passing therapy in inhibitor plasma from patients with HA (26-28). However, a multi-centre randomized trial performed recently, to compare TEG® and ROTEM® measurements in patients receiving by-passing therapy, showed such a large intra- and inter-patient variability following *in vivo* administration of recombinant activated FVII that the authors concluded that the use of either method was unsuitable for dose-response prediction in the clinical setting (26). In other studies, the important issue of the ability to predict clinical phenotype has been addressed. Chitlur and colleagues found patients with a mild bleeding phenotype to have better clot forming capacity than patients with a tendency for severe bleeding (29). The major advantage of modern thromboelastographic methods is that they can be performed as point-of-care testing. However, blood samples should be analysed within 2 hours, and frozen samples cannot be analysed. Furthermore, inter- and intra-individual variations make the results unreliable.

The thrombin generation assay (TGA) was developed by Hemker and colleagues to visualize the dynamics of continuous thrombin production (30). A solution of a fluorogenic substrate that is cleaved by thrombin is used, liberating a fluorophore. The intensity of the fluorescence is proportional to the amount of thrombin present, and a characteristic curve is obtained from which various parameters can be derived (Figure 1.3). The time before the reaction starts (the lag phase) is comparable in plasma from healthy individuals and FVIII-deficient individuals, while the propagation phase (reflected by  $T_{max}$  and the endogenous thrombin potential) is severely impaired in patients with haemophilia (31).





**Figure 1.3** The thrombin generation assay. The two curves illustrate the principle difference in thrombin formation in a healthy subject and a patient with severe haemophilia A. Parameters of the thrombogram: Lag time (min); Time to peak (min); Peak thrombin production ( $T_{max}$ ); Endogenous thrombin potential (ETP).

The method has been widely used in several areas of application, including venous thrombosis, arterial vascular disease and bleeding disorders (25). As with thromboelastography, there is a lack of standardized protocols for TGA, and the method suffers from large inter-centre variability (32). Considerable effort has been devoted to solving these problems to allow the use of TGA in clinical trials, and possibly in routine clinical practice (33). In research on haemophilia, TGA has been applied to several basic research questions (34-38), as well as clinically relevant issues associated with bleeding phenotype (39-41) and the monitoring of replacement and by-passing therapy (35, 42-45). By-passing agents are important in the treatment of inhibitor patients, especially those with high-responding inhibitors, where saturation with FVIII concentrates is not possible (46). Promising results have been presented supporting the use of TGA in the monitoring of therapy with by-passing agents (42, 47-52). For example, Dargaud

and colleagues recently showed that the use of TGA allowed individual tailoring of by-passing therapy in inhibitor patients during surgery, and that the *in vivo* clinical response was correlated to *in vitro* outcome measures of the assay (53).

TGA can be performed using platelet-rich (PRP) or platelet-poor plasma (PPP). PPP can be frozen and therefore has a great practical advantage. TGA is most often run with TF as the activator, and many different TF substrates are available at varying concentrations. A higher concentration of TF in the assay gives a more stable assay, but at the cost of sensitivity (15, 51) (Ref. 15, p. 265). Alternative protocols have been reported, for example, inducing thrombin generation by recalcification in order to increase the sensitivity (49). One important methodological concern has been raised regarding FXII-induced contact activation in test-tubes. To prevent contact activation, it has been suggested that corn trypsin inhibitor (CTI) can be added to reduce the variability in the assay, especially in low-TF assays (15, 25) (Ref. 15, p. 265). However, in assays using higher concentrations of TF ( $\geq 5$  pM) the addition of CTI was shown to be unnecessary (54, 55).

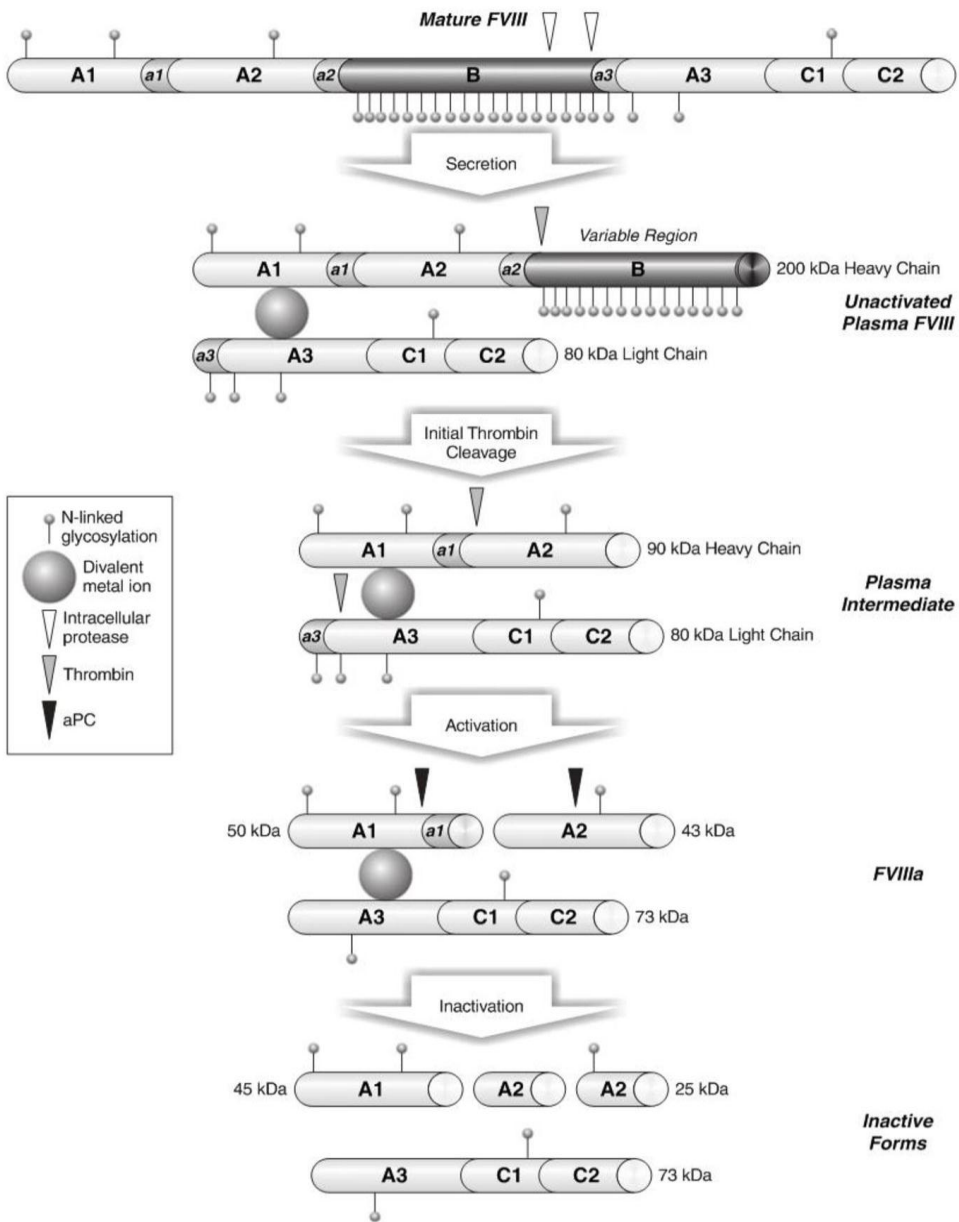


# Factor VIII in Health and Disease

## Factor VIII Protein Synthesis *In Vivo*

The factor VIII gene (*F8*) is located on the X chromosome (Xq28). It was cloned in the early 1980s, and is one of the largest genes described (186 kb) (56, 57). It contains 26 exons that encode a precursor protein of 2351 amino acids, including a 19-amino-acid-long signal peptide. The mature FVIII protein is a 2332-amino-acid-long glycoprotein with the domain structure A1-a1-A2-a2-B-a3-A3-C1-C2 (Figure 2.1). Both the A domains and the C domains show structural homology to factor V (FV), while the structure of the B domain is unique to FVIII. The acidic sequences *a1*, *a2* and *a3* play significant roles in thrombin activation, VWF and FX binding, and stabilization of the FVIII heterotrimer (see below) (15, 58) (Ref. 15, p. 24). The liver is considered to be the primary source of FVIII, and both hepatocytes and sinusoidal endothelial cells synthesize FVIII. However, a variety of organs and cells have been shown to be of great importance in maintaining FVIII synthesis, *viz.* the spleen, kidneys, lymph nodes and lungs, as well as endothelial and mesenchymal cells (58, 59).

Before being secreted into the circulation, the single-chain FVIII protein undergoes extensive post-translational intracellular processing, resulting in a heterodimeric structure containing a heavy chain with the domains A1-a1-A2-a2-B (amino acids 1-1648), and a light chain consisting of a3-A3-C1-C2 domains (amino acids 1649-2332). Upon secretion, the heavy chain and light chain are non-covalently connected through the A1 and A3 domains, and in the circulation the FVIII heterodimer is stabilized by binding non-covalently to its carrier protein VWF. VWF protects FVIII from proteolytic degradation and from binding to phospholipids and FIX, but also directs FVIII to the site of injury (58, 60). The haemostatic importance of VWF binding to FVIII is demonstrated in von Willebrand disease, in which either no, or a defective, VWF is expressed, resulting in a lack, or decrease in, both VWF and FVIII levels in plasma (61).



**Figure 2.1** The factor VIII protein from intracellular full-length protein, through processing to active and inactive forms. Letters A-C and a indicate the different regions of the FVIII protein (Reprinted with permission from Wiley-Blackwell.)(62)

The role of FVIII in haemostasis is to serve as a cofactor for FIXa by forming the tenase complex, within which the catalytic activity of FIXa is amplified many-fold. To enable complexation with FIXa, FVIII must be activated and released from VWF, a process carried out through proteolysis by thrombin and FXa. There are three cleavage sites for thrombin on FVIII: after Arg372, Arg740 and Arg1689. Proteolysis of the Arg1689 site in the light chain releases FVIII from VWF, exposing binding sites on FVIII. These bind to negatively charged phospholipids on the surface of activated platelets, as well as to FIXa (63). Furthermore, upon thrombin activation, the B domain is released, initiating the formation of an active FVIII heterotrimer (Figure 2.1). The B domain has previously been shown to be redundant for the coagulant activity of FVIII. However, recent studies have revealed important functional properties of the B domain, including roles in intracellular trafficking, the prevention of premature thrombin activation, a decrease in the affinity of inactivated FVIII for activated platelets, and a reduction in FVIII proteolysis by aPC in plasma (62).

Down-regulation of FVIII can proceed through two possible pathways. The protein may undergo proteolytic inactivation of aPC, FIXa and FXa, resulting in inhibition of the binding of FVIIIa to FIXa and FX, together with interruption of intramolecular binding of the different protein domains, initiating dissociation of the protein. The other pathway involves spontaneous dissociation, made possible by the fact that activated FVIII is a much less stable molecule than inactivated FVIII (64). FVIII is cleared from plasma by pinocytosis or phagocytosis, or receptor interaction with scavenger cells such as macrophages (60).

## Haemophilia A

### Clinical Disease

The first clinical report of haemophilia dates from 1803, when Dr John Otto of the New York Hospital described a female carrier with bleeding diathesis. In 1830, a boy with a family history of haemophilia, had a post-operative haemorrhage which was successfully treated with life-saving blood transfusions (15) (p. 1). As the *F8* gene is located on the X chromosome the recessive trait penetrates in males, affecting about 1 in 5000 males. The clinical diagnosis of haemophilia is determined by the level of FVIII activity (measured in kilo international units (kIU)/L) in plasma: severe haemophilia <0.01 kIU/L, moderate haemophilia 0.01-0.05 kIU/L and mild haemophilia >0.05-0.40 kIU/L (reference in a healthy population: 0.50-1.50 kIU/L). Clinical disease should be suspected in patients

showing easy bruising in early childhood, spontaneous bleeding (especially in joints or soft tissues) and/or post-operative or trauma-induced haemorrhage (65, 66).

The clinical phenotype varies considerably between patients, but patients with severe haemophilia usually experience their first bleeding during the first year of life. Moderately affected patients show a more diverse bleeding pattern, where some patients present with a similar phenotype to severely affected patients, requiring FVIII replacement therapy. However, at levels of FVIII:C above 0.02-0.03 kIU/L, patients rarely bleed. In congenital haemophilia A the most common site of bleeding is the joints, leading to the development of arthropathy, which may severely impair mobility and quality of life. Bleeding may also occur in soft tissues and (rarely) in the central nervous system (15, 66) (Ref. 15, p. 33). In acquired haemophilia A, which usually occurs in the elderly and is caused by the development of auto-antibodies to FVIII, bleeding in soft tissue is the most common clinical presentation (67).

Based on the observation that patients with FVIII:C above 0.02-0.03 kIU/L (i.e. moderate haemophilia A) bleed less and are less prone to develop chronic arthropathy, it was reasoned already in the 1950s that continuous replacement therapy with FVIII (prophylaxis) could potentially change the phenotype in severely affected patients to that more like moderate haemophilia. Prophylaxis was introduced in Malmö, Sweden, by Professor Inga Marie Nilsson in 1958, in patients with severe haemophilia A, and is today the treatment recommended by the World Federation of Hemophilia (66, 68). Already in the late 1960s, the follow-up of patients being treated in this way confirmed that the frequency of bleeding was reduced, joint status improved, and that the patients could lead a more normal life than before (15, 68) (Ref. 15, p. 38). It was also observed that arthropathy could be prevented by early prophylaxis, referred to as “primary prophylaxis”, prior to the first case of joint bleeding (haemarthrosis), i.e. before the age of 2 years, or after the first haemarthrosis (66, 69). Several dosage strategies are used in primary prophylaxis. The Swedish model prescribes high-dose prophylaxis (25-40 IU/kg of FVIII concentrate, three times weekly), starting prior to the occurrence of haemarthrosis, which is usually around the age of one year. In the Netherlands, the recommendation is an intermediate dose (15-25 IU/kg, three times weekly), whereas the Canadian approach is to use dose escalation (starting at a dose of 50 IU/kg once weekly), both introduced after the first haemarthrosis (70). However, a major drawback of this treatment is the high cost of clotting factors, which reduces the availability of adequate treatment, not least in developing countries. Therefore, on-demand treatment, given after the occurrence of bleeding, is often given (70).

When prophylaxis was introduced in Sweden in 1958, FVIII was administered to haemophilic patients as cryoprecipitated anti-haemophilic factor, which is a blood product prepared from plasma, and includes FVIII, fibrinogen,

VWF and FXIII. The first specific factor concentrates became available during the 1970s as intermediate-purity factor concentrates, which made self-administered home treatment possible (70, 71). With the more extensive use of plasma-derived products came the disastrous setback in the 1980s, with the transmission of the human immunodeficiency virus (HIV) and hepatitis C virus. However, as a result of improvements in virus-inactivation methods, and more careful screening of donated blood, modern plasma-derived clotting factors are considered safe (71). Advancements in developing recombinant FVIII, which started 20 years ago, provided an alternative to plasma-derived products, and today the majority of patients in developed countries are treated with recombinant products (71-75). Several recombinant FVIII (rFVIII) products are available today, the majority of them containing full-length FVIII molecules (Figure 2.1), stabilized either by human serum albumin or sucrose solution. The other type, B domain-deleted rFVIII (BDD-rFVIII), comprises a shorter protein sequence lacking most of the B domain, in which the N- and C-terminals are spared and fused by a short amino acid peptide linker. The advantage of shortening the FVIII molecule is increased stability, and BDD-rFVIII is stabilized by sucrose. The manufacturing processes of the different products are similar, and include viral inactivation and purification with monoclonal FVIII antibodies. However, different cell lines are used: baby hamster kidney cells for Kogenate<sup>®</sup>, and Chinese hamster ovary cells for Advate<sup>®</sup> and the BDD-rFVIII ReFacto<sup>®</sup> (76). Comparisons between full-length and BDD-rFVIII have shown the products to be equivalent in terms of efficacy and inhibitor development (77-80). However, the results of a recent meta-analysis raised some concern since the risk of *de novo* inhibitor development in previously treated patients was increased in patients who had received BDD-deleted rFVIII (81).

In patients with mild haemophilia A with a baseline FVIII:C  $\geq 0.15$  kIU/L, desmopressin (DDAVP), a synthetic anti-diuretic hormone analogue, can be given as an injection or as a nasal spray, for example, in dental extraction or other minor surgical procedures. DDAVP stimulates the endogenous release of FVIII and VWF, and is therefore also suitable in patients with von Willebrand disease. However, not all patients respond to DDAVP, and the effect must be tested in each patient prior to treatment. In addition, anti-fibrinolytic drugs, for example, tranexamic acid, are often used as complementary treatment for mucosal bleeding and in dental procedures (66, 70).

The most severe complication associated with FVIII replacement therapy is the development of neutralizing anti-FVIII antibodies, or inhibitors, which have serious effects on haemostasis (discussed in more detail in the following chapter). In patients with low-responding inhibitors (titre  $< 5$  BU/mL) the general strategy is to use FVIII products to treat haemorrhages, with increased dosage in order to reach a FVIII:C plasma level that will saturate the inhibitor and ensure haemostasis. This approach is usually not sufficient in patients with high-responding inhibitors ( $> 5$  BU/mL), and so-called by-passing agents are therefore



used in these patients. Two by-passing products are currently available: activated prothrombin complex concentrate (aPCC) and recombinant activated FVII (rFVIIa); and both induce thrombin production independently of FVIII and FIX. Use of a by-passing product is indicated in situations of acute haemorrhage, and as prophylaxis during surgery in inhibitor patients. Activated PCC was developed during the late 1970s and contains several inactivated (FII or prothrombin, FVII, FIX, FX and protein C) and activated (thrombin, FVIIa, FIXa and FXa) coagulation proteins, including trace amounts of FVIII. Prothrombin and FXa are considered to be the main active components, and can contribute directly to clot formation since they act downstream of the inhibited tenase complex (FIXa-FVIIIa). Furthermore, since aPCC contains both activated enzymes and zymogens, the overall level of substrate needed for thrombin production is increased. The half-life of aPCC (FEIBA<sup>®</sup>) is about 4-7 hours (15, 82). The rFVIIa (NovoSeven<sup>®</sup>) was launched during the 1990s. The haemostatic effect involves two main pathways. Firstly, the TF-dependent activation of FX on the surface of TF-bearing cells, which results in the formation of the prothrombinase complex with FVa (Figure 1.1 & 1.2), and secondly, through the activation of FX on the surface of activated platelets. Hence, both the initial, minute thrombin production and the thrombin burst are promoted by rFVIIa. Since rFVIIa is dependent on TF and phospholipids to exert a pharmacological effect, rFVIIa acts selectively at the site of injury. Its half-life is approximately 2-3 hours (15, 83). The clinical response to by-passing agents may be somewhat unpredictable in patients, however, both products are considered efficacious in treating haemorrhages. A comparative evaluation of aPCC and rFVIIa was performed in the FEIBA NovoSeven Comparative study FENOC study, showing comparable efficiency in treatment outcomes. However, a substantial proportion of the patients rated the efficacy differently, especially during the first 12 hours of treatment (84).

## **Genetic Defects in Haemophilia A**

Genetic defects causing impaired FVIII function can be divided into three categories: (i) gross gene rearrangements; (ii) deletions or insertions; and (iii) point mutations (single DNA base substitution) leading to premature peptide chain termination (“nonsense”), amino acid replacement (“missense”), or mRNA splicing defects, respectively (15) (p. 27). Although all categories of mutations are found in patients with severe haemophilia A, 50% of the severe cases are caused by inversion of intron 22, which separates exons 1-22 from exons 23-26 (85). Inversion of intron 1, and a combination of intron 22 and intron 1 inversions, have also been found in patients with severe haemophilia A (86).

As of 30 November 2012, 2107 unique mutations (apart from inversions) were registered in the Haemophilia A Mutation Database (HADB) (87). The

majority of the patients inherit the mutated gene from their mother, who is the carrier, but spontaneous mutations occur in about 30 percent of the affected individuals (65, 88). Regarding the single-base substitutions in the *F8* gene, the vast majority of the defined mutations are unique (only reported once). However, some appear in several patients, and are represented in patients with severe, moderate and mild disease.

A genetic defect may result in different pathologies. Decreased FVIII:C in plasma may be caused by the production of a defective protein with decreased functional FVIII:C in the circulation. Alternatively, a fully normal protein is produced, but secretion is impaired, resulting in a lower level of FVIII in plasma. Furthermore, the interaction of FVIII with its carrier protein, VWF, or with other partner molecules involved in coagulation (e.g. FIXa and FX) may be impaired, as well as the stability of the heterotrimeric form of FVIIIa, resulting in reduced functional activity of FVIII (15) (pp. 28-29).

### **Phenotypic Heterogeneity in Severe Haemophilia A**

It is a well-established observation among clinicians caring for patients with haemophilia A that the clinical presentation (phenotype), even in patients carrying inversion mutations, varies considerably. Some patients start bleeding early, while others, yet a minority of severe haemophilia A patients, present with a phenotype of mild haemophilia A and rarely bleed. The degree of arthropathy may also vary. Several factors, other than *F8* genotype, have been reported to influence the phenotype, including variable levels of other coagulant and anticoagulant factors, and the pharmacokinetics of the infused clotting factor (89). Carriers of haemophilia A may also present with various bleeding symptoms, which have been suggested to correlate with both the genotype and the phenotype of the haemophilic relative (90). These observations emphasize the fact that even monogenic diseases have multifactorial pathophysiology (91).



# Immune Response in Haemophilia A

Apart from protecting us from invasive pathogens, the immune system has evolved the capacity to discriminate foreign molecules from self, referred to as “tolerance to self”. In thymus T cells expressing a T cell receptor (TCR) with high affinity to self-antigens undergo negative selection (clonal deletion). Though, it has been demonstrated that some of the high-affinity T cells instead of being deleted, trigger development of CD4<sup>+</sup> immunosuppressive T cells, termed naturally occurring regulatory T cells (T<sub>regs</sub>) (92). The B cell repertoire is continuously renewed during life through rearrangements of the B cell receptor of B cells in the bone marrow. Self-reactive B cells are eliminated through clonal deletion before entering the periphery. However, B cells may diversify in the periphery through random mutations in antibody hypervariable regions (somatic hypermutation) (93) (p. 248). However, self-reactive T and B cell clones are detected in the periphery under both physiological and pathological conditions, resulting in, for example, FVIII inhibitor formation, both in patients with congenital haemophilia, and in previously healthy individuals (acquired haemophilia). Moreover, circulating anti-FVIII antibodies with antibody fractions with both inhibitory and non-inhibitory capacities have been detected in healthy individuals (94, 95). Naturally occurring T<sub>regs</sub> have been demonstrated to regulate T cell response to FVIII *in vitro*, and, together with anti-idiotypic antibodies, are believed to play a role in controlling the anti-FVIII immune response *in vivo* (15, 96) (Ref. 15, p. 52).

## FVIII Antigen Recognition

Upon the administration of exogenous FVIII, the antigen is recognized as foreign, and is internalized by antigen-presenting cells, such as dendritic cells and macrophages. The protein undergoes intracellular processing to provide linear peptide fragments that can be presented on the cell surface by MHC (major histocompatibility complex) class II molecules (97). MHC class II molecules consist of two transmembrane chains ( $\alpha$  and  $\beta$ ), and humans have three different types of MHC class II proteins: HLA (human leukocyte antigen)-DR, HLA-DQ and HLA-DP. Polymorphism of the genes encoding the  $\alpha$  and  $\beta$  chains of the

respective MHC class II molecules leads to great diversity of the allelic combinations of  $\alpha$  and  $\beta$  chains, resulting in a broad repertoire of MHC class II molecules. Hence, the ability of the immune system to recognize an antigen is increased immensely (98). Studies on autoimmune diseases (e.g. ankylosing spondylitis and diabetes type I) have demonstrated that certain HLA class I and class II alleles are more frequent in patients than in the general population, and inhibitor development in haemophilia may also be influenced by HLA allele genotype (99, 100).

When the peptide fragment is presented on the surface of the antigen-presenting cell, FVIII-specific CD4<sup>+</sup> T cells are activated through binding of the TCR to the MHC class II molecule. This interaction is dependent on co-stimulator molecules, including CD28 on the T cell and B7.1 (CD80) or B7.2 (CD86) on the antigen-presenting cells, in order to achieve full T cell activation (98). Most CD4<sup>+</sup> cells in the blood and secondary lymphoid organs are termed T helper cells, and are involved in both humoral and cellular immune responses. However, a great number of studies have shown that T<sub>regs</sub>, which form a subset of the CD4<sup>+</sup> cells, play an important role in suppressing immune responses. Two types of T<sub>regs</sub> have been identified. The first are natural T<sub>regs</sub>, developed in the thymus, which express CD4 and CD25 surface antigens, the forkhead box protein 3 (Foxp3) transcription factor, cytotoxic T lymphocyte-associated antigen (CTLA)-4 and glucocorticoid-induced tumour necrosis factor receptor (GITR). The second type of T<sub>regs</sub>, adaptive T<sub>regs</sub>, are CD4<sup>+</sup> and CD25<sup>-</sup>, which circulate in the blood as naive T cells, and differentiate into T<sub>regs</sub> upon stimulation with a specific antigen and cytokines. They exert immune suppression by the secretion of interleukin (IL)-10, transforming growth factor (TGF)- $\beta$ , and infrequently Foxp3 (101, 102). Mutations in genes encoding the effector or co-stimulatory molecules of T<sub>regs</sub>, leading to modification or deficiency in the T<sub>reg</sub> response have been identified (98). In haemophilia A polymorphic alterations in the IL-10, TNF- $\alpha$  and CTLA-4 genes have been investigated and correlated, however, not consistently, with the risk of inhibitor development (103-107). Furthermore, the FVIII-specific T cell repertoire has been investigated, showing that T<sub>regs</sub> from haemophilia A patients with and without inhibitors can, in some cases, recognize some of the same T cell epitopes. In other cases the T<sub>reg</sub> repertoire differs between the patient groups, in that T<sub>regs</sub> from inhibitor patients do not recognize, for example, immunodominant T cell epitopes on the A3 domain (residues 1691-1710 and 1941-1960), both of which are recognized by T<sub>regs</sub> from inhibitor-negative haemophilia patients. The same pattern has been demonstrated for a great number of T cell epitopes (97). Hence, the immune system fails to suppress a pathological response in inhibitor patients. In HIV-infected haemophilia patients with high-titre inhibitors, the antibody titre decreased, or even disappeared, as the CD4<sup>+</sup> T cell count decreased, a clinical observation that confirms the role of T<sub>regs</sub> in inhibitor development (60).

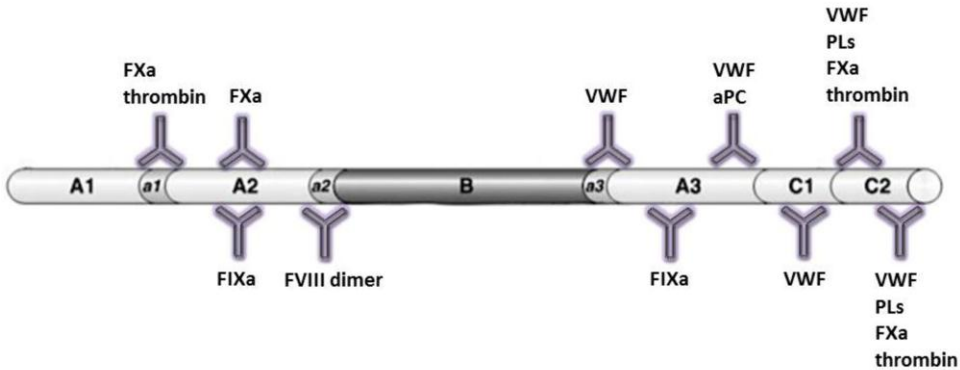
## The role of B cells

During the primary immune response, CD4<sup>+</sup> T cells activate naive FVIII-specific B cells which differentiate into antibody-secreting cells (plasmocytes), or into FVIII memory B cells. Upon re-exposure to FVIII (secondary immune response) memory B cells can serve as antigen-presenting cells to CD4<sup>+</sup> T cells through the expression of high-affinity antigen receptors, MHC class II and co-stimulatory signals (108). Following the secondary immune response, some of the memory B cells differentiate into short-lived antibody-secreting cells, enabling rapid elimination of the antigen, while others re-enter the peripheral lymphoid organs. A subset of B cells migrates to the bone marrow as long-lived plasma cells (109). In haemophilia A, FVIII-specific memory B cells have been found in the circulation of patients with FVIII inhibitors. Moreover, in inhibitor patients who were successfully treated with immune tolerance induction (ITI), FVIII-specific memory B cells were shown either to be absent, or present at very low levels (110).

## Inhibitory Anti-FVIII Antibodies

The development of inhibitory antibodies against FVIII (inhibitors) is the most serious complication in patients with haemophilia A, and approximately 20-35% of patients with severe haemophilia A develop inhibitors as a result of replacement therapy (111). FVIII inhibitors are IgG antibodies, initially characterized as belonging to subclasses IgG1 and IgG4 (112, 113). However, although IgG1 and IgG4 are the most abundant in inhibitor patients, further studies have shown that the FVIII inhibitor response is not isotypically restricted, but also involves the subclasses IgG2 and IgG3 (112, 114-116).

There are binding sites throughout the FVIII protein allowing interactions between FVIII and its partners in the coagulation process: VWF, phospholipids, FIX and FX (Figure 3.1) (117). Inhibitors obstruct these binding sites, impeding coagulation, thus presenting a challenge to haemostasis. In addition, inhibitors interfering with FVIII activation (by thrombin or FXa) have recently been characterized (118, 119). Although FVIII inhibitors (both in congenital and acquired haemophilia) recognize epitopes in all domains, the A2 domain and the light chain (C2, C1, A3 and *a1*) appear most immunogenic (118-121).



**Figure 3.1** Epitope regions of FVIII inhibitors and effects on binding of FVIII ligands. Letters A-C and a indicate regions of the FVIII protein; Roman numerals indicate coagulation factors; VWF= von Willebrand factor; aPC=activated protein C; PLs=phospholipids.

The mechanisms of inhibition have been widely investigated, revealing a number of major pathways. Inhibitors directed against most regions (A1, A2, C2, A3, C1, a1, and a3) block epitopes through steric hindrance<sup>2</sup>. (117, 122, 123). Other mechanisms of inactivation involve degradation of FVIII through hydrolysis, where some of the hydrolytic antibodies are exclusively found in inhibitor patients (124, 125), while others have also been detected in inhibitor-negative patients (126). The formation of immune complexes of FVIII and the inhibitor, and interference with the release of FVIII from VWF have also been suggested (127, 128).

The inactivation pattern of FVIII inhibitors is not uniform, and they are usually divided into types I or II, based on the kinetics of inhibition. The majority of inhibitors found in congenital haemophilia A inactivate FVIII:C completely in a linear fashion (type I, or first-order kinetics) in a manner that is dependent on both concentration and time. However, FVIII allo-antibodies, which arise in acquired haemophilia A, do not inhibit FVIII:C completely, and show a more complex kinetic pattern (type II, or second-order kinetics), which explains why a residual plasma level of FVIII can be detected in these patients, even in cases of high-titre inhibitors (129, 130). An explanation of the difference in kinetics was suggested by Gawryl and colleagues, who observed distinct recognition sites for

<sup>2</sup> The binding site of a FVIII partner molecule in coagulation is obstructed by the inhibitor.

type I and type II antibodies (129). However, type II inhibitors recognizing the A2 and C2 domains have been characterized in congenital haemophilia A patients, once again emphasizing the complexity of inhibitor biology (119, 131).

The immune response to FVIII within an individual is heterogeneous, and the antibody response is polyclonal in that several epitopes are recognized. This was demonstrated by Prescott and colleagues when comparing the epitope specificity in haemophilia patients with anti-FVIII auto-antibody response in patients with acquired haemophilia A (allo-antibodies), who found that heterogeneous epitope recognition was greater in the patients with congenital haemophilia A (132). In addition, a transient immune response has been reported in several studies evaluating the long-term immune response in both haemophilia A patients and in healthy individuals (133, 134). Interestingly, in a patient with mild haemophilia A, a transiently increased immune response to endogenous (self) FVIII was observed, while a sustained elevation of the immune response to wild-type FVIII was observed (133).

## Risk Factors in Inhibitor Development

Over the years, numerous studies have been carried out on factors that may predict, or increase the risk of, inhibitor development. Both treatment-related and patient-related factors have been identified, and the most important are highlighted below.

### **Treatment-Related Risk Factors**

#### *Treatment Regimen - Prophylactic and On-demand Treatment*

In a systematic review recently performed by Coppola and colleagues, 27 clinical studies from 1996 to 2011 were scrutinized, and the authors found evidence of protective effects of prophylaxis in both low- and high-responding inhibitors in patients exposed to primary prophylaxis, i.e., in previously untreated patients (135). This agrees with findings in other uncontrolled studies (137, 138), a case-control study (139), and the retrospective CANAL study (Concerted Action on Neutralizing Antibodies in severe haemophilia A) (136). However, preliminary data obtained in the on-going prospective RODIN study (Research Of Determinants of INhibitor development among Previously untreated patients with haemophilia) indicate a protective effect of prophylaxis, but not during the initial period of treatment (during the first 20 exposure days) (136-140). In previously treated patients, prophylaxis does not prevent inhibitor development (135).



### *Age at Start of Treatment and Treatment Intensity*

The majority of inhibitors develop before the age of 10 years and, according to the United Kingdom Hemophilia Centre Doctors' Organization (UKHCDO) database, the incidence is highest in the lower ages, 0-4 years (141). It has been discussed whether starting FVIII treatment early increases the risk of inhibitor formation. A prospective study on 62 children with severe haemophilia A demonstrated an inhibitor risk of 41% in patients exposed to prophylaxis before the age of 6 months, compared with a risk of 12% when treatment start was delayed until >12 months of age (142). During the past decade, several retrospective and prospective clinical trials, and case-control studies, have been carried out, and today the accumulated knowledge suggests that age at first exposure is not an independent risk factor for inhibitor development (143-145). For example, in the retrospective CANAL study, the association between age at first exposure and inhibitor development was explained by treatment intensity (136). The impact of treatment intensity as a risk factor has also been evaluated, confirming the finding of the CANAL study, that treatment intensity should be considered in clinical praxis (143, 145-148).

### *Type of Clotting Factor*

Controversial findings have been reported on the correlation between the source of FVIII (plasma-derived FVIII (pdFVIII) or rFVIII) used for treatment and thus causing inhibitor development. Following the introduction of rFVIII products in the late 1980s, the inhibitor incidence was unexpectedly high compared with prior observations in pdFVIII-treated patients. However, it has been suggested that this difference could be explained by more careful inhibitor monitoring than previously (149). In addition, differences in recovery of FVIII:C depending on the type of factor used have been observed (150). Unlike rFVIII, pdFVIII contains VWF, and VWF could hypothetically reduce the immunogenicity of infused FVIII by reducing the endocytosis of FVIII by dendritic cells, and by shielding epitopes on the protein. Several *in vitro* studies have been performed to test these hypotheses, and the findings support this hypothesis. Lacroix-Desmazes and colleagues demonstrated both reduced FVIII uptake in dendritic cells and decreased CD4+ T cell activation upon pre-incubation with FVIII and recombinant VWF (151, 152). Epitope mapping in combination with the assessment of inhibitor reactivity to different FVIII products, and also inhibition of the thrombin-generating effect on different FVIII products, have shown some reduction in the incidence of inhibition against epitopes in the A2 and light chain by VWF, however, the results were not consistent (44, 150, 153, 154). Experimental *in vivo* studies are scarce, but it was recently demonstrated that haemophilic mice with

maintained endogenous VWF production survived a tail clip challenge, while those lacking endogenous VWF did not, supporting the protective effect of VWF (155).

In the clinical setting, the results of two independent studies have shown significantly higher inhibitor incidence in rFVIII-treated patients than in those treated with pdFVIII, but the correlation was not statistically significant in the subgroup of patients with high-titre inhibitors (>5 BU/mL) (156, 157). This finding was, however, not confirmed in the CANAL study or in a recent meta-analysis of clinical trials performed by Franchini and colleagues (158, 159). In conclusion, it is still not clear whether or not inhibitor patients would benefit from the concomitant use of VWF, or alternatively, the use of pdFVIII.

### *Influence of Immune System Challenges*

More than 20 years ago, Matzinger proposed the idea that immune responses are not only triggered by foreign antigens, but also by “danger signals” produced by endogenous cells. According to this “danger theory”, situations may occur, for example, in oxidative cellular stress, where the body’s own cells are dangerous to self, but the opposite may also occur, where foreign antigens are tolerated (160). As a result of recent advances in the field, it has been suggested that the danger, or damage, system is part of an organism’s defence and repair system, and helps discriminate dead cells from living cells. Danger signals from both exogenous pathogens and endogenous cells can induce an inflammatory immune response and up-regulate antibody production (161, 162). In haemophilia A, it has been speculated that FVIII treatment given during potential immune system challenges, for example, surgery, trauma or infection, increases the risk of inhibitor development. This would explain why on-demand treatment, used in situations of acute bleeding, leads to a higher risk of inhibitor development than prophylaxis. Several studies have evaluated this hypothesis, and are presented below.

After systematically reviewing the literature, Eckhardt and colleagues found that patients with severe haemophilia A ran a higher risk of developing inhibitors if their first exposure to FVIII was during surgery, than if FVIII was administered to treat a haemorrhage (163). Moreover, there are indications that surgery also increases the risk of inhibitor development in previously treated patients, for example in patients with moderate and mild disease (146). Interestingly, Kurnik and colleagues showed that a low-dose prophylactic regimen (250 IU once weekly, approx. 25 U/kg/w) introduced in concert with minimization of immunological danger signals reduced the risk of inhibitors in severe haemophilia A patients. Danger signals were actively avoided by, for example, not introducing prophylaxis during an infection, by giving vaccinations subcutaneously and not on FVIII treatment days, and by avoiding surgery during the first 20 exposure days

(164).

## **Patient-Related Risk Factors**

### *Race*

Patients of African-American and Latino origin are more susceptible to inhibitor development than Caucasians (72, 145, 165). It was recently suggested by Viel and colleagues that a mismatch of the wild-type FVIII alleles prominent in African-Americans with the alleles in transfused FVIII, contributes to the higher risk of inhibitor development seen in African-Americans (166). The study has, however, been criticized since no adjustment was made for *F8* mutation in the analysis (167, 168). In contrast, the HIGS study (Hemophilia Inhibitor Genetics Study) provided no support for increased inhibitor incidence due to haplotype mismatch after adjustment for *F8* mutation type (169).

### *Genetic Alterations*

Mutations that result in large alterations of the FVIII gene are associated with the highest risk of developing inhibitory antibodies (111, 165, 170, 171). This is partly explained by failure in the synthesis of detectable FVIII protein, hence, infused FVIII will be regarded as foreign by the immune system. In a recently published meta-analysis on 5383 patients, including 1029 inhibitor patients, the risk of inhibitor development was related when compared to the reference of intron 22 inversion (which is correlated to inhibitor development). Large deletions and nonsense mutations were found to be associated with the highest risk (higher than intron 22 inversions), while intron 1 inversions and splice-site mutations rendered the same risk as intron 22 inversions; the risk was lower for small deletions/insertions and missense mutations (172). However, inhibitors may develop in patients with moderate or mild haemophilia A, mainly caused by *F8* missense mutations, at levels sometimes comparable to those observed in severe haemophilia A patients. Missense mutations found in these cases are mainly located within exons encoding for the light chain (173, 174).

Several investigations have found that the risk of developing an inhibitor is greatly increased in families where one member has already exhibited an inhibitor, compared with families with no previous incidence of inhibitors (175, 176). In the Malmö International Brother Study (MIBS) the relative risk of a sibling developing an inhibitor was 3.2 (95% confidence interval (CI) 2.1-4.9) if the older brother exhibited an inhibitor. The overall concordance regarding inhibitor development within families included in the MIBS study was reported to be

78.3%, and somewhat higher in twins (88.2%) (175). Assessment of the mutation-type-dependent concordance rate in a subset of MIBS families confirmed the influence of genetic and familial predisposition to inhibitor development. However, since families with members carrying high-risk mutations (see above) were also spared from inhibitor development, it has been concluded that both genetic and non-genetic risk factors must be included in risk assessment (177).

Today, the general understanding of the genetic influence on inhibitor development includes not only the mutation causing the disease, but also genetic determinants other than the *F8* gene. The HLA class I alleles A3, B7 and C7 and the class II alleles DQA0102, DQB0602 and DR15 have been demonstrated to increase the risk of developing inhibitory anti-FVIII antibodies, while other HLA alleles (HLA C2, DQA0103, DQB0603, and DR1) have been suggested to provide protection against inhibitor development (99, 100). However, inconsistent associations with HLA type have also been found (145). Regulatory proteins and cytokines (immune response genes) and their influence on inhibitor development have been studied recently. Astermark and colleagues reported a higher risk of inhibitor formation in patients with single nucleotide polymorphism of the TNF- $\alpha$  gene (-308 A/A genotype) (103), and of the microsatellite *IL10G* in the promoter region of the IL-10 gene (104). Increased risk has also been reported for single nucleotide polymorphism of the genes encoding IL-1 $\alpha$  and IL-12 (178). A protective association has been observed for the CTLA-4, IL-1 $\beta$ , IL-2 and TGF- $\beta$  genes (105, 178, 179). A broad range of single nucleotide polymorphisms in immune-modulating genes has been evaluated in the Combined Cohort of the HIGS study, and 53 SNPs were recently reported to significantly influence the risk of inhibitor formation (107).

## Non-Neutralizing Anti-FVIII Antibodies

Not all anti-FVIII antibodies detected in haemophilia A patients have neutralizing capacity. Gilles and colleagues characterized anti-FVIII antibodies against non-neutralizing epitopes of the FVIII protein by purifying antibodies from haemophilia A patients with inhibitors. These antibodies escape detection by the functional Bethesda assay, which is used to estimate the neutralizing capacity of an antibody, consequently, the most common designation used today is non-neutralizing antibodies (NNAs) (114). Non-neutralizing anti-FVIII antibodies have also been detected after induction of immune tolerance in haemophilia A patients, although with different specificity from the pre-tolerant anti-FVIII antibodies (180).

As for FVIII inhibitors, a heterogeneous epitope recognition profile has been

demonstrated for NNAs. One specific region of the A3 domain has been identified as being especially immunogenic to NNAs, whereas the recognition of epitopes in the heavy chain (A1-a1-A2-a2-B domains) seems to be more variable (95). Since NNAs are presumed to lack neutralizing capacity it has been commonly accepted that the main recognition site would be the redundant B domain in the heavy chain (181). In a more recent study, the recognition of epitopes was demonstrated to be predominantly in the heavy chain (73.7%), although NNAs were also detected against the light chain (13.2%). In addition, only 18.4% of the NNAs recognized the B domain, indicating that a large proportion of NNAs recognizing the heavy chain are directed against its functional domains (182).

The isotypic distribution of anti-FVIII antibodies was recently investigated, showing significant differences in Ig subclass distributions between different cohorts. In inhibitor patients with haemophilia A, and in patients with acquired haemophilia A, IgG1 and IgG4 were the predominant subclasses. Interestingly, in inhibitor-negative haemophilia A patients and in patients successfully treated with ITI, as well as in healthy subjects, IgG4 was completely absent. Instead, IgG1 and IgG3 were the dominant subclasses in inhibitor-negative haemophilia A patients, IgG1 in ITI-treated haemophilia A patients, and IgG1, IgG3 and IgA in healthy individuals (116). Regarding ITI-treated patients, previous evaluation of subclass distribution has demonstrated the presence of IgG4 antibodies in a large proportion of patients, especially in those with high-titre inhibitors and poor outcome of ITI (183). It may be that this patient population is immunologically different from patients who respond successfully to ITI. Differences in methodology may also explain some of the discrepancy in the findings. The prevalence of NNAs in haemophilia A patients has been evaluated in different cohorts of varying sample sizes, using different laboratory techniques, which may explain the variation in results, ranging from 5.0 to 53.8% (116, 181, 182, 184-189). Moreover, NNAs have also been detected in healthy individuals, but the prevalence seems to be much lower than in haemophilia patients: approximately 2-3% (95, 116, 189-191).

The question of whether NNAs play a clinically important role in haemophilia is still under debate. In the early 1980s it was suggested that NNAs were able to form immune complexes with circulating FVIII, thereby enhancing the clearance of FVIII from plasma (117, 128). Dazzi and colleagues assessed 23 patients with haemophilia A with both the Bethesda assay and ELISA (enzyme-linked immunosorbent assay), and found only one patient to be positive in both assays, and 39% to be ELISA-positive only. In addition, after evaluating pharmacokinetic parameters in a subset of the patients, they reported that the clearance rate increased in NNA-positive individuals, while the half-life was not affected (185, 192, 193). Other groups have investigated if NNAs were present in patients with a low FVIII recovery (< 66 % of the expected increase in FVIII concentration after administration of FVIII), by utilizing immunoprecipitation for the detection of NNAs (192, 193). However, no association was found, a finding that was recently

confirmed by Kempton and colleagues (194).

Whether there are risk factors that may predict, or increase the risk of (as in inhibitor development), NNA development, or not, is not clear. In a study cohort of 25 patients, Vianello and colleagues demonstrated an increased risk in patients carrying the intron 22 inversion mutation (188). However, this finding was not confirmed in a larger study population of 57 patients carrying the same mutation (182). Furthermore, discrepancies have been reported in associations with age in NNA-positive patients (182, 184), as well as the mode of treatment. Zakarija and colleagues found a lower NNA prevalence in patients on continuous prophylaxis, which could not be confirmed in the French cohort investigated by Lebreton and colleagues (182, 189).

## Immune Tolerance Induction to FVIII

### **Immune Tolerance in Theory**

The theoretical basis of ITI has been widely investigated, and pathways of special interest have been identified, including the involvement of B and T cell response, the role of anti-idiotypic antibodies, and potential mechanisms preventing inhibitor development. In a mouse model, high, but not physiological, levels of FVIII:C were demonstrated to irreversibly inhibit the differentiation of memory B cells into plasma cells (195). Moreover, B cell differentiation, demonstrated in several antigenic systems, can be inhibited through blocked cell signalling mediated by anti-idiotypic antibodies that cross-link the B cell receptor and the Fc part of the anti-FVIII antibody on the B cell surface. In addition to cross-linking capacity, anti-idiotypic antibodies act through the binding of circulating FVIII inhibitors (196). Depletion of CD20+ cells with rituximab has been used in ITI in haemophilia, mostly in patients in which previous ITI attempts have failed. CD20 is expressed on all kinds of B cells, but not on plasma cells, and rituximab depletes peripheral B cells through antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity and phagocytosis (197). The relapse rate in haemophilia A patients is high; a sustained tolerance being demonstrated in only 14% of the patients (198).

The role of T cells in immune tolerance induction is less well characterized. Studies have been performed on preventing a T cell response, either by blocking of co-stimulatory molecules (e.g. CD40 and CTLA-4), or by modifying TCR-CD3 signalling. Anti-CD40L was used in isolated haemophilia A patients, resulting in decreased inhibitor titre (199). However, this approach is deemed unsuitable due to reports of an increase in thrombotic events caused by platelet activation (200).

An interesting report from Brazil recently provided new insight into the natural course of inhibitory antibodies, showing that 56% of the patients with low historical peak titres (<5 BU/mL) achieved tolerance to FVIII within 10.3 years (range 6-11.8 years), without ITI treatment, despite the fact that FVIII on-demand therapy was reinstated (201).

## Immune Tolerance in Clinical Practice

Several ITI protocols are currently available for clinical use. The most common are listed in Table 2.1. The Bonn and Malmö protocols are high-dose FVIII regimens, while the Dutch protocol is based on a low-dose FVIII regimen (202-204). In addition, the Malmö protocol includes IgG adsorption and the immunomodulating agents (cyclophosphamide) and intravenous immunoglobulin. The reported success rate varies, depending on whether a high-purity FVIII product (e.g. rFVIII, lacking VWF) or a VWF-containing FVIII product was used for ITI (205). However, since this issue remains unresolved, the recommendation is to use the same product against which the inhibitory response was observed (206).

**Table 2.1** *The most commonly used protocols for ITI. (Modified after Benson et al. (206) and reprinted with permission from Wiley-Blackwell.). BID=twice daily.*

<b>The Bonn protocol</b>	<b>The Malmö protocol</b>	<b>The van Creveld (Dutch) protocol</b>
High-dose regimen that includes a bypassing agent	High-dose FVIII plus immunomodulation (adsorption and suppression) Cyclophosphamide 12–15 mg/kg IV daily for 2 d, then 2–3 mg/kg PO daily for 8–10 d	Lower-dose/adaptive dosing of FVIII: neutralizing dose and tolerizing dose
FVIII 100–150 U/kg BID		FVIII 25–50 IU/kg BID
pd-aPCC 50–100 U/kg BID	FVIII to achieve a 40–100% FVIII level, followed by FVIII infusion every 8–12 h to achieve a 30–80% FVIII level	for 1–2 wk, then 25 IU/kg every other day
Reported success rate, 92–100%	IVIG 2.5–5 g IV immediately after the first FVIII infusion, followed by 0.4 g/kg daily on days 4–8	Reported success rate, 61–88%
Median time to success, 14 months		Median time to success, 1–12 months
	Reported success rate, 59–82%	
	Median time to success, 1 months	

The goal of ITI treatment is to restore the clinical response to FVIII. The definition of a successful outcome is a negative Bethesda titre (undetectable levels of inhibitor) together with normalized FVIII pharmacokinetics (see specified definitions in Table 2.2).

**Table 2.2** Accepted definitions of success, partial success, failure and relapse during and after ITI in patients with haemophilia A with inhibitors. (Modified after Benson et al. (206) and reprinted with permission from Wiley-Blackwell.

Success	Partial success	Failure	Relapse
Inhibitor titre <0.6 BU/mL at 2 or more consecutive monthly measurements	Reduction in inhibitor titre to $\leq 5$ BU/mL	Failure to attain defined success or partial success within 33 months of uninterrupted ITI	Inhibitor recurrence within 12 months of successful ITI
FVIII recovery $\geq 66\%$ of expected value	FVIII recovery <66% of predicted	Failure to demonstrate on-going inhibitor titre reduction $\geq 20\%$ during each interim, non-overlapping 6-month period of uninterrupted ITI, beginning 3 months after initiation to allow for expected anamnesis (reasonable duration of unsuccessful ITI: minimum 9 months, maximum 33 months)	
FVIII half-life $\geq 6$ h after 72-h FVIII washout, and no anamnestic response upon subsequent FVIII exposure	FVIII biological half-life <6 h after 72-h FVIII washout associated with clinical response to FVIII therapy, and no increase in inhibitor titre >5 BU over 6 months of on-demand treatment or 12 months of prophylaxis		

Both patient-related factors and treatment-related factors have been found to affect the outcome of ITI. Patient age, ethnicity and disease-causing *F8* mutation have been investigated, but the results regarding their association with ITI outcome are inconclusive. However, patients with a peak historical titre <200 BU/mL, pre-ITI titre <10 BU/mL and <5 years since inhibitor diagnosis are considered to be “good-risk” patients with a higher probability of exhibiting tolerance. For this



reason, it is desirable to reach a pre-ITI titre of <10 BU/mL also in “poor-risk” patients, if time and the clinical condition of the patient allow (15, 206) (Ref. 15, p. 67). Results were recently reported from the first prospective randomized study on ITI, namely The International Immune Tolerance Study. This study was designed to compare a high-dose ITI protocol with a low-dose protocol. The findings were in favour of the high-dose regimen, as successful ITI was achieved faster than with the low-dose protocol, although the overall success rates in the two groups were comparable (207). However, a very important finding of this study was that patients on low-dose ITI bled more frequently, which led to the early interruption of the study.

# Aims of the Present Studies

The work presented in this thesis was focused on two main areas, each with specific aims.

## I. Monitoring of by-pass therapy *in vitro* in plasma from patients with haemophilia A using thrombin generation assay:

- to evaluate thrombin formation in brothers with haemophilia A, and
- to investigate the effect of a combination of FVIII concentrate and by-passing agent on thrombin formation in unrelated patients exhibiting FVIII inhibitors.

## II. Non-neutralizing anti-FVIII antibodies in congenital haemophilia A:

- to evaluate the prevalence of NNAs in siblings and unrelated patients with haemophilia A,
- to examine the specificity of NNAs against recombinant FVIII concentrates,
- to explore the long-term development of NNAs in haemophilia A patients receiving continuous replacement therapy, and
- to evaluate clinical impact of NNAs.



# Materials and Methods

This chapter briefly describes the study populations and laboratory methods utilized in the present investigations. More detailed information and study protocols can be found in the respective papers appended at the end of this thesis.

## Study Populations

### **The Malmö International Brother Study**

The Malmö International Brother Study (MIBS) was initiated in 1996 in order to establish an international register of twin- and non-twin brother pairs with haemophilia A and B, with and without inhibitory antibodies against FVIII (175, 208). The main aim of the study was to explore genetic and compound factors predisposing to inhibitor development. Data were collected regarding ethnic background, type of haemophilia, treatment history, inhibitor data (including titre at detection expressed in Bethesda units (BU), peak titre, current titre and immune tolerance protocols), as well as information regarding hepatitis C and HIV. In total, data were collected from 962 subjects in 460 families (388 with haemophilia A and 72 with haemophilia B) from 20 haemophilia centres in Europe and North America. A history of inhibitory anti-FVIII antibodies was found in 133 subjects in 100 families (22%) (175). The MIBS study was performed according to the declaration of Helsinki, and was approved by the Ethics Committee at Lund University. The study is registered at [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov) (ID NCT00231751). Plasma samples and clinical data from severe haemophilia A subjects were utilized in the investigations presented in Paper I and Paper III.

### **The Haemophilia Inhibitor Genetics Study**

The Haemophilia Inhibitor Genetics Study (HIGS) started in 2003 and is on-going. The main objective of the study is to explore whether genetic factors apart from the *F8* gene are associated with inhibitor development and immunogenic responses to the FVIII protein. Plasma samples and clinical data have been, and are being, collected from brothers with haemophilia A, at least one of whom has a

history of an anti-FVIII inhibitor (209). The HIGS study is being performed according to the declaration of Helsinki, was approved by the Ethics Committee at Lund University, and is registered at [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov) (ID NCT00166387). Plasma samples and clinical data were utilized in the study presented in Paper III.

## **The Biobank and UMAS Hemophilia Database at the Centre for Coagulation Disorders at Skåne University Hospital in Malmö**

Blood samples have been obtained from subjects with congenital bleeding disorders (haemophilia A and B, von Willebrand disease and rare bleeding disorders) since November 2003 in connection with the patient's scheduled routine follow-up at the Centre for Coagulation Disorders at Skåne University Hospital in Malmö. Plasma samples are stored in a local biobank. Approval was obtained from each patient and the procedure adopted is in accordance with the Swedish Act on Biobanks. Furthermore, a database has been established (the UMAS Hemophilia Database 4.1) for the collection of clinical data such as treatment regimens, incidences of bleeding, surgery etc., and the results of the SF-36 Health Survey. Plasma samples from the biobank and clinical data from the database were utilized in the investigations presented in Papers IV (case report) and V.

## Laboratory Methods

### **Factor VIII Activity in Plasma**

At the Coagulation Unit of the Department of Laboratory Medicine, Skåne University Hospital in Malmö, FVIII clotting activity in plasma has been measured with the two-stage chromogenic assay since the beginning of the 1990s. Prior to that, the one-stage FVIII:C assay was utilized. Plasma samples from the patient described in the case report (Paper IV) were consequently analysed with both methods, depending on the time of measurement.

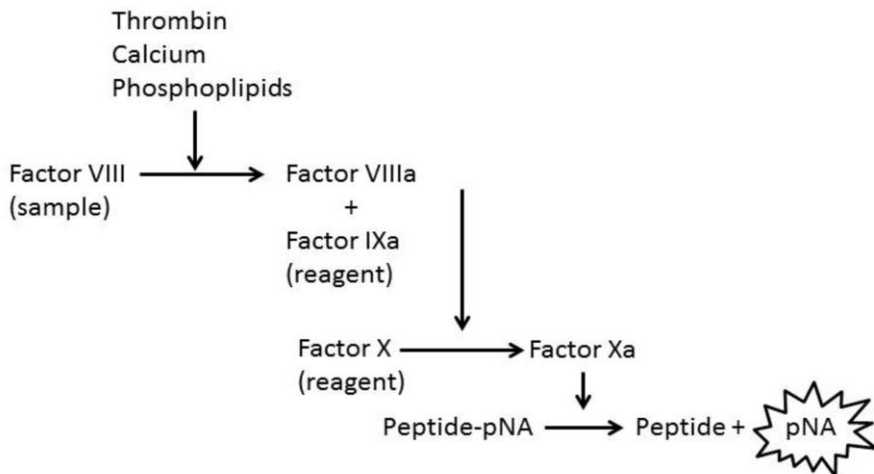
#### *One-stage FVIII:C Assay*

The one-stage assay is based on the premise that the clotting time of a sample with a constant and normal level of all coagulation factors except FVIII, will depend on the level of FVIII in the plasma sample, in a clot test with FVIII-depleted plasma.

The test base is FVIII-depleted plasma (<1% FVIII:C), and a primary standard reference plasma of known concentrations is established by serial dilution of standard plasma with the FVIII-depleted test bases. The clotting times obtained (APTT) are plotted against the different dilutions in a lin-log graph. The test sample is diluted in a similar fashion to the standard plasma, and the values of APTT are compared with the clotting times of the standard. The FVIII activity in the sample is given in percent (%) or kilo international units/litre (kIU/L) (6, 15, 130) (Ref. 6, p. 13; Ref. 15, pp. 248-249). The standard reference plasma should be the current International Standard for Plasma FVIII:C Concentrate, established by the Expert Committee on Biological Standardization of the World Health Organization.

### *Chromogenic Two-stage FVIII:C Assay*

The two-stage FVIII:C assay is based on the assumption that the amount of FXa generated is proportional to the FVIII activity in the sample. FX is first activated by FVIIIa and FIXa, and the FXa activity is determined in the second step. Today, the assay is performed using the chromogenic substrate S-222-pNA, and the FXa activity is determined by hydrolysis of the chromogenic peptide in which the chromophoric pNA is liberated. The colour intensity is read photometrically at 405 nm, and is proportional to FVIII:C (Figure 5.1). The Coatest<sup>®</sup> Factor VIII kit (Chromogenix, Mölndal, Sweden) is currently utilized.



**Figure 5.1** The principle of the chromogenic two-stage assay for the measurement of FVIII:C.

## The Bethesda Assay for FVIII Inhibitor Quantification

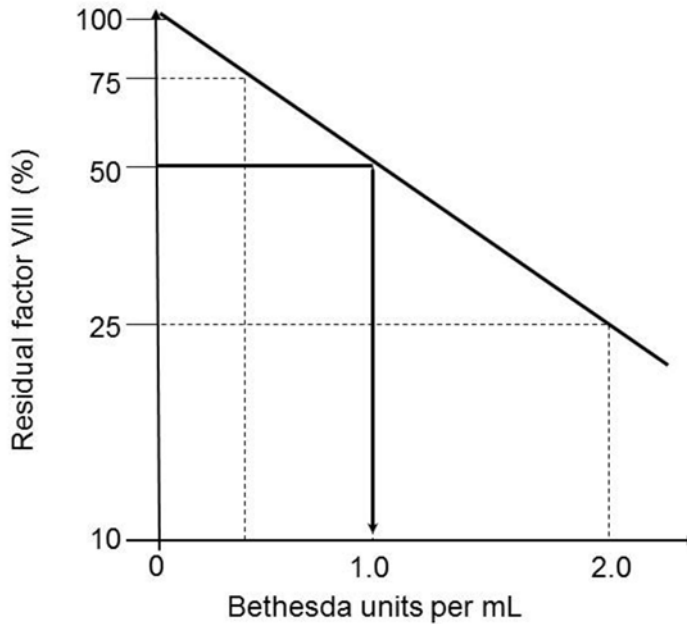
The Bethesda assay was used to quantify the neutralizing FVIII inhibitor level in plasma (Papers I, III, IV and V). One BU is defined as the amount of inhibitor that neutralizes 50% of FVIII:C in normal plasma after 2 hours' incubation in a water bath at 37°C. The clinical rationale for performing the FVIII Bethesda assay is either poor response to replacement therapy in a haemophilia A patient, or the suspicion of acquired inhibitory anti-FVIII antibodies (e.g. acquired haemophilia A) in a non-haemophilic subject with a prolonged coagulation time according to the APTT screening test.

At the Coagulation Unit of the Department of Laboratory Medicine, Skåne University Hospital in Malmö, the protocol used for the Bethesda assay includes the Nijmegen modifications with buffering of normal plasma with imidazole in order to stabilize the level of FVIII:C in the sample (210). Normal plasma from healthy donors is obtained from the blood bank at Skåne University Hospital, and the content of FVIII is defined as approx. 1 kIU/L, or 100%, of FVIII coagulant activity.

Assay procedure: 0.1 mL patient plasma is mixed with 0.1 mL normal pooled plasma (together with 0.1 M imidazole) and incubated for two hours at 37°C. As a control, commercially available FVIII-depleted plasma (Siemens Healthcare Diagnostics, Erlangen, Germany) is used, and prepared as the patient plasma. If the patient plasma contains a high titre of FVIII inhibitors, the patient plasma sample is diluted with FVIII-depleted plasma by serial dilution: 1/1, 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 and 1/200. The diluted plasma samples are thereafter prepared as described above. After two hours of incubation, the FVIII:C is measured in the patient and control plasma samples using the Coatest<sup>®</sup> Factor VIII kit (Chromogenix, Mölndal, Sweden), and the residual FVIII:C in the patient plasma is calculated as a percentage of the FVIII:C in the control plasma (Eq. (1)):

$$\frac{\text{Patient FVIII:C (kIU/L)} \times 100}{\text{Control FVIII:C (kIU/L)}} \quad (\text{Eq. 1})$$

In samples with a residual FVIII activity between 25 and 50% of the control, the inhibitor titre is derived (in BU/mL) from a graph of residual FVIII activity (y-axis) versus inhibitor units (x-axis) (see below). When serial dilution has been performed, multiplication by the dilution is carried out to give the final inhibitor titre. The detection limit is by definition <0.4 kBU/L, which corresponds to a residual FVIII:C in the patient plasma of 75% of the normal control (see Figure 5.2 below).



**Figure 5.2** The logarithm of the remaining FVIII activity is plotted on the y-axis and Bethesda units (BU) on the x-axis. One BU is defined as the amount of an inhibitor that neutralizes 50% of FVIII:C in the incubation mixture.(Graph obtained from Andreas Hillarp)

### The Malmö Inhibitor Assay

In the *in vitro* investigation of the treatment combining by-passing agents and FVIII clotting factors, the Malmö inhibitor assay was used to establish a uniform inhibitor level (Paper II). Here, the inhibitory activity in plasma is expressed as the number of IU of FVIII:C that are neutralized by 1 mL of patient plasma. One Malmö inhibitor unit (MIU) corresponds to about three BU (211).

Patient plasma (0.6 mL) is mixed with 0.2 mL FVIII concentrate (4 IU/mL) and incubated for two hours at 37° C. If a high-titre inhibitor is expected, the plasma sample is diluted in Tris buffer with 1% bovine serum albumin before the FVIII concentrate is added to the sample. Factor VIII-depleted plasma (FVIII:C <0.01 IU/mL) is used as reference plasma, and 0.6 mL reference plasma is mixed with 0.2 mL FVIII concentrate (4 IU/mL) and incubated as in the case of the patient plasma. After incubation, the residual FVIII:C is measured using the



standard method for FVIII:C (here the chromogenic assay).

The inhibitor level (inhibitor units/mL) is measured using one of the following equations:

$$\frac{(\text{Reference plasma \%} - \text{Patient plasma \%}) \times 0.8 \times \text{dilution}}{100 \times 0.6} = \text{IU/mL}$$

(Eq. 2)

or

$$(\text{Reference plasma IU/mL} - \text{Patient plasma IU/mL}) \times 1.33 \times \text{dilution} = \text{IU/mL}$$

(Eq. 3)

## **The Thrombin Generation Assay**

For the thrombin generation assay used in the studies presented in Papers I and II, two different protocols were developed (see Papers I & II for details). The TGA was performed ad modum Hemker, using a phospholipid/recombinant tissue factor (rTF) substrate to trigger the reaction (30, 212). Ten  $\mu\text{L}$  rTF was added to 50  $\mu\text{L}$  of a solution of a fluorogenic substrate (1 mM Z-Gly-Gly-Arg-AMC) and 15 mM  $\text{CaCl}_2$ . Forty  $\mu\text{L}$  of patient plasma was then supplemented with clotting factors and/or a by-passing agent according to the specific experimental protocol. The samples were incubated at 37°C in a Microplate Fluorescence Reader (Tecan Genios, Tecan Austria GmbH, Grödig, Austria), with which the fluorescence intensity was monitored for 90 min. The fluorescence intensity is measured in relative fluorescence units and is proportional to the amount of thrombin produced in the assay (31). A reference curve for thrombin concentration is established, and the thrombin concentration in each experiment is calculated by comparison with the standard curve. The thrombin concentration is expressed as the rate of development of fluorescence intensity (fluorescence units /min). The thrombin potential corresponds to the amount of thrombin formed during 60 minutes, and is expressed in relative fluorescence units. The inter-assay variability of the method has been evaluated at the our local coagulation research laboratory, showing a coefficient of variance of <5%-7.3% (44, 45).

## **Enzyme-Linked Immunosorbent Assay**

An ELISA was developed to detect binding antibodies against FVIII (Papers III-V). As the FVIII antigen, three different commercially available recombinant FVIII concentrates were used in separate solutions at a FVIII concentration of 2 $\mu$ g/mL, and also in a mixture containing all three concentrates at a combined final concentration of 2 $\mu$ g/mL. The cut-off for a positive result was calculated based on the mean and +3 standard deviations (SD) of ten healthy individuals used as controls on each plate. All positive samples were analysed at least twice. Antibody specificity was confirmed by pre-incubating the positive samples with excess antigen prior to reassessment with ELISA. In positive plasma samples where the inhibition by excess FVIII was reduced, but not completely inhibited, adsorption to Protein G Sepharose was used to confirm that the positive signal was dependent on IgG in the sample. The detailed protocol can be found in the sections on Materials and Methods in Paper III and Paper V.

## **Statistics**

### **Mann-Whitney U test**

The Mann-Whitney U test was performed to test for differences in median values (Papers I, III & V). A  $p$ -value  $<0.05$  was considered statistically significant.

### **Variance Component Analysis**

In the study on the thrombin-generating capacity in siblings (Paper I), the variances in the maximum thrombin production between and within the families were analysed by variance component analysis (ANOVA), in which the variance is given as an intra-class coefficient, with a 95% confidence interval (CI). An F-test ( $F = \text{between-group variability} / \text{within-group variability}$  (Eq. 4)) of the intra-class coefficient was performed, and the 95% CI was calculated to test the difference between the variances (213). A  $p$ -value  $<0.05$  was considered statistically significant. The statistical analysis was performed with support from the Competence Centre for Clinical Research, at Region Skåne (the regional public body responsible for health and medical services).

## **Analysis of Covariance**

In the study on the thrombin-generating effect of the combination of by-passing agents and FVIII, presented in Paper II, ANCOVA models were used to test for equality of the slopes (i.e., possible differences in the linear rates of change of  $T_{\max}$  with increasing dose of each FVIII factor) and for the equality of their intercepts (Figure X). P-values <0.05 were considered to indicate statistical significance. Data were prepared at the coagulation research laboratory at the Skåne University Hospital in Malmö. The statistical analysis was carried out at the contract research organization RHO Inc., Chapel Hill, North Carolina, USA.

## **Evaluation of Risk**

In the long-term study on NNA development (Paper V), chi-squared analysis was performed to evaluate the risk of antibody development in subjects infected with hepatitis C virus (HCV) vs. non-infected subjects, and is presented as a value with a 95% confidence interval.

## **Statistical Software**

Statistical tests were performed using the following software: SPSS (SPSS Corporation, Chicago, IL, USA), IBM SPSS Statistics 20 for Windows (IBM Corporation Armonk, NY, USA), Excel for Windows (Microsoft, Redmond, WA, USA) and r 2.6.2 (<http://www.r-project.org>). For specific information, the reader is referred to the papers appended at the end of this thesis.

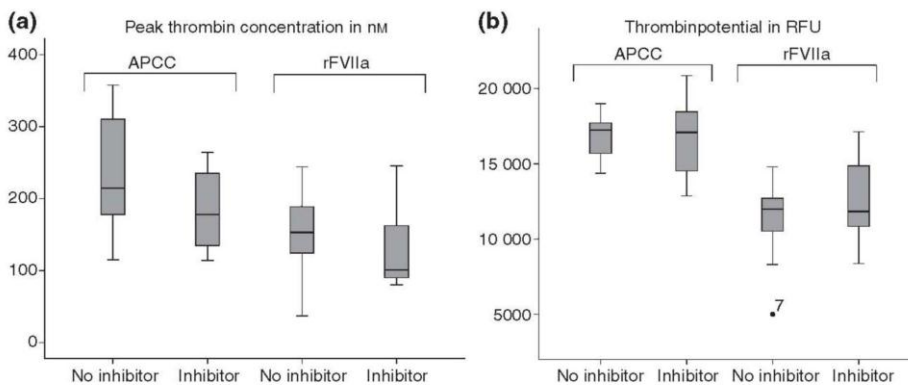
# Results and Discussion

## Monitoring By-passing Therapy *In Vitro* in Plasma from Patients with Haemophilia A

### **The Thrombin Generation Assay in Haemophilia A Siblings**

The thrombin-generating capacity of an individual has been demonstrated to be consistent, while the variation between individuals is large, resulting in a wide normal range (214). In addition, a well-known clinical observation in haemophilia care is that the FVIII:C level in plasma does not necessarily correlate to the thrombin production measured *in vitro*, or to bleeding phenotype (22, 38). This pattern may be explained by genetic determinants other than the specific disease-causative mutation causing modified expression or penetrance of *F8* (91, 215). Moreover, in the FENOC (FEIBA NovoSeven Comparative) study, the use of by-passing agents in inhibitor patients was evaluated in a comparative way, showing an inter-individual response to the products, especially during the first 12 hours from the start of symptoms. However, the effects of the two products on joint bleeding appeared to be similar (84). The aim of the study presented in Paper I was thus to explore the *in vitro* thrombin producing capacity in siblings enrolled in the MIBS study.

The mean maximum thrombin concentration in plasma from patients exhibiting inhibitors at the time of investigation was  $182.0 \pm 52.8$  mmol/L in experiments based on aPCC (FEIBA<sup>®</sup>), and  $130.7 \pm 54.9$  mmol/mL in rFVIIa-based assays (NovoSeven<sup>®</sup>), and was somewhat higher in inhibitor-negative plasma,  $222.7 \pm 85.5$  mmol/mL (aPCC) and  $142.8 \pm 53.6$  mmol/mL (rFVIIa) ( $p=0.16$  and  $0.29$ ) (Figure 6.1). Thus, as shown, for example, in the FENOC study (84), the presence of inhibitors had no detrimental influence on the effect of by-passing agents.



**Figure 6.1** (a) Peak thrombin concentration (nM) and (b) thrombin potential (RFU: relative fluorescence units) in patients without inhibitors ( $n = 30$ ) and in those with positive inhibitor titre ( $n = 12$ ). aPCC = activated prothrombin complex concentrate.; rFVIIa= activated recombinant FVII.

The thrombin concentration reached higher levels in the presence of aPCC than in the assays based on rFVIIa, but it should be stressed that the mode of action of the products differs, and that the effects of TF and platelets *in vivo* are not the same. The action of recombinant FVIIa is dependent on TF and phospholipid membranes, obtained *in vivo* by TF-bearing cells or platelets. The results of *in vitro* TGA presented here were obtained in PPP spiked with phospholipid micelles with low TF concentration, which may be a weakness when monitoring the effect of rFVIIa, and the protocol therefore included a supraphysiological dose of rFVIIa. However, when measurements were made with the opposite proportions of substrates, i.e., low rFVIIa (6  $\mu\text{g}/\text{mL}$ ) and high TF concentration, similar levels of thrombin were observed. In addition, it has been demonstrated that TF and phospholipids have an additive effect on outcome measures in TGA, and that a low concentration of TF can be compensated for by the presence of the phospholipid substrate (216). Moreover, the object of the study was not to compare the products with each other, but to compare measures of the response to by-passing therapy *in vitro* within and between individuals. Hence, the experimental pathways should be evaluated separately.

ANOVA was used to evaluate the variation in thrombin production within and between families. The variance (intra-correlation coefficient (213)) in the maximum thrombin concentration *between* families was 0.69 (95% CI 0.38-0.86) compared with 0.31 *within* a family using aPCC. When using rFVIIa, the variance was 0.71 (95% CI 0.41-0.87) *between* families and 0.29 *within* families. In conclusion, the variance in the maximum thrombin concentration was significantly

lower *within* a family than *between* families ( $p < 0.001$ ) for both aPCC (FEIBA<sup>®</sup>) and rFVIIa (NovoSeven<sup>®</sup>).

Based on these *in vitro* findings, the observed efficacy of by-passing therapy in a previously treated family member may help clinicians in the choice of product for a sibling. However, the results of this *in vitro* study must be confirmed by future *in vivo* studies.

### ***In Vitro* Effect of FVIII on By-passing Therapy in Plasma from Haemophilia A patients**

By-passing therapy is usually employed as monotherapy in patients exhibiting inhibitors of FVIII. However, clinical situations may occur in which patients present with haemorrhages refractory to monotherapy, and several case reports have suggested that a sequential treatment regimen, using both aPCC and rFVIIa, could be useful in such instances (50, 217, 218). For example, haemophilia A patients with inhibitors and a history of severe bleeding, and who were scheduled for orthopaedic surgery, were treated according to a protocol including preoperative immunoadsorption, followed by sequentially administered aPCC and rFVIIa (42, 219). A strategy in which aPCC and rFVIIa are added concomitantly to inhibitor plasma has also been explored, demonstrating a synergistic effect on *in vitro* thrombin generation (51). In a clinical setting, Martinowitz and colleagues used TGA to establish the individual doses of a combination of aPCC and rFVIIa required to maintain haemostasis. Synergy between the products was observed, in that far lower doses than those recommended for each product in single therapy could be used with concomitant administration (220). It was suggested that the prothrombin content of aPCC improves the effect of rFVIIa, and thus the overall effect of the combination of the two preparations (221). Furthermore, a decreased bleeding tendency has been observed in patients undergoing immune tolerance induction, as recently shown in the International Immune Tolerance Study, where patients randomized to the high-dose arm bled less than patients receiving low-dose ITI (207).

Concomitant treatment using FVIII concentrates and by-passing agents in inhibitor plasma has not previously been studied, and in order to do this a thrombin generation assay was developed. The plasma samples contained a high titre of inhibitory antibodies, 11 MIU/mL, which corresponds to approximately 33 BU/mL, and the samples were spiked with either one of the by-passing agents aPCC and rFVIIa, and five different FVIII clotting factors (for detailed information see Paper II). The experiments with Alphanate<sup>®</sup> included plasma from five patients, and in experiments assaying the combination of aPCC and rFVIIa (without FVIII added) plasma samples from four patients were used. All other experiments were based on plasma samples from 11 patients.

Even in the absence of by-passing agent, and with excess inhibitor, the thrombin production increased at the higher FVIII dose (Tables 6.1 and 6.2), which may be explained by the rather slow kinetics of type I inhibitors. In TGA, the reaction starts immediately when the FVIII concentrate is added. We have observed in our lab that the incubation of FVIII concentrate with inhibitor plasma for up to 2 hours still resulted in thrombin formation in the assay (unpublished observations). Doshi and colleagues recently showed that FVIII supplementation, alone or in combination with rFVIIa, resulted in improved thrombin-producing capacity in inhibitor plasma, depending on the kinetics of the inhibitor, rather than on the actual inhibitor titre (48).

**Table 6.1** Thrombin production in nmol/L for the assays combining aPCC and FVIII.

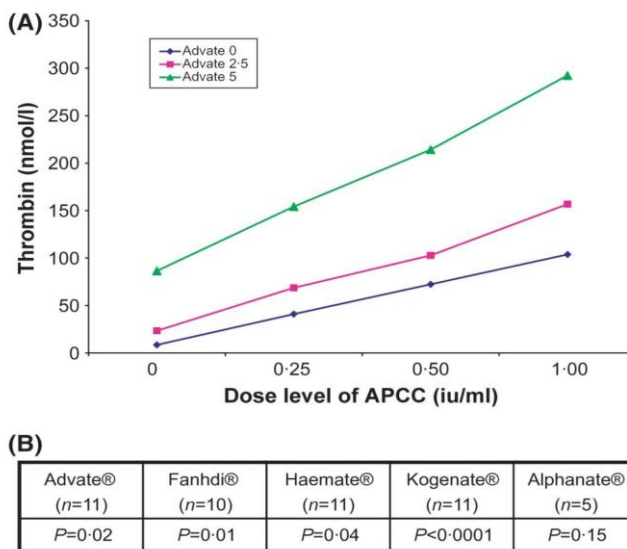
	aPCC 0	aPCC 0.25	aPCC 0.50	aPCC 1.0
FVIII dose level 0 IU/mL				
Advate <sup>®</sup>	8.5	40.9	72.3	103.8
Fanhdi <sup>®</sup>	10.6	46.2	71.3	115.7
Haemate <sup>®</sup>	8.8	44.1	70.4	112.0
Kogenate <sup>®</sup>	8.6	42.9	72.0	110.7
Alphanate <sup>®</sup>	10.7	44.9	64.0	100.7
FVIII dose level 2.5 IU/mL				
Advate <sup>®</sup>	23.4	68.6	102.8	156.8
Fanhdi <sup>®</sup>	67.0	135.0	179.0	255.5
Haemate <sup>®</sup>	68.9	134.1	173.2	248.8
Kogenate <sup>®</sup>	23.3	75.6	109.5	174.3
Alphanate <sup>®</sup>	49.4	115.7	150.7	215.1
FVIII dose level 5.0 IU/mL				
Advate <sup>®</sup>	86.5	154.2	214.2	292.4
Fanhdi <sup>®</sup>	139.5	221.4	282.1	376.6
Haemate <sup>®</sup>	131.2	202.7	252.2	363.97
Kogenate <sup>®</sup>	121.3	217.7	284.8	392.1
Alphanate <sup>®</sup>	122.3	200.3	257.4	355.7

**Table 6.2** *Thrombin production in nmol/L for the assay combining rFVIIa and FVIII.*

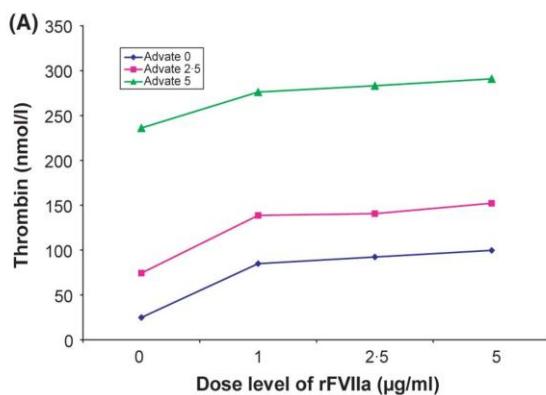
	rFVIIa 0	rFVIIa 1.0	rFVIIa 2.5	rFVIIa 5.0
FVIII dose level 0 IU/mL				
Advate <sup>®</sup>	24.8	84.9	92.3	99.7
Fanhdi <sup>®</sup>	22.7	80.4	88.2	94.9
Haemate <sup>®</sup>	25.4	87.2	93.9	100.9
Kogenate <sup>®</sup>	25.0	84.0	92.7	99.0
Alphanate <sup>®</sup>	17.9	68.0	74.8	81.8
FVIII dose level 2.5 IU/mL				
Advate <sup>®</sup>	74.3	138.7	140.6	152.2
Fanhdi <sup>®</sup>	159.9	205.0	219.8	224.7
Haemate <sup>®</sup>	204.2	239.8	246.3	246.3
Kogenate <sup>®</sup>	71.2	140.0	176.1	159.7
Alphanate <sup>®</sup>	147.5	201.2	203.9	205.4
FVIII dose level 5.0 IU/mL				
Advate <sup>®</sup>	236.1	276.2	283.2	290.9
Fanhdi <sup>®</sup>	284.2	322.1	325.7	343.0
Haemate <sup>®</sup>	278.5	328.3	338.3	339.6
Kogenate <sup>®</sup>	249.9	300.7	329.8	315.9
Alphanate <sup>®</sup>	316.1	344.2	346.1	370.3



Thrombin formation in samples in which a combination of aPCC and FVIII was used showed a potentiating effect, i.e., synergy, resulting in a higher level of thrombin than expected for all FVIII products except Alphanate® ( $p < 0.005$ , Alphanate®:  $p = 0.1470$ , Figure 6.2(B)). This was not the case in assays based on rFVIIa and FVIII, in which the combined effect of the preparations was additive (Figure 6.3). Finally, aPCC and rFVIIa were combined, without FVIII, resulting in an additive, but not synergistic, effect on thrombin production.



**Figure 6.2** The interaction between aPCC and FVIII clotting factor. The curves of the diagram should be parallel in cases where the pharmacological effect of increased dose level is additive. In the aPCC assay, here exemplified with Advate® (A), the curves are not parallel, indicating synergistic interaction resulting in a significant increase in thrombin production for all FVIII products apart from for Alphanate® (B).



(B)

Advate® (n=11)	Fanhdi® (n=11)	Haemate® (n=11)	Kogenate® (n=11)	Alphanate® (n=5)
P=0.98	P=0.99	P=0.97	P=0.90	P=0.99

**Figure 6.3** The interaction between rFVIIa and FVIII clotting factor. The increase in thrombin generating capacity is additive but not synergistic, here exemplified with Advate® (A). (B) P-values for all FVIII clotting factors included in the assay.

As mentioned above regarding the sibling cohort, the experiments were run using PPP, thus *in vitro* conditions may not result in the optimal effect of rFVIIa. Here, to mimic the *in vivo* situation as far as possible, TF substrates including phospholipid micelles were used to trigger the thrombin formation reaction (Technoclone TGA High reagent (TECHNOTHROMBIN® TGA RC) for the rFVIIa-based assays, and TECHNOTHROMBIN® TGA RB for the assays with aPCC). In addition, the outcome of the interaction analyses may depend on the different formulations of aPCC and rFVIIa. aPCC contains zymogens, coagulation factors and phospholipids, including FIX, FIXa and FXa (222). The extra phospholipid content in aPCC could be an advantage in this *in vitro* setting.

Unlike previous findings of a suggested protecting role of VWF, we observed no difference in product interaction between VWF-containing products (Alphanate®, Haemate® and Fanhdi®) and the high-purity concentrates (Advate® and Kogenate®). Epitope mapping was not performed in this study, and thus the major recognition sites of the inhibitors may not compete with VWF binding. Moreover, the by-passing agents may be more important contributors to thrombin formation in this setting, thus surpassing a hypothetical VWF mechanism.

## Methodological Considerations

As mentioned in the first chapter, an important methodological concern is FXII-induced contact activation in the test-tube, which can be reduced by adding corn trypsin inhibitor (15, 25) (Ref. 15, p. 265). The experiments performed in both studies discussed above (Papers I & II), were run with citrated PPP without the addition of corn trypsin inhibitor, which could be a disadvantage. However, the protocol has been evaluated previously, and was found to be highly reproducible, with a coefficient of variance of <5%-7.3% (44, 45). In addition, in TGAs based on a high concentration of TF ( $\geq 5$  pM) the addition of corn trypsin inhibitor was shown to be unnecessary (54, 55).

Another factor that has been discussed is whether the lack of platelets in platelet-poor plasma resulting in a lack of phospholipid membranes, would have a detrimental effect on the analytic conditions, especially when monitoring the effect of rFVIIa. Livnat and colleagues performed TGA on both PRP and PPP without finding any differences between the outcomes of the experiments (50). Furthermore, in the investigation of siblings with haemophilia discussed here, comparative pilot experiments were carried out using PRP, showing no significant differences in outcome (results not shown).

The levels of FVIII:C in the *in vitro* studies discussed here are higher than the doses used in a clinical context. aPCC is normally administered at a dose of 75 IU/kg, which corresponds to an *in vitro* dose of  $\sim 1.5$ -2 IU/mL. Finally, rFVIIa is commonly used at a dose of 90  $\mu\text{g}/\text{kg}$  in clinical practice and corresponds to an *in vitro* dose of 2.5  $\mu\text{g}/\text{mL}$ . However, it should be remembered that the work described here was performed *in vitro*, and that the conditions can never be exactly the same as those *in vivo*.

## Non-neutralizing Anti-FVIII Antibodies in Haemophilia

Non-neutralizing anti-FVIII antibodies have been investigated in a number of cohorts (see chapter on FVIII), most of which have been small, including 20 to a maximum of 39 haemophilia A patients, often with disease of different severity (185, 186, 188). Lebreton and colleagues investigated a French cohort of 210 haemophilia A patients without inhibitors, 144 of whom had severe haemophilia A, and found an overall prevalence of NNAs of 18.8% in patients with severe disease. In addition, the specificity of the NNAs was characterized using the X-map technique, revealing heterogeneous epitope recognition, with predominance towards the heavy chain. However, as mentioned in Chapter 3, functional parts of the heavy chain were also recognized by NNAs, and not only the, sometimes

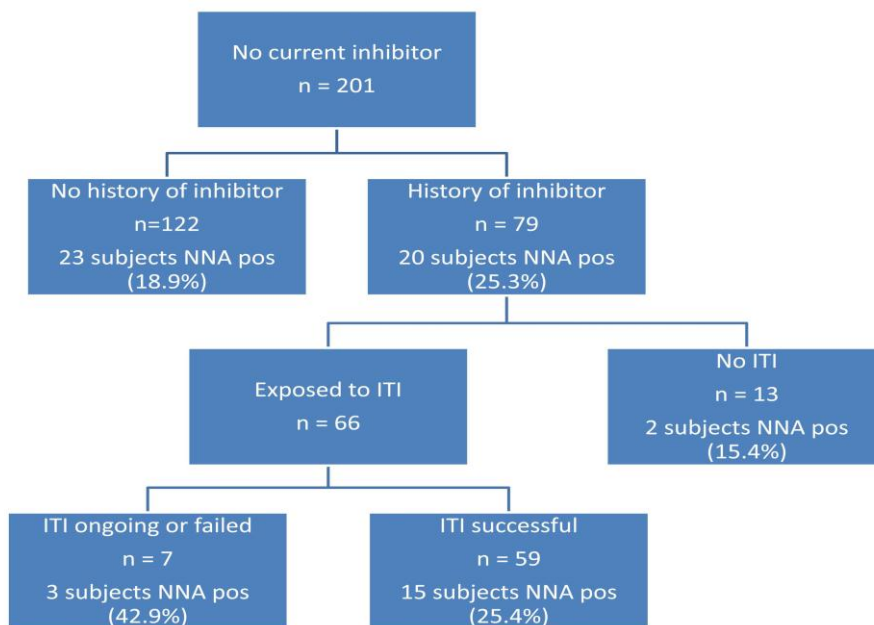
suggested, redundant B domain (182). In addition, a meta-analysis of studies on inhibitor development associated with different recombinant FVIII products, revealed an increased *de novo* inhibitor risk in patients receiving BDD-rFVIII (81). Apart from these studies, no large cohorts have been investigated regarding the specificity of the immune response to FVIII products used in the treatment of haemophilia A patients.

The development of NNAs within families, and the long-term development of NNAs in patients receiving continuous replacement therapy have not previously been evaluated. Therefore, a combined cohort of 201 haemophilia A brothers, and a single-centre-based cohort of 78 patients were followed over a period of approximately four years, to address these questions, utilizing an ELISA based on three commercially available FVIII products. The results of the investigations, presented in paper III-V, are discussed below.

### **Prevalence of Non-neutralizing Anti-factor VIII Antibodies**

The prevalence of NNAs was evaluated in 201 currently inhibitor-negative brothers with severe haemophilia A (Paper III). Seventy-nine subjects (39.3%) had a history of positive inhibitor titre verified by the Bethesda assay, and anti-FVIII antibodies were detected in 20 of them (25.3%) with ELISA. The majority of the subjects (n=122) had not been diagnosed as having an inhibitor in the past, but a positive anti-FVIII antibody titre was detected in 23 of them, corresponding to a frequency of NNAs of 18.9% (Figure 6.4). The cohort of unrelated patients (n=78) (Paper V) was followed for approximately four years, including a minimum of three plasma samples (for detailed information on inclusion, see Paper V). Ten of the 78 subjects (12.8%) raised an immune response on at least one occasion.

The prevalence of NNAs observed in the long-term study was lower than in the sibling cohort, and also in the lower range of values reported in other studies. Previous studies from which a high prevalence has been reported (39.0%-53.8%) have been based on smaller study cohorts, from 12 to 26 subjects. Furthermore, no adjustment for FVIII specificity of the ELISA was performed in these studies, which could result in a higher proportion of positivity due to unspecific binding (185, 188, 223). This phenomenon was clearly shown by Whelan and colleagues, who recently reported an overall NNA prevalence of 33%, which decreased to 5% when only samples with defined FVIII specificity were considered (116).



**Figure 6.4** NNA development in the sibling cohort (Paper III)

Moreover, regarding the long-term study on unrelated subjects (Paper V), 65.5% of the inhibitor-negative patient cohort at our centre met the inclusion criteria of at least three plasma samples available in the biobank, hence a selection bias influencing the NNA outcome cannot be excluded. In addition, based on the fact that family history influences the risk of inhibitor development (175), it may be relevant to assume a genetic predisposition affecting NNA development, thus explaining a higher prevalence of NNAs in the brother cohort.

Unfortunately, no comprehensive information about treatment regimen (prophylaxis or on-demand) was collected in the MIBS and HIGS studies, thus no analysis regarding FVIII product and NNA development could be performed in the sibling cohort. However, as was described in Chapter 3, it is reasonable to assume that prophylactic treatment with FVIII provides some kind of protection against the development of inhibitors, although the mechanism behind tolerance is still largely unclear. Starting continuous prophylaxis at an early age is standard in Sweden, and the median age at the start of treatment was 3.0 years in the cohort of unrelated patients investigated here. The relatively low NNA prevalence in this cohort may be explained to some extent by continuous replacement therapy.

## NNAs in Patients Treated with ITI and in Previously Inhibitor-positive Patients

Seventy-nine patients in the sibling cohort had been found to exhibit an inhibitor in the past, and 66 of them had been, or were presently, exposed to ITI (Figure 6.4). According to reports from the centres at which the patients were treated, it was considered that 59 patients had been successfully treated with ITI. In 25.4% of these, an antigenic response was verified with ELISA. It should be emphasized, however, that the definition of success was not specified for all patients, and in 24 patients ITI was confirmed solely by a negative Bethesda titre, or the method used for confirmation was not specified. This is, of course, a shortcoming of the evaluation of NNA prevalence in the ITI patients. Data were available on the confirmatory method and the product used for inhibitor detection in nine subjects included in the HIGS cohort (Table 6.3), in three cases showing a negative ELISA outcome only regarding the products the patients had received for treatment (Nos. 1, 7 and 9).

**Table 6.3** Subjects from the HIGS cohort successfully tolerized with ITI according to defined criteria, but with detectable FVIII antibodies according to ELISA. The type of FVIII product at the time of inhibitor detection is given. No. = subject number; Y/N = yes or no;

No.	ITI Y/N	Confirmation of ITI outcome	Peak titer (BU/mL)	Type of FVIII-product	FVIII-mix	FL-rFVIII A	FL-rFVIII B	BDD-rFVIII
1	Y	T1/2	110	PD	pos	neg	neg	pos
2	Y	T1/2, IVR, BU	36	BDD-rFVIII	pos	neg	pos	pos
3	Y	T1/2, IVR, BU	8.8	FL-rFVIII B	pos	pos	pos	pos
4	Y	BU	1	FL-rFVIII A	pos	pos	pos	pos
5	Y	T1/2, IVR, BU	12	FL-rFVIII	pos	pos	pos	pos
6	Y	T1/2, IVR, BU	13	PD	pos	pos	pos	pos
7	Y	T1/2, BU	62	BDD-rFVIII	pos	pos	pos	neg
8	Y	T1/2, BU	90	No data	pos	pos	pos	neg
9	Y	T1/2, IVR, BU	7	PD & BDD-rFVIII	pos	pos	pos	neg

The IgG subclasses were not determined in this study, but previous reports have demonstrated a loss of IgG4 antibodies in patients after exposure to ITI, at least in those showing a successful outcome (116). In addition, Nilsson and colleagues found the epitope specificity of antibodies remaining post-ITI to be different from the pre-tolerant antibody population (180). It is therefore probable that the antibodies detected here are different from the inhibitory antibodies that were

previously detected in these patients, at least in the subjects showing well-defined ITI success. However, the question as to whether NNA-positive subjects are at a higher risk of inhibitor relapse post-ITI than NNA-negative subjects remains to be answered.

Thirteen patients with a reported historic inhibitor titre were currently Bethesda-negative despite no exposure to ITI (Figure 6.4). Six of them (46.2%) had had a high-responding inhibitor with a mean peak titre of 15.6 BU/mL (range 5.0-37.5 BU/mL), and the remaining seven subjects had exhibited a low-responding inhibitor. Two of the low-responding subjects showed increased antigenic responses using ELISA. This finding suggests that tolerance to FVIII may evolve naturally in inhibitor patients, and our observation is confirmed by results from a Brazilian study on a haemophilia A cohort recently presented. Caram and colleagues followed inhibitor patients who did not receive ITI for a median time of 10.3 years, and found sustained tolerance in 56% of the low-responding (<5 BU/mL) patients, 50% of the high-responders with a titre of 5-9.9 BU/mL, and 3% of patients with a titre  $\geq 10$  BU/mL. They concluded that ITI may not be necessary for all patients identified as having inhibitors (201).

## **Specificity to Recombinant FVIII**

In the sibling cohort (Paper III) all plasma samples were initially tested using a mixture of all three FVIII products. A positive outcome led to further testing of immunogenicity against the separate products. The antibody response to the different FVIII products was heterogeneous and was raised not only against the non-functional B domain, but against both full-length (FL)-rFVIII and BDD-rFVIII (Table 6.4).

In the investigation of the long-term development of NNAs (Paper V) all plasma samples were assayed against all four antigenic formulations simultaneously, which revealed an even more heterogeneous outcome than in the sibling study (Table 6.5). No antibodies against the FVIII mixture were detected in seven plasma samples, while antibodies were detected against the separate antigens (Nos. 3, 4 and 9). A reasonable explanation is that since the same final antigen concentration was used in all assays (2  $\mu\text{g/mL}$ ), the separate products in the mixture had lower relative concentrations than in the single-product solutions. Moreover, since the patients were receiving continuous replacement therapy, a residual FVIII:C was found in some plasma samples (Table 6.5), which could lead to the underestimation of NNAs due to decreased sensitivity of the ELISA. In retrospect, re-evaluating the outcome of the sibling cohort, where a positive result against the antigen mixture was required for further analysis of antigen recognition, it must be acknowledged that the prevalence of 18.9% may have been underestimated.

**Table 6.4** ELISA results from the sibling cohort (Paper III) for subjects with non-neutralizing antibodies (NNAs). ELISA-mix = mixture of three antigens; ELISA FL-rFVIII A/B = full-length recombinant FVIII product A & B; ELISA BDD-rFVIII = B domain-deleted recombinant FVIII product.

Subjects	ELISA mix	ELISA FL-rFVIII A	ELISA FL-rFVIII B	ELISA BDD-rFVIII
1	pos	neg	neg	pos
2	pos	pos	pos	pos
3	pos	pos	neg	pos
4	pos	pos	pos	pos
5	pos	pos	pos	pos
6	pos	pos	pos	pos
7	pos	pos	pos	pos
8	pos	pos	pos	pos
9	pos	pos	pos	pos
10	pos	pos	pos	neg
11	pos	pos	pos	neg
12	pos	pos	pos	neg
13	pos	pos	pos	neg
14	pos	pos	pos	neg
15	pos	pos	pos	neg
16	pos	pos	pos	neg
17	pos	pos	pos	neg
18	pos	pos	pos	neg
19	pos	pos	pos	neg
20	pos	pos	pos	neg
21	pos	pos	pos	neg
22	pos	pos	pos	neg
23	pos	pos	pos	pos

**Table 6.5 (following page)** ELISA results for subjects with non-neutralizing antibodies (NNAs) from the cohort of unrelated subjects with haemophilia A observed during a time-period of approximately four years (Paper V). Results are given for each blood sampling time-point.. Treatment indicate what regimen that was used (prophylaxis (P) or on-demand (OD)); FVIII:C=Residual FVIII activity;Time last inf=time since last FVIII infusion; Adv=Advate®; Kog=Kogenate®; RF=ReFacto®.



Subjects	Treatment	FVIII product	FVIII: C	Time last inf	ELISA mix	ELISA Adv	ELISA Kog	ELISA RF
1	P	Kogenate <sup>®</sup>	<0.01	no info	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
1	P	Kogenate <sup>®</sup>	0.62	5	neg	neg	neg	neg
1	P	Kogenate <sup>®</sup>	0.02	49	neg	neg	neg	neg
1	P	Kogenate <sup>®</sup>	0.22	26	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
2	P	ReFacto <sup>®</sup>	<0.01	70	<b>pos</b>	<b>pos</b>	neg	<b>pos</b>
2	P	ReFacto <sup>®</sup>	<0.01	no info	<b>pos</b>	neg	neg	neg
2	P	ReFacto <sup>®</sup>	<0.01	no info	<b>pos</b>	neg	<b>pos</b>	<b>pos</b>
2	P	ReFacto <sup>®</sup>	<0.01	>72	neg	neg	neg	neg
3	P	Recombinate <sup>®</sup>	0.07	50	<b>pos</b>	<b>pos</b>	<b>pos</b>	<b>pos</b>
3	P	Kogenate <sup>®</sup>	0.49	21	<b>pos</b>	<b>pos</b>	neg	<b>pos</b>
3	P	Kogenate <sup>®</sup>	0.06	no info	neg	<b>pos</b>	neg	<b>pos</b>
4	P	ReFacto <sup>®</sup>	0.01	68	neg	neg	neg	neg
4	P	ReFacto <sup>®</sup>	0.01	72	neg	neg	<b>pos</b>	neg
4	P	ReFacto <sup>®</sup>	0.17	16	neg	neg	<b>pos</b>	neg
4	P	ReFacto <sup>®</sup>	0.05	48	neg	neg	<b>pos</b>	neg
4	P	ReFacto <sup>®</sup>	<0.01	60	neg	neg	neg	neg
5	OD	Recombinate <sup>®</sup>	<0.01	no info	<b>pos</b>	<b>pos</b>	neg	<b>pos</b>
5	P	Advate <sup>®</sup>	<0.01	336	<b>pos</b>	<b>pos</b>	<b>pos</b>	<b>pos</b>
5	P	Advate <sup>®</sup>	<0.01	no info	<b>pos</b>	neg	<b>pos</b>	<b>pos</b>
5	OD	Advate <sup>®</sup>	0.01	120	<b>pos</b>	<b>pos</b>	<b>pos</b>	<b>pos</b>
6	P	Recombinate <sup>®</sup>	0.02	16	neg	neg	neg	<b>pos</b>
6	P	Kogenate <sup>®</sup>	0.05	16	neg	neg	neg	neg
6	P	Kogenate <sup>®</sup>	0.30	23	neg	neg	neg	neg
6	P	Kogenate <sup>®</sup>	0.24	28	neg	neg	neg	neg
7	P	Advate <sup>®</sup>	0.09	18	<b>pos</b>	<b>pos</b>	neg	neg
7	P	Advate <sup>®</sup>	<0.01	96	neg	neg	neg	neg
7	P	Advate <sup>®</sup>	0.51	3	<b>pos</b>	<b>pos</b>	neg	neg
7	P	Advate <sup>®</sup>	0.16	14	<b>pos</b>	<b>pos</b>	neg	neg
7	P	Advate <sup>®</sup>	0.11	29	neg	neg	neg	neg
8	P	Helixate <sup>®</sup>	<0.01	74	neg	neg	neg	neg
8	P	Helixate <sup>®</sup>	0.56	4	neg	neg	neg	neg
8	P	Helixate <sup>®</sup>	0.07	26	neg	neg	neg	neg
8	P	Helixate <sup>®</sup>	0.06	27	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
9	P	ReFacto <sup>®</sup>	0.05	48	neg	<b>pos</b>	neg	neg
9	P	ReFacto <sup>®</sup>	0.02	70	neg	<b>pos</b>	<b>pos</b>	neg
9	P	ReFacto <sup>®</sup>	<0.01	>96	neg	<b>pos</b>	neg	neg
9	P	ReFacto <sup>®</sup>	0.17	28	neg	neg	neg	neg
10	P	Monoclate <sup>®</sup>	0.11	28	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
10	P	Helixate <sup>®</sup>	0.16	18	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
10	P	Helixate <sup>®</sup>	0.08	38	neg	neg	neg	neg
10	P	Helixate <sup>®</sup>	0.07	40	neg	neg	neg	neg
10	P	Helixate <sup>®</sup>	0.32	24	neg	neg	neg	neg

Both the investigations discussed above demonstrated heterogeneous epitope recognition between individuals (as in a previous study (114)), as well as an intra-individual variation over time, in that the response pattern to different FVIII products varied (Tables 6.4 & 6.5 ). Since the B domain of the FVIII protein is assumed to be redundant, it has been suggested that NNAs are exclusively directed against this domain (116, 181). However, both studies from the early 1990s, as well as more recent ones, have shown reliable evidence that NNAs have a more complex recognition pattern directed against different domains of the protein (114, 182). Results from the investigations discussed here are in agreement with these later findings.

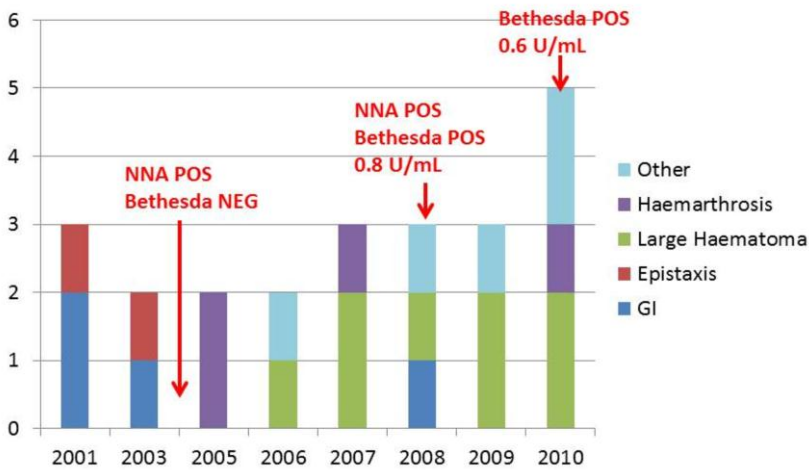
From a clinical point of view, it would be of interest to determine if one specific FVIII concentrate is more likely to induce an immune response in an individual patient, than another. An attempt was made to address this issue in the survey of the cohort of unrelated patients treated at our centre in Malmö. However, no such association was observed, except in one subject (No. 7, Table Y) who developed antibodies exclusively against the concentrate with which he had been treated. In the majority of samples showing a positive ELISA result regarding the FL-rFVIII products (56%), the immune response was not consistent in that antibodies against only one of the FL-rFVIII products were identified. This pattern was also found in the sibling cohort, but only in two cases (Table 6.4). One conceivable explanation could be assay-related in that the detection threshold of +3 SD for a positive outcome (see Material and Methods) was perhaps too high to detect low concentrations of antibodies against the other FL-rFVIII product. In addition, since different monoclonal antibodies are used in the manufacturing process of the two FL recombinant products, enrichment of different FVIII fragments in the products could have affected the ELISA results. A recent *in vitro* assessment of different recombinant FVIII concentrates revealed significant differences in protein content, FVIII activity and in thrombin formation capacity (224). However, it has also been reported that antibody response is not influenced by the actual protein content or FVIII activity (186). In addition, data on product specificity for the patients included in the long-term study were related to the study period of approximately four years. Products used by the patients prior this were not considered, and it cannot be ruled out that exposure to other sources of FVIII in the past may have influenced the immune response detected here.

### **Long-term Antibody Specificity To Recombinant FVIII**

Inhibitory anti-FVIII antibodies have been observed to appear transiently (133, 134). One of the objectives of the long-term follow-up conducted at Centre for Coagulation Disorders at Skåne University Hospital in Malmö (Paper V), was to clarify whether this was also the case with NNAs. Another important question is

whether NNAs, at least in some cases, are predictive of inhibitor development. As can be seen from Table 6.5, the appearance of NNAs is not consistent over time. Only in two cases (Nos. 3 and 5) were antibodies detected on all occasions, albeit with varying response patterns to the different antigens.

None of the subjects included in the long-term study developed an inhibitor during the study period. However, bearing in mind that the majority of the patients investigated had severe haemophilia A, it cannot be excluded that monitoring of patients with mild and moderate haemophilia A would show a different result. As presented in the case report on one patient with moderate haemophilia A (Paper IV), NNAs were observed as early as four years prior to the detection of an inhibitor. Despite having haemophilia of moderate severity, this patient had been on continuous replacement therapy for many years because of recurrent gastrointestinal bleeding. Interestingly, his bleeding phenotype changed during the last years of his life, becoming more similar to the phenotype normally observed in acquired haemophilia A (soft tissue bleeding), which also required episodes of increased administration of FVIII (Figure 6.5).



**Figure 6.5** Clinically significant haemorrhages occurring between 2001 and 2010. Type of haemorrhage is specified by colour. Detection of NNAs with ELISA and Bethesda-positive inhibitory anti-FVIII antibodies are indicated by red arrows.

This raised the suspicion of inhibitor development, which could, however, not be verified despite repeated Bethesda assays. Eventually, in 2008, a low-titre inhibitor was detected. Performing ELISA on a plasma sample from 2004 verified a FVIII-specific antibody response coinciding with the clinical observation of changed

bleeding phenotype. Since patients with moderate or mild disease have endogenous FVIII synthesis, the baseline measurement of FVIII:C may indicate levels that should theoretically ensure haemostasis, even in the presence of an inhibitor (225). As mentioned previously, observations in a patient with mild haemophilia A revealed a transient increase in immune response to endogenous (self) FVIII, while the immune response to wild-type FVIII showed a sustained elevation (133). Therefore, when a change in phenotype is seen in a clinical situation, additional assessment of the immune response may be justified despite the fact that the FVIII:C level seems sufficient to ensure haemostasis.

### **Role of Family History and *F8* Mutation in NNA Development**

This is the first time NNA development has been evaluated in families with haemophilia A (Paper III). When the ELISA-positive subjects were added to those already found to be Bethesda-positive in the past, the proportion of families with an antibody response in all siblings increased (Table 6.6). Since it is well-established that a positive family history of inhibitors leads to a higher risk of other family members with haemophilia developing inhibitors (175), it is reasonable to assume that genetic predisposition influences not only inhibitor development, but the overall immune response of an individual.

**Table 6.6** Evaluation of antibody response in families included in the sibling cohort (n=78). Results from antibody detection by Bethesda assay (BU) alone, as well as the combined outcome of Bethesda assay and ELISA are shown.

Concordant No Antibody Response		Concordant Positive Antibody Response		Discordant Antibody Response	
BU	BU & ELISA	BU	BU & ELISA	BU	BU & ELISA
17	11	8	20	53	47

The influence of the intron 22 inversion mutation on NNA development has been investigated previously in two small cohorts of 26 subjects each, showing conflicting associations with increased risk (187, 188). In the analysis of a French cohort of 144 severe haemophilia A subjects no association with *F8* mutation was found, and neither in any of the cohorts studied in this work discussed here. This is

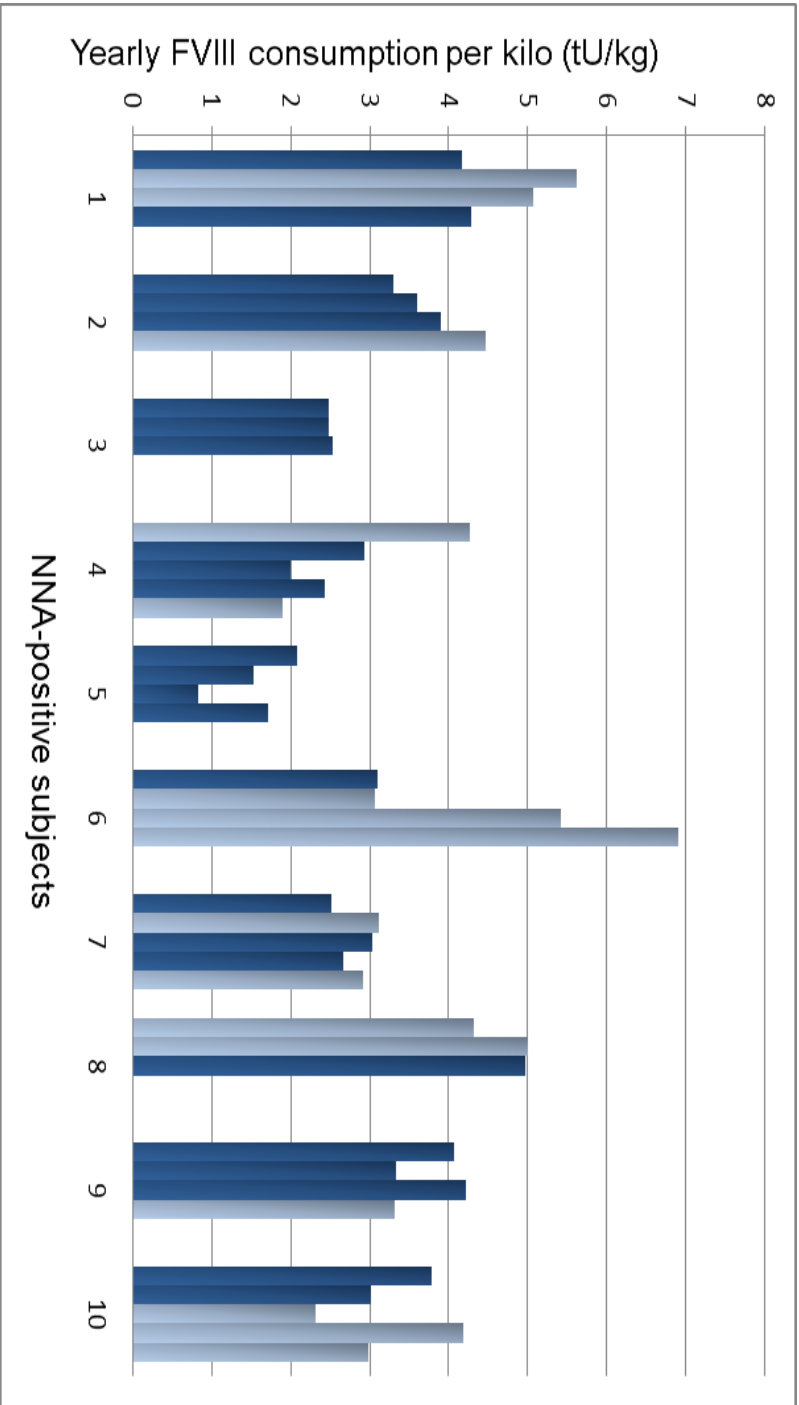
a contradictory finding compared with what is known about inhibitor development, although not all haemophilia A subjects with high-risk mutations develop inhibitors.

## **Role of NNAs in Clinical Care**

Considerable effort has been devoted to defining risk factors that predict inhibitor development. As previously reviewed (pp. 28), both treatment-related and patient-related factors have been scrutinized. Not many studies have been performed on risk factors for NNA development and, therefore, one of the objectives of the long-term study (Paper V) was to evaluate possible risk factors, as well as the clinical effects of NNAs.

As mentioned above, no association with *F8* mutation was found. Moreover, in the sibling cohort, patients with NNAs were significantly older than those in the NNA-negative cohort ( $p=0.021$ ). This association was not revealed in the unrelated cohort followed over four years (Paper V), or in the French cohort investigated by Lebreton and colleagues (182). Furthermore, in an attempt to evaluate the influence of potential challenges of the immune system on NNA development, data were collected regarding central venous access devices (Port-a-Cath, PAC) and the incidence of HCV and HIV infections. None of the subjects with a PAC developed NNAs. Moreover, a non-significant trend towards an increased risk of NNA development was observed in currently HCV-positive subjects, and in those who had previously been exposed to HCV eradication therapy. The latter is a known risk factor for FVIII inhibitor development (226). Eleven subjects were currently HIV-positive, however, ten of them were, or had been, co-infected with HCV. NNAs were identified in four patients with HIV, however, all of them were co-infected with HCV. The preliminary results show no association between NNA development and potential danger signals, as has been suggested in inhibitor patients. However, the data have not yet been evaluated with regard to major surgery or acute infections, so no definite conclusions can be drawn.

Pharmacokinetic variables, such as FVIII clearance rate and FVIII recovery, have previously been analysed in NNA-positive patients (185, 192-194), showing no convincing evidence of detrimental effects of NNAs. In the present investigation, information was collected on bleeding frequency and factor consumption. Interestingly, the bleeding frequency was significantly higher in NNA-negative patients ( $p=0.048$ ). Data on FVIII consumption was only available for the NNA-positive subgroup, which prevents comparison with the NNA-negative cohort. However, when comparing FVIII consumption with the antibody status at each point in time throughout the study, it was observed that the FVIII consumption decreased in the presence of NNA (Figure 6.6).



**Figure 6.6** Annual FVIII consumption per kilogram body weight in the ten subjects with an immune response to FVIII on at least one occasion during the study period. Bars in dark blue indicate a positive response using ELISA assay, while bars in light blue indicate a negative ELISA response. (No data were available on FVIII consumption on the third (of four) blood sampling occasions for subject No. 8).

This observation has not previously been reported, and requires further confirmation. Hypothetically, it could be the result of the presence of antibodies with enhanced FVIII activity. It was recently reported on a monoclonal anti-FVIII antibody recognizing a distinct A2 epitope, which is able to enhance the thrombin-catalysed FVIII activation (227, 228). However, further characterization of NNAs is required, and based on the observation presented here, a broader approach will be required to elucidate the function of NNAs.

## **Methodological Considerations**

The ELISA was designed to detect IgG antibodies against FVIII, and the results are qualitative as the exact antibody concentration is not determined. Several studies on the relationship between NNAs and FVIII have been performed by other groups using the same type of ELISA, thus the method is well established (116, 181, 185, 188). Recently, Mann and colleagues developed a quantitative assay using a fluorescence-based immunoassay (Luminex<sup>®</sup>) allowing quantification of anti-FVIII antibody levels. However, large amounts of recombinant FVIII are required to obtain a calibration curve based on affinity-purified human anti-FVIII antibodies from haemophilia A patients, which is a drawback of the method. Furthermore, the immunogenicity with regard to different kinds of FVIII products is not as easily evaluated with this method, since separate standards would be needed for each FVIII product (186). Lebreton and colleagues used the same fluorescence-based immunoassay (Luminex<sup>®</sup>) as Mann and colleagues, however, applying a qualitative approach similar to the one used with ELISA in the present investigations. One advantage of the Luminex<sup>®</sup> technique demonstrated by Lebreton and colleagues is that several antigens can be assayed simultaneously by coupling the antigens to microbeads (182).

The cut-off used to define a positive result was +3 SD above the mean optical density of plasma from ten healthy individuals used as controls on each plate. Since the optical density varies between test runs, the absolute optical density obtained in one experiment cannot be compared with that obtained in other experiments. Furthermore, as the control plasma was collected from ten healthy individuals, it is possible that one or more of the control samples may contain FVIII antibodies (95), resulting in a false negative result when FVIII antibodies in low-titre test plasma escape detection. Likewise, samples with low-titre FVIII antibodies may be evenly distributed around the cut-off of +3SD in repeated assays. This is a weakness of this experimental design that could maybe be overcome with a quantitative assay.

# Conclusions and Future Perspectives

Two main areas have been discussed in this thesis: the use of thrombin generation assay for monitoring the effect of by-passing agents in plasma from patients with haemophilia A, and the prevalence, specificity and potential clinical impact of non-neutralizing anti-FVIII antibodies in congenital haemophilia A. The main conclusions from the studies presented in the thesis are summarised here.

- Thrombin levels in siblings with haemophilia A are similar following treatment with by-passing agents, irrespective of the product used. This suggests that genetic determinants other than *F8* may influence the amount of thrombin formed in an individual. The choice of product in families with haemophilia could thus be based on the effect observed in other family members (Paper I).
- The amount of thrombin formed *in vitro* when adding a by-passing agent (rFVIIa or aPCC) to haemophilia A inhibitor plasma was increased in the presence of FVIII. This finding supports the use of FVIII concentrates in by-passing therapy to optimize the haemostatic effect (Paper II).
- The specificity of non-neutralizing antibodies *in vitro* towards different rFVIII products is heterogeneous within an individual, and NNAs are directed against both functional and non-functional regions of the FVIII protein (Papers III and V).
- The immune response to FVIII and the specificity of the immune response to different rFVIII products vary over time (Papers IV and V).
- The detection of non-neutralizing anti-FVIII antibodies may, in certain individuals, precede the appearance of a Bethesda-positive neutralizing antibody (inhibitor) against FVIII (Paper IV).
- For complete evaluation of the immune response to FVIII, both neutralizing and non-neutralizing anti-FVIII antibodies should be analysed (Papers III-V).



## Future Perspectives

Methods of monitoring global haemostasis have been developed, improved and widely evaluated since they were first introduced in the 1980s, however, standardization has yet to be achieved, to enable introduction of thrombin generation assay in clinical practice. Data recently presented by Dargaud and colleagues on a standardized TGA protocol seem promising, and it has been suggested that the use of TGA in clinical trials may be appropriate (33). The potentiation of by-passing agents when adding FVIII concentrates, supported by the findings presented in this thesis (Paper II), is a promising approach in inhibitor patients with refractory haemorrhaging. However, *in vivo* assessment must be performed in a clinical setting to confirm whether the clinical response observed in patients agrees with the *in vitro* findings. As was observed in the FENOC study, the clinical response to by-passing agents may be perceived differently by patients (84), and this issue could also be addressed with combination therapy. An advantage of potentiating by-passing agents with FVIII concentrates is that by-passing agents are more expensive than FVIII concentrates, and the cost of treatment could thus be reduced. However, thrombotic complications have been reported from clinical observations when using combination therapy (aPPC with rFVIIa) (229). This is a serious issue that must be thoroughly investigated before combined therapy is introduced in the clinic.

Inhibitor development is the major complication associated with FVIII replacement therapy, and although considerable advances have been made in treatment regimens and patient care, inhibitor patients still suffer from higher morbidity than non-inhibitor patients. There are no preemptive measures available for use in clinical practice, however, recent reports support the concept of minimizing potential immune system challenges when introducing or increasing FVIII treatment (146, 164, 230). As shown in the studies presented in this thesis, as well as those by others, the Bethesda assay is not a comprehensive method for monitoring the immune response that may be developed in patients with haemophilia A. Although the clinical importance of non-neutralizing anti-FVIII antibodies still remains unclear, the findings in the case report (Paper IV) support the use of an immunoassay as a complement to the Bethesda assay when the clinical situation is unclear.

An interesting question for future research is whether there are anti-FVIII antibodies with protective or potentiating effects on FVIII. In the long-term study performed on haemophilia A patients in Malmö, NNA-positive patients bled significantly less than those showing no FVIII immune response (Paper V). Epitope mapping and further characterization of antibodies from haemophilia A patients would provide new insights into the biology of FVIII immune response and also potential new approaches to treatment.

Based on the findings summarized in this thesis, anti-FVIII antibodies should not simply be defined on the basis of their neutralizing or non-neutralizing capacity as measured with the Bethesda assay. There is a broad repertoire of anti-FVIII antibodies circulating in the body, and their roles remain to be elucidated.



# Populärvetenskaplig sammanfattning

Hemofili A är en form av blödarsjuka som beror på en medfödd defekt av äggviteämnet faktor VIII (FVIII). FVIII spelar en viktig roll för att kroppens blodlevringsförmåga (s.k. koagulation) ska fungera optimalt och med nedsatt funktion hos FVIII riskerar man att drabbas av allvarliga blödningar i framför allt leder, men även i muskler, slemhinnor (t.ex. näsblödning) och i hjärnan. Ledblödningarna leder till kronisk nedbrytning av leden, s.k. artropati, vilket påverkar patientens rörelseförmåga och även livskvalitet avsevärt. Sjukdomen finns i tre svårighetsgrader baserat på vilken halt av FVIII man kan uppmäta i blodplasman hos patienten: svår, moderat och mild hemofili. Patienter med svår hemofili löper en påtagligt högre risk för spontana svåra blödningar än de övriga grupperna.

Den gen som styr produktionen av FVIII är belägen på den kvinnliga könskromosomen, X-kromosomen, och därmed är det (i princip) endast män som drabbas av sjukdomen hemofili (ca 1 av 5000 män). En kvinna kan dock ärva det defekta genanlaget från sin mor och kan, som bärare, i vissa fall drabbas av blödningssymptom. I ca 30% av hemofilifallen finns inte sjukdomen i familjen sedan tidigare, utan beror då på att en spontan nymutation uppstått i FVIII-genen hos den drabbade individen.

Sverige har varit ett föregångsland när det gäller hemofilisjukvård. Redan på 1950-talet började Professor Inga Marie Nilsson på Malmö Allmänna sjukhus att introducera förebyggande behandling med FVIII (s.k. profylax) till de blödarsjuka patienterna. Hon och hennes kolleger resonerade så att då patienter med moderat och mild hemofili blöder betydligt mer sällan och inte så allvarligt som patienter med svår hemofili, så borde en högre halt av FVIII i blodplasman hos de svårast drabbade patienterna göra att blödningssymptomen minskar och att sjukdomen då liknar den hos en patient med moderat/mild hemofili. Ganska snart stod det klart att teorin stämde med den kliniska bilden; patienterna blödde mindre frekvent och ledsjukdomen kunde bromsas och t.o.m. förebyggas. Från att ha varit en sjukdom med svåra symptom och en medellivslängd på inte ens 20 år lever svenska hemofilipatienter idag ett väsentligen normalt liv, med normal livslängd och livskvalitet. Profylaxbehandling är idag rekommenderad över hela världen som förstahandsval till pojkar med hemofili. Ett stort hinder är dock att kostnaden för sådan behandling är mycket hög och profylax finns därför inte tillgängligt överallt.

FVIII-preparaten som används idag är antingen syntetiska eller framställda från blodplasma från blodgivare. På 1980-talet drabbades en stor del av de blödarsjuka av HIV och/eller gulsot p.g.a. att en del av blodplasman som användes för FVIII-framställning kom från HIV- och/eller gulsotsmittade individer. Katastrofen ledde till att bättre metoder för att rena och avdöda blodprodukter från

virus utvecklades och att donerat blod testas mer noggrant. De läkemedel som används idag bedöms vara säkra. En del hemofilpatienter drabbas dock av biverkningar till FVIII-behandlingen där antikroppsbildning (s.k. inhibitorer), som drabbar ca 30% av patienterna med svår hemofili, är den mest allvarliga. Antikroppar är äggviteämnen som cirkulerar i blodbanan och som där bryter ner effekten av FVIII-läkemedlet. Det leder till att patienterna på nytt löper en stor blödningsrisk, både spontant och t.ex. vid kirurgiska ingrepp eller olyckor. Blödningar hos patienter med FVIII-inhibitorer är ofta svårbehandlade. Det finns idag läkemedelsalternativ till FVIII som kan ges till dessa patienter och de preparaten kallas för by-passing-produkter.

När man behandlar patienter med FVIII kan man efter injektionen mäta den farmakologiska effekten i blodet. För by-passing-produkter finns inte någon metod tillgänglig i kliniken för att mäta effekten. Den kliniska erfarenheten av by-passing-behandling är att effekten kan variera mellan olika individer och därför är det önskvärt att finna en metod för att vägleda val av dosering och även i viss mån val av specifik by-passing-produkt. Ett annat problem med by-passing-behandling är att kostnaden per injektion är betydligt högre än FVIII-behandling.

I två av de delarbeten som diskuteras i den här avhandlingen har by-passing-behandling vid svår hemofili A utvärderats med hjälp av en metod som kallas för "thrombin generation assay" (TGA). Metoden presenterades första gången i mitten av 1980-talet och har sedan dess utvecklats och modifierats. Med TGA kan man mäta den sammanlagda blodlevringsförmågan i blodplasma från en individ, till skillnad från t.ex. FVIII-mätning där man endast mäter halten av just FVIII. På så sätt får man en mer fullständig bild av blodlevringsförmågan.

I det första delarbetet (Paper I) var frågeställningen om by-passing-behandling av patienter med hemofili påverkas av ärftliga faktorer. Blodplasma från brödrapar med hemofili blandades med två olika by-passing-produkter i två separata försök och analyserades med TGA. Resultaten visade att blodlevringsförmågan inom ett brödrapar når jämförbara nivåer vid tillsats av by-passing-produkter, men att två obesläktade individer varierar betydligt mer i blodlevringsförmåga vid by-passing-behandling. Resultaten indikerar att det kan finnas en ärftlig faktor som styr hur väl vår koagulation fungerar. Inom hemofilisjukvården skulle man kunna använda sig av kunskapen om hur en familjemedlem har svarat på behandling med by-passingprodukt om ytterligare en individ inom samma familj behöver liknande behandling. För att säkert veta detta behöver man dock utvärdera effekten i större patientmaterial.

I det andra delarbetet (Paper II) användes TGA för att utvärdera behandlingseffekten av en kombination av by-passing-behandling och FVIII. Det har tidigare observerats hos patienter med inhibitorer att de, trots närvaro av de FVIII-hämmande antikropparna, blöder mindre om de behandlas med FVIII. Hypotesen i delarbete två var därför att tillsats av FVIII eventuellt kan förstärka effekten av by-passing-produkten. Flera olika FVIII-produkter testades som tillägg

till två olika by-passing-produkter. Resultaten från försöken visade att tillägget av FVIII gav en förstärkt, additiv, effekt på blodlevringsförmågan mätt med TGA (additiv: effekten av läkemedel 1 + effekten av läkemedel 2 = dubbel effekt; jfr  $1+1=2$ ). I vissa fall var effekten t.o.m. synergistisk, vilket innebär att tillsatsen av FVIII gav en extra skjuts till blodlevringen utöver den uppmätta additiva effekten (jfr.  $1+1>2$ ). Resultaten talar för att denna strategi skulle kunna användas för behandling hos i alla fall patienter med svåra blödningar där endast by-passing-produkt inte ger tillräcklig effekt.

Hämmande antikroppar mot FVIII (inhibitorer) mäts med en särskild metod som kallas för Bethesda-metod. Den metoden kan dock inte upptäcka alla sorters antikroppar. Man vet sedan tidigare att det bildas olika sorters FVIII-antikroppar i kroppen, både hos hemofilipatienter, men också hos friska individer. Vilken funktion eller effekt dessa antikroppar har på FVIII är oklart. Vissa studier har visat att de kan inverka negativt på effekten av FVIII-läkemedel, medan andra studier har visat att så inte är fallet. I delarbete III-V användes en metod som kallas för ELISA för att hitta FVIII-antikroppar i blodplasma från patienter med hemofili. Två stora patientgrupper undersöktes; en grupp av bröder med hemofili ( $n=201$ ) och en grupp med obesläktade individer med hemofili ( $n=78$ ). En frågeställning i båda studierna var om olika typer av FVIII-produkter kan påverka bildandet av FVIII-antikroppar på olika sätt och i olika grad. Därför testades plasmaproverna mot tre olika FVIII-produkter som är vanliga vid behandling av hemofili. Resultaten visar att ca 19% av bröderna och ca 13% av de obesläktade individerna bildar FVIII-antikroppar och att fynden av FVIII-antikroppar varierar beroende av vilken FVIII-produkt som används i experimenten. Teoretiskt kan man tänka sig att en individ bara producerar antikroppar mot just den produkt de själva använt, men något tydligt sådant mönster fann vi inte i studierna. I brödrastudien ingick ett antal individer som genomgått behandling syftande till att oskadliggöra inhibitoriska FVIII-antikroppar (ITI) och när prover från dessa individer analyserades fann vi att ca 25% av dem hade kvarstående FVIII-antikroppar, men av annan typ än de hämmande antikropparna. Vi fann också att en grupp patienter som hade drabbats av inhibitorer tidigare hade blivit av med dessa spontant utan aktiv ITI. Detta fynd är intressant då ITI-behandling är tidskrävande, omfattande för patienten och mycket kostsam. Om man bättre kan välja ut patienter som verkligen behöver den skulle mycket vara vunnet.

Den obesläktade patientgruppen följdes över en tidsperiod av ca fyra år och flera blodprover insamlades under den tiden. Analys av proverna visade att produktionen av FVIII-antikroppar varierade över tiden på så sätt att en individ uppvisade ett positivt svar vid ett provtagningstillfälle för att sedan inte producera några antikroppar vid nästa provtagningstillfälle. Flera, redan kända, riskfaktorer för bildande av inhibitoriska FVIII-antikroppar (t.ex. FVIII-mutation, ålder, kirurgiskt ingrepp och gulsotinfektion) utvärderades i relation till FVIII-antikropsutfallet utan att vi fann någon koppling till dessa riskfaktorer. Ett

mycket intressant fynd i långtidsobservationen av obesläktade individer var att patienter som någon gång under studietiden bildade FVIII-antikroppar blödde signifikant mindre än den grupp av patienter som inte bildade några antikroppar. Orsaken till detta fynd är ännu oklar, men kan indikera att det kan finnas en typ av FVIII-antikroppar som skyddar eller t.o.m. förstärker FVIII-effekten i blodet. Framtida studier får utvisa.

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## ORIGINAL ARTICLE

# Thrombin generation *in vitro* in the presence of by-passing agents in siblings with severe haemophilia A

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**Summary.** Previous data have shown an inter-individual difference in the thrombin generating capacity *in vitro* as well as phenotypic bleeding pattern among patients with severe haemophilia A (FVIII:C activity below 1%). The reason for this is not known. In addition, there are no reports on how thrombin generation may correlate between siblings. In this study, we evaluated and compared thrombin generation *in vitro* using plasma samples in the presence of by-passing agents (FEIBA<sup>®</sup> and NovoSeven<sup>®</sup>) in 21 unrelated brother pairs with and without inhibitors enrolled in the Malmö International Brother Study (MIBS). Mean maximum thrombin formation in patients with a current inhibitor titer was  $182.0 \pm 52.8$  mmol mL<sup>-1</sup> (FEIBA<sup>®</sup>) and  $130.7 \pm 54.9$  mmol mL<sup>-1</sup> (rFVIIa), respectively, and some-

what higher in those without inhibitors,  $222.7 \pm 85.5$  mmol mL<sup>-1</sup> (FEIBA<sup>®</sup>) and  $142.8 \pm 53.6$  mmol mL<sup>-1</sup> (rFVIIa) ( $P = 0.16$  and  $0.29$ ). The variance regarding the maximum thrombin production *within* a family was significantly lower compared with the thrombin production *between* families ( $P < 0.001$  for both FEIBA<sup>®</sup> and NovoSeven<sup>®</sup>). Our data indicate that genetically determined factors, other than the FVIII:C activity seems to influence the phenotypic variation in thrombin formation in the presence of by-passing agents. The nature of these determinants remains to be identified.

**Keywords:** haemophilia A, inhibitors, siblings, thrombin generation

## Introduction

Haemophilia A is an X-linked recessive disease caused by an impaired production of functional coagulation factor VIII [1]. Patients with haemo-

philia A are treated with concentrates of FVIII and for the majority of the patients such replacement therapy ensures haemostasis and prevents haemorrhage. However, as many as 30% of patients with severe haemophilia (FVIII:C < 1%) develop inhibitory antibodies against the given factor [2]. There are different possible strategies to manage this side-effect of treatment. The ultimate goal of these patients should be to eradicate the inhibitors and induce tolerance to FVIII [3,4]. However, haemostasis has to be controlled prior to and during immune tolerance induction (ITI), as well as in the case of ITI failures, by using saturating levels of the deficient factor or FVIII-bypassing agents, e.g. activated prothrombin complex concentrates (APCC) or recombinant FVIIa (rFVIIa). These latter agents induce thrombin formation independently of the deficient factor [3]. One of the major obstacles in the clinical use of by-passing agents is the lack of reliable monitoring assays. One option is the thrombin generation assay (TGA) introduced by Hemker *et al.* [5]. This assay allows continuous measurement

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of thrombin generation in a plasma sample [6] and the curve generated represents the result of prothrombin activation and thrombin inactivation [7]. It has been shown that there is a significant inter-individual discrepancy in the thrombin generating capacity among haemophilia patients even though they have the same plasma FVIII activity [8,9]. The reason for this is not known. In addition, there is no report in the literature on how the thrombin generation capacity may vary within families and between siblings. Therefore, we measured thrombin generation *in vitro* in the presence of FEIBA<sup>®</sup> and NovoSeven<sup>®</sup> in brother pairs with severe haemophilia A with and without inhibitors enrolled in the Malmö International Brother Study (MIBS) [10].

## Materials and methods

### Materials

FEIBA<sup>®</sup> (APCC) was purchased from Baxter (Baxter Healthcare Corporation, Chicago, IL, USA) and Novo Seven<sup>®</sup> from Novo Nordisk Scandinavia AB (Bagsvaerd, Denmark). Human thrombin from ERL Enzyme Research Lab (Dia-Service, Gothenburg, Sweden), freeze dried phospholipid/recombinant TF (3.2 µM phosphatidylcholine/phosphatidylserine (PCPS) 80/20 + 17.9 µM rTF in HNa buffer) was obtained from Technoclone and fluorogenic substrate solution (1 mM Z-Gly-Gly-Arg-AMC) from BACHEM Feinchemikalien AG (Bubendorf, Switzerland). FVIII-deficient plasma (immune depleted) was purchased from Biopool, Trinity Biotech, Ireland. Normal pooled plasma was collected from 20 apparently healthy individuals (laboratory staff). FVIII:C Chromogenic assay kits were purchased from Haemachrom Diagnostica (Möln dal, Sweden).

We used plasma samples from 42 patients belonging to 21 brother pairs with severe haemophilia A enrolled in the MIBS study with a FVIII-level of less than 0.01 IU mL<sup>-1</sup> [11]. Blood samples were collected into tubes containing 0.129 M citrate and platelet poor plasma (PPP) was prepared by centrifuging the tubes repeatedly two times for 20 min at 2000 g. The plasma samples were frozen at -80°C and thawed at 37°C just before starting the assay.

### Methods

TGA was performed using relipidated tissue factor (rTF) as a trigger of the reaction [12]. The phospholipid/tissue factor-mix contained 3.2 µM PCPS 80/20 and 17.9 µM rTF in HNa buffer. 10 µL rTF was added to 50 µL of a fluorogenic substrate solution

(1 mM Z-Gly-Gly-Arg-AMC) and 15 mM CaCl<sub>2</sub>. About 40 µL of patient's plasma was then supplemented with either FEIBA<sup>®</sup> (final concentration of 1 U mL<sup>-1</sup>) or NovoSeven<sup>®</sup> (final concentration of 60 µg mL<sup>-1</sup>). The samples with or without by-passing agents were incubated at 37°C in a Microplate Fluorescence Reader (Tecan Genios; Tecan Austria GmbH, Grödig, Austria) where the fluorescence intensity was monitored for 90 min. The fluorescence intensity [measured in relative fluorescence units (RFU)] is proportional to the amount thrombin generated in the assay [6]. Calculation of the thrombin concentration is made from comparison to a reference curve which is established from known concentrations of thrombin. The thrombin concentration is expressed as the rate of development of fluorescence intensity [fluorescence units (FU)] calculated for each reading (FU min<sup>-1</sup>). Thrombin potential corresponds to the amount of thrombin formed during 60 min and is expressed in RFU. Evaluation of the inter-assay variability of the method has earlier been performed, showing a coefficient of variance of <5–7.3% [13,14].

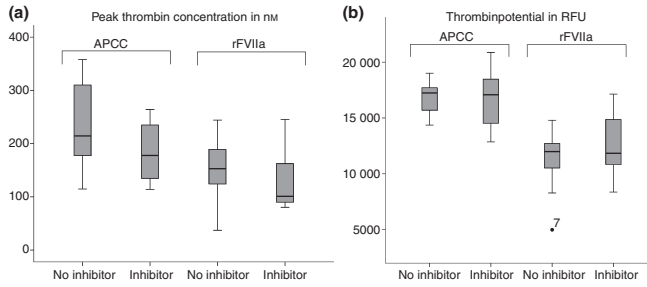
FVIII:C activity was measured using the Chromogenix COATEST<sup>®</sup> FVIII:C/4 (Instrumentation Laboratory, Breda, the Netherlands) according to the manufacturer's instructions. For detection of inhibitory antibodies, we used the Bethesda assay with Nijmegen modifications [15].

### Statistics

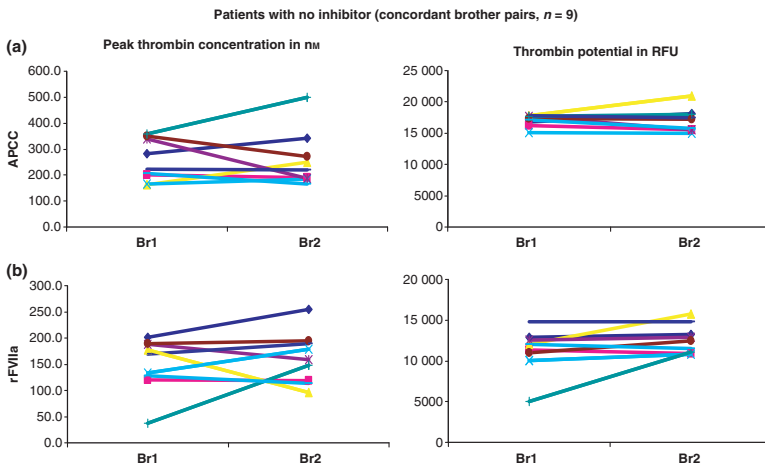
Mean and median values of the peak thrombin concentration and potential are given with range. The Mann-Whitney *U*-test was used to test the levels of maximal thrombin production between samples with and without a current inhibitor titre. To calculate the variances between and within the families regarding the maximum thrombin production a variance component analysis was conducted. An *F*-test of the ICC (intra class coefficient) was performed and 95% CI was calculated to test the difference between the variances [16]. A *P*-value <0.05 was considered as statistically significant. The statistical tests were performed in spss 15.0 for Windows (SPSS Corporation, Chicago, IL, USA) and R 2.6.2 [17].

## Results

Forty-two plasma samples from 21 brother pairs with haemophilia A were evaluated. All samples had a FVIII:C activity below 0.01 IU mL<sup>-1</sup>, consistent with an untreated severe phenotype. Nine brother



**Fig. 1.** (a) Peak thrombin concentration (nM) and (b) thrombin potential (RFU: relative fluorescence units) in patients without inhibitors ( $n = 30$ ) and in those with a positive inhibitor titer ( $n = 12$ ). APCC, activated prothrombin complex concentrates (FEIBA<sup>®</sup> 1 U mL<sup>-1</sup>); rFVIIa (NovoSeven<sup>®</sup> 60 µg mL<sup>-1</sup>).



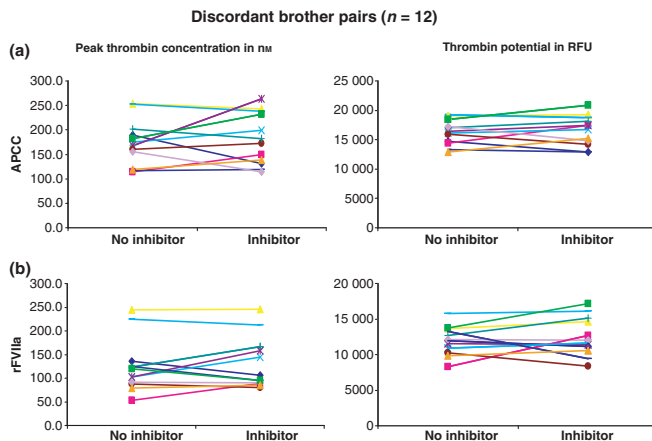
**Fig. 2.** Thrombin formation in concordant brother pairs lacking inhibitory antibodies. Br, brother; APCC, activated prothrombin complex concentrates (FEIBA<sup>®</sup> 1 U mL<sup>-1</sup>); rFVIIa (NovoSeven<sup>®</sup> 60 µg mL<sup>-1</sup>).

pairs were inhibitor concordant in that none of the siblings had experienced an inhibitor. The remaining 12 pairs were discordant with a current inhibitor present in one of the brothers in each family. No significant thrombin was formed in the absence of by-passing agents (not shown). After spiking the plasma with either FEIBA<sup>®</sup> or NovoSeven<sup>®</sup>, various amounts of thrombin was formed. The mean peak thrombin concentration (Tmax) in the presence of FEIBA<sup>®</sup> was 211.1 nM (range: 114.0–499.3 nM, median: 190 nM) and the corresponding thrombin potential 16.684 RFU (range: 12 860–20 940 RFU, median: 16 790 RFU) (Fig. 1a and b). The mean peak thrombin concentration in samples spiked with NovoSeven<sup>®</sup> was 139.3 nM (range: 37.1–254.2 nM, median: 125.9 nM) and the mean thrombin potential 12.129 RFU (range: 4990–17 140 RFU, median: 12 016 RFU). When comparing the levels of maximal thrombin production in samples with and

without a current inhibitor titre, we found a trend, but not significantly, towards a difference both in the presence of FEIBA<sup>®</sup> ( $P = 0.16$ ) and in the presence of NovoSeven<sup>®</sup> ( $P = 0.29$ ) (Fig. 1).

When comparing the amount of thrombin formed within families between concordant (Fig. 2) as well as discordant (Fig. 3) siblings, there was a clear tendency for siblings to produce a similar amount of thrombin after the addition of each agent. Among the pairs with a variation in thrombin formation, no common trend was to be found. Variance components analysis show that the variance (intra correlation coefficient) regarding the maximum thrombin production *between* families was 0.69 [95% confidence interval (95% CI), 0.38–0.86] compared to 0.31 *within* a family for APCC (FEIBA<sup>®</sup>). For rFVIIa (NovoSeven<sup>®</sup>) the variance was 0.71 (95% CI 0.41–0.87) *between* families and 0.29 *within* families. These differences in variance





**Fig. 3.** Thrombin formation in discordant brother pairs. Br, brother; APCC, activated prothrombin complex concentrates (FEIBA<sup>®</sup> 1 U mL<sup>-1</sup>); rFVIIa (NovoSeven<sup>®</sup> 60 µg mL<sup>-1</sup>).

between and within families were clearly significant ( $P < 0.001$  for both APCC and rFVIIa).

Interestingly, in some families, the maximal thrombin formed in the plasma from the brother with an inhibitor was even higher than in the plasma sampled from the non-inhibitor sibling. In addition, the plasma with a current inhibitor produced in some families more thrombin in the presence of FEIBA<sup>®</sup> and at the same time less in the presence of NovoSeven<sup>®</sup>, whereas in other families, the thrombin formed with NovoSeven<sup>®</sup> exaggerated that of FEIBA<sup>®</sup> (Fig. 3). Similar results were seen for the thrombin potential.

## Discussion

Several studies have described that the thrombin generation, and also the levels of separate coagulation factors, vary between healthy individuals [18]. This may be due to genetic factors including polymorphisms and modifier genes known to be involved in monogenic disorders with the capacity to modify penetrance, dominance, or expression of the disease causing gene [19,20]. In addition, for unknown reasons, patients with haemophilia A, although having the same basal FVIII activity, experience a variation in bleeding phenotype. No comparison of the thrombin formation capacity between siblings with haemophilia has been performed. Therefore, in order to shed some more light on the putative inheritance of factors located outside the factor gene that might influence the haemostatic balance as well as the therapeutic effect of various haemostatic agents, we evaluated the thrombin formation capacity *in vitro* in the presence and absence of by-passing agents in siblings enrolled in the MIBS study.

Interestingly, we found that the thrombin formation *in vitro* seems to be similar within families, supporting the assumption from earlier studies that there are genetic determinants influencing thrombin generation. Furthermore, this concordance in thrombin generating capacity seems to be independent of which bypassing product that is used. Our data also indicate, earlier shown in for example the FENOC study [21], that thrombin generation in the presence of by-passing agents is not significantly affected by the presence of inhibitory antibodies.

In our experimental set-up we used a supra-physiologic concentration of rFVIIa which does not correspond to the clinical dosage used in the treatment of patients. Since we used tissue factor of low concentration and PPP in the assay we found the high concentration of rFVIIa valid to use to be able to perform a stable assay. We have re-analysed some samples using a high concentration of tissue factor and 1/10 of the concentration of rFVIIa (6 µg mL<sup>-1</sup>) resulting in a similar level of thrombin production (data not shown).

The amount of thrombin formed was higher in the presence FEIBA<sup>®</sup> than in the presence of NovoSeven<sup>®</sup>, but it is important to point out that these levels should not be compared, since the mode of action of the products differs and the impact of tissue factor and platelets *in vivo* are not the same. On the other hand, an intra- as well as inter-family comparison with each product is valid to perform. In agreement with the present data the FENOC study showed that there are a disparity in the clinical response in patients treated with FEIBA<sup>®</sup> and NovoSeven<sup>®</sup> [21]. None of the products was however superior to the other, but the clinical response to these agents differed.

The variation in TGA parameters might be influenced by preanalytical conditions, for example in the preparation of PPP [8] and also by prolonged storage of PPP [9,22]. In addition, the use of PPP may be a disadvantage since platelets play a major role in haemostasis and thrombin generation [23,24], and one cannot assure the PPP to be free from residual platelets that could interfere with the result of the assay [22]. All samples within a family were collected at the same time and all samples were taken care of by the same laboratory and technician. Consequently, the variation in the preanalytical conditions within families will be minimized. Evaluation of the inter-assay variability has been performed in earlier studies showing a variability coefficient of <5–7.3% [13,14].

In summary, our study indicates that siblings with haemophilia A might produce a similar amount of thrombin when treated with bypassing agents irrespective of which product that is used. The results have to be correlated to and compared with the clinical effect of these agents *in vivo*. However, the data suggest that there are genetic determinants, other than FVIII, that influence the amount of thrombin formed in these patients. There are several candidates for this and the nature of them remains to be identified. However, keeping the FENOC study in mind, the current data encourage further studies and suggest that the product choice in families with haemophilia might be based on the previous effect observed in other family members.

### Acknowledgements

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### Disclosures

JA has received research grants from and served as consultant for both NovoNordisk and Baxter. BE has

served as consultant for both NovoNordisk and Baxter.

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# Paper II



## Combination of FVIII and by-passing agent potentiates *in vitro* thrombin production in haemophilia A inhibitor plasma

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### Summary

The by-passing agents, recombinant activated factor VII (rFVIIa) and activated prothrombin complex concentrate (APCC), are important tools in the treatment of patients with haemophilia A and high-responding inhibitory antibodies. It has been observed clinically that in some patients undergoing immune tolerance induction the bleeding frequency decreases, hypothetically caused by a transient haemostatic effect of infused FVIII not measurable *ex vivo*. We evaluated how by-passing agents and factor VIII (FVIII) affect thrombin generation (TG) *in vitro* using plasma from 11 patients with severe haemophilia A and high titre inhibitors. Samples were spiked with combinations of APCC, rFVIIa and five different FVIII products. Combination of APCC and FVIII showed a synergistic effect in eliciting TG ( $P < 0.005$ ) for four FVIII products. When rFVIIa and FVIII were combined the interaction between the preparations was found to be additive. APCC and rFVIIa were then combined without FVIII, resulting in an additive effect on thrombin production. Each product separately increased TG above baseline. In conclusion, the amount of thrombin formed *in vitro* by adding a by-passing agent, was higher in the presence of FVIII. Our findings support the use of FVIII in by-passing therapy to optimize the haemostatic effect.

**Keywords:** thrombin generation, inhibitors, haemophilia A, factor VIII concentrates, by passing agents.

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The majority of patients with congenital haemophilia A are successfully treated with prophylactic replacement therapy with factor VIII (FVIII) products. The ultimate goal is to prevent bleeding, mainly joint bleeds and to maintain haemostasis (Pipe & Valentino, 2007). However, as many as 30% of patients with severe haemophilia A (FVIII plasma level <1% of normal concentration) develop inhibitory antibodies against FVIII (Berntorp, 2007). In order to control haemostasis there are different clinical strategies to handle these antibodies; for example giving saturating levels of FVIII (Astermark, 2003), or the eradication of inhibitors by immune tolerance induction (ITI) (Tellier *et al*, 2009). FVIII-bypassing agents, which induce thrombin production independently of FVIII, can also be used. These include the activated prothrombin complex concentrates (APCC) and recombinant activated factor VII (rFVIIa), products with different modes of action (Gomperts *et al*, 2008). APCC (FEIBA<sup>®</sup>) contains, among other zymogens and coagulation enzymes, factor II (FII) and activated factor X (FXa), which initiate thrombin formation downstream of the

inhibited FVIII (Varadi *et al*, 2003; Turecek *et al*, 2004a,b). Recombinant FVIIa (NovoSeven<sup>®</sup>), in pharmacological doses, activates, in a tissue factor-dependent pathway, FX leading to thrombin production (Monroe *et al*, 1997, 1998; Mackman, 2009). These products are normally used as monotherapy. However, occasionally patients develop haemorrhages refractory to monotherapy. Several case reports indicated that a sequential combination therapy of FEIBA<sup>®</sup> and NovoSeven<sup>®</sup> was efficacious in such cases (Key *et al*, 2002; Schneiderman *et al*, 2007; Livnat *et al*, 2008). van Veen *et al* (2009) have shown, in an *in vitro* setting, a synergistic effect on thrombin generation when combining APCC and rFVIIa. There are also studies showing that adding FX to FVIIa increases the haemostatic potency both *in vitro* and *in vivo* compared with utilization of FVIIa alone (Tomokiyo *et al*, 2003). Moreover, published case reports have presented clinical protocols for patients with a history of complicated bleeds scheduled to undergo orthopaedic surgery, where preoperative immunoadsorption for elimination of inhibitory antibodies are followed

by sequential administration of either FEIBA<sup>®</sup> or NovoSeven<sup>®</sup> and FVIII concentrates (Habermann *et al*, 2004; Dargaud *et al*, 2005). Recently, Martinowitz *et al* (2009) reported on the use of thrombin generation assay to monitor patients with refractory bleeds in order to find individual dose levels of by-passing agents to maintain haemostasis. When using combinations of APCC and rFVIIa the patients could receive a concomitant treatment regimen at doses far lower than recommended for the individual product (Martinowitz *et al*, 2009).

It has been observed clinically for some years that in some patients undergoing immune tolerance induction therapy the bleeding frequency seems to decrease during the treatment period. The International Immune Tolerance Study was recently terminated because of less frequent haemorrhages in the high-dose arm (<http://www.itistudy.com/>; Clinical Trials Gov ID # NCT00212472; and Dr Charles Hay, Department of Clinical Haematology, Manchester Royal Infirmary, Manchester, UK [personal communication]), which the Data Safety Monitoring Board considered to be a safety concern. We therefore wanted to investigate if there is an advantage *in vitro* of combining a by-passing agent with FVIII clotting factor concentrates in order to increase the thrombin generating capacity in plasmas from haemophilia A patients with an inhibitor.

## Materials and methods

### *Patient's plasma samples*

Plasma was obtained from patients with severe congenital FVIII deficiency (<0.01 IU/ml), and with a measurable titre of inhibitory FVIII antibodies. The experiments with Alphanate<sup>®</sup> included five patients, and 10 patients were included in the experiments with APCC and Fanhdi<sup>®</sup>. Plasma samples from four patients were spiked with a combination of APCC and rFVIIa without the additive of FVIII. In all other experiments plasma samples from 11 patients were used.

Blood samples were obtained by venepuncture and blood collected into tubes containing 0.129 mol/l citrate; platelet-poor plasma was prepared by centrifuging the tubes twice for 20 min at 2000 g. Samples were frozen at -80°C and thawed at 37°C just prior to assay.

### *Reagents*

The APCC, FEIBA<sup>®</sup> and the rFVIII-product, Advate<sup>®</sup>, were obtained from Baxter, Baxter Healthcare Corporation, Deerfield, IL, USA. Kogenate<sup>®</sup> (rFVIII) was obtained from Bayer, Bayer Schering Pharma, Leverkusen, Germany. rFVIIa, NovoSeven<sup>®</sup>, was obtained from Novo Nordisk, Bagsvaerd, Denmark. The plasma-derived von Willebrand factor (VWF) containing FVIII concentrates Fanhdi<sup>®</sup> and Alphanate<sup>®</sup> were obtained from Grifols, Barcelona, Spain and Haemate<sup>®</sup> (Humate-P), from CSL Behring, King of Prussia, PA, USA. The VWF:FVIII ratio is close to 1 in Fanhdi<sup>®</sup> and Alphanate<sup>®</sup>

and 2.5 in Haemate<sup>®</sup> (Ristol *et al*, 1996; Berntorp, 2009; Logan, 2009). Human thrombin was obtained from ERL Enzyme Research Lab, Dia-Service, Gothenburg, Sweden. Freeze-dried phospholipid/recombinant high relipidated high tissue factor (rhTF) in Tris-HEPES-NaCl buffer, containing a high concentration of phospholipid micelles with tissue factor (TECHNOTHROMBIN<sup>®</sup> TGA RC for the rFVIIa-assays and TECHNOTHROMBIN<sup>®</sup> TGA RB for the assays with APCC), was obtained from Technoclone GmBH, Vienna, Austria. Fluorogenic substrate solution (1 mmol/l Z-Gly-Gly-Arg-AMC) was purchased from BACHEM Feinchemikalien AG, Bubendorf, Switzerland. FVIII-deficient plasma (immune-depleted) was purchased from Biopool, Trinity Biotech, Dublin, Ireland, and used for dilution of patient samples and factor concentrates. Plasma for normal pool was collected from 20 individuals (laboratory staff) and used as positive control in the thrombin generation assay. FVIII:C Chromogenic assay kits were purchased from Haemachrom Diagnostica, Mölndal, Sweden.

### *Inhibitor test*

Patient plasma was diluted to give an inhibitor titer of 11 Malmö inhibitor units (MIU/ml) in the thrombin generation assay. One Malmö inhibitor unit corresponds to the number of IU of FVIII coagulant (FVIII:C) that are neutralized by 1 ml of patient plasma. One MIU corresponds to about three Bethesda inhibitor units (BIU) (Nilsson & Hedner, 1976).

### *Thrombin generation assay*

The thrombin generation assay was performed using a rhTF (see *Reagents* above) as a trigger of the reaction (Varadi *et al*, 1999). Ten microlitres of rhTF was added to 50 µl of a fluorogenic substrate solution and 15 mmol/l CaCl<sub>2</sub>. To start the reaction, 40 µl of patient plasma was supplemented with either 5 µl rFVIIa or APCC and 5 µl of FVIII-dilution. The final concentrations of rFVIIa were 0, 1.0, 2.5 and 5.0 µg/ml, and for APCC 0, 0.25, 0.5 and 1 IU/ml. FVIII:C was added to reach final concentrations of 0, 2.5 and 5 IU/ml. The doses used in this study were based on pilot experiments in our laboratory, and were found to reach an optimal stability and sensitivity in the assays. Compared with doses used in clinical context, the dose levels of FVIII:C in the study were higher than standard dosing. APCC is normally administered at a dose of 75 IU/kg, which corresponds to an *in vitro* dose of c. 1.5–2 IU/ml. Finally, rFVIIa, at a dose of 90 µg/kg, which corresponds to the medium dose level (2.5 µg/ml) used in the experiment, is commonly used for treating patients. However, the work is performed *in vitro*, hence the conditions can never be exactly the same as *in vivo*.

All samples were analysed in duplicates. Four patients were analysed in the experiment with only APCC and rFVIIa in combination without the addition of FVIII. The experiment was repeated two times for one patient and three times for the

others. The samples were incubated at 37°C in a Microplate Fluorescence Reader (Tecan Genios, Tecan Austria GmbH, Grödig, Austria) where the fluorescence intensity was monitored every minute for 90 min. The fluorescence intensity (measured in relative fluorescence units (RFU)) is proportional to the amount thrombin generated in the assay (Varadi *et al*, 2004). Calculation of the thrombin concentration was made from comparison to a reference curve that was established from known concentrations of thrombin. The thrombin concentration is expressed as the rate of development of fluorescence intensity [fluorescence units (FU)] calculated for each reading (FU/min).

**Statistics**

The analysis of covariance (ANCOVA) statistical program was used to analyse the interactions between the products included in this study. The statistical calculations were performed at RHO Inc, Chapel Hill, NC, USA. *P*-values <0.05 were considered to indicate statistical significance.

**Results**

*APCC–FVIII interaction*

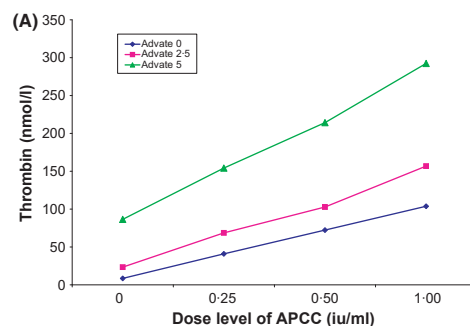
Thrombin generation assay was performed on the plasma samples with different dose levels of FVIII and APCC (see *Materials and methods*). This experimental design created 12 possible treatment combinations for each patient plasma sample. The samples without any APCC or FVIII concentrate added produced small amounts of thrombin (8.5–10.7 nmol/l) in the thrombin generation assay. Despite the presence of inhibitors in each plasma sample, the thrombin production increased in a dose-dependent fashion when FVIII was added to the sample (see Table I).

For each separate dose level of APCC, the thrombin production (Tmax) was shown to increase significantly with increased FVIII dose level, irrespective of the FVIII product added. Similarly, for each dose level of the different FVIII products, the thrombin production increased significantly when the APCC dose was increased. When comparing the maximum thrombin production between the different FVIII products all products seemed to reach approximately the same level (355.7–392.1 nmol/l), apart from Advate®, which reached a maximum thrombin production of 292.4 nmol/l (Table I).

To validate whether the combination of the two products potentiated the thrombin generation *in vitro* or whether the increase of the dose level only produced an additive pharmacological effect, the ANCOVA statistical program was used. Analysis of the interaction between APCC and the FVIII products showed that the combination of the preparations potentiated each other in a synergistic way, resulting in a significant increase in thrombin production for all products apart from for Alphanate® (*P* = 0.1470), as exemplified with Advate® in Fig 1.

**Table I.** Thrombin production in nmol/l for the assay combining APCC and FVIII.

	APCC 0 iu/ml	APCC 0.25 iu/ml	APCC 0.5 iu/ml	APCC 1 iu/ml
FVIII dose level 0 iu/ml				
Advate	8.5	40.9	72.3	103.8
Fanhdi	10.6	46.2	71.3	115.7
Haemate	8.8	44.1	70.4	112.0
Kogenate	8.6	42.9	72.0	110.7
Alphanate	10.7	44.9	64.0	100.7
FVIII dose level 2.5 iu/ml				
Advate	23.4	68.6	102.8	156.8
Fanhdi	67.0	135.0	179.0	255.5
Haemate	68.9	134.1	173.2	248.8
Kogenate	23.3	75.6	109.5	174.3
Alphanate	49.4	115.7	150.7	215.1
FVIII dose level 5 iu/ml				
Advate	86.5	154.2	214.2	292.4
Fanhdi	139.5	221.4	282.1	376.6
Haemate	131.2	202.7	252.2	364.0
Kogenate	121.3	217.7	284.8	392.1
Alphanate	122.3	200.3	257.4	355.7



**(B)**

Advate® (n=11)	Fanhdi® (n=10)	Haemate® (n=11)	Kogenate® (n=11)	Alphanate® (n=5)
<i>P</i> =0.02	<i>P</i> =0.01	<i>P</i> =0.04	<i>P</i> <0.0001	<i>P</i> =0.15

**Fig 1.** The interaction between APCC and FVIII clotting factor. The curves of the diagram should be parallel in cases where the pharmacological effect of increased dose level is additive. In the APCC-assay, here exemplified with Advate (A), the curves are not parallel, indicating synergistic interaction resulting in a significant increase in thrombin production for all FVIII products apart from for Alphanate (B).

*rFVIIa–FVIII interaction*

As shown with APCC, thrombin production was significantly increased in the absence of rFVIIa when additional FVIII was added to the sample. The maximum thrombin production ranged from 290.9 to 370.3 nmol/l, where Advate® produced



**Table II.** Thrombin production in nmol/l for the assay combining rFVIIa and FVIII.

	rFVIIa 0 µg/ml	rFVIIa 1 µg/ml	rFVIIa 2.5 µg/ml	rFVIIa 5 µg/ml
FVIII dose level 0 iu/ml				
Advate	24.8	84.9	92.3	99.7
Fanhd	22.7	80.4	88.2	94.9
Haemate	25.4	87.2	93.9	100.9
Kogenate	25.0	84.0	92.7	99.0
Alphanate	17.9	68.0	74.8	81.8
FVIII dose level 2.5 iu/ml				
Advate	74.3	138.7	140.6	152.2
Fanhd	159.9	205.0	219.8	224.7
Haemate	204.2	239.8	246.3	246.3
Kogenate	71.2	140.0	176.1	159.7
Alphanate	147.5	201.2	203.9	205.4
FVIII dose level 5 iu/ml				
Advate	236.1	276.2	283.2	290.9
Fanhd	284.2	322.1	325.7	343.0
Haemate	278.5	328.3	338.3	339.6
Kogenate	249.9	300.7	329.8	315.9
Alphanate	316.1	344.2	346.1	370.3

the lowest amount of thrombin and Alphanate® generated the highest level (see Fig 2).

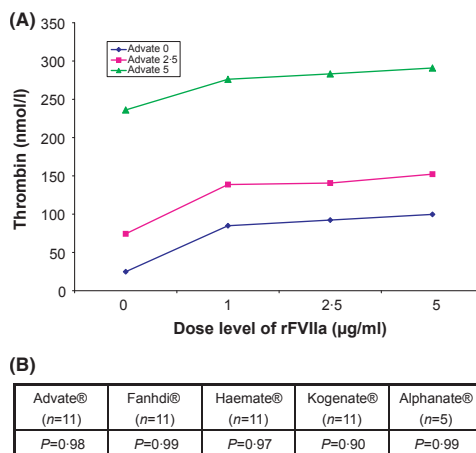
In the interaction test for rFVIIa and the different FVIII concentrates it was found that thrombin generation increased with increased dose level of either rFVIIa or FVIII. However, in contrast to the findings in the APCC experiment, the increase in thrombin generating capacity was additive but not synergistic (see Table II).

*APCC-rFVIIa interaction*

We further wanted to evaluate whether the combination of APCC and rFVIIa would give a synergistic effect on the maximum rates of thrombin generation (Tmax). Four patients were included in this experiment. The experimental design used was the same as in the experiments shown above, apart from that no FVIII was added. Increasing the dose of rFVIIa ( $P = 0.01$ ) and APCC ( $P < 0.0001$ ) gave a significant increase in the maximum thrombin concentration produced. However, we could not find any synergistic interaction between the two products ( $P = 0.9289$ ) (see Table III).

**Discussion**

This study investigated whether there is an advantage in combining a by-passing agent [APCC (FEIBA®) or rFVIIa (NovoSeven®)] with a FVIII clotting factor (Advate®, Haemate®, Fanhd®, Kogenate® and Alphanate®) in order to increase the thrombin generating capacity *in vitro* in plasmas from haemophilia A patients with an inhibitor. Occasionally, patients with haemophilia A and inhibitory antibodies develop



**Fig 2.** The interaction between rFVIIa and FVIII clotting factor. The increase in thrombin generating capacity is additive but not synergistic, here exemplified with Advate (A). (B) P-values for all FVIII-clotting factors included in the assay.

**Table III.** Thrombin production in nmol/l for the assay combining rFVIIa and APCC.

rFVIIa (µg/ml)	APCC (iu/ml)			
	0	0.25	0.5	1
0	11.5	44.1	66.8	136.5
1	28.5	55.3	91.4	131.3
2.5	32.2	59.8	96.7	138.4
5	36.6	61.9	100.6	151.7

haemorrhages that cannot be controlled by monotherapy with either APCC or rFVIIa. There are case reports showing that it is efficacious to treat such bleeds with a sequential administration of a combination of APCC and rFVIIa (Key *et al*, 2002; Schneiderman *et al*, 2007; Livnat *et al*, 2008). There are also *in vitro* studies indicating a synergistic pharmacological effect between APCC and rFVIIa, resulting in increased thrombin generation (van Veen *et al*, 2009).

We showed that a combination of by-passing agent and FVIII has a positive effect on *in vitro* thrombin generation in plasma from patients with severe haemophilia A and excess of inhibitory antibodies. As the patient plasma samples included other coagulation factors in normal concentrations a background activity of thrombin production was generated in the assays (see Tables I and II). All plasma samples were diluted to reach inhibitory antibody levels of 11 Miu/ml (c. 33 Bethesda u/ml). Despite the presence of inhibitory antibodies at a concentration enough to neutralize 11 iu/ml of FVIII:C, i.e. in excess to the FVIII added, the thrombin production increased

in a dose-dependent way when adding FVIII alone to the plasma samples. This might be explained by the kinetics of the inhibitory antibody. The thrombin generation reaction starts immediately when the FVIII-concentrate is added to the plasma sample. Since the binding of the antibody to FVIII, and inhibition of its function are relatively slow processes it may explain why thrombin is produced. In experiments with incubation times up to 2 h thrombin production still occurred in samples with excess of inhibitor (unpublished observations), which corroborates with the clinical observation that some patients experience less bleeding during ITL. The reason for this is not known. Hypothetically, the explanation could be a transient haemostatic effect of infused FVIII not measurable *ex vivo*.

We could not demonstrate any clear difference in FVIII interaction with regard to concentrate content of VWF. This is somewhat surprising as it has been proposed by several authors that VWF may compete with inhibitor binding (Gensana *et al*, 2001; Kallas & Talpsep, 2001; Goudemand *et al*, 2006) and also impact on thrombin generation. As an example, FVIII concentrates containing VWF were shown to elicit more thrombin in inhibitor plasma (Salvagno *et al*, 2007). However, when present together with APCC, other mechanisms affecting thrombin generation (see below) could tentatively be more important and overshadow a hypothetical VWF mechanism.

Analysis of the interactions between APCC and the FVIII products show that the combination of the preparations potentiates each other in a synergistic way, resulting in a significant increase in thrombin production for all products apart from Alphanate® ( $P = 0.1470$ ). Alphanate®, Haemate® and Fanhdi® are all plasma-derived concentrates that contain VWF (Ristol *et al*, 1996; Berntorp, 2009; Logan, 2009). It was previously shown that the immunoreactivity appears to be higher towards Advate® compared with Haemate® and Fanhdi® in plasmas with inhibitors directed mainly to light chain epitopes, resulting in decreased thrombin generation (Salvagno *et al*, 2007). Hypothetically, one would therefore expect a higher thrombin generation and a better synergistic or additive effect of the VWF-containing concentrates in the present study, although this was not seen. The lesser effect elicited by Alphanate® may partly be explained by the lower number of inhibitor plasmas studied with that product. As we have not performed epitope mapping in this study we could not eliminate the possibility that different epitopes affect the outcome of the thrombin generation (see Fig 1). Furthermore, APCC (FEIBA®) contains, among other zymogens and coagulation enzymes, FIX and FIXa, to which FVIII acts as a co-factor (Varadi *et al*, 2003; Turecek *et al*, 2004a,b), and this could explain why the outcome of a combination of the products potentiates thrombin generation.

When combining rFVIIa with a FVIII concentrate, the effect, in our study, was additive and not synergistically potentiated (see Fig 2). The thrombin generation assay used is performed with platelet poor plasma (PPP). It is well known that rFVIIa needs the presence of phospholipids or platelets to reach its full

pharmacological capacity (Monroe *et al*, 1997; Livnat *et al*, 2008). In order to improve the conditions for the experiments containing rFVIIa we used a reagent with a high concentration of phospholipid micelles with tissue factor as a trigger of the thrombin generation reaction (TECHNOTHROMBIN® TGA RC). This should be able to compensate, at least partly, for the absence of platelets in the plasma samples. Anyhow, it is known from earlier studies that the phospholipid content of APCC contributes to the effect on the thrombin generation assay (Livnat *et al*, 2008). This is another possible explanation for the difference between APCC and rFVIIa that we found in the outcome of the interaction tests. Further more, the addition of FX to a plasma sample has been shown to increase the thrombin generation capacity of rFVIIa both *in vivo* and *in vitro* (Tomokiyo *et al*, 2003). As mentioned above, APCC consist of e.g. coagulation enzyme FXa and several case reports describing sequential therapy with APCC and rFVIIa propose an administration protocol where APCC is given prior to rFVIIa (Key *et al*, 2002; Economou *et al*, 2008). By using such protocol the FXa-content of APCC could, hypothetically, improve the effect of rFVIIa. However, since the experimental conditions for monitoring rFVIIa were not optimal, this study did not aim to compare the effect of APCC and rFVIIa. We believe that the improved effect of adding FVIII to a by-passing agent in the presence of a high-responding inhibitor, could, potentially, be a very useful treatment option in clinical situations where monotherapy with by-passing agent has failed.

Livnat *et al* (2008) has shown *in vitro*, both in platelet rich plasma and in PPP, a synergistic effect between APCC and rFVIIa on thrombin generation. We performed similar experiments in this study, with thrombin generation assay of PPP samples with the addition of APCC and rFVIIa in combination. In contrast to what is referred to above we did not observe any synergy between the two products, but a significant increase in thrombin generation where the interactive effect was additive (see Table III).

The development of inhibitory antibodies is the major complication for patients with haemophilia. As mentioned above, occasionally the use of by-passing agents (APCC or rFVIIa) as monotherapy fails to control haemorrhages in patients with severe forms haemophilia A or B, and case reports as well as *in vitro* data suggest a combination of the two products in such cases. However, this study shows for the first time that a combination of FVIII and a by-passing agent enhances thrombin generation. Our findings could be useful for the development of clinical strategies for patients with inhibitors and severe haemorrhages refractory to monotherapy with by-passing agents.

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# Paper III



ORIGINAL ARTICLE *Inhibitors*

## Antibody formation and specificity in Bethesda-negative brother pairs with haemophilia A

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**Summary.** Antibodies directed towards non-neutralizing epitopes on the factor VIII protein (FVIII) may be detected in patients with haemophilia A. We evaluated the prevalence of non-neutralizing antibodies, in 201 inhibitor-negative brother pairs with severe haemophilia A, enrolled in the Malmö International Brother Study and the Haemophilia Inhibitor Genetics Study. To evaluate binding specificity of the antibodies, ELISA plates were coated with two recombinant full-length (FL) FVIII-products and one recombinant B-domain-deleted (BDD) product. Seventy-nine patients (39.3%) had a history of positive inhibitor titre measured by Bethesda assay, and FVIII antibodies were detected in 20 of them (25.3%). Additional 23 samples from subjects without a history of FVIII inhibitors were ELISA-positive corresponding to a frequency of non-neutralizing antibodies of

18.9%. The antibody response towards the different FVIII products was heterogenous, and was raised not only towards the non-functional B-domain but also towards both FL-rFVIII and BDD-rFVIII. In patients considered successfully treated with immune tolerance induction, 25.4% had remaining FVIII antibodies. The number of families with an antibody response in all siblings was increased when the total antibody response was taken into account, further supporting the concept of a genetic predisposition of the immune response. Further studies and careful monitoring over time are required to appreciate the immune response on the risk of inhibitor development or recurrence in the future.

**Keywords:** antibody specificity, factor VIII, haemophilia A, immune tolerance induction, non-neutralizing antibodies

## Introduction

Antibodies directed towards non-neutralizing epitopes on the factor VIII protein (FVIII) have been described in a substantial fraction of patients with haemophilia A [1–7] and in healthy individuals [8–10]. These antibodies are detected by immunoassays, such as enzyme-linked immunosorbent assay (ELISA) [1–3,11], fluorescence-based immunoassay [12,13] and immune-precipitation assay [4], but escape detection by the functional Bethesda assay. The frequency of non-neutralizing antibodies (NNA) in patients with haemophilia varies among studies from 12.2% to 53.8% [1,3,7,11–15]. Several possible functions of these antibodies have been discussed, for example,

their potential influence on pharmacokinetic parameters. Dazzi *et al.* [1] showed an increase in clearance rate of infused FVIII in patients with antibodies detected by ELISA, but negative in the Bethesda assay. Scandella *et al.* further investigated whether patients with low recovery (<66% of expected raise in FVIII concentration after administration of FVIII) and negative Bethesda assays had positive NNA titres detected by immune-precipitation [6]. No clear relationship between low recovery and the presence of such antibodies could be shown, a result confirmed by others [4,16]. Recently, it was suggested that NNA have restricted binding specificity towards full-length FVIII products in NNA-positive plasma samples [14]. However, Lebreton *et al.* [13] showed, in a French cohort, epitope specificity of NNA mainly towards the heavy chain of the FVIII protein, with 18.4% of the antibodies directed towards the B-domain. In addition, other factors, such as epitope spreading and ageing, might influence the entire antibody response [17,18]. To improve the understanding for inhibitor development, it is important to evaluate the entire antibody

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response. Many questions remain regarding NNA formation and their possible clinical impact.

Most studies aimed at investigating non-neutralizing FVIII antibodies have been performed with small numbers of patients. There are no data from large cohorts on specific immunogenicity towards different FVIII products used in the treatment of patients with haemophilia A. In addition, there are no studies on the entire antibody response, including both neutralizing and NNA antibodies, within families containing multiple members with haemophilia A. We have analysed the prevalence of NNA towards FVIII in 201 patients with haemophilia A, with and without a history of inhibitors, from two study cohorts of brothers: the Malmö International Brother Study (MIBS) [19] and the Haemophilia Inhibitor Genetics Study (HIGS) [20]. Three different recombinant FVIII products were used, separately or pooled together, as antigen to evaluate differences in antigenicity between them. We further evaluated the presence of FVIII antibodies in subjects exposed to immune tolerance induction therapy (ITI).

## Materials and methods

### Patient population

Plasma samples were obtained from 259 patients in 123 families with severe haemophilia A (factor VIII level  $<0.01$  IU mL<sup>-1</sup>) from two cohorts: the MIBS ( $n = 90$ ) and the HIGS ( $n = 169$ ). From the cohort of 259 patients, 201 Bethesda-negative subjects, including 78 brother pairs, were further evaluated for the presence of non-neutralizing FVIII antibodies, defined as a Bethesda-negative but ELISA-positive antibody response (see Fig. 1).

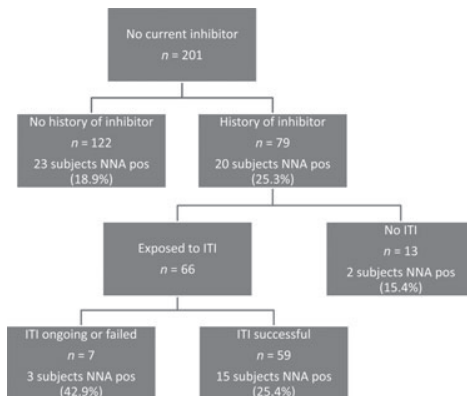


Fig. 1. Characteristics of the combined study cohort.

The median age was 14.0 years (mean: 17.3 years, range: 0–74 years), and the majority of patients were Caucasians (84.1%). In addition, there were nine Hispanics, two Asians, eight of African descent and 13 of other ethnic origins. The disease-causative mutation was identified in 190 patients (94.5%), with 110 patients carrying an inversion mutation, seven patients a large deletion, 17 patients a nonsense mutation, 36 patients a small deletion/insertion, two patients a splice-site mutation and 18 patients a missense mutation.

Seventy-nine (39.3%) patients were reported to have a history of an inhibitor (see Fig. 1), but had no current titre, with a median historical peak titre of 13.0 BU mL<sup>-1</sup> (mean: 152.4 BU mL<sup>-1</sup>, range: 1.0–3000 BU mL<sup>-1</sup>). The majority (68.4%) of the subjects were high-responders, ranging from 5.0 to 3000 BU mL<sup>-1</sup> (median: 34.0 BU mL<sup>-1</sup>, mean: 216.3 BU mL<sup>-1</sup>). ITI was initiated in 83.5% (66/79) of these patients, and the treatment was reported to be successful in 89.4% (59/66) of them. Three patients were undergoing ITI at the time of plasma sample collection, and failure of ITI was reported in four patients. Eight (8/78; 10.3%) families were concordant for a history of positive inhibitor titres in all siblings, 53 families (67.9%) were discordant and 17 families (21.8%) were concordant for no history of inhibitory FVIII antibodies.

### Enzyme-linked immunosorbent assay

The immunoassay was performed using three different commercially available recombinant FVIII concentrates: the full-length recombinant preparations Advate® (Baxter, Deerfield, IL, USA) and Kogenate® (Bayer AG, Leverkusen, Germany), and the recombinant B-domain-deleted ReFacto® (Wyeth, Maidenhead, UK). The FVIII concentrates were used in separate solutions with a final FVIII concentration of 2 µg mL<sup>-1</sup>, and also in a mixture where all three concentrates were combined to reach the same final concentration, 2 µg mL<sup>-1</sup>, of FVIII-antigen. Microtitre plates (Nunc-Immuplate, Roskilde, Denmark) were plated with 50 µL per well of FVIII-solution (2 µg mL<sup>-1</sup>) and incubated at 4°C overnight. The plates were washed three times each with washing buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 with 0.1% Tween 20) using a plate washer (Nunc-Immuno Wash 8) before non-specific sites were blocked by adding 100 µL of 1% bovine serum albumin (BSA; ICN Biomedicals inc., Irvine, CA, USA) in quench buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 with 1% BSA). The plates were incubated for 60 min at room temperature. After incubation, the plates were washed three times each with washing buffer. Patient plasma samples were thawed at 37°C for 5 min and diluted in quench buffer to 1/100 before 50 µL of the

sample was plated in duplicate on the microtitre plate. The plates were incubated at room temperature for 2 h and after a new wash cycle (three times with washing buffer) a 1/100 000 dilution of an antihuman IgG antibody (Dako P0214, Glostrup, Denmark) conjugated with horseradish-peroxidase was added (50  $\mu$ L per well). After 60 min of incubation at room temperature, the plates were washed three times and 100  $\mu$ L of ortho-phenylenediamine substrate (OPD; Dako) containing H<sub>2</sub>O<sub>2</sub>, (4 OPD tablets were dissolved in 12 mL water and 5  $\mu$ L 25% H<sub>2</sub>O<sub>2</sub> was added just before use) was added to each well. After incubation for 18 min at room temperature, the reaction was stopped by adding 100  $\mu$ L of 0.5 M H<sub>2</sub>SO<sub>4</sub> (Merck, Whitehouse Station, NJ, USA) to each well. The optical density (OD) in the wells was read at 490 nm wavelength using a Tecan Infinity F200 plate reader (Tecan, Männedorf, Switzerland).

Samples from 10 healthy individuals, a positive control sample and two blank controls (quench buffer instead of sample) were analysed on each plate. All samples were run in duplicate and the mean OD was calculated after subtraction of the mean OD of the blank control. The cut-off for positivity was calculated based on the mean and +3 standard deviation (SD) of the 10 healthy individuals. Thus, on each plate, the OD readings are expressed as no. of SD above the mean of the 10 healthy individuals, which results in a plate-specific cut-off that defines a positive sample. This is a necessary measure to avoid differences in classifications, as the absolute optical density will vary between test runs. To approve a test run, the positive control sample had to be within specified limits (95 percentile based on repeated measurements). To be classified as NNA in this study, a positive sample has to be positive on at least two independent test runs.

To control for antibody specificity, the positive samples were mixed with excess antigen prior to the analysis. This was performed by pre-incubating the 1/100 diluted plasma sample for 2 h at 37°C with 0.5  $\mu$ g of recombinant FVIII in a volume 200  $\mu$ L. After incubation, the samples were re-analysed with ELISA assay according to the protocol described above. For four samples with a remaining positive, although significantly weaker, signal after pre-incubation with soluble FVIII, and for seven samples not assayed with pre-incubation with soluble FVIII, Protein G Sepharose adsorption was performed to decide if the positive signal was dependent on IgG in the sample or not. Five-hundred microlitre of Protein G Sepharose (Fast Flow; GE Healthcare, Uppsala, Sweden) was mixed with 100  $\mu$ L plasma and 400  $\mu$ L of quench buffer (see above) and incubated at room temperature for 2 h on a platform rotator. After incubation, the solution was diluted in quench buffer to reach a final plasma concentration of 1/100. The ELISA assay was then performed as described above.

#### *Bethesda assay*

Inhibitory antibodies towards FVIII were analysed using the original Bethesda and/or the Nijmegen modified assay [21–23] at the local laboratories according to the study protocols with a cut-off for a positive inhibitory antibody set at >0.9 BU in the HIGS study and >0.6 BU in the MIBS study.

#### *Genotyping*

Genotyping of the F8 mutation was performed as previously described [24,25].

#### *Statistical analysis*

Statistical tests were performed in PASW 18.0 for Windows (SPSS Corporation, Chicago, IL, USA) and in Excel 2007 for Windows (Microsoft, Redmond, WA, USA). The Mann–Whitney *U* test was used to test the difference in median age between patients with and without non-neutralizing FVIII-antibodies. A *P*-value less than 0.05 was considered statistically significant.

## **Results**

### *Non-neutralizing FVIII-antibodies in patients without a current inhibitor*

ELISA assays were performed in the 201 patients without a current inhibitor. Antibodies towards a mixture of all three rFVIII products were found in 43 (21.4%) patients, of whom 23 had no previous history of an inhibitor, corresponding to a frequency of NNA of 18.9% (23/122) (see Fig. 1). Within this subgroup of 23 subjects, eight were ELISA-positive towards both the mixture of coating antigens and each antigen alone (see Table 1). The remaining 15 subjects showed a heterogeneous antibody response. In all but two cases, antibodies were identified against both full-length molecules, whereas only 10 of the plasma samples contained antibodies against the BDD-molecule. With subject plasma No. 1, the ELISA was negative in the presence of both full-length molecules, but in No. 3, with only one of them.

### *Non-neutralizing FVIII-antibodies in patients treated with ITI*

Immune tolerance induction had been initiated in 66 of the 79 subjects with a history of inhibitory FVIII antibodies (see Fig. 1). ITI was on-going in three cases at the time of blood sampling. All three of these were reported by the investigator to have a negative Bethesda titre; however, one had a positive ELISA assay. Failed ITI treatment was reported in four subjects,



**Table 1.** ELISA results for subjects with non-neutralizing antibodies (NNA). The immunoassay was performed using three different recombinant FVIII concentrates: two full-length recombinant preparations (FL-rFVIII A/B) and one recombinant B-domain-deleted preparation (BDD-rFVIII). The FVIII concentrates were used in separate solutions, but also in a mixture where all three concentrates were combined. The antibody response towards different FVIII-products was heterogeneous, and was raised towards both FL-rFVIII and BDD-rFVIII.

Subject	FVIII-mix	FL-rFVIII A	FL-rFVIII B	BDD-r FVIII
1	Pos	Neg	Neg	Pos
2	Pos	Pos	Pos	Pos
3	Pos	Pos	Neg	Pos
4	Pos	Pos	Pos	Pos
5	Pos	Pos	Pos	Pos
6	Pos	Pos	Pos	Pos
7	Pos	Pos	Pos	Pos
8	Pos	Pos	Pos	Pos
9	Pos	Pos	Pos	Pos
10	Pos	Pos	Pos	Neg
11	Pos	Pos	Pos	Neg
12	Pos	Pos	Pos	Neg
13	Pos	Pos	Pos	Neg
14	Pos	Pos	Pos	Neg
15	Pos	Pos	Pos	Neg
16	Pos	Pos	Pos	Neg
17	Pos	Pos	Pos	Neg
18	Pos	Pos	Pos	Neg
19	Pos	Pos	Pos	Neg
20	Pos	Pos	Pos	Neg
21	Pos	Pos	Pos	Neg
22	Pos	Pos	Pos	Neg
23	Pos	Pos	Pos	Pos

even though all four had a negative Bethesda titre. In two of these, an antigenic response was detected with the ELISA assay. Fifty-nine (89.4%) subjects were considered successfully treated with ITI. In 35 of the subjects, success was defined as having a negative Bethesda titre, a normal half-life ( $T_{1/2}$ ) and/or a normal FVIII recovery. In the remaining patients, ITI outcome was either confirmed exclusively with a negative Bethesda titre, or the confirmatory method was not specified. Overall, antibodies towards the FVIII mixture were found in plasma samples from 15 (25.4%) of the 59 subjects considered successfully treated. In Table 2, the antigenic responses of the nine HIGS patients with data available on the defined success criteria and the product used at inhibitor detection are

shown. In 3 (33.3%) of the subjects, the ELISA assays were negative only towards the product the patient had been treated with, that is the BDD-rFVIII in two cases (patients No. 7 and 9), and full-length in one (patient No. 1). In this latter patient, it is noteworthy that a Bethesda titre of 0.7 BU was stated despite a successful ITI treatment. Likewise, a titre of 0.8 BU was reported in patient No. 4. For subject No. 2, the ELISA was clearly positive with FL-rFVIII B and BDD-rFVIII, but negative in the assay using FL-rFVIII A as coating antigen. The binding was only partially possible to inhibit with the addition of each molecule, however, and was therefore considered primarily unspecific.

Thirteen patients with a current negative Bethesda titre had a previous history of inhibitors, but no exposure to ITI-therapy (see Fig. 1). Six (46.2%) of these subjects had experienced high-responding inhibitors with a mean peak titre of 15.6 BU mL<sup>-1</sup> (range: 5.0–37.5 BU mL<sup>-1</sup>, median: 11.5 BU mL<sup>-1</sup>), whereas the others were low-responders. In two of the plasma samples of the latter subjects, an antibody response was detected in the ELISA assay, whereas this was not the case for the others.

#### Age, mutation type and race in patients with NNA

The median age did not differ significantly ( $P = 0.29$ ) in patients without (median: 13.5 years, mean: 15.7 years, range: 1–68 years) and with (median: 14.0 years, mean: 23.2 years, range: 0–74 years) Bethesda-negative ELISA-positive antibodies. However, within the subgroup *without* a history of inhibitory antibodies ( $n = 122$ ), there was a significant difference in median age of patients with NNA (median: 30.0 years, mean: 28.0 years, range: 1–65 years) compared with patients without NNA (median: 14.0 years, mean: 17.0 years, range: 0–74 years) ( $P = 0.021$ ). This was not the case in the subgroup with a history of inhibitors ( $n = 79$ ) ( $P = 0.43$ ).

No significant difference in NNA prevalence was found, in either the total cohort ( $n = 201$ ) or any of the two subgroups (with and without history of inhibitors,

**Table 2.** Subjects from the HIGS cohort successfully tolerized with ITI according to defined criteria, but with detectable FVIII antibodies in the ELISA assay. The type of FVIII product at the time of inhibitor detection is given.

Subject	ITI Y/N	Confirmation of ITI outcome	Peak titre (BU mL <sup>-1</sup> )	Type of FVIII-product	FVIII-mix	FL-rFVIII A	FL-rFVIII B	BDD-rFVIII
1	Y	T1/2	110	PD	Pos	Neg	Neg	Pos
2	Y	T1/2, IVR, BU	36	BDD-rFVIII	Pos	Neg	Pos	Pos
3	Y	T1/2, IVR, BU	8.8	FL-rFVIII B	Pos	Pos	Pos	Pos
4	Y	BU	1	FL-rFVIII A	Pos	Pos	Pos	Pos
5	Y	T1/2, IVR, BU	12	FL-rFVIII	Pos	Pos	Pos	Pos
6	Y	T1/2, IVR, BU	13	PD	Pos	Pos	Pos	Pos
7	Y	T1/2, BU	62	BDD-rFVIII	Pos	Pos	Pos	Neg
8	Y	T1/2, BU	90	No data	Pos	Pos	Pos	Neg
9	Y	T1/2, IVR, BU	7	PD and BDD-rFVIII	Pos	Pos	Pos	Neg

BDD-rFVIII, B-domain-deleted recombinant FVIII; BU, Bethesda assay; FL-rFVIII, full-length recombinant FVIII; ITI, immune tolerance induction; IVR, *in vitro* recovery; T1/2, half-life; PD, plasma-derived FVIII.

see Fig. 1), when comparing patients carrying a high-risk mutation, defined as inversions, nonsense mutations or large deletions, with those with a low-risk mutation. Likewise, there was no correlation between race and NNA development in any cohort analysed (data not shown).

#### *Non-neutralizing FVIII antibodies in families*

As noted above, 53 (67.9%) families had been previously characterized as discordant with respect to an inhibitory antibody response, 8 (10.3%) families concordant with a positive inhibitor titre found in all siblings and 17 families (21.8%) concordant without a history of inhibitors. However, when considering the total FVIII antibody response, the number of discordant families was reduced to 47 (60.3%) and the number of families with an antibody response in all siblings increased to 20 (25.6%), as 10 of the discordant and two of the concordant negative (i.e. no previous antibody response) families showed a FVIII antibody response in all subjects. For example, in one family with three siblings carrying a small deletion mutation, only one had a history of inhibitory FVIII antibodies, but with inclusion of results from the ELISA assays, all three brothers showed an antigenic response towards FVIII (data not shown). In four of the 17 families without a previous inhibitor, at least one sibling had a positive antibody response.

#### **Discussion**

In our cohort of 201 patients with severe haemophilia A, the prevalence of NNA (i.e. a Bethesda-negative but ELISA-positive antibody response with FVIII-specific competition) was 18.9%. In comparison, the prevalence of NNA in previously published works using ELISA assay has varied between 12.2% and 53.8% [1,3,7,14], and in studies using fluorescence-based immunoassays (x-MAP), a prevalence of 18.1% to 45.4% [12,13,15] has been reported. Several factors could explain this large variation: sample size, differences in patient characteristics (disease severity, age, mutation) and experimental design, thus, comparison of prevalence with previously studied cohorts should be done with caution. In addition, our combined study cohort includes brother pairs, which could bias the outcome. Furthermore, an objective of HIGS is to study families in which one or more member has an inhibitor, yielding a higher prevalence of inhibitors in the overall patient population. Importantly, using cohorts of sibling pairs, we have, for the first time, been able to evaluate and compare the entire antibody response within families. Our data clearly show that the interpretation of the immune response towards the deficient factor in terms of antibody formation will be clearly influenced by the use of an immunoassay like

ELISA, instead of just capturing the immune response by the Bethesda assay. In addition, in our cohort, the proportion of families in which all siblings developed antibodies, either of the inhibitory or non-neutralizing type, was relatively higher, supporting the concept of a genetic predisposition for the immune response to occur.

When characterizing the non-neutralizing antibodies, we found that their specificity was not consistent against all three products tested in our assays. It has been suggested that NNA towards FVIII are exclusively directed towards epitopes in the non-functional B-domain of the FVIII protein [3,14]. Thus, plasma with such antibodies should test positive to FL-rFVIII preparations, but negative to the BDD-rFVIII. In agreement with this, 13 of our plasma samples (56.5%) were positive towards FL-rFVIII, but not towards BDD-rFVIII (Table 1). However, in the remaining 10 samples, NNA were found positive towards the BDD-rFVIII product, indicating antibody reactivity towards the functional domains as well. Unfortunately, all products used for treatment in the patients were not known, but the findings are in agreement with those of Lebreton *et al.* who recently showed that 73.7% of NNA were restricted towards epitopes of the heavy chain (A1-, A2- and B-domains) and 18.4% were directed towards the B-domain [13]. Interestingly, one of the plasma samples (subject No. 1 in Table 1) did not show any reactivity towards the FL-rFVIII, but only towards the BDD-rFVIII. The reason for this is not known and requires further study, but might be due to the flanking sequences introduced in the construct. The plasma of subject No. 15 showed an IgG-mediated, but unspecific binding to the FVIII molecule, as the binding was not possible to inhibit with excessive amounts of the molecule. This emphasizes the importance of testing for specificity when performing ELISA measurements. Our findings also underline the importance of using a mixture of products in the assay and, if possible, a quantitative approach, as even a discrepant outcome for the FL-rFVIII products may be observed, as in the case of plasma No. 3. This consistent but surprising finding in repeated assays was IgG-mediated and specific, in that it was possible to inhibit with only the product it bound to. We cannot, however, rule out the possibility that smaller amounts of antibodies towards the other full-length molecules were also present, and that these were simply not detected in our assay at the cut-off of +3 SD. The concern of a cut-off-related outcome should be further discussed when evaluating any antibody response, as in patients without an identified inhibitor as well as in those with or without NNA, antibodies of both neutralizing and non-neutralizing capacity may be present, but at lower concentrations than those corresponding to the defined cut-off of the assay.

There are discrepant data on the influence of the disease-causative mutation and NNA prevalence. The intron 22 inversion mutation of *F8* was shown in one study to increase the risk of NNA response [7]; however, we found all genetic alterations represented in our cohort producing NNA. When comparing patients carrying one of the high-risk mutations for inhibitor development with patients carrying low-risk mutations, no significant difference in NNA prevalence was observed, a finding which agrees with other studies [3,13]. In contrast with the French study by Lebreton *et al.* we found, in the subgroup without a history of inhibitors ( $n = 122$ ), a significant difference in median age of patients with NNA (30.0 years) compared with patients without NNA (14.0 years) ( $P = 0.021$ ).

Fifty-nine patients in our study had, according to the investigators caring for them, been successfully treated with ITI. However, 25.4% still had detectable antibodies in the ELISA assays, but the binding specificity of these antibodies varied. In five patients, antibodies were detected although both half-life and *in vivo* recovery were considered normal. In addition, in three cases, the ELISA assay was only negative against the product used at detection of the inhibitor. Unfortunately, the specific drug used for ITI was not captured, but it is reasonable to believe that the product used for ITI was the same as that in use at time of inhibitor detection. This is more or less a general approach and may indicate an as yet uncharacterized and undefined clinical difference between the molecules. The risk for recurrence of the inhibitory antibodies after ITI among patients with NNA remains to be determined, but it is reasonable to believe that it may be higher than without a detectable response, although the characteristics of these remaining antibodies have been suggested to change during ITI [5]. Therefore, the evaluation of all antibodies should be performed in patients on ITI. In this context, it is also worth pointing out that a normal PK – predefined as  $>66\%$  *in vivo* recovery and  $>6$  h T1/2 – does not necessarily reflect a normal PK for that patient. This is clearly demonstrated in our study by patient No. 1 in Table 2, who was reported to be successfully treated based on a normal T1/2, but had a positive ELISA, as well as a low Bethesda titre below the cut-off. The second patient (see Table 2, patient No. 4) with a similar outcome was only defined by a negative Bethesda titre. Altogether these findings point out the importance for strict criteria for defining ITI success, as well as standardization of the Bethesda assay.

Eleven of the 13 (84.6%) inhibitor patients without ITI exposure, including six high-responders with peak titres between 5 and 37.5 BU mL<sup>-1</sup>, were also negative in the ELISA assay. This reflects the natural course of the inhibitor response, and should be compared with the report by Caram and colleagues describing a spontaneous remission in approximately

50% of patients with a peak titre below 10 BU mL<sup>-1</sup>, but very rare above that level [26]. Some of our patients may have become tolerant as well, but complete information on treatment regimens, including time since last exposure to FVIII, was not available and hence, full evaluation of the antibody response is not possible.

In conclusion, among a large cohort of brother pairs, we describe heterogeneous antibodies, not captured in the Bethesda assay, directed towards the entire full-length FVIII molecule in patients without a previous history of inhibitors. To fully appreciate the clinical implications of these antibodies, additional studies are required. Our findings, however, indicate the importance of evaluating all antibodies towards a mixture of products when seeking to understand the immune response to the deficient factor in both related and unrelated subjects, as the immunogenicity may differ between products. It is too early to conclude that these antibodies, usually classified as NNA, will become more prevalent at higher ages, but our data suggest that it is important to evaluate a possible relationship with age. In addition, we have shown that NNA are often present in cases where ITI has been considered successful and the defined PK parameters have been normalized. These subjects should be carefully monitored over time to appreciate the impact of this immune response on the risk of inhibitor recurrence in the future.

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## Author contributions

Jenny Klintman, Jan Astermark and Andreas Hillarp designed the research study and analysed the data. Jenny Klintman performed the

ELISA assays in concert with lab technician Kerstin Fridh, and with technical support from Andreas Hillarp. Jan Astermark, Sharyne Donfield and Erik Berntorp designed the HIGS Study and contributed with essential data. Jenny Klintman wrote the manuscript with appreciated support from all co-authors.

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## Paper IV



**Letter to the Editor:**

**Clinically relevant non-neutralizing anti-FVIII antibodies in a patient  
with haemophilia A**

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We present a report of a patient with moderate haemophilia A who, until he died in 2010, was cared for at the Haemophilia Centre in Malmö, Sweden. We have performed retrospective analysis of plasma samples from several time points using a FVIII ELISA assay (as previously presented (1)). In this patient, anti-FVIII antibodies were detected as early as four years prior to the development of detectable FVIII inhibitors.

The patient was born in 1928 and was diagnosed with haemophilia A in 1962 with a FVIII:C level of 0.035 IU/mL using a coagulation-based one-stage FVIII:C assay. Over the years his basal FVIII levels varied between 0.02 IU/mL and approximately 0.15 IU/mL. One explanation for this variation is the introduction and use of different methods for measuring FVIII:C (initially one-stage assays, and since beginning of 1990's the chromogenic assays), known to result in varying FVIII levels. Genetic analysis identified a unique, and previously unknown, missense mutation in exon 6 His 209>Arg (nt 683 A>G). Apart from haemophilia A, the patient suffered from asthma, insulin-dependent diabetes, hypertension, and hepatitis C.

In the early 1980's the patient presented with a gastrointestinal (GI) haemorrhage (melena). Colonoscopy showed tubulovillous adenomas in the colon and a ventricular ulcer. He had recurrent gastrointestinal bleeding which, during episodes, were treated with prophylactic FVIII concentrates and tranexamic acid. In 1995 he once again presented with a gastrointestinal haemorrhage. Gastro- and colonoscopy confirmed a ventricular ulcer and adenomas in the colon, but also a generalized vulnerability of the mucous membranes in the gastrointestinal tract. FVIII prophylaxis twice weekly was started, since the risk of re-bleeding was considered imminent. In 1996 eradication therapy with interferon and Ribavirin for the HCV infection was begun. However, the eradication therapy was interrupted when, concurrent with the interferon therapy, he developed a low-titre inhibitor. Immune tolerance induction, using the Malmö protocol (2), was initiated within one week after detection of the inhibitor. The response was positive and immediate based on a negative inhibitor titre and a T<sub>1/2</sub> of about 6 hours. No immunoassay was available at this time. The patient was able to continue with prophylactic FVIII treatment.

Until the beginning of 2000 he presented with recurrent GI bleeds but had almost no other sites of spontaneous bleeding. He had no joint disease, and throughout his life he had reported very few haemarthrosis. In 2005, however, he had two spontaneous haemarthrosis, and from that point on his bleeding phenotype changed in character (Fig. 1). GI bleeding decreased in frequency. Instead, he suffered from repeated large haematomas, haemarthrosis and a variety of other haemorrhages (e.g. eye and tooth). During the period 2001 to 2010 the patient received prophylactic FVIII treatment with 2000 U twice weekly, and the basal FVIII level varied between mild and moderate levels (Figure 2). The Bethesda titre was measured at repeated intervals and eventually, in 2008, a low-titre inhibitor (0.8 BU/mL) was detected. FVIII treatment was continued with the dose increased to 3000 U three times weekly in order to saturate the inhibitor. Even though the inhibitor was of low-titre and post-infusion levels of FVIII reached acceptable levels to ensure haemostasis, the patient presented with severe spontaneous haemorrhages during the last years of his life. In 2009 and 2010 his condition required increased FVIII treatment and blood transfusions on several occasions. In July 2010 he presented with a large thoracic haematoma and was hospitalized for several weeks until he died due to cardiac arrest.

Inhibitor development is the most severe complication of FVIII treatment. It most commonly affects those with severe haemophilia A (basal FVIII-level  $<0.01$  IU/mL), but about 25% of newly diagnosed inhibitor patients have mild or moderate disease (3, 4). Type of *F8* mutation, and intensive FVIII use have been shown to increase the risk of inhibitor development in this patient-group (5, 6). The patient that we present here received intensive FVIII therapy for many years to treat recurrent gastrointestinal haemorrhages. In the last 5-6 years of his life his bleeding phenotype changed in character to a phenotype more commonly found in patients with acquired haemophilia A, with an increase in soft tissue haemorrhages (Fig. 1). On one occasion the bleeding was likely associated with trauma (2009), but otherwise no trauma was confirmed. Other blood parameters including blood count and liver function, were normal during the time period presented here. This may indicate development of an inhibitor, but it could not be

confirmed by repeated Bethesda assays. Furthermore, the patient's FVIII levels during the period varied between mild and moderate levels, a pattern observed over many years in this patient. For those reasons, alternate treatment options (e.g. FVIII by-passing products) were not considered and the FVIII prophylaxis continued unchanged. However, in patients with moderate or mild disease an inhibitor towards exogenous FVIII can be present even though the baseline measurement of FVIII:C indicates levels that should theoretically ensure haemostasis (7). When we retrospectively analyzed plasma samples from this patient with a FVIII ELISA, we were able to detect anti-FVIII antibodies as early as four years (in 2004) before an inhibitory antibody was detected (in 2008) (Fig. 2). Interestingly, the development of the ELISA-positive anti-FVIII antibody in 2004 coincided with the changed bleeding phenotype observed, indicating a potential clinical impact of the antibody. Whether this antibody is a non-neutralizing anti-FVIII antibody (NNA) with a potential, not yet identified, haemostatic effect or a low-titre FVIII inhibitor is not clear. No immune-based assay was done to confirm complete eradication of the immune response after immune tolerance induction in 1996, and a remaining immune response with no clinical impact may have been present. However, it cannot be excluded that the change of bleeding pattern can be explained by an altered kinetic or a pre-existing antibody and not a de novo-inhibitor.

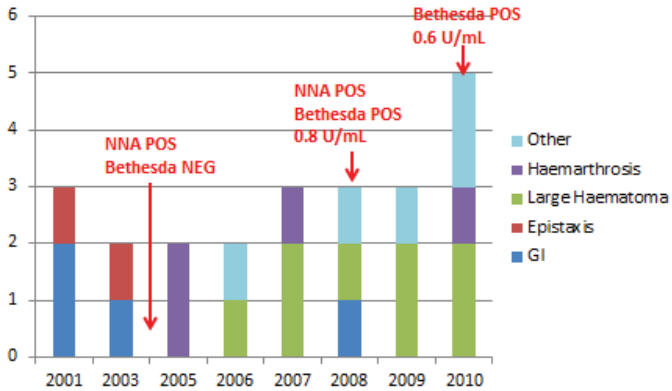
We have recently evaluated the prevalence of non-neutralizing anti-FVIII antibodies (NNA) in a large cohort of inhibitor-negative brothers with severe haemophilia A, finding a prevalence of NNA of 18.9% (1). Whether such antibody response varies over time and if NNA can transform into inhibitory anti-FVIII antibodies remain to be explored. However, the observation made in the patient presented here indicates that this might be the case, at least in patients with mild or moderate disease. In addition, our findings suggest that the kinetics of these antibodies may vary over time and in their early appearance influence the bleeding phenotype. Careful continuous monitoring of the immune response in patients with haemophilia is a valid approach, particularly in patients where the clinical phenotype changes in character. In such cases, the Bethesda assay should be supplemented by additional immune assays, and treatment modifications considered.

## Disclosures

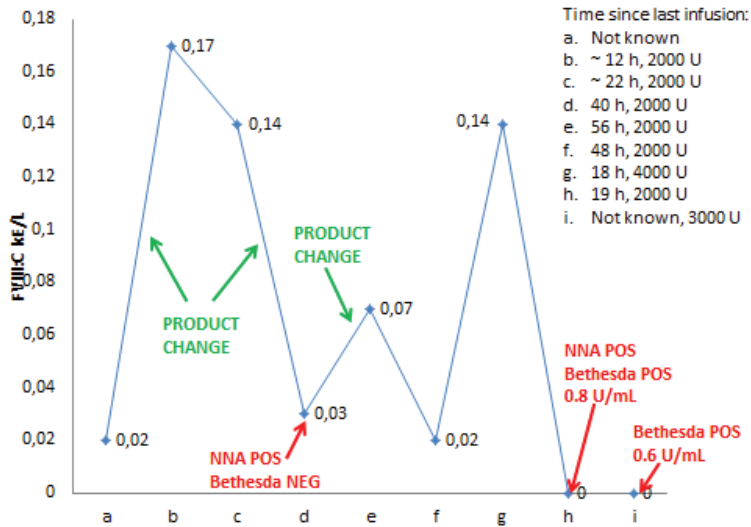
The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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**Figure 1** Clinically significant haemorrhages occurring between 2001 and 2010. Type of haemorrhages is specified by color. Detection of NNA with ELISA assay and Bethesda-positive inhibitory anti-FVIII antibodies is indicated with red arrows. NNA = non-neutralizing anti-FVIII antibodies.



**Figure 2** FVIII activity in plasma (FVIII:C kU/L) and time since last infusion at different time points: 2001 (a), 2002 (b), 2002 (c.), 2004 (d), 2005 (e), 2006 (f), 2007 (g), 2008 (h) and 2010 (i). Detection of NNA with ELISA assay and Bethesda-positive inhibitory anti-FVIII antibodies is indicated with red arrows. NNA = non-neutralizing anti-FVIII antibodies.



# Paper V





# **Long-term anti-FVIII antibody response of Bethesda-negative haemophilia A patients receiving continuous replacement therapy**

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Key words: haemophilia A, factor VIII, immunogenicity, clotting factors, non-neutralizing anti-FVIII antibodies

## **Abstract**

It has previously been shown that patients with haemophilia A may develop non-neutralizing anti-FVIII antibodies (NNA) that escape detection by the Bethesda assay, but are detected using immune-based assays. Recently, we and others found NNAs to be directed, not only towards non-functional parts of the protein, but towards all regions of the FVIII protein. We also showed in a cohort of brother pairs with haemophilia A, a heterogeneous antibody response towards different FVIII products. However, the clinical relevance and the natural history of NNA remain unclear. Therefore, we have followed a cohort of unrelated subjects with haemophilia A, on continuous replacement therapy, for four years with the goal of exploring the long-term development of NNA using an ELISA assay. Ten of 78 subjects (12.8%) exhibited an immune response that was transient and heterogeneous, and none of the subjects developed an inhibitor towards FVIII during the study period. In order to investigate the potential clinical relevance of NNA, the result of the ELISA assay was examined in relation to clinical variables. No associations between a positive ELISA assay and age, *F8* mutation and PAC implantation were shown. Interestingly, patients with NNA had significantly fewer bleeding episodes ( $p=0.048$ ) compared with NNA-negative subjects. The results indicate that the immune response towards FVIII within an individual may vary over time. However, the clinical impact of NNA remains unclear and requires further investigation.

## Introduction

Development of neutralizing anti-factor VIII (FVIII) antibodies (inhibitors) during replacement therapy is considered the most serious complication of the management of patients with haemophilia A. In patients with severe haemophilia A (FVIII level < 0.01 U/mL) about 30 % of patients develop such an immune response (1). However, antibodies directed towards non-neutralizing epitopes (NNA) may be detected in both patients with haemophilia A (2-9) and in healthy individuals (4). The frequency of non-neutralizing antibodies (NNA) in patients with haemophilia varies among studies from 12.2% to 53.8% (2-9). We have recently evaluated the prevalence of NNA with an enzyme-linked immunosorbent assay (ELISA) in a large cohort of inhibitor-negative brother pairs with severe haemophilia A, finding anti-FVIII antibodies in 18.9% of subjects (10). In contrast to previous ELISA-based studies (8), an immune response was raised not only towards the two full-length rFVIII (FL-rFVIII) preparations used in the assay, but also towards the B-domain-deleted rFVIII (BDD-rFVIII) product. This immune response pattern was also shown in a French cohort using the x-MAP methodology (5). Further, we found the immune response to be heterogeneous with an inter-individual response variation towards the three FVIII products in a substantial number of subjects (10).

The potential clinical relevance of NNA remains unclear. Even though observations have been made in Bethesda-negative patients supporting the hypothesis that NNA increases FVIII clearance (3), evaluation of NNA-influence on other pharmacologic parameters (e.g. recovery) has not shown any association (11-13). Defined as non-neutralizing anti-FVIII-antibodies, the presumption is that the antibodies do not have the capacity to inhibit FVIII activity, and hence cannot influence haemostasis. However, the natural history of NNA has not been evaluated. In several autoimmune diseases the phenomenon of epitope spreading, with a shift or increase in antigen recognition, has been suggested both to be involved in the pathogenic development of a disease (e.g. diabetes mellitus type 1 and arthritis) (14, 15), and also in modifications of clinical manifestations of a disease (e.g. autoimmune bullous skin diseases) (16). Epitope spreading is a potential explanation for the heterogenic antibody response we have previously shown, as well as that described by others (5, 10), and could, hypothetically, induce transformation of NNA to

neutralizing anti-FVIII antibodies. Whether these aspects occur, or not, in a clinical setting is thus far unexplored.

This investigation studied the natural history of NNA in a cohort of unrelated subjects with haemophilia A receiving continuous replacement therapy with FVIII. The immune response towards three recombinant FVIII concentrates was evaluated. Further, to address the potential clinical relevance of NNA, the outcome of the ELISA assays were examined in relation to clinical variables of importance to patients with haemophilia A: FVIII product used for treatment, use of Port-à-Cath (PAC), presence of HCV infection, bleeding frequency and FVIII consumption. Since the primary aim was to explore the long-term course of NNA development, multiple blood samples were collected from each subject during a time-period of approximately four years.

## **Materials and methods**

### ***Patient material***

A total of 130 subjects with severe and moderate haemophilia A were cared for at the out-patient clinic for Coagulation Disorders at Skåne University Hospital in Malmö, Sweden between 2003 and 2009. All subjects on continuous prophylactic replacement therapy, without a current inhibitor titre, and with at least three plasma samples collected between the period November 2003 and April 2009 were eligible for inclusion in the study: in total 78 subjects and 327 plasma samples (Figure 1). The mean number of samples obtained per subject was 4.1 samples (median: 4.0; range: 3-6 samples per subject), and the mean time between first and final blood sampling for the patients was 46.7 months (median 49.0 months; range: 9-63 months). The median age at the time of first blood sampling (individual dates) 25.5 years (range: 1-68 years). Clinical variables were collected from the UMAS Haemophilia Database and from the computerized medical records at Skåne University Hospital. Seventy-four subjects (94.9%) were diagnosed

with severe haemophilia A and 4 subjects (5.1%) had moderate haemophilia A. The disease causative mutation was known for 93.6% (n=73) of the patients: 25 (34.2%) patients were carrying an inversion mutation, 18 (24.7%) subjects a missense mutation, 9 (12.3%) a splice site mutation, 14 (19.2%) subjects a large deletion, 8 (11.0%) patients a nonsense mutation, 3 (4.1%) subjects an insertion mutation, and 1 (1.4%) patient a small deletion. The majority of the patients, 89.7% (70/78), were on continuous prophylactic treatment with FVIII, and the remaining eight (10.3%) had intermittent prophylactic treatment during the study period. The mean residual FVIII:C level was 0.13 IU/mL (median: 0.05 IU/mL; range: <0.01-1.61 IU/mL). The median age at the time prophylactic treatment had been started was 3.0 years (range: 1-52 years). The majority of the patients used recombinant FVIII products throughout the study period (Table 1). Information about yearly FVIII consumption was obtained from the pharmaceutical registry at the Hospital Pharmacy at Skåne University Hospital in Malmö. The study was approved by the regional research ethics committee for southern Sweden.

### ***Bethesda assay***

Inhibitory antibodies towards FVIII were analysed at the Coagulation Unit of the Department of Laboratory Medicine, Skåne University Hospital, Malmö, using the Nijmegen modified Bethesda assay (17-19). The cut-off for a positive inhibitory antibody was set at >0.4 kBU/L.

### ***Enzyme linked immunoassay (ELISA)***

The immunoassay was performed as previously described (10), using three commercially available recombinant FVIII concentrates in separate solutions with a final FVIII concentration of 2µg/mL, but also in a mixture where all three concentrates were combined to reach the same final concentration, 2µg/mL, of FVIII antigen. The cut-off for positivity was calculated based on the mean +3 standard deviations (SD) of samples from ten healthy individuals used as controls on each plate. All positive samples were analyzed at least twice. Antibody specificity was controlled for by incubating the positive samples with excess antigen prior to re-analysis with the ELISA assay described above. In eight of the 24 ELISA-positive plasma samples the inhibition by excess of FVIII was reduced, but not completely inhibited. For those eight plasma samples, Protein G

Sepharose adsorption was used to confirm that the positive signal was dependent on IgG in the sample.

### ***Genotyping***

Genotyping of the *F8* mutation was performed as previously described (20).

### ***Statistical analysis***

Statistical tests were performed in IBM SPSS Statistics 20 for Windows (IBM Corporation Armonk, NY, USA) and in Excel 2010 for Windows (Microsoft, Redmond, WA, USA). The Mann–Whitney U test was used to test the difference in age and median numbers of samples between patients with and without non-neutralizing FVIII antibodies. A *p*-value less than 0.05 was considered statistically significant. Chi-square analysis was performed for evaluation of the risk of antibody development in HCV-infected vs. non-infected subjects.

## **Results**

### ***Non-neutralizing antibodies (NNA) towards FVIII***

All 327 samples were analyzed with ELISA. Anti-FVIII antibodies towards at least one antigen (FVIII mixture and/or separate FVIII product) on at least one occasion were found in 10 subjects (12.8 %) and in 25 (7.6%) plasma samples (Table 2). The mean duration of study for NNA-positive subjects was 46.2 months (median: 47.0; range: 25-61), and 46.8 months (median: 49.0; range: 9-63) for the group without detectable NNAs. In general, a more frequent antibody response was observed towards the recombinant full-length (rFL) FVIII products compared with the recombinant B-domain deleted (rBDD) FVIII product (Table 2). The majority of the NNA-positive subjects (8/10) showed a transiently positive immune response towards FVIII throughout the study period. In subjects no. 3 and no. 5, antibodies were found in all plasma samples. There was variation in antigenic response, however, on the different occasions. Three NNA-positive subjects were treated with the recombinant B-domain deleted (rBDD) FVIII product and the others received full-length (FL) FVIII products. Among the rBDD-FVIII-treated

subjects only one (no. 2) raised antibodies towards this product. Further, subject no. 6 was treated with rFL-FVIII products during the entire study period, but raised antibodies only towards the rBDD-FVIII product, and not towards the rFL-FVIII antigens. In 14 of 25 (56.0%) ELISA-positive plasma samples, antibody responses were captured towards only one of the rFL-FVIII products. Finally, in seven plasma samples no antibodies were detected towards the FVIII mixture, but the assays were positive against separate FVIII antigens (no. 3, no. 4 and no. 9).

### ***Age, mutation type and immune system challenges in patients with NNA***

Nine of the patients with NNA development had severe haemophilia A and one had moderate haemophilia A. There was no significant difference in age between subjects with and without NNA. The median age in the NNA-positive group (n=10) was 37.5 years (mean: 31.9, range: 1-50), while the median age was 22.0 years (mean 27.0, range 2-68) in the NNA-negative group (n=68) ( $p=0.31$ ).

The *F8* mutation was known for nine of 10 NNA-positive subjects: two had an inversion mutation, three a missense mutation (of which one has been associated with inhibitor development: Arg 2163>His (nt 6545 G>A, (21)), another two had a deletion mutation, one a splice site mutation, and one a nonsense mutation. The distribution of mutations were comparable in the two groups (that is, NNA-positive vs. NNA-negative subjects) (data not shown), but there were too few subjects in the NNA-positive group to permit statistical comparisons.

None of the eight subjects who either had or had had a Port-à-Cath (PAC) implanted developed NNA (data not shown). Further, 23 (23/68, 33.8 %) subjects without NNA and five (50.0%) of 10 NNA-positive subjects were infected with HCV, an odds ratio for NNA development in currently HCV-positive subjects of 2.0 (95% CI 0.5-7.5). Sixteen subjects had received HCV eradication therapy (including interferon) in the past, and were currently HCV-negative. Among those 16, three had detectable NNA. Considering both patients who had a currently detectable, and those with a historic, HCV infection (total of 44 subjects), the odds ratio for NNA development was 3.6 (95% CI 0.7-18.0) in HCV-exposed subjects. Eleven subjects were HIV-



positive, among which ten subjects were currently, or had been, co-infected with HCV. Four of the HIV-positive subjects developed NNA, but all of them were co-infected with HCV.

### ***Bleeding phenotype and FVIII consumption in patients with NNA***

Information about bleeding frequency during the three months prior to and three months after each blood sampling was collected from the Malmö Haemophilia Database in which haemorrhages requiring treatment are registered. In the group without NNA, 37.3% of the subjects were “non-bleeders”, that is, they did not report any bleeding events in the interval prior to and following the blood sampling during the study period. In the NNA-positive group, 70.0% were “non-bleeders”. The mean bleeding frequency in patients without NNA was 1.3 bleedings/  $\pm$  3 months (range of means: 0-11.3) and in subjects with NNA 0.4 reported bleedings/  $\pm$  3 months (range of means: 0-3.5) ( $p=0.048$ ). Three subjects with NNA-production reported hemorrhages during the study period (no. 1, no. 7 and no. 8), but only 50.0% of the reported bleeding episodes coincided with a positive ELISA assay (data not shown). We also evaluated the yearly FVIII requirement of each patient. Most subjects varied moderately in their FVIII consumption during the study period, and no consistent association could be observed between the occurrence of anti-FVIII antibodies and increased dosing of FVIII (Figure 2).

## **Discussion**

Non-neutralizing anti-FVIII antibodies (NNA) have been explored in a number of cohorts of patients with haemophilia A. The prevalence varies, ranging from 12.2-53.8 % in ELISA-based studies (3, 6-8). We have previously reported an NNA prevalence of 18.9 % in inhibitor-negative subjects in a large cohort of brothers with severe haemophilia A (10), similar to that observed in a recently described French cohort (5). The long-term NNA prevalence in the current investigation of unrelated subjects with continuous replacement therapy was 12.8% (10/78), in the lower range of previous findings. However, in studies finding a high prevalence (38.0% and 53.8%) sample sizes have been small, 20 to 30 patients (3, 7), compared to our larger study group of 78 patients cared for at one centre. Since the aim of our study was to explore the long-term development of

NNA, only subjects with a minimum of three plasma samples available for analysis were included. This comprised the majority of the patients followed at our centre (60.0%), and 65.5% of the inhibitor-negative cohort, but a selection-bias influencing the outcome of NNA frequency cannot be excluded.

It was previously suggested that NNAs are exclusively directed towards the non-functional B-domain of the FVIII protein (6, 8). Even though a more frequent immune response was observed towards the rFL-FVIII products here, a substantial proportion of the subjects (4/10) developed NNA towards the rBDD-FVIII product. This was similar to our recently presented brother cohort (10) where NNAs were found towards the rBDD-FVIII product in 43.5% of the subjects with anti-FVIII NNA. This was also shown in 18.4% of the subjects in a French cohort (5). Whether the recognition sites for anti-FVIII NNA in the functional domains are functional or non-functional epitopes remains to be defined. Moreover, the immune response was transient in eight NNA-positive subjects, of whom four raised an inconsistent immune response with a positive outcome towards different antigens in the different assays. These findings stress the importance of repeated analyses of the immune response in an individual, since a negative assay on one occasion does not guarantee lack of immunologic response in the future. In a recent report on a patient with moderate haemophilia A, we described a change from a moderate towards a more severe bleeding phenotype that coincided with the detection of Bethesda-negative anti-FVIII-antibodies using ELISA. It was fully four years later that a Bethesda-positive FVIII inhibitor could be detected (Klintman, submitted). In the present study, none of the ELISA-positive subjects developed a Bethesda-positive anti-FVIII antibody.

Consistent with that previously shown in patients with inhibitory anti-FVIII antibodies (22), our ELISA assays showed a heterogeneous immune response towards the different rFVIII products. It would be tempting to believe that an immune response within an individual patient develops exclusively towards the actual protein he has previously been exposed to. However, this was not observed in the cohort presented here, where only one subject developed antibodies exclusively towards the product with which he had been treated. Moreover, in a majority of the ELISA-positive plasma samples (56.0%) the immune response towards the two rFL-FVIII products was

not consistent in that NNAs were detected only towards one of the rFL-FVIII. One possible explanation is assay-related in that the detection threshold of +3 SD for a positive outcome might be set too high to enable detection of low concentrations of antibodies towards the other rFL-FVIII molecule. Further, since different monoclonal antibodies are used in the manufacturing process of the two rFL-products, enrichment of differing amounts of FVIII fragments in the products can potentially modify the ELISA result. In addition, the potential impact of non-haemophilia related antibodies towards FVIII requires consideration. Using the same ELISA method as used here, we have found anti-FVIII antibodies in 1.3% of 155 healthy individuals (unpublished data), a level previously observed in healthy subjects (4), but lower than observed in patients with haemophilia. Hypothetically, a similar natural FVIII immune response may also occur in some people with haemophilia A. If an additional immune response occasionally is raised in such patients, a threshold for antibody detection can be reached. A heterogeneous response pattern was observed in the brother cohort previously described by us (10), and in work by others (8). Our findings might raise concern about the specificity of the assay. To ensure a specific reaction, a positive outcome was controlled for with repeated testing, by pre-incubation of an excess of antigen prior to additional re-testing, and in eight plasma samples an assay with Protein G Sepharose adsorption was added. In seven plasma samples no antibodies were detected towards the FVIII mixture, but the assays were positive against separate FVIII antigens. This is most likely a matter of antigen concentration, since the same final concentration of FVIII antigen (2µg/mL) was used in all assays, giving a higher concentration of an individual product in the assays using a single antigen. Further, since the plasma samples were obtained from subjects on replacement therapy some plasma samples will have had a residual FVIII:C level which could, potentially, result in a less sensitive assay, and an under-estimation of the NNA prevalence. Regarding product-specific antibody response, it is important to consider that associations between antibody response and product used for treatment considered only products used during the specific study period – products used in the prior years were not considered. Previous exposures to other FVIII sources might of course potentially influence an individual's immune response.

In agreement with previous findings by others, but in contrast to results in our brother cohort

study, no relationship between NNA development and age was found. Equally, there were no differences between NNA-positive subjects and NNA-negative subjects regarding the *F8* mutation distribution (5, 10). In addition, a potential immune system challenge, such as PAC implantation, was not associated with NNA development. None of the patients with a PAC developed NNA. We found a non-significant trend towards an increased risk of antibody development in patients currently HCV-positive, as well as in those who had undergone HCV eradication therapy. Whether interferon therapy included in the HCV-eradication therapy (a known risk factor for FVIII inhibitor development (23)) influenced NNA development is not clear, but needs further evaluation.

Of primary importance to both patients with haemophilia and their professional caregivers is reduction of risk for haemorrhages. The potential effect of NNA on FVIII pharmacokinetics (e.g. increased clearance and low recovery) has been studied without consistent results (3, 11, 12). Interestingly, we found bleeding frequency to be significantly higher in NNA-negative subjects ( $p=0.048$ ), and in individual subjects the FVIII consumption was somewhat decreased in the presence of NNA. We observed in one subject a decrease in FVIII:C level from a FVIII:C of 0.02 IU/mL prior to study start to FVIII:C <0.01 IU/mL, in conjunction with an increase in FVIII consumption and the occurrence of NNA. This subject belongs to a family with moderate haemophilia A with a missense mutation in exon 23 Arg 2163>His (nt 6545 G>A) shown to be associated with inhibitor development in moderate hemophilia (21). Since the patient was continuously Bethesda-negative, it can be conjectured whether the decrease in FVIII levels was related to NNA or not.

In conclusion, we followed a cohort of unrelated subjects with severe and moderate haemophilia A in order to explore the long-term course of non-neutralizing anti-FVIII antibody (NNA) development using an ELISA assay. The immune response was found to be transient and heterogeneous in 10 of 78 subjects (12.8%) and none of the subjects developed an inhibitor towards FVIII during the approximately four years of study. No association between a positive ELISA assay and increased bleeding frequency or FVIII consumption was shown. On the contrary, patients with NNA development had significantly fewer bleeding episodes ( $p=0.048$ )

compared to NNA-negative subjects. Our data suggest that the specificity of the immune response can vary over time, but the clinical significance remains unclear.

## **Acknowledgement**

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## **Author contribution**

Jenny Klintman (JK), Jan Astermark and Erik Berntorp designed the research study and analysed the data. JK performed the ELISA assays in concert with Kerstin Fridh, and with technical support from Andreas Hillarp. JK collected the clinical data and wrote the manuscript with appreciated support from all co-authors.

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## Figure legends

### Figure 1 Characteristics of the study cohort

**Table 1** FVIII products used for continuous prophylaxis at start and end of inclusion. Monoclate and Octonativ are plasma-derived products. ReFacto is a recombinant B-domain deleted FVIII product, while the others are recombinant full-length FVIII products.

**Table 2** ELISA results for the ten subjects with an immune response towards FVIII on at least one occasion during the study period. The immunoassay was performed using three different recombinant FVIII concentrates: two full-length recombinant concentrates (FL-rFVIII) Advate® (Adv) and Kogenate® (Kog), and one recombinant B-domain-deleted concentrate (BDD-rFVIII) ReFacto® (RF). The FVIII concentrates were used in separate solutions, but also in a mixture where all three concentrates were combined. The antibody response towards different FVIII products was heterogenous, varied over time and was raised towards both FL-rFVIII and BDD-rFVIII. All but one ELISA-positive subject were continuously on prophylactic treatment during

the study period. Residual FVIII:C level (IU/mL) in the plasma sample and time since last FVIII infusion (hours) is listed.

**Figure 2** Chronologic yearly FVIII consumption per kilogram bodyweight for the ten subjects with an immune response towards FVIII on at least one occasion during the study period. Bars in **dark blue** indicate occurrence of a positive ELISA assay and bars in **light blue** indicate a negative ELISA assay. For subject no. 8, data on FVIII consumption was missing for the third (out of four) blood sampling occasion.



130 subjects with severe and moderate hemophilia A

Exclusions:

- 11 inhibitor positive subjects
- 35 subjects < 3 plasma samples
- 6 subjects with on-demand therapy

Inclusion:  
78 subjects  
plasma samples n=327

severe hemophilia A  
n=74

moderate hemophilia A  
n=4

**Table 1**

<b>FVIII-products</b>	<b>Number of subjects (% of total N=78) at start</b>	<b>Number of subjects (% of total N=78) at end</b>
Kogenate®	15 (19.2%)	22 (28.2%)
ReFacto®	14 (17.9%)	14 (17.9%)
Recombinate®	32 (41.0%)	0
Advate®	9 (11.5%)	35 (44.9%)
Helixate NexGen®	6 (7.7%)	7 (9.0%)
Monoclate®	1 (1.3%)	0
Octonativ®	1 (1.3%)	0

**Table 2**

Subjects	Treatment	FVIII product	FVIII:C	Time last inf	ELISA mix	ELISA Adv	ELISA Kog	ELISA RF
1	P	Kogenate	<0.01	no info	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
1	P	Kogenate	0.62	5	neg	neg	neg	neg
1	P	Kogenate	0.02	49	neg	neg	neg	neg
1	P	Kogenate	0.22	26	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
2	P	ReFacto	<0.01	70	<b>pos</b>	<b>pos</b>	neg	<b>pos</b>
2	P	ReFacto	<0.01	no info	<b>pos</b>	neg	neg	neg
2	P	ReFacto	<0.01	no info	<b>pos</b>	neg	<b>pos</b>	<b>pos</b>
2	P	ReFacto	<0.01	>72	neg	neg	neg	neg
3	P	Recombinate	0.07	50	<b>pos</b>	<b>pos</b>	<b>pos</b>	<b>pos</b>
3	P	Kogenate	0.49	21	<b>pos</b>	<b>pos</b>	neg	<b>pos</b>
3	P	Kogenate	0.06	no info	neg	<b>pos</b>	neg	<b>pos</b>
4	P	ReFacto	0.01	68	neg	neg	neg	neg
4	P	ReFacto	0.01	72	neg	neg	<b>pos</b>	neg
4	P	ReFacto	0.17	16	neg	neg	<b>pos</b>	neg
4	P	ReFacto	0.05	48	neg	neg	<b>pos</b>	neg
4	P	ReFacto	<0.01	60	neg	neg	neg	neg
5	OD	Recombinate	<0.01	no info	<b>pos</b>	<b>pos</b>	neg	<b>pos</b>
5	P	Advate	<0.01	336	<b>pos</b>	<b>pos</b>	<b>pos</b>	<b>pos</b>
5	P	Advate	<0.01	no info	<b>pos</b>	neg	<b>pos</b>	<b>pos</b>
5	OD	Advate	0.01	120	<b>pos</b>	<b>pos</b>	<b>pos</b>	<b>pos</b>
6	P	Recombinate	0.02	16	neg	neg	neg	<b>pos</b>
6	P	Kogenate	0.05	16	neg	neg	neg	neg
6	P	Kogenate	0.30	23	neg	neg	neg	neg
6	P	Kogenate	0.24	28	neg	neg	neg	neg
7	P	Advate	0.09	18	<b>pos</b>	<b>pos</b>	neg	neg
7	P	Advate	<0.01	96	neg	neg	neg	neg
7	P	Advate	0.51	3	<b>pos</b>	<b>pos</b>	neg	neg
7	P	Advate	0.16	14	<b>pos</b>	<b>pos</b>	neg	neg
7	P	Advate	0.11	29	neg	neg	neg	neg
8	P	Helixate	<0.01	74	neg	neg	neg	neg
8	P	Helixate	0.56	4	neg	neg	neg	neg
8	P	Helixate	0.07	26	neg	neg	neg	neg
8	P	Helixate	0.06	27	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
9	P	ReFacto	0.05	48	neg	<b>pos</b>	neg	neg
9	P	ReFacto	0.02	70	neg	<b>pos</b>	<b>pos</b>	neg
9	P	ReFacto	<0.01	>96	neg	<b>pos</b>	neg	neg
9	P	ReFacto	0.17	28	neg	neg	neg	neg
10	P	Monoclata	0.11	28	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
10	P	Helixate	0.16	18	<b>pos</b>	<b>pos</b>	<b>pos</b>	neg
10	P	Helixate	0.08	38	neg	neg	neg	neg
10	P	Helixate	0.07	40	neg	neg	neg	neg
10	P	Helixate	0.32	24	neg	neg	neg	neg

