Haemophilia B

Diagnostic Insights, Genetic Aspects and Clinical Outcomes

KRISTINA KIHLBERG
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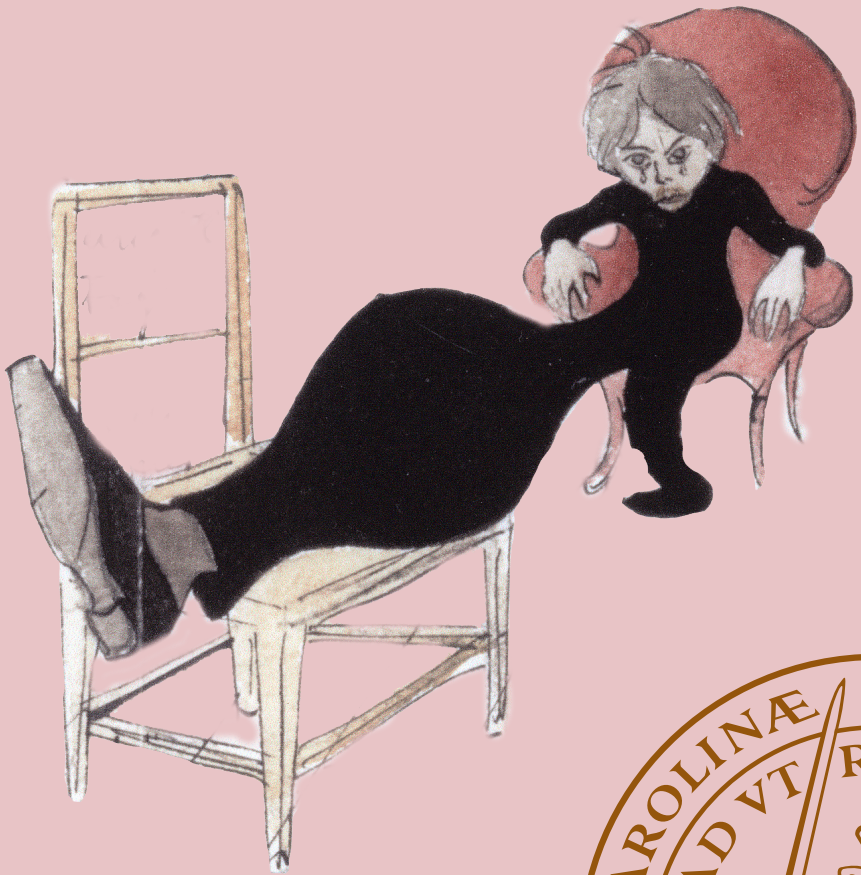

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Kristina Kihlberg

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DOCTORAL DISSERTATION

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Abstract

Haemophilia B (HB) is a rare inherited bleeding disorder caused by the deficiency of coagulation factor IX (FIX). The major clinical issues are bleedings, often targeting the joints, and the development of neutralising antibodies, i.e. inhibitors, to the FIX replacement therapy. Historically HB has been seen as identical to the more common haemophilia A (HA), i.e. deficiency of coagulation factor VIII (FVIII), but important differences between the two diseases exist. As a result of the rarity of HB, much of our knowledge of HB has been extrapolated from what is known about HA. To improve the care for persons with HB (PwHB), studies focusing on HB are of importance. The aim of this thesis was to characterise HB regarding its diagnostic challenges, treatment, clinical outcomes, and the quality of life of PwHB, and to compare some of these aspects to those of HA.

Paper I describes the comparison of the one-stage and the chromogenic assays in measuring the FIX activity level. In HA, a discrepancy between the two methods in measuring FVIII has been reported in approximately one-third of persons with non-severe HA; however, this has not previously been evaluated in HB. We found that 25% of persons with non-severe HB had discrepant results between the two methods, with higher values recorded when the chromogenic method was used. All but one of these persons had the same FIX gene (*F9*) mutated amino acid. This was the first study to show that assay discrepancy occurs in HB and we concluded that both the one-stage and the chromogenic assays are needed for the correct diagnosis and classification of HB.

Papers II-IV describe a cohort of 79 persons with severe HB from the Nordic countries, and 79 matched controls with HA. In *Paper II*, joint assessment using ultrasound and haemophilia joint health score (HJHS) was conducted and showed that despite the fact that 95% of PwHB were treated with prophylaxis, 37% reported joint bleedings during the prior year. Ultrasound scores were overall low and HJHS scores were significantly lower among PwHB compared with persons with HA (PwHA), indicative of a milder arthropathy in patients with severe HB than in PwHA. Treatment adherence was evaluated using Validated Haemophilia Regimen Treatment Adherence Scale (VERITAS) questionnaires and showed overall good adherence.

Paper III presents information on *F9* variants, inhibitors, and immune tolerance induction (ITI) therapy in PwHB. We found a high proportion of severe *F9* gene defects and a relatively high prevalence of inhibitors of 15%. Of inhibitor patients, 92% had experienced allergic manifestations and 25% nephrotic syndrome. ITI success was independent of the *F9* variant and was attained despite allergic reactions and previous ITI failures. Immunosuppression included in the ITI regimen showed a high beneficial rate and may enhance the chances of success. Analyses of non-inhibitory anti-FIX antibodies (NNAs) with a multi-analyte profiling-based fluorescence immunoassay (xFLI) and an enzyme-linked immunosorbent assay (ELISA) were conducted, but no NNAs were identified.

In *Paper IV*, health-related quality of life (HRQoL) was assessed using the EQ-5D-3L questionnaire and showed a high frequency of pain, mobility problems and anxiety/depression in PwHB, indicating that areas of insufficient care exist. No significant differences in HRQoL between PwHB and PwHA were found, and impaired joint health assessed by the HJHS was found to have a significant negative impact on HRQoL.

Key words Coagulation factor IX; Haemophilia B; Haemophilia A; *F9* variant; Assay discrepancy; Chromogenic assay; One-stage assay; Bleeding; Arthropathy; Treatment adherence; Inhibitors; Non-neutralising antibodies; Immune tolerance induction; Quality of life

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To Niklas,
Oskar, Edvin and Ivar

"We will not have failure – only success and new learning"
Queen Victoria
(Queen of the United Kingdom of Great Britain 1836-1901, and carrier of haemophilia B)

Table of Contents

Abstract

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

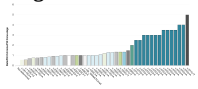
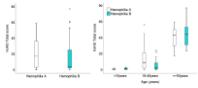
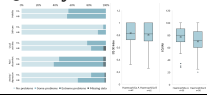
I. Kihlberg K, Strandberg K, Rosén S, Ljung R, Astermark J.
Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B.
Haemophilia. 2017 Jul;23(4):620-627. doi: 10.1111/hae.13219.

II. Kihlberg K, Baghaei F, Bruzelius M, Funding E, Holme PA, Lassila R, Nummi V, Ranta S, Osooli M, Berntorp E, Astermark J.
Treatment outcomes in persons with severe haemophilia B in the Nordic region: The B-NORD study.
Haemophilia. 2021 May;27(3):366-374. doi: 10.1111/hae.14299.

III. Kihlberg K, Baghaei F, Bruzelius M, Funding E, Holme PA, Lassila R, Martin M, Nummi V, Ranta S, Strandberg K, Andersson NG, Berntorp E, Astermark J.
Factor IX antibodies and tolerance in hemophilia B in the Nordic countries – The impact of *F9* variants and complications.
Thromb Res. 2022 Sep;217:22-32. doi: 10.1016/j.thromres.2022.06.015.

IV. Kihlberg K, Baghaei F, Bruzelius M, Funding E, Holme PA, Lassila R, Nummi V, Ranta S, Andersson NG, Berntorp E, Astermark J.
No difference in quality of life between persons with severe haemophilia A and B.
Haemophilia. 2023 Feb 15. doi: 10.1111/hae.14759. Epub ahead of print.

Thesis at a Glance

	I	II	III	IV
Paper	**Diagnostics** 	**Treatment outcomes** 	**FIX antibodies** 	**Quality of Life** 
Title	Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B	Treatment outcomes in persons with severe haemophilia B in the Nordic region: The B NORD study	Factor IX antibodies and tolerance in hemophilia B in the Nordic countries – The impact of *F9* variants and complications	No difference in quality of life between persons with severe haemophilia A and B
Objective	To compare the results from the one-stage and the chromogenic assays in PwHB.	To characterise treatment outcomes in persons with severe HB, with a focus on joint health, compared with matched controls with HA.	To investigate the presence of FIX antibodies in severe HB and to evaluate ITI outcome and complications in relation to the *F9* variant.	To assess HRQoL in PwHB and to compare this to data on PwHA, as well as to evaluate the impact of joint health on HRQoL and to identify areas of insufficient care.
Methods	Data from the one-stage and the chromogenic assays from the same blood samples were collected. Information on the *F9* variant and bleeding frequency was retrieved from medical records.	PwHB attending HTCs in the Nordics were enrolled and matched with controls with HA. Joint assessment using HJHS and ultrasound according to the HEAD-US protocol was conducted. Adherence was evaluated using VERITAS.	Information on *F9* variants, inhibitors, ITI and complications were collected in PwHB. Analyses of non-inhibitory anti-FIX antibodies with an xFLI and an ELISA method were conducted.	PwHB attending HTCs in the Nordic countries were enrolled and matched with controls with HA. HRQoL was assessed using the EQ-5D-3L questionnaire and joint health was assessed using HJHS.
Results	25% of patients with non-severe HB had discrepant results between the two methods, with higher values recorded when the chromogenic method was used. All but one of these patients had a variant affecting the same amino acid at the N-terminal cleaving site of the activation peptide.	79 PwHB were enrolled. 95% were on prophylaxis. 37% reported joint bleeds during the prior year. Only two patients had a VERITAS score corresponding to 'non-adherence'. HEAD-US protocol scores were overall low. HJHS scores were significantly lower among PwHB compared with PwHA.	Null variants were seen in 42%. 15% had a current or former inhibitor: of these, 92% had experienced allergic manifestations and 25% nephrotic syndrome. Eight of 10 PwHB with at least one ITI attempt were tolerant. IS were included in seven of eight successful or partially successful ITIs. No NNAs were identified.	63 PwHB and 63 PwHA completed the EQ-5D. No significant difference was seen between PwHA and PwHB in EQ-5D profiles, LSS, EQ-5D index or EQ VAS score. Linear regression adjusted for age demonstrated that an increase in HJHS score was associated with a significant decrease in both EQ-5D index and EQ VAS scores.
Conclusions	Assay discrepancy occurs for FIX activity and both one-stage and chromogenic assays are needed for the correct diagnosis and classification of HB.	The Nordic cohort of PwHB is well treated by prophylaxis, but the goal of zero bleeds for all has not been reached. The HJHS results suggest that patients with severe HB suffer from a milder arthropathy than patients with severe HA.	A high proportion of severe *F9* gene defects may explain the high prevalence of inhibitors. ITI success was independent of the *F9* variant and was attained despite allergic reactions and previous ITI failures. Inclusion of IS may enhance the chances of ITI success.	A high frequency of pain, mobility problems and anxiety/depression was reported and indicates that areas of insufficient care exist. No significant differences in HRQoL were found between PwHB and PwHA. Impaired joint health had a significant negative impact on HRQoL.

Abbreviations: HB, Haemophilia B. HA, Haemophilia A. FIX, coagulation factor IX. HTC, Haemophilia Treatment Centre. HJHS, Haemophilia Joint Health Score. HEAD-US, Haemophilia Early Arthropathy Detection Ultrasound. VERITAS, Validated Haemophilia Regimen Treatment Adherence Scale. ITI, Immune Tolerance Induction. *F9*, coagulation factor IX gene. IS, immunosuppresants. xFLI, Fluorescence Immunoassay. PwHB, Persons with Haemophilia B. PwHA, Persons with Haemophilia A. NNAs, Non-Neutralising Antibodies. HRQoL, Health-Related Quality of Life. LSS, Level Sum Score.

Abbreviations

AAV	Adeno-associated viral vectors
ACMG	American College of Medical Genetics and Genomics
ADP	Adenosine diphosphate
APC	Activated protein C
aPCC	Activated prothrombin complex concentrate
aPTT	Activated partial thromboplastin time
BAT	Bleeding assessment tool
BMI	Body mass index
BU	Bethesda units
CRM+	Cross-reacting material positive
CV	Coefficient of variation
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor-like domain
EHL	Extended half-life
ELISA	Enzyme-linked immunosorbent assay
F8	Coagulation factor VIII gene
F9	Coagulation factor IX gene
FDPs	Fibrin degradation products
FLI	Fluorescence-based immunoassay
FV	Coagulation factor V
FVII	Coagulation factor VII
FVIII	Coagulation factor VIII
FIX	Coagulation factor IX
FX	Coagulation factor X
FXI	Coagulation factor XI
FXII	Coagulation factor XII
FXIII	Coagulation factor XIII
Gla	γ-carboxyglutamic domain
GPIb/IIb/IIIa	Glycoprotein Ib/IIb/IIIa
HA	Haemophilia A
HB	Haemophilia B
HCV	Hepatitis C virus
HEAD-US	Haemophilia Early Arthropathy Detection Ultrasound protocol
HGVS	Human Genome Variation Society
HIV	Human immunodeficiency virus
HJHS	Haemophilia joint health score
HRQoL	Health-related quality of life
HTC	Haemophilia treatment centre
IgG1/4	Immunoglobulin G1/4
IL	Interleukin
ISTH	International Society on Thrombosis and Haemostasis

ITI	Immune tolerance induction
IVIG	Intravenous immunoglobulin
LSS	Level sum score
MFI	Median fluorescence intensity
MIU	Malmö inhibitor unit
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
NGS	Next-generation sequencing
NNA	Non-neutralising antibodies
PAI1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Plasma derived
PF4	Platelet factor 4
PwH	Persons with haemophilia
PwHA	Persons with haemophilia A
PwHB	Persons with haemophilia B
rFVIIa	Recombinant activated factor VII
RNA	Ribonucleic acid
SD	Standard deviation
SHL	Standard half-life
TAFIa	Activated thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TNF	Tumour necrosis factor
t-PA	Tissue type plasminogen activator
TTO	Time trade off
u-PA	Urokinase-type plasminogen activator
VAS	Visual analogue scale
VERITAS	Validated Haemophilia Regimen Treatment Adherence Scale
VWF	Von Willebrand factor
WFH	World Federation of Haemophilia
xFLI	Multi-Analyte Profiling based fluorescence immunoassay

Context of this thesis

This thesis was carried out within the Department of Translational Medicine, Faculty of Medicine, Lund University and the Centre for Thrombosis and Haemostasis, Skåne University Hospital in Malmö, Sweden. The Centre for Thrombosis and Haemostasis in Malmö is one of three haemophilia treatment centres (HTCs) in Sweden and in this thesis aspects of HB were investigated and evaluated in a Nordic setting.

Together with my supervisors and Associate Professor Karin Strandberg at the Division of Laboratory Medicine, Coagulation, Region Skåne, I designed the assay discrepancy study, wrote the protocol and ethical application, analysed the data, and wrote the paper (Paper I).

Papers II-IV are based on the B-NORD study, for which I have had a role as project manager and I have, with the support of my supervisors, performed much of the administrative work, including writing the protocol and ethical applications, designing the case report form (CRF), patient information, referral forms, laboratory instructions and newsletters, as well as developing research agreements with the participating centres, agreement licences for the usage of evaluation tools, and the developing of an electronic database. I also enrolled some of the patients, analysed the data and performed the statistical work with support from the Unit of Medical Statistics and Epidemiology at Region Skåne. With the support of my supervisors, I interpreted the data and wrote the papers.

Introduction

Normal haemostasis

Haemostasis is the system that arrests blood flow in the event of injury to a vessel wall, but which also restores normal blood flow after the damaged vessel is remodelled. Haemostasis is a complex system comprising many components. Steps of normal haemostasis include: vasoconstriction, platelet plug formation and fibrin clot formation, see Figure 1.[1,2]

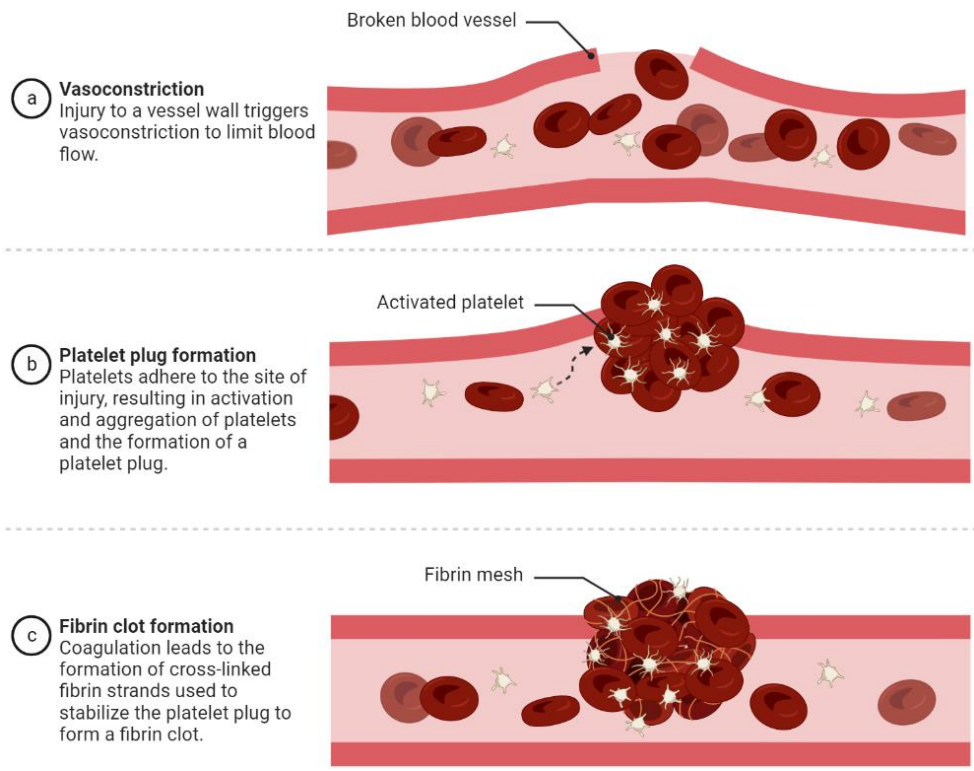


Figure 1. Normal haemostasis.
Figure adapted from "Blood Clot Formation in Broken Vessel", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

a. Vasoconstriction

When a vessel wall becomes injured, a reflex vasoconstriction occurs and the blood flow to the injured area is reduced.

b. Platelet plug formation

Adhesion. In the event of endothelial damage to a vessel, platelets from the blood come into contact with collagen in the subendothelial tissue. The platelets adhere to this collagen directly through platelet collagen receptors, but also with the help of von Willebrand factor (VWF) mediated by glycoprotein Ib (GPIb). The VWF is a large protein synthesised in the endothelium and megakaryocytes, and is present in both plasma and in the subendothelial vessel wall.

Activation. Platelet adhesion results in platelets spreading over the subendothelial tissue, as well as the activation of platelets, which causes the platelets to change shape and cytoplasmic granulae are then released. The granulae include dense bodies (containing adenosine diphosphate [ADP], thromboxane A2 and serotonin) and α-granulae (containing platelet factor 4 [PF4], fibrinogen, and VWF).[2] The normally inactive glycoprotein IIb/IIIa (GPIIb/IIIa) receptor on the platelet's surface is activated by the release of ADP and allows it to bind fibrinogen and VWF.

Aggregation. Platelet-platelet interaction (aggregation) is induced by the activated GPIIb/IIIa binding to fibrinogen and VWF and links the platelets together, resulting in the formation of a platelet plug.

c. Fibrin clot formation

Blood coagulation leads to the formation of cross-linked fibrin strands which are used to stabilise the platelet plug, and a fibrin clot is formed (see Figure 2). Plasma coagulation proteins (clotting factors) are synthesised mainly in the liver and normally circulate in plasma in their inactive forms. The coagulation factors work as enzymes or co-factors (coagulation factor V [FV] and FVIII). Originally the coagulation sequence of enzymatic reactions to form fibrin was described as a cascade with two pathways: the intrinsic (contact activation pathway) and the extrinsic (tissue factor pathway) pathways. Today this is seen as unrepresentative *in vivo* and coagulation is normally initiated through the exposure of tissue factor (TF) and activated through the classical 'extrinsic pathway'. The reactions in the coagulation pathway normally mainly take place on the activated platelet surface, which localises the blood clotting to the site of the injury. Roman numerals are usually used for most of the coagulation factors and an 'a' after the numerals indicates the active forms. Factor I is most often referred to as fibrinogen, factor II as prothrombin and FIIa as thrombin.

Initiation – on TF-bearing cells. Coagulation is initiated when TF is exposed to blood. TF is expressed on subendothelial parts of the vessel wall, such as fibroblasts and smooth muscle cells, and is exposed to blood in the event of vascular damage. In inflammation, activated endothelium and monocytes can also express TF. TF binds to activated coagulation factor VII (FVIIa) and the complex activates coagulation factor X (FX) to FXa. Non-activated FVII can also be activated to FVIIa by binding to TF. The TF/FVIIa complex also activates FIX and FIXa then activates FX with FVIIIa as a cofactor. FXa converts a small amount of prothrombin to thrombin with FVa as cofactor.[1,3]

Amplification and propagation – on activated platelets. Thrombin can be seen as the key enzyme of the coagulation process and the small amount of thrombin produced by the initiation of the coagulation pathway further activates platelets and FV, FVIII and coagulation factor XI (FXI). FXI can not only be activated by thrombin, but also by FXIIa; however, today the main role of FXI is not believed to be in the initiation of the coagulation process, but rather in the amplification after feedback activation by thrombin[1,3] and the role of activated coagulation factor XIIa (FXIIa) *in vivo* activation of FXI is not understood fully. FXI further activates FIX. FVIIIa serves as a cofactor to FIXa and, together, they form the 'tenase complex' which strongly enhances the activation of FX. FVa binds to FXa and forms the 'prothrombinase complex', which dramatically increases the activation of prothrombin to thrombin. The large amount of thrombin produced by the prothrombinase complex converts soluble fibrinogen to fibrin. Thrombin activates FXIII, and the activated FXIIIa cross-links adjacent fibrin molecules and thereby stabilises the fibrin clot.[1-3]

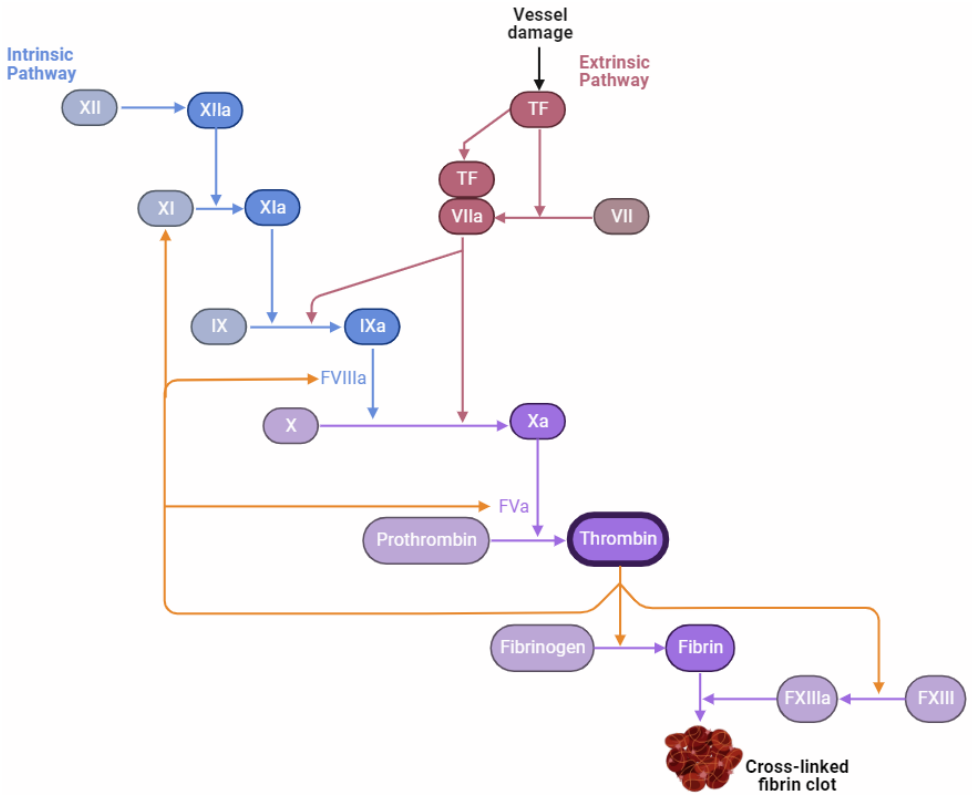


Figure 2. Simplified illustration of the procoagulant pathway in blood coagulation.
Figure adapted from "Coagulation Cascade", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Systems limiting coagulation

A physiological system to limit the coagulation process prevents clotting when vessel injury is not present, and limits clotting to sites of injury. Endothelial cells are important in this system and produce factors that inhibit platelet binding, secretion, and aggregation as well as anticoagulant factors (including heparan sulphates) and factors to activate fibrinolysis.[1,3,4] Plasma proteins important in the coagulation limiting system include:

- *Antithrombin*. Antithrombin can inhibit several procoagulant factors in the coagulation process, but it primarily inhibits thrombin, FXa and FIXa and mainly does so when heparan sulphates or other heparin substances are present, which is the case for normal intact endothelium.[1,5]
- *Tissue factor pathway inhibitor (TFPI).* TFPI is released by endothelial cells and platelets and inhibits the TF/FVIIa initiation of the coagulation process by inhibiting the TF/FVIIa complex.[1,3,4]

- *Protein C.* Protein C is activated by a complex consisting of thrombin bound to thrombomodulin, an endothelial cell receptor. Activated protein C (APC) inhibits FVa and FVIIIa.[1,3,4]
- *Protein S.* Protein S works as a co-factor and accelerates the inhibiting reaction mediated by APC.[1,3,4]

Fibrinolysis

Once the fibrin clot is formed, the fibrinolytic system is activated to dissolve fibrin to help maintain or re-establish circulation patency after vascular injury. Fibrin is dissolved into soluble fibrin degradation products (FDP), including D-dimers, which are produced when cross-linked fibrin is degraded (see Figure 3). Plasmin is the key enzyme in fibrinolysis and is the enzyme that digests fibrin. The inactive plasminogen is activated to plasmin by tissue-type plasminogen activator (tPA) (released from endothelial cells) and urokinase-type plasminogen activator (uPA) (produced in the kidneys). Control systems exist to supress the fibrinolytic system in the absence of a clot, including α2-antiplasmin, which inactivates free plasmin in the blood rapidly, as well as plasminogen activator inhibitor 1 (PAI1) and thrombin-activatable fibrinolysis inhibitor (TAFI). Fibrin binds both plasminogen and tPA on its surface and the fibrinolytic system is thereby localised to the site of the blood clotting. Fibrin also enhances the activation of plasminogen by tPA and thus regulates its own degradation.[1,2,6]

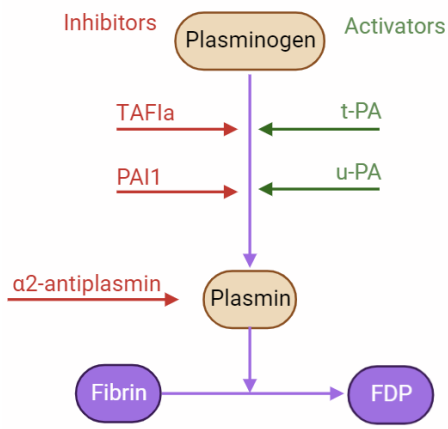


Figure 3. Fibrinolysis.
TAFIa, activated thrombin-activatable fibrinolysis inhibitor. PAI1, plasminogen activator inhibitor 1. t-PA, tissue-type plasminogen activator. u-PA, urokinase-type plasminogen activator. FDP, fibrin degradation products. Created with BioRender.com

Coagulation factor IX

HB is caused by the deficiency of functional FIX and its deficient participation in normal haemostasis.

FIX is a vitamin K-dependent glycoprotein synthesised in the liver by hepatocytes. It circulates in blood as a single-chain inactive precursor (zymogen). The pre- and pro-peptides are removed to form the mature protein and the zymogen is converted to an active serine protease, active FIX, FIXa, by the cleavage of two peptide bonds at arginine 145 and arginine 180, leading to the removal of an activation peptide (see Figure 4). The activation is performed by FXIa or the TF/FVIIa complex, as described in the haemostasis chapter of this thesis. The active enzyme is a two-chain serine protease: the chains are linked to each other by a disulphide bridge. The light chain is 145 amino acids long and contains three domains: a γ-carboxyglutamic domain (Gla) and two epidermal growth factor-like domains (EGF1 and EGF2). The heavy chain consists of 235 amino acids and contains the catalytic domain, the serine protease.[7,8] The Gla domain binds calcium ions, as a result of which a conformational change is induced, and the domain becomes positively charged, potentiating an interaction with negatively charged phospholipid membranes, the anchoring of which increases the catalytic activity of the enzyme. The serine protease performs the catalytic activity and the EGF domains form, together with the serine protease, the interface to FVIII and mediate binding to platelets and TF.[7,9,10]

The main role of FIXa in the coagulation process is that it activates FX to FXa by hydrolysis with FVIIIa as a cofactor and in the presence of calcium ions and membrane phospholipids. FIXa is inhibited by antithrombin.[8]

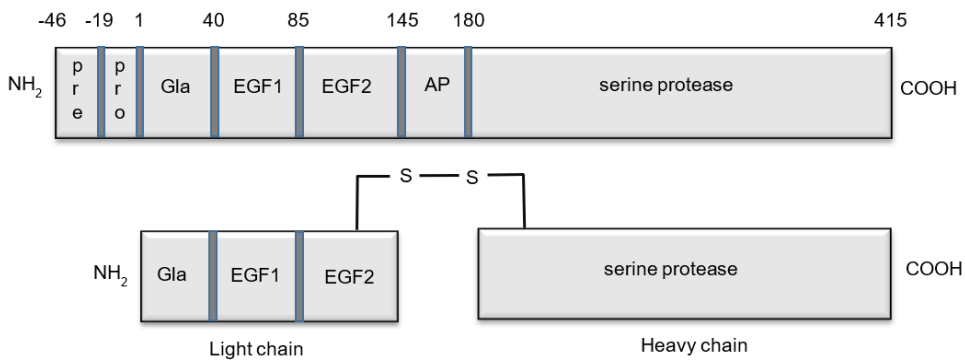


Figure 4. The domain structure of FIX and FIXa.
The zymogen is shown at the top and the active enzyme at the bottom. AP, activation peptide. Gla, γ-carboxyglutamic. EGF, epidermal growth factor.

Haemophilia B

Historical perspectives

The oldest found description of a bleeding disorder, which may have been haemophilia, is from as early as the 2nd century A.D. describing a female carrier and her two sons who died of bleeding after being circumcised.[11] Despite the rarity of the disease, many people know of it since Queen Victoria of the UK, who reigned from 1837-1901, was a carrier of the disease and passed the condition through her daughters to royal houses across the continent, hence the condition was popularly known as 'the royal disease'. It was, however, not until 2009 that it was confirmed that the royal disease in fact was haemophilia B. This was done by Rogaeve et al.[12] who performed genotype analyses on the remains of the family of tsar Nicholas II of Russia, including analyses on Crown Prince Alexei, who suffered from severe bleedings beginning at infancy, and his mother Empress Alexandra, granddaughter of Queen Victoria.

FIX was first described in 1952 when Biggs *et al.* [13] found this factor to be lacking in a young boy named Stephen Christmas, who was suffering from bleeding symptoms. Hence, FIX was first called Christmas factor and accordingly HB was once known as Christmas disease.

Haemophilia without treatment results in severe morbidity and early mortality with many children dying before the age of 15 years of trivial injuries or small operations, such as circumcision or tooth extractions.[14] The first blood transfusion for the treatment of haemophilia was reported in *The Lancet* in 1840.[15] The development of the process of fractionation of human plasma during World War II led in its extension to the treatment of the first HB patient with human FIX concentrate in 1961.[16] During the 1960s cryoprecipitate was developed and used for treating HA and in the 1970s human freeze-dried factor concentrates were developed and these latter products made it possible for the patients to treat themselves intravenously at home. This resulted in a dramatic improvement in the treatment of persons with haemophilia (PwH) since they now could treat themselves as soon as bleeds occurred and later use the products prophylactically, to decrease the risk of bleeding. However, no viral inactivation was present for the products at the time and transmission of both hepatitis C virus (HCV) and human immunodeficiency virus (HIV) to the recipient became a feared complication to the treatment. The epidemic of HCV in haemophilia patients occurred during the years of 1961-1985 and for HIV during 1978-1985 and resulted in great morbidity and mortality. In 1985, plasma-derived products using viral inactivation processes were introduced, as a result of which the viral epidemics ended.[7] After the discovery and publication of the molecular structure of FIX, the manufacture of recombinant FIX concentrates was enabled and, in 1997, the first commercial product was launched.

Part of my thesis work has been to go through the medical records of the HB patients enrolled in the various studies. In some cases, in the older patients, the records go back a long way and the history of haemophilia can be followed in the individual patients.

Epidemiology

Correct numbers on the prevalence of HB are hard to find and depend on access to treatment and life expectancy data. Today, haemophilia is thought to affect ethnic groups equally and the World Federation of Haemophilia (WFH) estimates that over 75% of people worldwide with haemophilia have not yet been identified and diagnosed correctly. The incidence of HB at birth is, according to the WFH, estimated to be 5.0 per 100,000 males born for all severities of HB, and 1.5 per 100,000 males born for severe HB. The distribution of disease severity in HB is somewhat uncertain and differs between reports; however, frequencies of 24-32% for severe disease, 21-23% for moderate and 50-53% for mild disease have been reported.[17]

The mortality rate is unfortunately higher for people with haemophilia than for the general population and the WFH estimates the prevalence of people living with haemophilia to be 3.8 per 100,000 males for all severities of HB and 1.1 per 100,000 for severe HB. With a current world male population of 3.9 billion, the expected number of persons living with HB is 148,200 for all severities, and 42,900 with severe HB. In the annual global survey from WFH in 2020, only 37,076 persons with HB were reported, of whom 209 live in Sweden.[18]

Access to treatment and treatment intensity determines life expectancy of persons with haemophilia. Before the introduction of replacement therapy, life expectancy of a person with haemophilia was less than 20 years.[17] A recent report from the Netherlands – a country where persons with haemophilia normally receive intensive prophylactic treatment – stated that the overall life expectancy of a person with haemophilia was reduced by 6 years compared to the median life expectancy of the general male population. Intracranial bleeding and malignancies were reported as the most common causes of death.[19]

Genetics and inheritance

HB is a recessive inherited disorder with *F9* located on the X chromosome. Males are affected predominantly since they only carry one X chromosome and are hemizygous for *F9*. Women are usually heterozygous carriers, in which case they may have reduced FIX levels and mild bleeding symptoms. Rarely, a more severe phenotype can occur in females as a result of skewed chromosome X-inactivation, co-inheritance from an affected father and a carrier mother, or when the normal X

chromosome is missing or partially missing, as in Turner syndrome.[17] Because males transmit their Y chromosome to their sons and the X chromosome, carrying the disease-causing variant, to their daughters, affected males do not transmit the disease to their sons but to all of their daughters, who are obligate carriers (see Figure 5). A female carrier has a 50% risk of passing the disease to her children: 50% of her sons will have haemophilia and 50% of her daughters will be carriers. A *F9* variant is the same throughout the family.

Sporadic cases are common in which a non-carrier female without a family history of haemophilia gives birth to an affected male. The prevalence of sporadic cases is dependent on the disease severity and the population tested. In 2007, Kasper *et al.* reported that 43% of severe HB and 30% of mild and moderate HB cases were sporadic.[20]

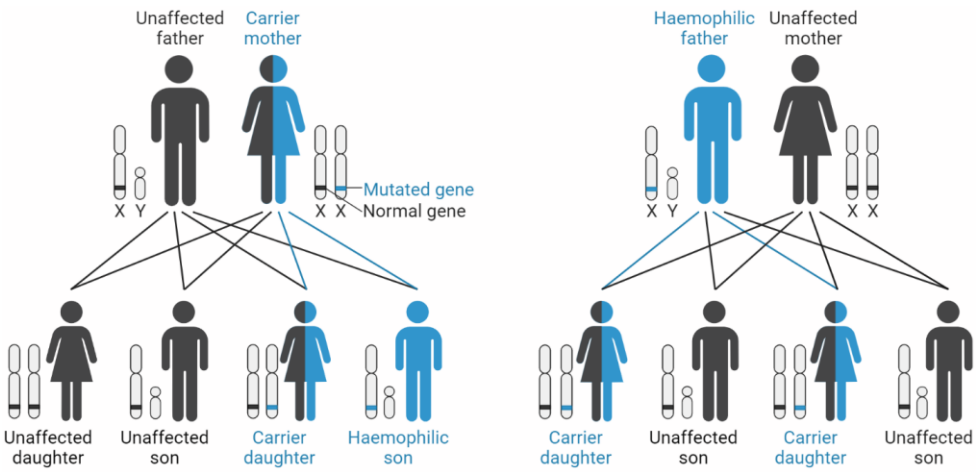


Figure 5. The X-linked recessive inheritance pattern of haemophilia B.
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The *F9* is 34kb, contains eight exons and seven introns and is located on the long arm of the X chromosome. Variants in all regions of the gene have been described, with the majority being point variants. Variant types described in the *F9* include:[21-23]

- Substitutions (point variants) – one nucleotide in the DNA is replaced with another;
- Deletions – loss of a large or small part of the gene;
- Insertion – one or more nucleotides are added to the gene;
- Duplications – one or more nucleotides are copied and repeated in the gene;

- Insertions and deletions (indel) – a deletion and insertion happen at the same time at the same location in the gene but with a more complex change than in a substitution;
- Complex changes.

The variants can be described further by the effect that they have on the formed protein. In this way, substitutions can be further classified as missense variants (the nucleotide change results in the exchanging of one amino acid for another in the protein), nonsense variants (the nucleotide change results in the creation of a premature stop signal in the translation to protein) and splice variants (the genetic alteration occurs at the splice site, i.e. the boundary of an exon and an intron). Also, deletions can be splice variants. Frameshift variants are a loss or addition of nucleotides with the result of a shift to another reading frame and a change of the code for all downstream amino acids. Deletions, duplications, and insertions can all be frameshift variants. In-frame variants do not disrupt the translational reading frame.

A rare form of HB is caused by a variant, not in the coding region, but in the F9 promoter and is referred to HB Leyden. Persons having the Leyden phenotype normally have increasing FIX levels after puberty and often develop a milder phenotype with increasing age.[24]

Some variants affecting the F9 can result in a reduction in FIX activity, but not in antigen, and are then referred to as cross-reacting material-positive (CRM+) or type II. In the same way, a variant can be classified as CRM-reduced or CRM-negative or type I, with parallel antigen and activity reduction.[23] Variants preventing the synthesis of FIX antigen are referred to as null variants. In Paper III, we recorded variants as null variants if they were nonsense variants, frameshift outside poly-A runs, large structure deletions or splice-site variants involving conserved nucleotides.[25] This classification is, however, not as established in HB as in HA.

Symptoms

Bleedings

Various bleeding episodes are the most common symptoms of haemophilia, and the clinical characteristics generally correlate with the residual factor activity. People with the severe form of the disease suffer from more frequent, more severe, as well as seemingly spontaneous bleeding episodes, and even a small laceration can be life-threatening. Persons with mild haemophilia have a milder phenotype but are at risk of major bleedings after trauma or surgery.

Common symptoms that lead to the diagnosis of HB include soft tissue bleedings, bleedings after blood sampling, injections or surgery, and bleedings of the oral

cavity. Rarer causes are cephalic or subgaleal haematomas, as well as intracranial haemorrhages. Bleedings into muscles and joints are characteristic of the disease, but do not as often form the diagnostic bleeding. Mucocutaneous bleedings, such as bruising and epistaxis, are reported commonly in persons with haemophilia, but are also common in healthy individuals and only a minority of children referred for investigation because of mucocutaneous bleedings are diagnosed with an underlying bleeding disorder.[7,26] The most characteristic symptom of haemophilia is joint bleedings, especially into elbows, knees, and ankles. Other bleedings seen include bleedings from the renal- and gastrointestinal tract and bleedings in the abdomen and vital organs. Complications to bleedings into soft tissues and muscles are not only pain and the loss of blood but can also result in neural defects caused by pressure from the haematoma, as well as pseudotumours. Pseudotumours are rare expanding destructive encapsulated blood-filled cysts.[7]

Generally, the factor activity level correlates well with the bleeding phenotype. However, it is known that the frequency and severity of bleedings can differ in persons with the same factor activity level.[27]

Haemophilic arthropathy

Joint bleeding usually begins when the child with haemophilia starts walking. Recurrent joint bleedings eventually cause arthropathy which can be both painful and disabling. Bleedings into the large synovial joints, especially the ankles, knees and elbows, are most commonly seen. A combination of exposure to great mechanical stress, rich vascularisation of synovial tissue and the haemostatic balance with a low expression of TF in joints, are suggested explanations for the predilection for these locations. The ankles and knees are weight-bearing joints and are affected more commonly than the elbows, which are often secondarily affected as a result of increased use when arthropathy has developed in the lower limbs.[17] A target joint is often defined as a joint that is subject to three or more bleeding episodes in a consecutive 3- or 6-month period.[28,29]

The pathophysiology of haemophilic arthropathy is multifactorial and both inflammatory and degenerative mechanisms contribute (see Figure 6). Synovitis, cartilage destruction, bone destruction and osteopenia result from the intra-articular bleedings, leading to the development of arthropathy. Iron and inflammatory cytokines, especially interleukins IL-1β, IL-1α, IL-6 and tumour necrosis factor (TNF), have been suggested to be central to the development of arthropathy.[17,30-32]

- *Synovitis* in haemophilic arthropathy is related to an increased iron load in the joint, which causes an inflammatory process characterised by hypertrophy and neo-angiogenesis. Haemosiderin deposition into the joint is thought to play a role in the induction of synovial proliferation. After a first bleed, the formation of brittle new blood vessels increases the risk of recurrent bleeding.

- *Cartilage damage* results from a direct effect of the presence of intra-articular blood and secondary effects of synovial changes with the production of pro-inflammatory cytokines. Inflammation and direct contact between blood and cartilage have been reported to lead to the development of hydroxy radicals, resulting in chondrocyte apoptosis and inhibition of cartilage matrix synthesis leading to irreversible cartilage damage. Results from *in vitro* models suggest that one single bleed may be sufficient to induce damaging effects on joint cartilage.[33,34]
- *Bone damage* seen in persons with haemophilic arthropathy is often the result of local joint bone destruction (characterised clinically by cyst formation, subchondral sclerosis, osteophyte formation and epiphyseal enlargement) as well as systemic osteoporosis. The pathophysiology underlying bone damage has not been elucidated fully, but the changes seen can be secondary to cartilage degeneration and associated with low physical activity level, vitamin D deficiency and the presence of other risk factors, such as HIV and HCV infection.[17,30-32]

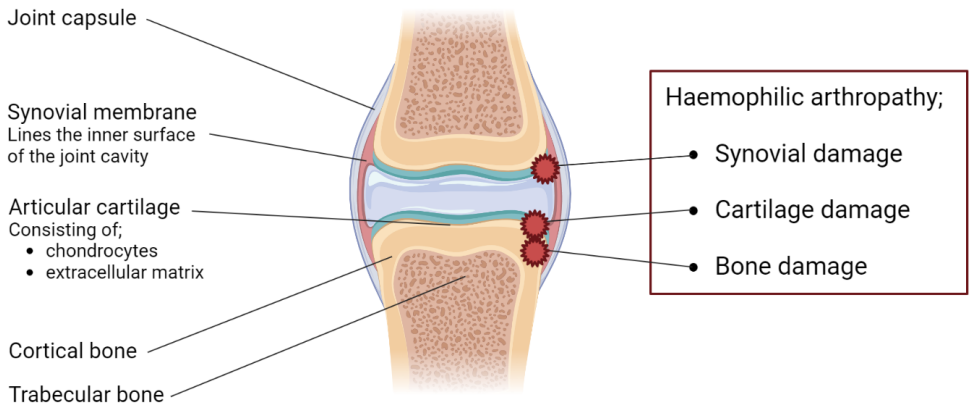

Figure 6. Overview of structures damaged in haemophilic arthropathy.
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Diagnostics and instruments for follow up

Laboratory methods

Laboratory testing is crucial for the diagnosis and follow up of PwH. The activated partial thromboplastin time (aPTT) is prolonged in persons with severe and moderate haemophilia but, in those with mild haemophilia, the aPTT may either be prolonged or normal. Additional screening tests of haemostasis including prothrombin time and platelet count are normal. Specific analysis for FIX activity

level, as well as genetic testing, takes the diagnostics further. Analyses for FIX activity and FIX inhibitor quantification are also required in the follow up of the haemophilia patient.

FIX activity analyses

The diagnosis and classification of disease severity of HB are based on the measured FIX activity level. Different assays based on different analytical principles to measure the FIX activity are in use. The most commonly used assays include the one-stage assay, based on the aPTT, and the chromogenic assay, a two-stage assay based on the development of colour.[35] The FIX one-stage assay is available in most HTCs worldwide, and has been the most used assay for several years. The chromogenic assay for FIX activity is newer, has not been available for as long as the one-stage assay, and is therefore used to a lesser extent.

Regardless of which method is used, the FIX activity can be expressed as a percentage or as a result given in kilo international units per litre (kIU/L) or international units per decilitre (IU/dL) or millilitre (IU/mL). Detailed descriptions of the one-stage and chromogenic assays are presented in the Methods chapter of this thesis.

Severity classification of HB is based on the FIX activity level:[36]

Severe	<0.01	IU/mL
Moderate	0.01 - 0.05	IU/mL
Mild	>0.05 <0.4	IU/mL

FIX inhibitor quantification

Inhibitors to FIX are measured by the Bethesda assay or the Nijmegen-modified Bethesda assay and the result is given in Bethesda units (BU). A detailed description of this assay is given in the Methods chapter of this thesis.

One Bethesda unit is defined as the amount of inhibitor in 1 millilitre of patient plasma that would destroy 50% of the factor activity of an equal amount of normal plasma in 2 hours at 37°C.[37] The WFH defines a positive FIX inhibitor as having a Bethesda titre of ≥0.3 BU; however, the detection limit may be slightly higher and vary between different laboratories. A low-responding inhibitor is defined as an inhibitor <5.0 BU at different time points, and a high-responding inhibitor is consequently ≥5.0 BU. A transient inhibitor is an inhibitor that falls below the defined threshold within 6 months without any change in treatment and despite the use of FIX replacement products.[38]

F9 variant detection

Since for a long time haemophilia has been recognised as an inherited disorder, family history and pedigrees have been a part of the diagnostic work-up for many years. Today, several methods for direct genetic testing are available to search for

variants in *F9*. Depending on the resources available, a full *F9* screening with amplification of the *F9* by polymerase chain reaction (PCR) followed by Sanger sequencing, or next-generation sequencing (NGS) can be used. If resources are limited, a screening approach prior to Sanger sequencing can be chosen. In case of no amplification in an exon during PCR or when a disease-causing variant cannot be found, a large deletion, duplication or insertion can be suspected, and multiplex ligation-dependent probe amplification (MLPA), quantitative real-time PCR or gap-PCR can be used.[7,38] The approach to variant analysis for the studies included in this thesis is described in the 'Methods' section of this thesis.

Clinical assessment instruments and imaging

For a more objective quantification of a person's bleeding symptoms, a validated bleeding assessment tool (BAT) such as the International Society on Thrombosis and Haemostasis (ISTH) BAT can be used.[39] The use of a "log-book" for documentation of bleedings and treatment in the everyday lives of the persons with haemophilia B (PwHB) can further ease the clinical evaluations.

In the evaluation and monitoring of joint status, a physical examination is performed on routine follow-up visits to the clinic and the use of a scoring method to document and evaluate changes objectively is recommended. The HJHS[40-42] is used widely for this purpose and was employed in the evaluation of joint health in Papers II and IV in this thesis. A detailed description of HJHS 2.1 is given in the 'Methods' section of this thesis and a copy of the summary score is attached as Appendix 1.

Different instruments for joint imaging in the evaluation of complications of haemophilia are being used. Radiography is the most common imaging technique of joint structure, and the Pettersson score,[43] a scoring system based on the degree of joint damage assessed by radiological changes in the knees, elbows and ankles, has been used widely. However, X-ray imaging is not sensitive to early arthropathic changes in PwH and, therefore, a need for more sensitive instruments for the evaluation of joint health has driven the development of new ways to assess haemophilic arthropathy. Magnetic resonance imaging (MRI) is a sensitive imaging instrument in the evaluation of joint structure and arthropathy. However, MRI is expensive, time consuming and can be difficult to perform on children. Lately, ultrasound has been gaining popularity in the evaluation of early signs of joint damage in the follow up of joint health in PwH. Ultrasound scoring systems are available to assess haemophilic arthropathy and, in Paper II in this thesis, the Haemophilia Early Arthropathy Detection Ultrasound (HEAD-US) scoring system[44] was used in the evaluation of joint health. A detailed description of it is given in the 'Methods' section of this thesis and a reprint of the scoring method is attached as Appendix 2. The limitations to ultrasound imaging are that it is operator dependent and only gives a partial visualisation of the joints. However, the advantages are a much lower cost and easier access than MRI, as well as it being easier to perform on children.[7,38]

Treatment

The haemophilia team

The optimal treatment for PwH consists of a multidisciplinary team located at an HTC (see Figure 7). This team might differ between different centres depending on the organisation, but the core team often consists of:

- *A physician* with training in managing haemophilia (often a paediatric or adult haematologist), who monitors the patient's health and prescribes treatment;
- *A haemophilia nurse*, who coordinates the haemophilia care and educates the patient and their family;
- *A physiotherapist* with knowledge of haemophilia, who assesses joint health, educates the patient on preventive measures and counsels on recovery after bleeds;
- *A coagulation laboratory and laboratory specialist* for the performance of diagnostics and monitoring of treatment;
- *An orthopaedic surgeon* for the assessment of musculoskeletal complications and to carry out orthopaedic surgery.

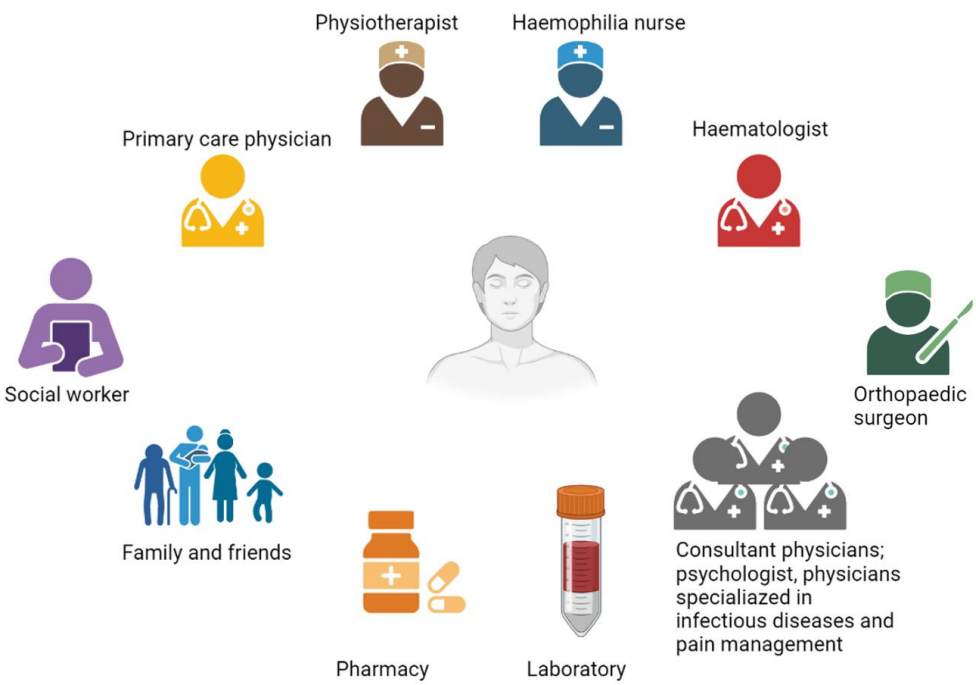

Figure 7. The haemophilia team.
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Except for these core functions, the care surrounding a PwH often includes the primary care physician and a social worker for counselling on community resources, consultant physicians for the handling of comorbidities (for example chronic pain and infectious diseases) and the pharmacy for the provision and distribution of pharmaceutical products. Finally, and very important, is the critical function of the person's family, not only in a psychosocial aspect, but also as caregivers since a family member is often taught to administer the patient's factor replacement therapy intravenously before the patient is old enough to learn how to do this for himself.

Pharmacotherapy

Coagulation FIX concentrate

Replacement therapy with intravenously administered FIX concentrates is still the treatment of choice for both treating and preventing bleedings in PwHB. In the section 'Historical perspectives' in this thesis, a short overview is given on the development of these products. Today, two main types of factor concentrates are used:

- Plasma-derived (PD) FIX concentrates – products made from plasma donated by human blood donors;
- Recombinant FIX concentrates – products manufactured in a laboratory using recombinant technology, and not made from human blood.

  In recent years, new FIX concentrates with longer half-lives than the traditional products have been developed and today recombinant replacement products are further divided into:

  - Standard half-life (SHL) products – with a half-life of approximately 18-24 hours;
  - Extended half-life (EHL) products – with a half-life at 3- to 5-fold that of SHL products. Strategies to extend the half-life of factor products include PEGylation and fusion technologies (Fc-fusion, albumin-fusion).[38]

The FIX concentrates raise the plasma FIX level by approximately 0.01 IU/mL per each infused IU FIX product per kilogram body weight. Recombinant SHL FIX products have a somewhat lower recovery than PD FIX ones; however, this effect is not observed with EHL products.[38,45,46]

Replacement therapy can be used episodically, i.e. 'on-demand', to stop a current bleeding but, to prevent bleedings from occurring, in 1958 Nilsson et al. introduced prophylactic treatment with factor concentrate for PwHA.[47] Later, in the 1960s and 70s prophylactic treatment with FIX concentrates was also introduced for PwHB[47,48] and this approach is still today considered the gold standard of care in HB.[38]

Different approaches on when to start the prophylaxis exist. Definitions by the WFH regarding the start of prophylaxis are:

- Primary prophylaxis – prophylaxis begun in the absence of joint disease and before the second joint bleeding and 3 years of age;
- Secondary prophylaxis – prophylaxis initiated after two or more joint bleedings, but before the onset of joint disease;
- Tertiary prophylaxis – prophylaxis initiated after the onset of joint disease.

WFH also defines prophylaxis by its intensity and is divided into high dose (>4000 IU/kg per year), intermediate dose (2000-4000 IU/kg per year) and low-dose prophylaxis (1000-2000 IU/kg per year).[38]

Access to FIX concentrates is unfortunately limited in many parts of the world. In such cases, less effective options, such as fresh frozen plasma and cryoprecipitate, are used in the treatment of haemophilia.

Bypassing agents

In patients with inhibitors, the function of FIX concentrates is typically neutralised and the treatment is rendered ineffective. To treat and prevent bleedings in such patients, so-called bypassing agents can be used. Bypassing agents bypass the FIX step in the coagulation process to achieve haemostasis. Today, two different bypassing agents are used in the treatment of haemophilia:[38,49]

- Recombinant activated factor VIIa (rFVIIa) – which binds to TF and activates FX (and FIX);
- Activated prothrombin complex concentrate (aPCC) – which contains mainly non-activated prothrombin, FIX, FX and mainly activated FVII. However, as aPCC contains FIX, use of this product can lead to an increased inhibitor level in HB and cannot be used in patients with HB and a history of allergic reactions to FIX concentrates. As a consequence, aPCC is mainly used in PwHA and inhibitors.

Tranexamic acid

Tranexamic acid works as an antifibrinolytic agent and promotes clot stability by binding reversibly to plasminogen, thereby blocking the binding of plasminogen to fibrin. By so doing, it competitively inhibits the activation of plasminogen to plasmin.[50,51] The drug can be used locally or systemically (oral or intravenously) and is particularly useful in the treatment of superficial soft tissue and mucosal bleedings where it can be used alone or as additional therapy to factor concentrates. The use of tranexamic acid alone is, however, not sufficient in the treatment or prevention of joint bleedings in patients with severe haemophilia.

Orthopaedic treatment

In the management of arthropathy, several conservative treatments including prophylactic treatment with factor products, physiotherapy, treatment with a short course of oral glucocorticoids or anti-inflammatory agents, and joint injection with a glucocorticoid, are used. Sometimes, however, such an approach is not sufficient and orthopaedic treatment, such as synovectomy, arthrodesis or arthroplasty, is carried out to reduce pain, frequency of bleedings and impaired mobility.[7]

Synovectomy. Chronic hypertrophic synovitis and recurrent bleedings are the main indications for synovectomy. Surgical synovectomy can be performed by open or arthroscopical surgery. Medical synovectomy (synoviorthesis) is performed by injecting a material into the joint with the aim of stabilising the synovium. The two main types of medical synovectomy are chemical synovectomy, in which rifampicin and oxytetracycline clorhydrate are used commonly, and radiosynovectomy, where a radioisotope is injected into the joint.

Arthroplasty. Joint replacement can be performed in persons with chronic arthropathy, and the main objective is usually to reduce the level of pain, but often there is an accompanying increase in function and mobility. The surgery requires intensive factor replacement to prevent bleeding during and after the surgery, and post-surgery rehabilitation.

Arthrodesis. Sometimes arthrodesis, can be the preferred surgical technique in PwH suffering from chronic arthropathy and severe pain. Arthrodesis of the ankle is often the favoured treatment over ankle arthroplasty in patients with painful end-stage ankle arthropathy.

Complications of treatment

FIX inhibitors

The development of neutralising antibodies (inhibitors) against FIX is a very serious complication in HB, since it can cause the given replacement treatment to be ineffective. The occurrence of inhibitors in HB is rare, and an incidence of up to 5% is often reported.[7,21,52,53] In HB, inhibitors occur almost exclusively in persons with the severe form of the disease and only very rarely in those with mild or moderate HB.[52] In the majority of cases, the inhibitors appear early in life and before 20 exposures of FIX treatment.[38,54,55] Possible risk factors for the development of inhibitors are discussed frequently, but are generally less well understood in HB compared to HA. Genetic factors are thought to influence inhibitor development and *F9* null variants have been shown to be associated with inhibitors to a greater extent than non-null variants.[38,54,56-58]

The neutralising antibodies appear to be polyclonal in nature, and are predominantly composed of immunoglobulin G4 (IgG4); however, immunoglobulin G1 (IgG1)

subclass antibodies have been reported to appear transiently in association with allergic reactions. The antibodies show specificity against the serine protease and the Gla domains of FIX. They inhibit FIX binding to FVIII, as well as the binding of FIX to phospholipids, and thus prevent the activation of FX.[7]

Inhibitor levels are measured using Bethesda units and the peak inhibitor titre classifies inhibitors as 'low-responding' (low titre), with a peak titre of <5 BU, or 'high-responding' (high titre) with a peak titre of ≥5 BU. Some 'low responding' inhibitors are transient and disappear spontaneously.

The management of PwHB and FIX inhibitors includes strategies for bleeding control, as well as treatment attempting to eradicate the inhibitor, referred to as immune tolerance induction (ITI). The experience with ITI treatments in PwHB is limited, and there is no established consensus on how such patients should be managed. The basis of ITI therapy is frequent doses of FIX concentrates, but dosing and frequencies vary and different regimens with or without the addition of immunosuppressive agents have been attempted.[54,59-63] The success rate for ITI attempts in HB is often reported to be only 30-40%.[7,38,55] Since FIX replacement products have insufficient efficacy in patients with HB and inhibitors, bypassing agents, described above, are used to treat and prevent bleedings in these patients. However, these products are not as efficient and are less predictable in outcome with an inter-individual response variability, in comparison to FIX concentrates.

Allergic reactions and nephrotic syndrome

The development of inhibitors in PwHB can further be complicated by allergic reactions to the FIX replacement products, as well as the development of nephrotic syndrome. Anaphylaxis is reported to occur in approximately 50-60% of PwHB with inhibitors and more often in persons carrying *F9* null variants.[7,54] Allergic reactions can sometimes precede the occurrence of inhibitors and can be the first sign of antibody development. The aetiology of the allergic reactions in association with inhibitor development is not clear. However, there is some support for the theory that the extravascular distribution of the small FIX protein leads to mast cell activation and IgE-mediated hypersensitivity response.[54,64] Complement activation by transient IgG1 antibody formation has been suggested as an immune trigger,[65] as well as the theory that the immune response is triggered by immune complex formation as a result of the large amounts of infused exogenous FIX. Higher factor concentrations are seen in PwHB than in those with HA because of the higher normal concentrations in plasma and the much higher standard dosing of FIX replacement products in PwHB in comparison to FVIII products in PwHA.[66] Patients with complete gene deletions are reported to have the greatest risk of anaphylactic reactions, and it has further been suggested that deletion of neighbouring immune response modifier genes in these patients might contribute to the development of anaphylactic reactions.[67] Attempts of desensitisation therapy with small doses of intravenous or subcutaneous FIX has been tried to allow FIX

treatment to continue.[68,69] In PwHB with inhibitors and allergic reactions to FIX products, bleedings are preferably treated with the bypassing agent rFVIIa and not aPCC, since aPCC contains FIX and can cause or worsen an allergic reaction.[38]

PwHB treated with ITI are at risk of developing nephrotic syndrome, which most commonly occurs approximately 7-9 months into the ITI therapy attempt. Nephrotic syndrome is recognised by oedema, proteinuria and low serum albumin and occurs more commonly in inhibitor patients with allergic reactions. The aetiology of nephrotic syndrome in inhibitor patients still remains unclear. In a couple of patients, renal biopsy has revealed an appearance consistent with that of membranous glomerulonephritis.[7,70] The main treatment strategy in the event of nephrotic syndrome is to discontinue the FIX treatment, after which an improvement in oedema and proteinuria may be seen.

Non-neutralising antibodies to FIX

Not all antibodies directed towards FIX are believed to have a neutralising effect on FIX, and hence they escape detection by the Bethesda assay. Instead, these antibodies are traditionally detected using immunoassays such as the ELISA technique. In HA, the presence of non-neutralizing (non-inhibitory) antibodies (NNAs) and their possible role has been discussed and studied over the years. A prevalence of NNAs towards FVIII in PwHA was reported as 25% in a recent meta-analysis by Abdi *et al*.[71] The consequences of the presence of these NNAs and their clinical significance are still being discussed and are not yet understood fully. It has been suggested that NNAs in PwHA can form immune complexes with FVIII and thereby enhance the clearance of the administered FVIII concentrates from plasma.[72-74] However, contradictory results, showing no correlation between recovery and the presence of NNAs have also been presented.[75] It has also been suggested that NNAs in PwHA may predict the development of an inhibitor.[76] Studies on NNAs in PwHB are few, and data are sparse. Boylan *et al.* evaluated the association between anti-FIX antibody profiles and the development of inhibitors using a fluorescence-based immunoassay (FLI). They presented results of one or more classes of anti-FIX antibodies in 40% of plasma samples which tested negatively using the Nijmegen-Bethesda assay.[77] However, much is still unknown regarding NNAs and their clinical significance.

Transfusion-transmitted diseases

The development of cryoprecipitate and FIX concentrates in the 1960s and 1970s revolutionised the treatment of haemophilia. However, at this time, the products available were not virally inactivated and the transmission of both hepatitis C virus (HCV) and human immunodeficiency virus (HIV) via the products to the receiving patient became a tragic reality.

An epidemic of HCV in PwH started in 1961 and did not end until viral inactivation processes for the PD FIX concentrates were introduced in 1985. It was known

previously that the transfusion of blood or plasma could transfer hepatitis infection and the large pooling of plasma to derive the factor concentrates constituted an increased risk of disease transmission. It was however hard to characterize this 'non-A non-B hepatitis' in patients since a test for HCV first became available in 1991. HCV can result in death from liver disease; however, today interferon-based therapies for HCV are available and have helped many PwH who contracted HCV.[7]

During the years 1978-1985, an epidemic of HIV in haemophilia patients occurred and was caused largely by a commercial concentrate from the USA.[7] Darby *et al.* reported that out of the 6278 males with haemophilia in the UK during 1977-1991, 20% (1227) were infected with HIV as a result of replacement products and this resulted in a significant increase in mortality.[78] Most patients with HIV were coinfected with HCV, and approximately one-third of those with HCV were also infected with HIV. In the early 1990s, antiretroviral therapies for the treatment of HIV became available and the mortality rate was thereafter reduced.

After the introduction of viral inactivation processes in the development of replacement products, no HIV or HCV transmissions have occurred as a result of factor treatment. However, the possibility of transmission of variant Creutzfeldt-Jakob disease has been discussed as cases of transmission by blood transfusion have been reported.[7]

Quality of life

The risk of bleedings for PwHB, both traumatic bleeds as well as spontaneous ones, causes uncertainty and psychological strain and the bleedings as well as the development of arthropathy can lead to pain and impairment of mobility. Even though the current treatment for haemophilia has improved greatly over the years, to the extent that today it is believed that PwHB can have a normal, or close to normal, life expectancy, therapy with FIX concentrates still needs to be administered intravenously, which impacts upon the patient's daily life and can potentially affect the quality of life of PwHB.

Health-related quality of life (HRQoL) can be referred to as how well a person functions in his/her life and his/her perceived well-being in physical, mental, and social domains of health.[79] High frequencies of pain and functional impairment, as well as an increased prevalence of anxiety and depression in PwH with significant impairment of HRQoL, have been reported.[80-85] Even though a good HRQoL is an important goal in the management of PwHB, this is an area with limited data and in need of further evaluation to identify areas of insufficient care and to improve the understanding and responsiveness to signals of ill health in these patients.

Future aspects and novel therapies

The great improvements in the treatment of haemophilia that have taken place during the past 60 years have reduced the morbidity and mortality dramatically for PwHB. As a result, the life expectancy for PwH now approaches that of the healthy male population. With current research, it is hoped that further improvements and more individualised treatment possibilities will manifest. Software programs to help personalise factor dosing based on pharmacokinetic analyses have been developed for use in the clinical setting at the HTCs and may, in the future, come to replace the weight-based dosing regimens that are usually used today.

Novel therapies

New treatment possibilities include EHL FIX products, which are already on the market, as well as novel non-factor replacement products and emerging gene therapy.

- *Extended half-life FIX products* have already been discussed in the section on 'Pharmacotherapy' in this thesis. They include factor products manufactured using either PEGylation or fusion technologies to extend their half-life, enabling longer intervals between injections and/or the maintenance of a higher trough FIX level with the same frequency of factor administration.
- *Non-factor replacement products* are products that induce haemostasis independently of the lacking coagulation factor and have the advantage of subcutaneous, instead of intravenous, administration. However, the occurrence of thrombotic complications is a theoretical possibility when balancing feedback mechanisms in the coagulation system are not in place, and cases of thromboses have been reported in early clinical trials. In HA, the first non-factor replacement product, emicizumab, is already on the market and consists of a bispecific antibody which mimics the role of the lacking FVIII by bridging FIXa and FX.[86,87] However, this drug is only for the treatment of HA and has no effect in PwHB. Products that have currently progressed furthest in the development of new treatment options of HB are the following:
    - Fitusiran, an investigational ribonucleic acid (RNA) interference therapy that targets antithrombin messenger RNA and suppresses the production of antithrombin in the liver. The reduced antithrombin levels are thought to improve thrombin generation and consequently improve haemostasis in PwH with or without inhibitors.[88] Phase 3 studies with the product are ongoing for the treatment of both HA and HB.

- Concizumab, a monoclonal antibody that binds to and inhibits tissue factor pathway inhibitor (TFPI). The drug is thought to be effective in both HA and HB regardless of inhibitor status. Phase 3 trials are ongoing.[89] Similar, Marstacimab, is an additional monoclonal antibody targeting TFPI which is currently being evaluated in an ongoing phase 3 study.[90]
- SerpinPC, an inhibitor of activated protein C (APC), which is being investigated for the treatment of PwHA and PwHB.[91] Phase 1 studies have been completed.
- VGA039, a monoclonal antibody inhibiting the cofactor activity of protein S, which is being developed as a universal haemostatic agent for various bleeding disorders. Approval has been obtained to launch a phase 1 clinical trial of the drug.[92]

- *Gene therapy* is an appealing future treatment for haemophilia, since the disease is caused by a single gene defect and a small expression of the deficient FIX protein is sufficient to bring about a significant reduction in bleeding episodes. In healthy individuals, FIX is produced in the liver and in the ongoing phase 3 HB gene therapy trials, the *F9* is introduced into hepatocytes in PwHB by adeno-associated viral vectors (AAV). The hepatocytes then start to produce the deficient FIX protein, and even though much is still to be explored, the potential benefit of gene therapy can be life changing for PwH. Concerns regarding gene therapy include pre-existing neutralising AAV antibodies, hepatotoxicity, variability of factor levels, uncertainties concerning the durability of response and the possibility of oncogenesis with the development of hepatocellular carcinoma.[93]

Despite the great progress that has already been made in the field, and the research opportunities in the pipeline, challenges remain in haemophilia care. The most important issue to address is how to enable reliable diagnostics globally and how to make treatment available for all PwH, regardless of which country they reside in.

Global aspects of haemophilia care

Haemophilia care is complex: diagnostics require specialist laboratory assays and knowledge, and today pharmacotherapy is costly and still not curative, as well as needs to be started early in life to prevent disabling complications and premature death. As discussed in the 'Epidemiology' section of this thesis, the WFH estimates that over 75% of PwH worldwide have not yet been diagnosed correctly. Great regional differences occur: only 8% of persons predicted to have haemophilia are identified in Africa, compared to 83% of expected PwH identified in Europe. Furthermore, only 34% of the world's population live in high- or upper middle-income countries (categories based on the World Bank Group rankings) but 91% of

the total amount of FVIII replacement products are being used in these countries.[94] In addition, prophylaxis frequency differs between regions and is as low as zero in some countries, in comparison to 100% in other countries.[94] In conclusion, haemophilia care today is not equal on a global basis and in parallel to research striving to improve treatment possibilities, much work needs to be done in order to provide knowledge and comprehensive care to PwH living in countries with fewer opportunities. Workshops and twinning programmes can contribute to this work, and novel therapies not requiring intravenous administration, or the development of a single treatment leading to sustainable expression of factor levels – as is the goal for gene therapy – might turn out to be suitable for countries with limited healthcare infrastructure, if the financing can be resolved.

Haemophilia B – what is the difference to haemophilia A?

Both HB and HA are rare, recessively inherited, X-linked bleeding disorders. The diseases have historically been considered as being identical; however, important differences between them do exist.

HB is caused by a deficiency in coagulation factor IX (FIX) and HA by a deficiency in coagulation factor VIII (FVIII). The protein FIX is an enzyme with a molecular weight of 55 kDa whereas FVIII works as a co-factor and is much larger than FIX, at 280 kDa. HA is more common than HB with a prevalence at birth of 25 in 100,000 males, in comparison to 5 in 100,000 males for HB.[18] Approximately 30-45% of PwHB have the severe form of the disease, whereas for PwHA the figure is 60%.

Both *F9* and the coagulation factor VIII gene (*F8*) are located on the long arm of the X chromosome. *F9* is smaller, approximately 34 kilobases long, and structurally simpler with only eight exons, compared to the much larger and structurally more complex *F8*, which is approximately 180 kilobases long and contains 26 exons. The relative occurrence of the different variants causing severe HB or HA differs between the two diseases with missense variants reported to constitute more than half of the genetic abnormalities in persons with severe HB, compared to only 15% in those with HA. In contrast, severe gene defects, such as large deletions and nonsense mutations, account for 80% of cases of severe HA, with the intron 22 inversion causing approximately half of the severe cases. The different prevalence of null variants (i.e. variants leading to the prevention of protein synthesis) between the two diseases explains the different frequency of CRM+. Almost one-third of PwHB are classified as CRM+ compared to only approximately 5% of PwHA.[95,96]

The clinical symptoms of HB and HA are similar, comprising joint bleedings, muscle haematomas and soft tissue bleeds; however, it is an ongoing debate as to whether there is a difference between the diseases in clinical severity in persons with the same residual plasma factor level. The first report suggesting a difference in phenotype between the diseases came in 1959 and reported HB to be less severe and less disabling than HA.[97] Further reports have been published suggesting that there are fewer bleeding episodes in PwHB compared to those in PwHA,[98-100] lower use of prophylaxis,[101,102] lower rate of hospital admissions[103] and lower orthopaedic joint scores and arthropathy,[98] as well as a lower frequency of joint surgery[99,100,104] in PwHB compared to PwHA. The data are, however, limited and the findings inconsistent. Reports showing no difference in phenotype, bleeding frequency or treatment intensity between PwHA and PwHB have also been published.[105,106]

The incidence of inhibitors is lower in HB compared to HA. An overall incidence of inhibitors is often reported to be approximately 3-5% for PwHB compared to 25-30% for PwHA. It has been debated whether the higher prevalence of less severe variants in PwHB can form the basis of a milder disease and, together with a smaller FIX molecule with fewer antigenic epitopes, extravascular distribution of FIX and

FIX's structural similarity to other vitamin K-dependent factors, be part of the explanation for the lower prevalence of inhibitors in PwHB compared to those with HA. In patients with mild or moderate HA, inhibitors have been reported at an incidence of 5-10%; however, in PwHB, inhibitors are reported almost exclusively in the severe form and are very rarely seen in the milder forms.[7,38] This observation also contributes to the lower number of FIX inhibitors compared to FVIII inhibitors. The phenotype and management of inhibitors also differ between the two diseases, as inhibitors in PwHB may also be associated with allergic reactions to FIX replacement therapy. In contrast to PwHA, in whom anaphylactic reactions almost never occur, anaphylaxis in more than 50% of PwHB and inhibitors has been reported.[54] The aetiology of the allergic reactions is not understood fully and hence it is not clear why PwHB are affected almost exclusively. This is discussed further in the section on allergic reactions in the 'Introduction' section of this thesis. In HB, the occurrence of inhibitors can also be followed by the development of nephrotic syndrome, but this is not seen in PwHA.[54,95] Finally, ITI therapy success rates seem to differ between PwHB and those with HA. In PwHB and inhibitors, ITI success rates of only 30-35% are usually reported, compared to the much higher success rates of 60-80% in PwHA and inhibitors.[38,55,95] In PwHB, the low success rates of ITI and frequent anaphylactic reactions have led to attempts of adding immunosuppressive treatment to the ITI therapy.

The pharmacokinetic properties of replacement therapy with FVIII and FIX differ and, although based on the same principles, the dosage schedules and treatment regimens differ slightly between the management of HA and HB. The *in vivo* recovery is lower for FIX replacement products compared to that for FVIII products, with a recovery of 0.8-1.0 (IU/dL)/(IU/kg) for FIX and 1.5-2.0 (IU/dL)/(IU/kg) for FVIII.[96] In contrast, the half-life is longer for FIX products compared to that of FVIII products, with half-lives of around 18 hours for FIX SHL products compared to approximately 12 hours for FVIII SHL products.[96] FVIII circulates in plasma in a complex with von Willebrand factor, which stabilizes FVIII but it has also been shown to limit the half-life extension of EHL products.[107,108] FIX is on the contrary independent of VWF. FVIII is found exclusively inside the blood vessels and the rate of plasma clearance alone determines the half-life. FIX, however is thought to also distribute extravascularly and bind to the extracellular matrix, in particular to type IV collagen. The distribution volume of FIX concentrates is thought to be around four times greater than the patient plasma volume and the total amount of endogenous FIX in a patient has been suggested to be three times greater than the level that can be measured in plasma. The clinical impact of this observation, and whether it has an impact on the phenotype, has not been determined and is still being discussed, but might be an explanation for the extended levels of active FIX.[95,109-111] A further consideration to this phenomenon is that when analysing plasma samples for functional FIX activity, the entire FIX capacity might not be measured, but only the intravascular FIX. As a result, the plasma samples might, in fact, lead to a misclassification of disease severity and a possible explanation of a milder

bleeding phenotype in HB patients compared to those with HA with similar measured factor activity.

Table 1. Comparison of characteristics of haemophilia B and haemophilia A.

Haemophilia B		Haemophilia A
	General characteristics	
X-linked recessive	Inheritance	**X-linked recessive**
5/100,000	Incidence	**25/100,000**
Bleedings in joints, muscles, soft tissues	Clinical symptoms	**Bleedings in joints, muscles, soft tissues**
	Characteristics of the missing coagulation factor and replacement therapy	
FIX	Deficient coagulation factor	**FVIII**
Enzyme	Molecular function	**Co-factor**
55 kDa	Molecular weight	**280 kDa**
8	Gene exons	**26**
Intra- + extravascular	Distribution of coagulation factor	**Intravascular only**
0.8-1	Recovery of replacement products (U/dL)/(U/kg)	**1.5-2**
18 h	T½ SHL products	**12 h**
	Inhibitor characteristics	
3-5%	Inhibitor incidence	**25-30%**
30-35%	ITI therapy success	**60-80%**
>50%	Anaphylaxis in inhibitor patients	**Rare**
Can occur in inhibitor patients	Nephrotic syndrome	**Not reported**

Rationale for this thesis

Studies focusing on HB are limited, and many reports on the disease derive from studies in which PwHB constitute a minor part of a larger cohort that mainly comprises PwHA. This is a result of the rarity of the disease and the similarities to the more common HA. As a consequence, much of our knowledge on HB has been extrapolated and transferred from data on HA; however, even though the diseases are similar, important differences do exist between them and there is a need for studies focusing solely on PwHB. An overview of the most important differences between HA and HB as known today are presented in this thesis in the chapter '*Haemophilia B – what is the difference to haemophilia A?'*

Paper I. The rationale for Paper I and the Assay Discrepancy study is based on the fact that in approximately one-third of persons with non-severe HA, a discrepancy between the one-stage and the chromogenic assays in measuring FVIII activity level has been reported.[112-116] The diagnosis and severity grading of haemophilia are based on the measured factor activity level and a correct classification is of importance in order to not miss or underestimate a risk of bleeding, as well as to be able to design a well-balanced treatment for the patient. In HA, the one-stage method usually provides the higher result. However, an 'inverse discrepancy', where the one-stage method gives a lower value than the two-stage or chromogenic method, has also been described, and the bleeding phenotype has been shown to correspond more accurately to the two-stage or chromogenic assay. Furthermore, different genetic variants have been associated with discrepant results in the assays.[112,113,115,117-119] The chromogenic assay for FIX activity has not been available for as long as the one for FVIII and is therefore not used as widely. In the laboratory of the HTC at Skåne University Hospital in Malmö, the one-stage clotting assay and the chromogenic assay for FIX had, before the start of the Assay Discrepancy study, been used in parallel over a period of time and signs indicative of assay discrepancy had been observed. However, to the best of our knowledge, FIX assay discrepancy had not previously been evaluated systematically and this forms the rationale for the Assay Discrepancy study and Paper I, where we aimed to evaluate the two assays to establish whether the discrepancies reported between the methods for FVIII also apply to those for FIX.

Papers II-IV. During the last few years, great progress has been made in the development of novel therapies for haemophilia, and some have been shown to be especially promising in HB. EHL factor products have already been introduced and

are gaining ground rapidly. Studies on non-replacement products and gene therapy are showing promising results. The new therapies will hopefully bring new opportunities to further individualise and optimise treatment for our haemophilia patients. However, with these new possibilities, the requirements to understand the disease better and to clarify which patients may benefit from a specific treatment increase. This is the rationale for the B-NORD study, on which Papers II-IV are based. As a result of the rarity of HB, multicentre studies are required to enrol sufficient study participants in order to establish meaningful results. The Nordic countries have a close collaboration through the Nordic Haemophilia Council[120], a network that aims to standardise and improve haemophilia care in the Nordic countries through studies and the development of guidelines. This homogenous area was thought to form a good basis for an HB study. The bleeding phenotype and development of arthropathy have considerable impact upon the haemophilia patient's life, they influence the choice of treatment and are important in order to understand and optimise therapy. These factors formed the rationale for Paper II.

Inhibitors in HB are a particularly complicated area with very limited treatment data, including a lack of consistent criteria for ITI success and treatment guidelines. In Paper III, we endeavoured to compile and present data on inhibitor patients, including detailed descriptions on ITI treatment attempts, treatment outcome and complications, all in relation to the *F9* variant, to increase the knowledge on these rare, but complicated and difficult-to-treat situations. Our hope is that this work will contribute to an increased understanding of how these patients are best treated.

The B-NORD study also presented us with the opportunity to study our patients' quality of life. Ultimately, what is most important is our patients' well-being, and we need to learn what our patients consider to be of importance for a good, healthy, and fulfilling life, in order to understand how we can improve the care that we provide and recognise signs of ill health. This formed the rationale for Paper IV.

Overall aim of the thesis and specific aims of the papers

The overall aim of this thesis:

To characterise HB regarding its diagnostic challenges, treatment characteristics and clinical outcomes including bleedings, arthropathy and FIX antibodies, as well as the quality of life of persons who have it. A further general aim was to compare the phenotype and quality of life of persons with HB to those with HA, to see if differences between the two diseases exist.

Specific aims of the individual papers:

Paper I – Diagnostics

- To assess and compare the results of the one-stage clotting assay with those of the chromogenic assay in PwHB, to investigate whether discrepancies between the two methods are present.
- In case of discrepancies between the two methods, a further aim was to investigate conceivable explanations underlying these observed discrepancies in FIX activity.

Paper II – Treatment, bleedings and arthropathy

- To characterise persons with severe HB in the Nordic countries concerning treatment and occurence of bleedings and arthropathy, and to compare their joint health with matched PwHA.

Paper III – Neutralising and non-neutralising antibodies

- To investigate the presence of neutralising and non-neutralising antibodies in persons with severe HB in the Nordic countries.
- To evaluate ITI outcome and complications in relation to the pathogenic *F9* variant.

Paper IV – Quality of life

- To assess HRQoL in PwHB and to compare this to data on matched individuals with HA.
- To evaluate the impact of co-morbidity and joint health on quality of life to identify any areas of insufficient care and to improve the understanding of health in a wider perspective in PwH.

Ethical considerations

The studies included in this thesis have ethical approval from the Regional Ethical Board in Lund, Sweden, Dnr 2015/894 (The Assay Discrepancy study) and Dnr 2016/1089 (The B-NORD study). For the B-NORD study, ethical approval was also obtained from the independent ethics committees in each participating country before enrolment began.

In B-NORD, written informed consent was collected from the PwHB or his legal representative in accordance with the Declaration of Helsinki. In the Assay Discrepancy study, we planned for informed consent forms to be completed by the participating PwHB, but the ethical board considered that an information letter to the participants with the possibility of opting out from the study was a more appropriate approach.

For the B-NORD study, three amendments to this first ethical approval were made. The first amendment concerned adding another study centre, and the addition of the International Physical Activity Questionnaire–Short Form (IPAQ-SF) on physical activity to the study. The second amendment concerned the addition of the questionnaires VERITAS-Pro and VERITAS-PRN, and to add a follow up of the patients who, after enrolment, changed their treatment to EHL factor products. The third amendment was a request to the ethical board for the study "KAPPA – Key Aspects of medical Practice in Patients with haemophilia A" to be able to collect information on patients with HA from the KAPPA database, to be used as control material for data collected in the B-NORD study.

In addition to the ethical approval from the ethical boards, the studies also have approvals from KVB (Samrådsgrupp för kvalitetsregister, vårddatabaser och beredning) to obtain information from the medical journals in Region Skåne and from PUL (PUL – anmälan om behandling av personuppgifter) for the usage of personal information.

None of the studies in this thesis involved any change to the patient's treatment, everyday life or surgical interventions. Information about the patients was taken from medical journals, questionnaires completed by the patients, blood samples, physical examinations and ultrasound examination of the joints. Instead, the biggest ethical issue, as I see it, concerns the fact that HB is a rare disease, and it is a challenge to report relevant information without risking the anonymity of the study

participants. In Paper III, this was particularly difficult since individual ITI therapies were reported, and there are only a very few PwHB who experience inhibitors.

The fact that haemophilia is an X-linked recessively inherited disease can bring further ethical concerns, and many PwHB have family members with the same disease. In both the Assay Discrepancy study and the B-NORD study, we identified the underlying *F9* variants in the study participants. Individual patients often have access to medical journals and, in many cases, their *F9* variant. In Paper I we discovered discrepant assay results in families with the same *F9* variant. We found this to be of scientific value and chose to publish details of the individual variants. However, when publishing genetic material in such small study cohorts, this is not without ethical concerns about maintaining the anonymity of the individuals concerned. Haemophilia is a lifelong disease and the staff at the HTCs have often known their patients for a long time, and know their medical history well. This makes it challenging to keep the study participants' details anonymous from the HTC staff in descriptive studies with small cohorts. Reflections on how to balance the risk of identification and the value of publishing data are unavoidable.

The rare occurrence of the disease often means that it is the patient's treating physician or nurse who is involved in the study enrolment. This is an additional ethical dilemma since it cannot be ignored that our patients are in a position of dependence on us as healthcare providers and that this can influence their willingness to participate in, or even feel forced to participate in, studies. It is of utmost importance that the patients are informed that their care and medical treatment is not affected, depending on whether they choose to participate in the study or not.

As discussed previously, haemophilia care today is unfortunately not equal globally, and this gives rise to ethical considerations. What difference does it make if the phenotype of HB is slightly milder than that of HA, if not all persons with haemophilia have treatment available to stop a life-threatening bleed? Or if a person suffers from haemophilia but they have not yet received a diagnosis as a result of an insufficiently developed health-care system? Haemophilia is, of course, not the only diagnosis in which healthcare today is unequal globally, this applies to many conditions. In parallel to the work that needs to be done to provide knowledge and care to all persons living with haemophilia, is it not also however, the duty of those who have the opportunity to conduct research to do so, and share the knowledge with all who might benefit from it?

Methods and Methodological considerations

Study design and study populations

This thesis is based on two different cohorts from the studies: the FIX Assay Discrepancy study and the B-NORD study. The FIX Assay Discrepancy study provides the basis of Paper I, and the results from the B-NORD study are presented in Papers II-IV (see Figure 8).

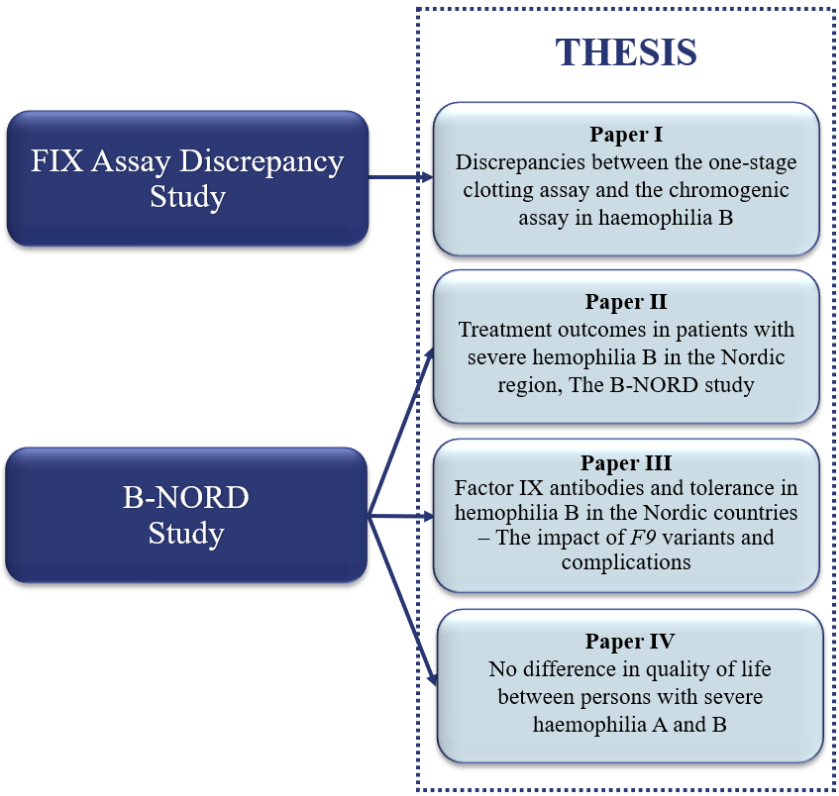


Figure 8. The structure of this thesis based on the Assay Discrepancy study and the B-NORD study.

The FIX Assay Discrepancy study (Paper I)

The study subjects for this study were identified using the local haemophilia register at the HTC at Skåne University Hospital in Malmö, Sweden. All patients diagnosed with HB, all levels of severity, registered in May 2015 were considered eligible to participate. Information on *F9* variant, treatment and bleedings was retrieved from the medical records. Results from the one-stage and the chromogenic assays from the same blood sample, after a wash-out period of more than 7 days, were recorded, and FIX activation kinetic analyses were performed.

Eighty-five PwHB were enrolled. Based on the one-stage assay, 26 of these PwHB had severe disease (FIX <0.01 IU/mL) and 59 non-severe HB (FIX 0.01-<0.40 IU/mL). Forty-nine PwHB were excluded as a result of insufficient data on FIX analyses or insufficient data to confirm a wash-out period of more than 7 days after FIX replacement therapy. The final study population consisted of 36 PwHB from 22 families (see Figure 9).

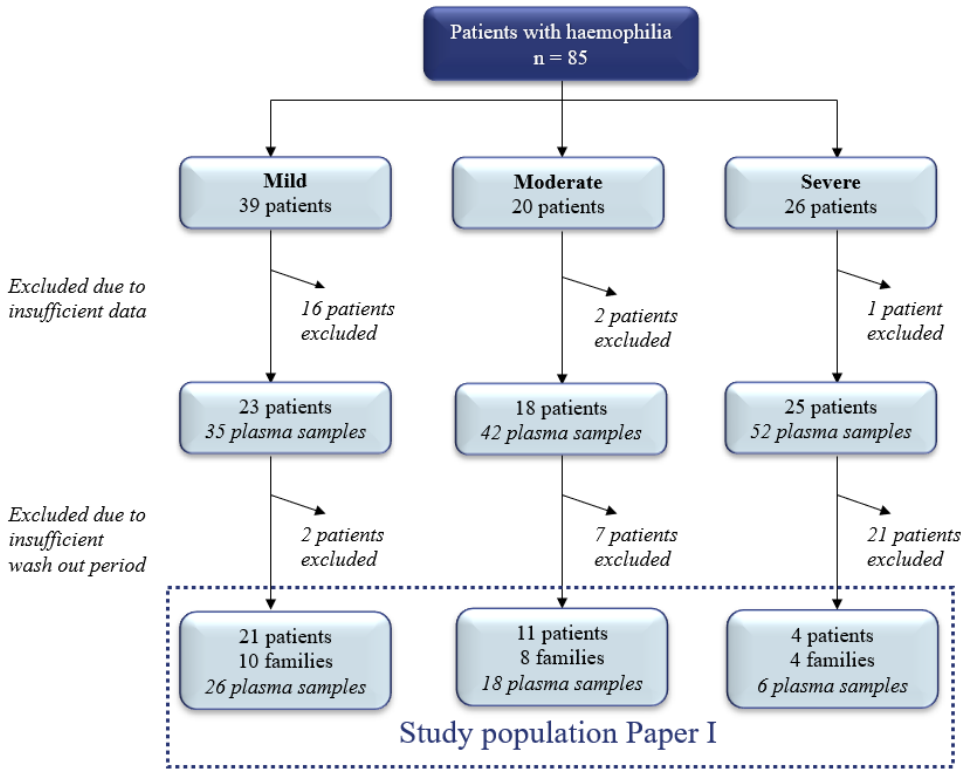


Figure 9. Study population and reasons for exclusions in the Assay Discrepancy study.

The B-NORD study (Papers III-IV)

The B-NORD study is a non-interventional, multicentre, cross-sectional study conducted in six HTCs in the Nordics. Three centres in Sweden (Malmö, Gothenburg, and Stockholm) participated, and one each in Norway (Oslo), Denmark (Copenhagen) and Finland (Helsinki). The goal was to enrol all patients with severe HB registered at the participating centres. In Norway and Sweden, the included centres care for all of the countries' PwHB. The HTC in Copenhagen cares for approximately half of Denmark's haemophilia population and the HTC in Helsinki provides care for approximately 60% of Finland's PwHB.

All males or females with congenital severe HB registered at one of the HTCs were eligible for inclusion in the study. Severe HB was defined as FIX activity level <0.01 IU/mL according to the one-stage or chromogenic assay. Exclusion criteria were concomitant bleeding disorders and the inability to provide informed consent. The study included a study visit for each of the PwHB at enrolment. The first patient was enrolled in June 2017 and enrolment ended in April 2020. Data collected are shown in Figure 10.

Procedure of the B-NORD study:

Clinical information gathered:

- Socioeconomics
- *F9* variant
- Details of haemophilia treatment
- Bleedings and previous joint procedures
- Inhibitor history, including information on ITIs, allergic reactions and nephrotic syndrome
- Comorbidities and medication

Physical examination including:

- Basic physical examination, height, weight
- HJHS version 2.1
- Ultrasound according to the HEAD-US

Questionnaires filled in by the patient/parent:

- VERITAS-Pro and VERITAS-PRN for the evaluation of treatment adherence
- EQ-5D-3L for the evaluation of quality of life

Lab assessments:

- Neutralising and non-neutralising antibodies

Figure 10. Procedure of the B-NORD study.

In PwHB with a history of inhibitors, the treating physician reported whether the person was considered to be tolerant or not to FIX at enrolment, and information on ITI treatment attempts was collected. The criteria used for ITI success/partial success/failure was at the discretion of the treating physician. The Nijmegen-modified Bethesda assay was performed for the evaluation of inhibitors, as well as an ELISA and an xFLI assay for the evaluation of NNAs.

One hundred and eight PwHB were registered at the six HTCs at the start of the study. Out of these, 79 (73%) males were enrolled in the study. No women fulfilled the inclusion criteria. Reasons for non-participation in the study are shown in Figure 11. As a result of local regulations, no ethical approval could be obtained for children in Denmark.

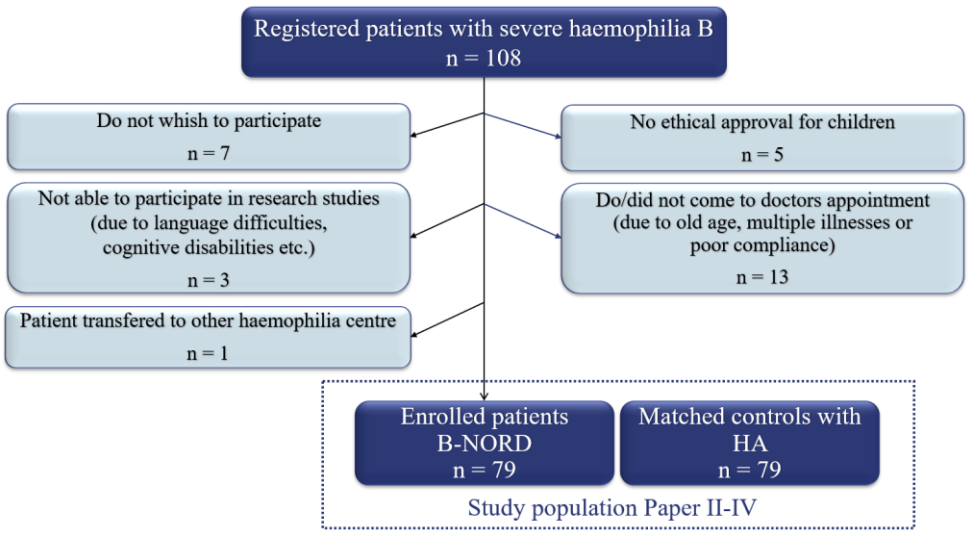


Figure 11. Study population and reasons for non-participation in the B-NORD study.

Each patient with severe HB was matched with a control person with severe HA from one of the included HTCs. The controls were taken from a web-based international register of PwHA, the KAPPA Registry,[121] developed by Haemophilia Systems (Munkeby Systems, Malmö, Sweden). The controls were enrolled between October 2013 and December 2017 and matched by gender, age, and treatment modality. The study was observational and no interventions were conducted in this cohort. Treatment decisions were at the discretion of the treating physician at the different HTCs.

Laboratory methods

FIX activity analyses

FIX one-stage assay

The one-stage clotting assay to measure FIX activity is the most widely used assay worldwide and has been so for many years. This assay is based on the aPTT. The FIX activity in plasma is calculated using the ability of a plasma sample to correct the clotting defect of a plasma sample which has a complete lack of FIX, i.e. FIX <0.01IU/mL (FIX-deficient plasma), but contains all the other factors required for normal clotting.[112,122] Factor-deficient plasma can be gathered from donated blood from persons with severe HB, which hence lacks FIX completely, or it can be generated by immunodepletion. The test plasma is diluted and mixed with FIX-deficient plasma and an aPTT is then performed on the mixture. The concentration of FIX is rate-limiting on the clotting time. The clotting time in the patient sample is compared to that of a standard or reference plasma of known FIX activity and is calibrated against an international calibrator. A standard curve is established, and the plotting of the clotting times enables a quantitative result.

The details on aPTT reagents, FIX-deficient plasma and coagulation analyser used in Paper I are presented in the original paper and are therefore not presented in detail in this thesis.

FIX chromogenic assay

The chromogenic assay is a two-stage assay measuring the generation of FXa, which is dependent on the FIX activity of the tested plasma sample (see Figure 12). In the first stage, the test plasma is mixed with reagents containing FVIII, FX, FXIa, thrombin, phospholipids and calcium. FXIa activates any FIX present to FIXa. Thrombin activates FVIII to FVIIIa which, together with the FIXa, calcium ions and phospholipids, activates FX to FXa. In the second stage, a chromogenic peptide substrate specific for FXa is added. Formed FXa cleaves the chromogenic substrate which leads to the release of pNA (para-nitroaniline) which, when released, gives rise to colour.[123] The colour intensity is read optically at 450 nm and is proportional to the FIX activity since the FIX concentration is the rate-limiting step.

The details on the chromogenic coagulation analyser used in Paper I with detection limit and details on imprecision are presented in the original paper and are therefore not presented in detail in this thesis.

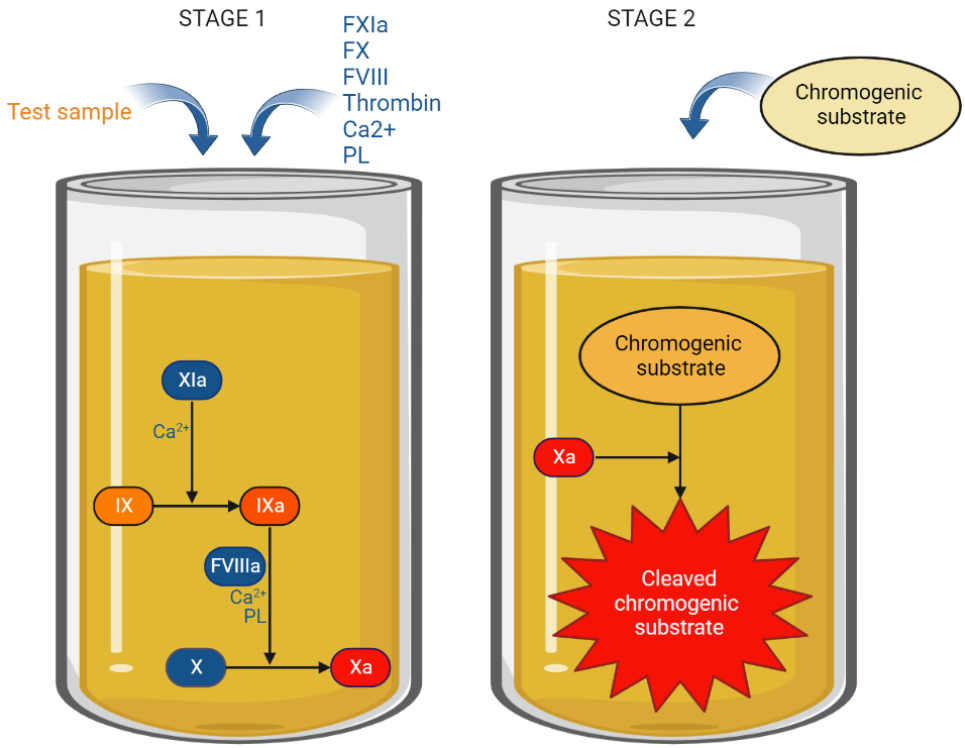


Figure 12. Schematic illustration of the principles of the chromogenic method for the measurement of FIX activity. Abbreviations: PL: phospholipids. Created with BioRender.com

FIX activation kinetics

In Paper I, we found that persons with the *F9* variants (*F9*: c.572G>A; p.Arg191His and *F9*: c.571C>T; p.Arg191Cys) at the N-terminal cleaving site of the activation peptide of FIX had discrepant FIX results in the one-stage and the chromogenic assays. These *F9* variants were not observed in any PwHB with non-discrepant results. We therefore hypothesised that a variant at this site is associated with discrepant results and that an impact of these variants on the activation process causes discrepant results. To investigate this further, manual tests for FIX activation kinetics were performed. These tests are not used routinely in clinical practice, but are experimental in order to investigate and try to find an explanation for the results we found with the one-stage and chromogenic assays.

Activation kinetics were performed for the one-stage method in a microplate and monitored after the addition of calcium ions. Subsampling was made at eight different time points and a stop solution was added to the subsample to end the reaction. Formed FIXa at the different time points was determined with the

chromogenic kit Rox FIX-A (Rossix, Mölndal, Sweden). Two independent runs were made on each of the plasma samples from three patients with discrepant assay results (*F9*: c.572G>A; p.Arg191His) and from two patients showing no assay discrepancy (FIX activities of 0.26 and 0.10 IU/mL, respectively), as well as on the sample with FIX activity of 0.10 IU/mL after predilution 1:5 with FIX-deficient plasma, in order to obtain a FIX activity of 0.02 IU/mL (an activity similar to the assigned FIX activities with the one-stage method for plasma with the *F9* variant: c.572G>A; p.Arg191His).

FIX activation kinetics using the chromogenic method were determined with the Rox Factor IX kit (Rossix, Mölndal, Sweden). Kit Reagent A (FVIII and FX) and diluted sample were mixed in a deep well plate. Subsampling of 50 µL was made at different time points into wells of a flat-bottomed microplate preheated to 37°C and containing 150 µL Reagent B (FXIa, prothrombin, phospholipids and $CaCl_2$). FIX activation was allowed to proceed for 0-12 minutes. Addition of chromogenic FXa substrate as well as ethylene-diaminetetraacetic acid (EDTA) to terminate the activation was added to the different wells. The generated FXa activity, proportional to the amount of generated FIXa, was determined through the release of pNA measured at 405 nm. The same plasma samples were used as for the activation kinetics for the one-stage method described above and two independent runs were made on each of the samples.

Anti-FIX assays for the detection of neutralising antibodies (inhibitors)

The Bethesda assay

The Nijmegen-modified Bethesda assay was used to quantify the FIX inhibitor level in the B-NORD study (Papers II-IV).

Patient plasma was mixed with normal buffered pooled plasma and incubated for 2 hours at 37°C. After 2 hours, the FIX activity was measured using a one-stage or chromogenic assay. At the coagulation laboratory in the HTC at Skåne University Hospital, this is carried out using a chromogenic assay. FIX activity in the patient plasma was compared to that of a control plasma sample with equal parts FIX-deficient plasma and normal pooled plasma. The residual factor activity was calculated as a percentage of the FIX in the control plasma. The residual factor activity was converted to Bethesda units derived from a graph or by formula NBU = (2-log RA)(0.301)-1.[124] The titre was given in kilo Bethesda Units per litre (kE/L) or Bethesda units per millilitre (BU/mL, BU).

The modification according to Nijmegen[125] was described initially in the anti-FVIII Bethesda assay and implies that the FVIII level in normal plasma is buffered (originally with imidazole) and that deficient plasma, rather than buffer, is used in

the control mixture. This is not as important for the FIX assay, but the same method is normally used as for FVIII.

In the B-NORD study, the assay was performed by the local laboratories and the cut-off levels were 0.4 or 0.5 BU/mL, depending on the individual laboratory. A level of 0.4 BU/mL corresponds to a residual FIX activity of 75% of the normal control plasma.

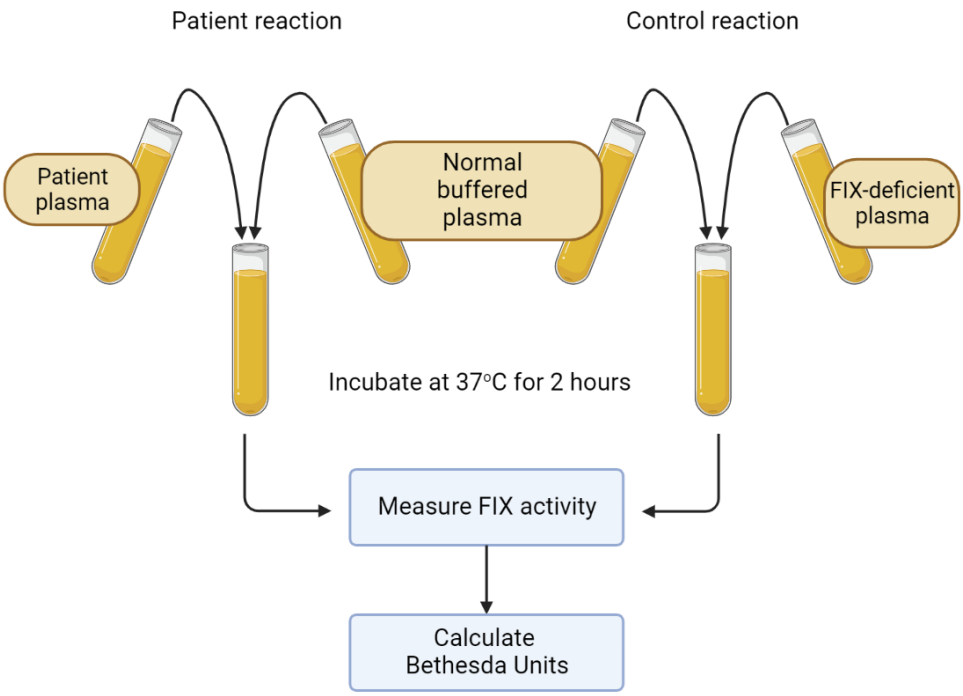


Figure 13. Schematic illustration of the Bethesda assay for the detection of neutralising FIX antibodies.
Created with BioRender.com

The Malmö inhibitor assay

The Malmö inhibitor assay was used previously to estimate the presence of inhibitors and expressed the inhibitor activity in plasma as the number of units of FIX inactivated by 1 mL of patient plasma.[126] One Malmö inhibitor unit (MIU) corresponds to about 3 BU. This assay was not used primarily for the detection of inhibitors in this thesis work, but older results from the Malmö inhibitor assay from medical journals were used in our study and, for comparative purposes, recalculated to Bethesda units.

Anti-FIX assays for the detection of non-neutralising antibodies

In the B-NORD study, two different methods were used to investigate the presence of NNAs: one ELISA and one Multi-Analyte Profiling (Luminex xMAP) based fluorescence immunoassay (xFLI). Results from the Nijmegen-Bethesda assay were used for comparison in order to distinguish inhibitors from NNAs. The results are presented in Paper III.

All analyses for NNAs were conducted at the coagulation laboratory at the HTC in Malmö, Sweden. Plasma samples from the PwHB enrolled at HTCs outside of Malmö were centrifuged and frozen, and transported in an unbroken freezing chain packed in dry ice.

Anti-FIX ELISA method

An in-house ELISA was used. Recombinant FIX (nonacog alfa, BeneFIX) was used as the FIX antigen and was coated overnight in a microtitre plate. Plasma samples were diluted 50-fold in a Tris-blocking buffer supplemented with 1 mM $CaCl_2$, incubated for 2 hours and then applied to the wells. Following washing, a secondary antibody (horseradish-peroxidase conjugated polyclonal rabbit anti-human IgG) with specificity for the primary antibody and with a colorimetric label attached, was added. To remove excess of the secondary antibody, the system was washed once more, after which absorbance was measured in a microplate reader (Tecan Infinite 200, Männedorf, Switzerland) to detect and quantify the colorimetric label. The cut-off for a positive result for each test run was determined by analysing normal plasma samples of 10-12 healthy individuals per test run and the cut-off was given as the mean + 3 standard deviations (SD). The inter-assay coefficient of variation (CV) was >50% for the positive control. This high CV is discussed further in the 'Results' and 'Discussion' sections of this thesis.

Anti-FIX xFLI method

Recombinant FIX (nonacog alfa, BeneFIX) was coated to MagPlex microsphere beads. Citrated plasma samples were diluted 100-fold in phosphate buffer saline (PBS) supplemented with 0.05% Tween-20 (PBST) and 0.1% ovalbumin (PBST-O), added to wells containing FIX-coupled microspheres and incubated for 2 hours. The samples were thereafter washed with PBST and incubated with R-phycoerythrin-labelled goat anti-human IgG for 1 hour. The samples were analysed in a MagPix instrument (Luminex, Corporation, Austin Texas, USA) and the readings were recorded as median fluorescence intensity (MFI). A general cut-off for positivity was determined by analysing samples from 26 healthy individuals and calculating the mean + 3 SDs. The inter-assay CV was 12.2% for the high positive control and 14.3% for the low positive control.

Variant analyses

Information on the *F9* variants in the enrolled individuals with HB was gathered in both the Assay Discrepancy study and the B-NORD study and presented in Papers I and III.

Variant analyses from PwHB in Sweden and Finland were performed at the genetic laboratory in collaboration with the HTC in Malmö, Sweden. *F9* variants for the enrolled individuals from Norway were performed at the HTC in Oslo, Norway. No data on *F9* variants were available for the patients from Denmark.

Variants were identified by Sanger sequencing. Large deletions and duplications were determined by Multiplex Ligation-dependent Probe Amplification (MLPA). The details on the variant analyses are presented in the original papers (I and III) and are therefore not presented in detail in this thesis. The variant reports were gathered and presented according to the recommendations of the Human Genome Variation Society (HGVS). The variants were interpreted for clinical significance according to the American College of Medical Genetics and Genomics (ACMG) guidelines applicable in 2021. The interpretation was made using an automated scoring system, and a manual review and adjustment of specific criteria, using the VarSome's ACMG implementation.[127] The FIX Gene Variant Database provided by the Structural Immunology Group, University College London, UK, was used for comparison.[128]

Instruments for joint evaluation

Haemophilia Joint Health Score

The HJHS version 2.1 was used in the B-NORD study for joint assessment, and the results are presented in Papers II and IV.

The HJHS is a joint examination tool developed by the International Prophylaxis Group[40,41] and is designed primarily for children with haemophilia aged 4-18 years, but its use has been extrapolated to adults and the tool was later also validated for the use in adults.[42] The HJHS 2.1 tool assesses six joints including the ankles, knees, and elbows. The assessment is made in nine areas and each area is assessed and scored using defined scoring criteria on an ordinal categorical scale. The areas scored include: swelling (score 0-3), duration of swelling (0-1), muscle atrophy (0-2), crepitus on motion (0-2), flexion loss (0-3), extension loss (0-3), joint pain (0-2) and strength (0-4). Higher scores indicate greater joint damage. Twenty is the maximum score per assessed joint. An additional assessment of global gait includes assessments of walking, walking up and down stairs, running, and hopping on one leg. Gait is scored as 0-4, with 0 being 'all skills within normal limits' and 4 being

equivalent to 'no skills within normal limits'. All of the joints' scores, together with the global gait score, are combined and added up to a total score ranging from 0 (best) to 124 (worst). A copy of the HJHS 2.1 summary score sheet is found in Appendix 1 in this thesis.

With regard to at what point an HJHS score indicates an affected joint and to define normative reference values for the HJHS 2.1, St-Louis *et al.* gathered data on a non-haemophilia population of 120 healthy adult participants in the HJHS 2.1 Validation in Adult Patients Study.[42] Healthy adults without haemophilia had significantly lower HJHS total scores compared to those with haemophilia. Median HJHS total scores for healthy adults were 2.0 (Q1-Q3: 0.75-5.0) in persons aged 18-29 years, 3.0 (Q1-Q3: 1.5-6.0) in persons aged 30-40 years, 3.0 (Q1-Q3: 2.0-6.0) in persons aged 41-50 years and 8.0 (Q1-Q3: 3.0-12.5) in persons aged >50 years. In this cohort, participants with a history of a minor joint injury or surgery were not excluded. Furthermore, Sluiter *et al.* assessed the joints of healthy young active adults using the HJHS version 2.1, and found that in the absence of clinical complaints, 40% of the participants showed scores of up to 3 as a result of crepitus or flexion loss.[129] This is discussed further in relation to the study results in the 'Results' section of this thesis.

The HJHS assessments for the PwHB in the B-NORD study and the PwHA in the KAPPA Registry were conducted by a physiotherapist or physician at the included HTCs. The HJHS 2.1 was chosen for joint assessment since it measures joint health status of the joints most commonly affected by bleeding in haemophilia and measures joint health not only with respect to structure, but also in terms of function/impairment. The tool is well known and used in routine follow up at all of the HTCs included in the study. The HJHS tool is believed to be relatively sensitive, it can identify early signs of joint damage and can also be used for monitoring joint changes over time. However, a limitation to the HJHS assessment tool, and a source of criticism, is that it takes a long time to perform and it is considered by some to be too time consuming for routine clinical practice.

HEAD-US

Joint evaluation with ultrasound according to the Haemophilia Early Arthropathy Detection protocol (HEAD-US) was performed on PwHB enrolled in the B-NORD study and the results are presented in Paper II.

It has been reported previously that early signs of arthropathy can be seen in PwH with asymptomatic joints after only one or a few bleeds, and that these signs are not always apparent on physical examination.[130] As discussed previously, joint imaging by MRI is considered to be a sensitive and radiation-free way of picturing the joints; however, the technique is not always readily available. The HEAD-US protocol was developed by Martinoli *et al.*[44] with the goal of developing a simplified ultrasound

scanning procedure and scoring method to assess the joints of PwH and to make ultrasound imaging of haemophilic joints easier. In the B-NORD study, we used ultrasound and the HEAD-US protocol as an additive tool in the evaluation of joint health.

The HEAD-US method evaluates elbows, knees and ankles for disease activity and disease damage according to a specific scanning procedure. Disease activity (synovitis) is assessed by the evaluation of hypertrophic synovium and disease damage by the evaluation of articular surfaces including cartilage and subchondral bone. Hypertrophic synovium is scored 0-2 by assessing joint recesses and the amount of synovial tissue contained within them. The assessment of osteochondral damage is made at predetermined surfaces, and is graded 0-4 for articular cartilage and 0-2 for bone structures. The scores for each joint are added together and the maximum score per joint is eight. A copy of the scoring method used in the HEAD-US protocol is found in Appendix 2.

In the B-NORD study, ultrasound scanning was performed by a physiotherapist or physician at the HTCs. The maximum score was given to joints with severe arthropathy and reduced joint mobility leading to difficulties in obtaining optimal ultrasound images. Joints with arthroplasties were recorded as missing data.

Ultrasound imaging has the limitations of operator dependency, and the fact that the joints are only partially visualised. However, ultrasound imaging has the advantages of being easier than MRI to perform on children, the evaluation can be carried out at the HTC at a routine visit, and the cost is much lower than for an MRI examination.

Evaluation of treatment adherence and quality of life

VERITAS

The Validated Haemophilia Regimen Treatment Adherence Scale (VERITAS) is a self-administered or parent-reported questionnaire to evaluate treatment adherence to factor replacement products in PwH. Adherence to treatment is important in order to reduce the risk of developing arthropathy, and in Paper II we used VERITAS to assess treatment adherence in the PwHB enrolled in the B-NORD study.

Two versions of the questionnaire have been developed: VERITAS-Pro, which is used for persons taking prophylactic treatment with factor products, and VERITAS-PRN, which is used for persons using episodic treatment.[131,132] The questionnaires comprise 24 questions divided into six subscales. The subscales ‘time’, ‘dose’, ‘plan’, ‘remember’ and ‘communicate’ are the same for the two versions of the questionnaire, but the last subscale differs between the two questionnaires, and for

VERITAS-Pro it is 'skip', while for VERITAS-PRN it is 'treat'. Response options for the questions are presented as five-point Likert scales ranging from 'Always' to 'Never'. Each answer is given a numerical score: the response indicating the 'best' adherence is scored as one point and the response for 'worst' adherence is scored as five points. The scores are summarised on each subscale and range from 4 (most adherent) to 20 (least adherent). A total score is calculated by summarising the subscale scores. The best possible score is 24 and the worst possible score is 120. A proposed cut-off score for 'non-adherence' is set at a score of ≥57.[131] A sample copy of the VERITAS-Pro questionnaire can be found in Appendix 3 in this thesis.

Permission for the use of the VERITAS questionnaires in the B-NORD study was obtained from the Indiana Hemophilia & Thrombosis Center, USA.

EQ-5D

EQ-5D is a standardised questionnaire developed by the EuroQol Group[133,134] to measure health related quality of life (HRQoL). The questionnaire is not specific to any particular health condition or patient group, and hence seeks to assess health status in a 'generic' manner.

There are two EQ-5D questionnaires available: a three-level version of the questionnaire (EQ-5D-3L) and a five-level version (EQ-5D-5L). In Paper IV, the three-level version of the questionnaire was used to assess the HRQoL of the PwHB aged 12 years and older[135] enrolled in the B-NORD study. This version was chosen, since it was used in the KAPPA database in which the information on the HA controls is held. The official language versions of the questionnaire corresponding to the spoken language at the individual included HTCs were used.

The EQ-5D questionnaire is self-administered and consists of two pages: the EQ-5D-3L descriptive system and the EQ visual analogue scale (EQ VAS). The descriptive system consists of the five dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. Each dimension in the EQ-5D-3L has three levels: no problems, some problems, extreme problems (levels 1-3). The participants are asked to check the box against the level that best describes their experience of each of the five dimensions: the result is referred to as the participant's 'EQ-5D profile'. The profiles can be summarised as a Level Sum Score (LSS) by adding up the levels (1, 2 or 3) for each dimension, treating each level as a number rather than as a categorical description. The LSS can, in this way, be used as a crude measurement of severity. The best EQ-5D profile (11111) represents 'having no problems' in all five dimensions and adds up to the LSS 5, the LSS for the worst health state (33333) is consequently summarised to 15. EQ-5D health state summary index score can be calculated by attaching values from a representative value set to each of the levels in the dimensions. The value sets have been obtained from a valuation exercise in which a sample of the general population in a region is asked

to place a value on EQ-5D health states using either the time trade-off (TTO) or the VAS valuation technique. In Paper IV, we calculated health state index scores from the health profiles using the TTO scores from the Danish value set.[136] This value set was chosen since it was believed to be representative of the Nordic cohort in our study. The index scores range from less than 0, to 1, with higher scores indicating higher health utility. The score of 1 is a health state equivalent to perfect health, while 0 is equivalent to death, and negative values are valued as worse than death.[135]

The second page of the EQ-5D-3L questionnaire includes the EQ VAS, a scale from 0 to 100, where the study subjects are asked to indicate their 'overall health state today' with 0 representing 'worst health imaginable' and 100 as 'best health imaginable'. The scale consists of a line with end-point descriptors, but also marks in units of ones and tens with number labels on the tens markers. This part of the questionnaire provides additional data to the EQ-5D profiles, since it reflects the patient's assessment of their overall health including dimensions that might not be included in the descriptive part of the questionnaire. A sample copy of the questionnaire can be found in Appendix 4 in this thesis.

Permission for the use of the EQ-5D-3L questionnaire was obtained from the EuroQol Research Foundation (agreement number 152708).

Data management and statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 23, IBM SPSS Statistics 25 (Armonk, NY, USA) and Microsoft Excel (Redmont, WA, USA). p values <0.05 were considered to be statistically significant. The statistical analyses were performed with advisory support from Enheten för Medicinsk statistik och Epidemiologi, Kliniska Studier Sverige, Forum Söder (Centre for Medical Statistics and Epidemiology, Clinical Studies Sweden).

The data management system was operated at the Centre for Thrombosis and Haemostasis, Malmö, Sweden. The data collection in the B-NORD study was performed using paper case report forms (CRFs). An electronic database was created in Epi Data Manager (EpiData Association, Odense, Denmark) and data from the CRFs were transferred into the database using the supplemental EpiData EntryClient. The data were exported to SPSS for statistical analyses. The HA controls were identified in the KAPPA Registry.[121]

Descriptive statistics are presented throughout the thesis. Continuous variables are described using means (M) ± standard deviation (SD) for normally distributed data and medians with first-to-third quartiles (Q1-Q3) for non-normally distributed data. Categorical data are reported as numbers and percentages.

The non-parametrical Wilcoxon matched pairs signed-rank test was used for the calculation of disparity between the FIX activity results from the one-stage and chromogenic assays in Paper I. In Papers II and III, the Mann–Whitney U test was used for comparisons for continuous, non-normally distributed variables when comparing two groups, and the Kruskal–Wallis test was used when comparing three or more groups. For binary or categorical variables, the Chi-squared test and Fisher's exact test were used to examine whether an association between the variables existed. Fischer's exact test is used in small sample sizes when more than 20% of cells have expected frequencies <5.[137]

In Paper IV, the questionnaire EQ-5D-3L was used to assess HRQoL. Data collected using EQ-5D can be presented and analysed in various ways.[135,138] The EQ-5D levels were dichotomised, as suggested in the EQ-5D user guidelines, into 'no problems' (level 1) and 'any problem' (levels 2 and 3), and McNemar's paired test was used to assess whether the number of persons reporting a problem differed between PwHB and PwHA. The EQ-5D health profiles were summarised to a Level Sum Score (LSS) and health state index scores were calculated from the profiles using valuation scores from the Danish value set.[136] A paired sample t-test was used to analyse differences in EQ VAS scores and EQ index values between PwHB and their matched HA controls. Differences in EQ-5D index values and EQ VAS scores between PwHB treated with SHL and EHL were assessed using the independent samples t-test. The Pearson correlation coefficient was used to examine the correlation between HJHS score and patient age in Paper IV. The relationship between a numerical outcome and a numerical exposure can be estimated using linear regression, which also gives an estimate of the correlation, or strength, of the linear association.[139] To examine the relationships between EQ-5D results, HJHS, patient age, and body mass index (BMI), linear regression was applied.

Whether the VAS scale should be considered to be an ordinal or a ratio scale can be debated, since this affects the choice of appropriate statistical methods for analysis of the results. No uniformity is found in the literature on this matter. [140] When examining our data, the EQ VAS scores were not concentrated around either extreme of the scale, and we therefore chose parametric tests for further statistical analyses.

Strengths and limitations

The strengths of this thesis include the focus on PwHB, which is in contrast to the majority of previous studies of haemophilia, in which mainly PwHA have been included. Further strengths are the international multicentre design of the B-NORD study, on which Papers II-IV are based, and the inclusion of closely matched HA controls taken from the same HTCs. The matching balances potential confounders

and avoids selection bias. A further strength is that the persons enrolled in the B-NORD study were from a homogenous geographical area, enrolled at HTCs with a close collaboration and common Nordic treatment guidelines.[120] In addition, the majority of the patients registered at the HTCs were enrolled in the study and the *F9* variants were identified in most of the PwHB.

However, the retrospective observational design with some of the data extracted from medical records brings limitations to the studies. Disadvantages of observational studies are the lower level of evidence, the issue of confounding and the fact that causal inference can be difficult to make. Advantages, however, are that observational studies are relatively inexpensive, often less limited by regulatory restrictions, and they are not as time-consuming to conduct as longitudinal experimental clinical trials.

A further limitation in Paper III is that no consistent criteria for ITI treatment success were used, thus comparisons of different ITI treatment regimens were difficult to make. A prospective study would enable easier the use of consistent criteria for ITI treatment success and comparisons between therapeutic regimens, but would, on the other hand, require either a much larger study with more HTCs enrolled, or a considerably longer time frame. Inhibitors in PwHB are rare, and data on ITI therapy in PwHB even more so. In fact, in the *WFH Guidelines for the Management of Hemophilia, 3rd edition*, published in 2020, the WFH did not even make a recommendation on the use of ITI in PwHB as a result of the limited experience. Therefore, even retrospective data on this topic are of importance to collect and share.

All studies of PwHB have the concern of a limited number of subjects and, despite the B-NORD study being an international multicentre study, the work in this thesis is no exception. The balance between the cost and implementation of a complex and large study, and the number of enrolled study subjects, is a reality for all researchers conducting studies of rare diseases. Considering the busy everyday work at the HTCs, the B-NORD study was, in comparison to many other studies, a relatively simple study for the centres to participate in, which was also the goal, since this enhanced the chances of including more patients. Seventy-nine persons with severe HB were finally enrolled in this study, and this is, compared with previous reports focusing on HB, a relatively high number of participants.

The HA controls in B-NORD were taken from the KAPPA Registry, a pre-existing register, which brings the limitation that missing data cannot be supplemented and there were unfortunately more missing data in the register than anticipated. Information on bleedings and joint surgery was incomplete in the KAPPA Registry and these important parameters could, therefore, unfortunately not be compared between PwHA and PwHB. A further disadvantage of using the KAPPA Registry was that the enrolment periods for PwHB and PwHA differed slightly.

In the Assay Discrepancy study, plasma samples were collected from PwHB and analysed with both the one-stage and the chromogenic assays in order to investigate whether discrepancies in the results were present. A limitation to this study is that the plasma samples were collected over a period of 8 years. Some samples were analysed on the same day of the sampling, but some were analysed after thawing of a frozen sample. The occurrence of degradation of sample quality associated with freezing and storing over a period of time cannot be excluded. However, the analyses by the one-stage and chromogenic assays were performed simultaneously and, therefore, the results from the same plasma sample were believed to be comparable.

Many limitations of this thesis could have been avoided if the studies included had been prospective and with a large number of subjects. A future scenario with a large international multicentre prospective study, enrolling PwHB and matched PwHA from birth and following them over time, with close follow ups and predetermined criteria and definitions on ITI treatment success and failure, as well as the inclusion of matched healthy controls without haemophilia, would bring further knowledge on the natural history and treatment outcomes of this rare disease. Such a study would, however, be complex in its implementation and in need of great financial support. However, the PedNet Haemophilia Registry has the aim of establishing a large birth cohort of patients with HA and HB. It has been collecting data on children with haemophilia born from the year 2000 and onwards.[141] On 1 January 2022, a total of 2759 children had been included in the Registry.[142]

Results and discussion

Assay discrepancy and diagnostic challenges in haemophilia B - Assay Discrepancy study (Paper I)

Different instruments and reagents are used worldwide in the measurement of FIX activity, which results in a variability in test results and difficulties in making comparisons between them. Today, the one-stage and the chromogenic assays are the most widely used methods for functional FIX activity analysis. It has been reported previously that in approximately one-third of persons with non-severe HA, a discrepancy between these two assays in measuring FVIII levels exists.[112-115] To our knowledge, assay discrepancy in HB had not previously been evaluated systematically when we began the work on the Assay Discrepancy study.

We analysed plasma samples from PwHB for FIX activity with both the one-stage and the chromogenic assays and the results are presented in Paper I. Fifty plasma samples collected between 2008 and 2016, from 36 PwHB representing 22 different families, were analysed using both methods. No difference was seen between the assay results in persons with severe haemophilia and this group was therefore not evaluated further. Only one individual, a person with severe HB, had a history of inhibitors.

The remaining group consisted of 32 persons with non-severe HB from 18 different families. From this group, 44 samples were analysed with both assays and showed mean values of $FIX_{one\text{-}stage}$ 0.09 (SD: 0.09 IU/mL, range: 0.01–0.35 IU/mL) and $FIX_{chromogenic}$ 0.11 (SD: 0.08 IU/mL, range: <0.01–0.34 IU/mL), respectively. The agreement between the two methods is shown in Figure 14. For individuals with more than one set of results, the plasma samples were collected independently on different dates.

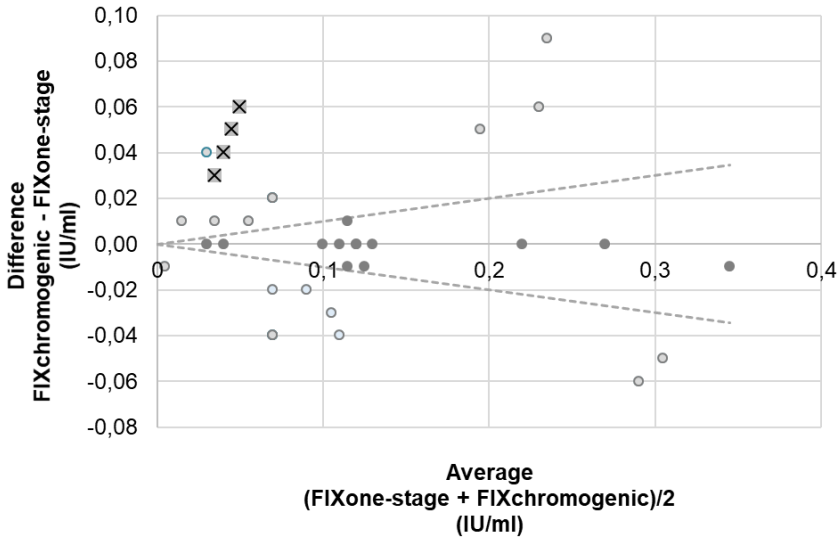


Figure 14. Agreement between $FIX_{chromogenic}$ and $FIX_{one\text{-}stage}$ in patients with non-severe haemophilia B shown in a Bland–Altman plot. The dotted lines are set at ± 10%. The values marked with an x are from patients with the *F9* variant c.572G>A; p.Arg191His.

Assay discrepancy

The ratio between the two FIX assays was calculated to compare the results of the two methods. In the calculation, results <0.01 were given a value of 0. There is no established definition of assay discrepancy in haemophilia, but a two-fold or greater difference between the two assays, resulting in a ratio of ≥2 or ≤0.5, is used commonly.[118] We found this definition to be appropriate, considering clinical relevance, and this definition has been used throughout Paper I.

Fifteen samples from eight patients, i.e. 25% of the patients with non-severe HB enrolled, were calculated to have a two-fold or greater difference between the results of the two assays. In these samples, the chromogenic assay showed the higher values (mean $FIX_{one\text{-}stage}$ 0.02, SD: 0.004 IU/mL and $FIX_{chromogenic}$ 0.06, SD: 0.01 IU/mL). No cases were found with a two-fold or greater result from the one-stage method compared to the chromogenic method, except for one sample, where the one-stage assay showed a higher value of 0.01 compared to the chromogenic assay result of <0,01, hence it was not possible to calculate a ratio. The individual results and ratios from the two methods from persons with mild or moderate HB are shown in Table 2.

Table 2. Results from the one-stage and chromogenic assays from persons with non-severe haemophilia B in the Assay Discrepancy study.

Family	ID	Year of birth	FIXone-stage (kIE/L)	FIXchromogenic (kIE/L)	Ratio (FIXchromogenic /FIXone-stage)	Mutation		Mutation effect	Domain
A	1	2004	0.01	<0.01			ND		
B	2	1949	0.09	0.05	0.56	c.1105C>G	p.Leu369Val	Missense	Serine protease
C	3	1987	0.08	0.06	0.75	c.391+5G>A	p.N/A	Splice	
	4	2000	0.09	0.05	0.56				
			0.05	0.06	1.20				
D	5	1961	0.13	0.09	0.69	c.835G>A	p.Ala279Thr	Missense	Serine protease
			0.11	0.11	1.00				
E	6	1951	0.12	0.09	0.75	c.835G>A	p.Ala279Thr	Missense	Serine protease
F	7	1991	0.12	0.11	0.92	c.835G>A	p.Ala279Thr	Missense	Serine protease
			0.10	0.10	1.00				
	8	1997	0.10	0.08	0.80				
			0.06	0.08	1.33				
G	9	1933	0.17	0.22	1.29	c.1265C>A	p.Thr422Asn	Missense	Serine protease
	10	1993	0.11	0.12	1.09				
	11	1940	0.20	0.26	1.30				
	12	1978	0.22	0.22	1.00				
	13	1968	0.33	0.28	0.85				
	14	1970	0.12	0.12	1.00				
	15	2010	0.13	0.13	1.00				
	16	1950	0.13	0.12	0.92				
	17	1967	0.35	0.34	0.97				
			0.26	0.32	1.23				
H	18	1989	0.12	0.12	1.00	c.168_169de	p.Gln57Lysfs*47	Deletion+insertion	GLA
I	19	1972	0.04	0.04	1.00	c.88+5G>A	p.N/A	Splice	
J	20	1989	0.03	0.03	1.00	c.1025C>T	p.Thr342Met	Missense	Serine protease
	21	2000	0.03	0.04	1.33				
K	22	1999	0.06	0.08	1.33	c.301C>T	p.Pro101Ser	Missense	EGF1
L	23	1982	0.19	0.28	1.47	c.459G>A	p.Val153Val	Silent	EGF2
	24	1979	0.27	0.27	1.00				
M	25	1962	0.01	0.02	2.00	c.127C>T	p.Arg43Trp	Missense	Pro-peptide
N	26	1962	0.02	0.05	2.50	c.572G>A	p.Arg191His	Missense	Linker
			0.02	0.08	4.00				
O	27	1999	0.02	0.05	2.50	c.572G>A	p.Arg191His	Missense	Linker
			0.02	0.06	3.00				
			0.02	0.07	3.50				
	28	1995	0.02	0.06	3.00				
			0.02	0.06	3.00				
			0.02	0.07	3.50				
			0.02	0.07	3.50				
P	29	1995	0.02	0.06	3.00	c.572G>A	p.Arg191His	Missense	Linker
			0.02	0.08	4.00				
	30	2000	0.02*	0.06*	3.00				
Q	31	1934	0.02	0.07	3.50	c.572G>A	p.Arg191His	Missense	Linker
R	32	1989	0.01	0.05	5.00	c.571C>T	p.Arg191Cys	Missense	Linker

ND, not determined. *Plasma from the same venepuncture was analysed on different occasions with the two methods. Normal range $FIX_{one\text{-}stage}$ 0.70-1.30 IU/mL and $FIX_{chromogenic}$ 0.80-1.50 IU/mL. For individuals with more than one set of results, the numbers refer to analyses of independently collected plasma samples.

In HA, classic assay discrepancy is defined as a lower value for the two-stage or chromogenic assay, compared to that for the one-stage assay. In contrast, inverse discrepancy is when the one-stage assay yields the lower result. Using these definitions, the findings in our material are equivalent to inverse discrepancy and we did not observe consistent results indicating the presence of classic discrepancy in our cohort.

At very low FIX levels, a ratio of ≥2 or ≤0.5 to define assay discrepancy can be misleading, and represents a very small difference, i.e. in the range 0.01-0.02 IU/mL, where this ratio definition can mean an actual difference of only 0.01 IU/mL. In our material, we did not find this to be a major problem since the discrepancies we found were at higher levels, ranging from 0.01 to 0.06 IU/mL.

F9 variant and assay discrepancy

In all but one of the patients with non-severe HB (97%), the causative *F9* variant was determined. Twelve different variants were identified: eight missense variants, two splice variants, one deletion and one silent variant. The variants are presented in Table 2 and Figure 15, together with the analysis results and the assay ratio. Ten of the variants were found to be registered in the Factor IX Gene (*F9*) Variant Database[128] or the CHBMP *F9* Mutation List.[143] The two remaining variants were not present in the databases: *F9*: c.168_169delTCinsA; p.Gln571Lysfs*47 and *F9*: c.1105C>G; P.Leu369Val. The first of these two variants occurred in a woman with mild haemophilia and led to a premature stop codon, and would, in a male, cause severe haemophilia. The significance of the second variant was assessed as "deleterious" and "probably damaging" by the prediction software Ensembl Variant Effect Predictor,[144] which reports SIFT and PolyPhen-2 scores.

In HA, different genetic variants have been shown to be associated with discrepant results in FVIII assays. Variants causing reduced stability of FVIII have been suggested as an explanation to the discrepant results.[112,113,115,117,118] In our HB cohort, 13 of the 15 discrepant samples were found to be from six individuals, from four different families, but all with the same *F9* variant (*F9*: c.572G>A; p.Arg191His). Interestingly, this variant was not found in any of the PwHB not showing an assay discrepancy between the two methods. All of the 13 samples had a result of 0.02 IU/mL with the one-stage assay, while the results from the chromogenic assay ranged from 0.05-0.08 IU/mL (mean: 0.06, SD: 0.01 IU/mL) (p = 0.001). All six PwHB from whom the samples were taken had, on at least one occasion, received results that would lead to different haemophilia severity classifications with the two assays, i.e. they would be classified as having moderate haemophilia if the sample had just been analysed with the one-stage assay, and mild haemophilia if it had been analysed with the chromogenic assay.

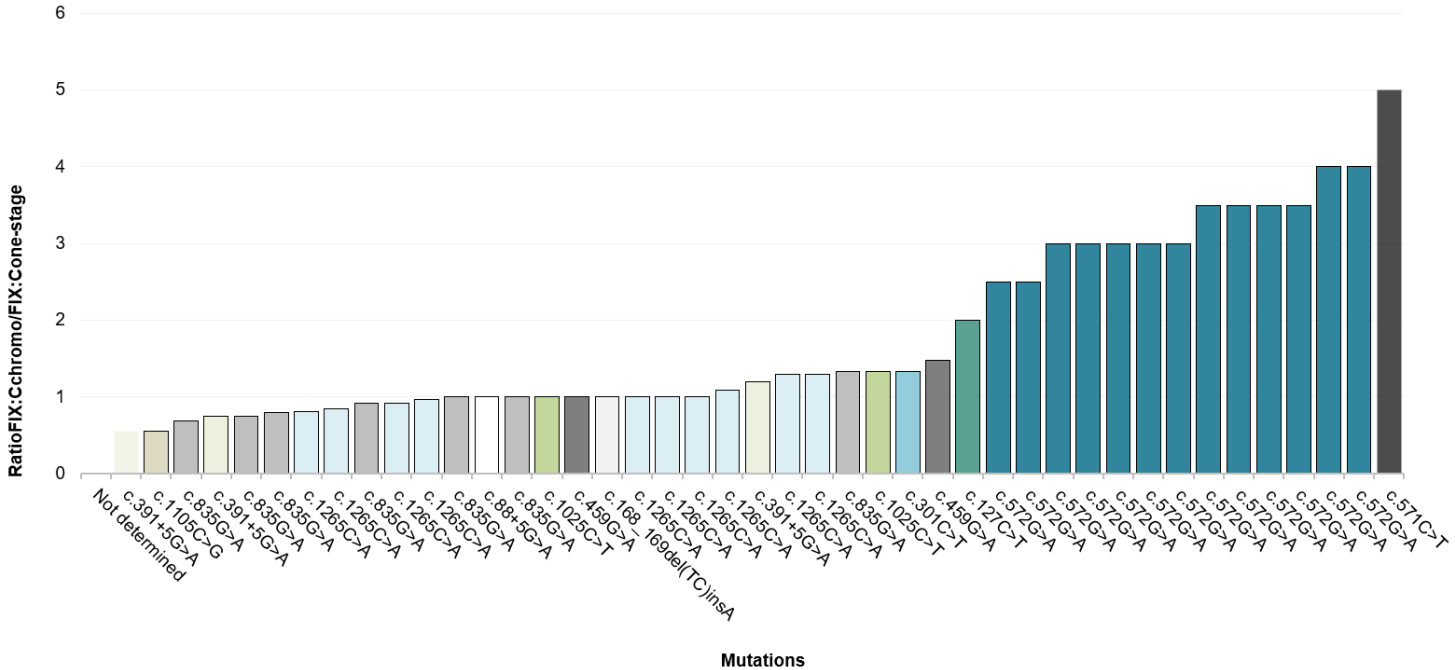


Figure 15. **Ratio $FIX_{chromogenic}/FIX_{one\text{-}stage}$ for samples from non-severe haemophilia patients in the Assay Discrepancy study and their *F9* variant.** One pile colour is designated for each separate F9 variant.

These discrepant findings lead to discussions on which method is 'the best' and gives the 'true' activity level of FIX. The *F9* variant in our cohort associated with assay discrepancy (*F9*: c.572G>A; p.Arg191His) was primarily reported in the Factor IX Gene (*F9*) Variant Database to give rise to mild-to-moderate disease severity. To investigate the bleeding phenotype further in the persons showing assay discrepancy, a careful review of their medical records was carried out. All but one of the patients were treated on-demand with a FIX concentrate. The remaining patient was on prophylaxis with replacement therapy: this is a young patient who had never had spontaneous bleedings, but was treated with prophylaxis before physical activities as a precaution. Out of the six patients, only one had, during the last 15 years, an episode of spontaneous bleeding (haematuria) requiring FIX replacement therapy. Our assessment of the six patients was that they appeared to have bleeding symptoms in concordance with a diagnosis of mild haemophilia, which would suggest that the chromogenic assay is better able to predict the bleeding phenotype in these cases. An apparent limitation to this deduction is that the finding was based on retrospective data, and the number of individuals was small. In addition, and as described earlier in the 'Introduction' section in this thesis, it has also been described previously that the bleeding phenotype can differ between persons with the same FIX activity level and no assay discrepancy.[27] Therefore, no conclusions on this matter can be drawn.

The HB population in Sweden has been investigated previously with the use of haplotyping. We found that three of the four families with the variant *F9*: c.572G>A; p.Arg191His had been included in this study and were shown to be identical by descent.[145] The relevance of this to our findings is, however, unclear.

The *F9* variant c.572G>A; p.Arg191His, discussed above, which is present in the individuals from whom 13 of the 15 discrepant samples were taken, is located at the N-terminal cleaving site of the activation peptide. One of the two remaining discrepant samples was also associated with a *F9* variant at the same location: the N-terminal cleaving site of the activation peptide (*F9*: c.571C>T; p.Arg191Cys). The ratio for this plasma sample was 5.0: the patient was taking on-demand treatment with a FIX replacement concentrate, but had due to epistaxis previously been on short-term prophylaxis. The last case of assay discrepancy was associated with FIX levels of 0.01 IU/mL from the FIX one-stage assay and 0.02 IU/mL from the FIX chromogenic assay and the patient had an *F9* variant in the propeptide (*F9*: c.127C>T; p.Arg43Trp). In Figure 16, the domain structure of FIX is shown with the *F9* variants marked, and the variants associated with discrepant assay results are highlighted.

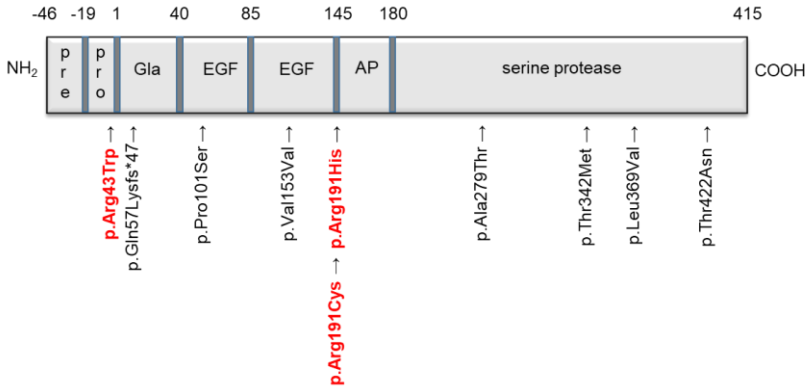


Figure 16. The domain structure of the FIX zymogen with the *F9* variants identified in the Assay Discrepancy study marked. The variants associated with discrepant assay results are highlighted in red. Variants not shown in the figure: *F9* c.391+5G>A; p.N/A, *F9*:c.88+5G>A; p.N/A.

Influence of FIX activation on assay discrepancy?

Since two *F9* variants located at the N-terminal cleaving site of the activation peptide were associated with assay discrepancy in seven different persons, but not in any persons not showing assay discrepancy, we speculated on the possibility that the activation process of FIX influences the results, leading to an assay discrepancy. This led us to investigate this theory further.

Different reagents with different compositions and activity can be used with the one-stage method and it has been reported that the results of FIX measurements in the presence of N-glycoPEGylated recombinant FIX concentrate is reagent-dependent in the one-stage assay.[146] Therefore, we analysed the FIX one-stage assay activity further with two different activators to investigate whether this had any relevance to our result. Plasma samples from five PwHB – three with the *F9*: c.572G>A; p.Arg191His variant and assay discrepancy, and two persons without assay discrepancy – were analysed with both the PTT-Automat reagent (silica activator) and the Actin FSL reagent (ellagic acid activator). However, no significant difference in results could be seen, which indicates that the discrepancies that occured in our cohort were not reagent dependent. Additional reagents to the ones we evaluated do exist; however, we do not consider that the reagent is likely to be the explanation for our findings.

Furthermore, we conducted manual tests for FIX activation kinetics, as described in the 'Methods' section of this thesis. We could thereby confirm the discrepant findings, but the activation kinetics were similar for the patients with and without assay discrepancy. Hence, we could not find an explanation for the discrepant results in the activation process, and the mechanism underlying the described assay discrepancy remains unclear and requires further evaluation.

Aspects of FIX activity assays

Considering the importance of the FIX analyses in diagnosing and classifying PwH, it is of great importance that we know and understand the methods used, their possible interferences and how to interpret the results.

Seven PwHB in our study had been at risk of being classified with different disease severities, i.e. mild or moderate haemophilia, if only one of the two FIX activity methods had been used at a certain time. The factor activity level is not the only consideration that a physician needs to take into account when devising the treatment plan for a PwHB, but it is an important and large piece of the puzzle. In case of trauma or prior to surgery, it is important not to underestimate the risk of bleeding, as well as to be able to trust that the methods will show reliable results, in order to design a well-balanced treatment regimen for the patient. An assay discrepancy can mean that some individuals with haemophilia will not be diagnosed with haemophilia if only one of the methods is used or, on the contrary, will be diagnosed as having haemophilia but might, in fact, have a normal FIX activity. Additionally, many young patients with moderate haemophilia with a residual factor activity of 1-2% and an active lifestyle, who live in areas with sufficient financial opportunities, are today considered for prophylaxis with factor products. However, prophylaxis is seldom given to persons classified as having mild haemophilia, and this further illustrates the importance of a correct disease severity classification.

As described in the 'Methods' section of this thesis, the one-stage assay measures the ability of a plasma sample to correct the clotting defect of FIX-deficient plasma, whereas the chromogenic assay measures the ability of FIX in the plasma sample to act as a cofactor in the activation of FX. Historically, the chromogenic method has been seen as the technically more complex assay to perform and more difficult to automate, compared to the one-stage assay.[147] The FIX chromogenic assay has not been available until recently, and is not used as widely as the chromogenic method for FVIII. The claimed higher cost for the chromogenic assay has also contributed to a lower use for this method; however, this may depend upon how use of the assay is implemented. In 2015, Kitchen *et al.*[35] reported from a survey of 30 laboratory scientists in seven countries that the one-stage assay for FIX activity was used in 88% of the included centres, but only 11% reported any use of the FIX chromogenic method.

Interference from lupus anticoagulant, heparin and other anticoagulants can affect the results in the one-stage assay. The chromogenic assay has the advantage of high dilution of the clotting factors in the initial plasma dilution and the inhibitory effect of lupus anticoagulant is diluted out, thus limiting the interference.[122,147] The presence of lupus anticoagulant is a potential factor that could have influenced the results in our study. However, some of the PwHB included in our study had been tested for lupus anticoagulant and did not show any positive results. Furthermore, we consider lupus anticoagulant to be an unlikely explanation for the results, since

the discrepancy was seen concordantly in six different PwHB, all with the same *F9* variant.

The precision of the chromogenic method is often seen as an advantage compared to the one-stage method. The imprecision (CV) in the one-stage assay used in Paper I was 12% at 0.3 IU/mL and 22% at 0.06 IU/mL, compared to the chromogenic assay used which had a CV of 10% at level 0.3 IU/mL and 8% at level 0.06 IU/mL. Furthermore, the one-stage assay requires FIX-deficient plasma for the analysis, as well as an aPTT reagent.

The discrepant findings in this study were consistent in one alternative clotting assay, as well as a manually performed chromogenic assay; however, whether the results will also be consistent in an alternative automated chromogenic assay has not been evaluated, since we did not have access to this method in our laboratory.

Assay influence on inhibitor testing?

In testing for inhibitors, the FIX activity is measured after the patient's plasma sample is mixed with normal plasma. This testing can be performed using either a one-stage or chromogenic assay. In HA it has been shown that lupus anticoagulant can influence the result and mimic FVIII inhibitors in clot-based assays, but also that a false-positive test result with inhibitor titres of 0.5-1.9 BU might occur in as many as 26% of PwHA, if the one-stage method for FVIII activity is used in the Bethesda assay.[148] This issue is outside the scope of our study but is, of course, of interest also in HB; to my knowledge, the two methods have not been evaluated head-to-head in inhibitor testing in HB.

Assay discrepancy following replacement therapy and gene therapy

The work in Paper I was conducted on plasma samples taken after a wash-out period of at least 7 days after treatment with SHL factor products. This was done to ensure that the FIX level measured was not affected by replacement therapy. The work was designed in this way in order to study the patient's baseline FIX values and not assay discrepancies following replacement therapy. However, the new modified FIX replacement products have brought a new dimension to the assay discrepancy discussion, since the modifications made to the FIX molecules to prolong the circulation time have also been shown to influence the functional assays. Discrepancies between the one-stage and the chromogenic assays in measuring FIX concentrate potency have been observed, as well as discordance when different reagents are used in the same type of assay.[149] Assay discrepancies have also been observed in haemophilia patients after being treated with gene therapy.[150,151] However, these aspects of assay discrepancy are not within the scope of this thesis and will not be discussed further here.

Severe haemophilia B in the Nordic countries and comparisons to haemophilia A – The B-NORD study (Papers II-IV)

Clinical characteristics of the B-NORD study cohort

In the B-NORD study, 79 persons with severe HB, 1-75 years of age, were enrolled from six Nordic HTCs. Median age was 30 years (Q1-Q3: 19-53 years) and 16 (20%) were children under 18 years of age. Current or former inhibitors were present in 12 PwHB (15%). As discussed in the 'Introduction' section of this thesis, the overall incidence of inhibitors in PwHB is often reported to be approximately 3-5%. A level of 15% is, therefore, somewhat higher than expected, and we also saw that all of the inhibitor patients were registered at HTCs in Sweden. Since inhibitors, and particularly the treatment of inhibitors in PwHB, is an area with limited knowledge, this motivated us to look further into our material and this formed the initiation of Paper III. Further clinical characteristics of the B-NORD cohort are presented in Table 3.

Table 3. Enrolment data and clinical characteristics for the B-NORD study cohort.

	HB n=79	HA n=79
Age at enrolment, years, median (Q_1-Q_3)	30 (19-53)	30 (20-53)
BMI, kg/m^2, median (Q_1-Q_3)	25 (22-28)	24 (21-27)
Age at diagnosis, years, median†(Q_1-Q_3)	0 (0-0.8)	1 (0-2)
Family history of haemophilia (%)	37 (47)	39 (49)
Unknown/missing data	5 (6.3)	34 (43)
History of, or current inhibitor (%)	12 (15)	9 (11)‡
Treatment modality (%)		
On demand*	2 (2.5)	1 (1.3)
Prophylaxis	75 (95)	76 (96)
ITI/Bypassing therapy	2 (2.5)	2 (2.5)
Age at start of prophylaxis, years, median§(Q_1-Q_3)	3 (1-16)	3 (2-12)
Previous joint surgery (%)¶	27 (35)	MD
HIV positive (%)	4 (5.1)	3 (3.8)
Unknown/not tested	16 (20)	15 (19)
HCV status (%)		
Never infected (Ab-/PCR-)	37 (47)	29 (37)
HCV positive (Ab+/PCR+)	4 (5.1)	12 (15)
Recovered infection (Ab+/PCR-)	27 (34)	23 (29)
Unknown/not tested	11 (14)	15 (19)

Numbers (%) or median (Q1, first quartile - Q3, third quartile). HB, haemophilia B. HA, haemophilia A. BMI, body mass index. MD, missing data. HCV, hepatitis C virus. HIV, human immunodeficiency virus. *One child, who had never had a joint bleed, currently on FIX on-demand treatment had stopped prophylaxis 7 months before study enrollment and was matched with a patient with HA on prophylaxis. The number of patients (n) was noted if it deviated from the total number: †n =76 (HB), n=65 (HA), ‡n=78, §n=71 (HB), n=51 (HA), ¶n=77.

F9 variants

The *F9* variants were analysed and identified in 64 (81%) of the PwHB in the B-NORD study. Forty-two different variants were found and all but one were classified as 'pathogenic' according to the ACMG classifying system. The remaining *F9* variant was classified as being 'likely pathogenic'. The different *F9* variants are presented in Paper III. Null variants were found in 33 PwHB (42%): of these, nine had a history of inhibitors.

In comparing the *F9* variants in the B-NORD cohort to the variants registered in the Factor IX Gene (*F9*) Variant Database, we found a higher occurrence of severe gene defects, i.e. large structure deletions (prevalence in B-NORD 10%, prevalence in the Factor IX Gene (*F9*) Variant Database 4,8%) and nonsense variants (B-NORD 30%, Factor IX Gene (*F9*) Variant Database 22%). The variant distribution is otherwise largely in agreement with the database (see Figure 17).

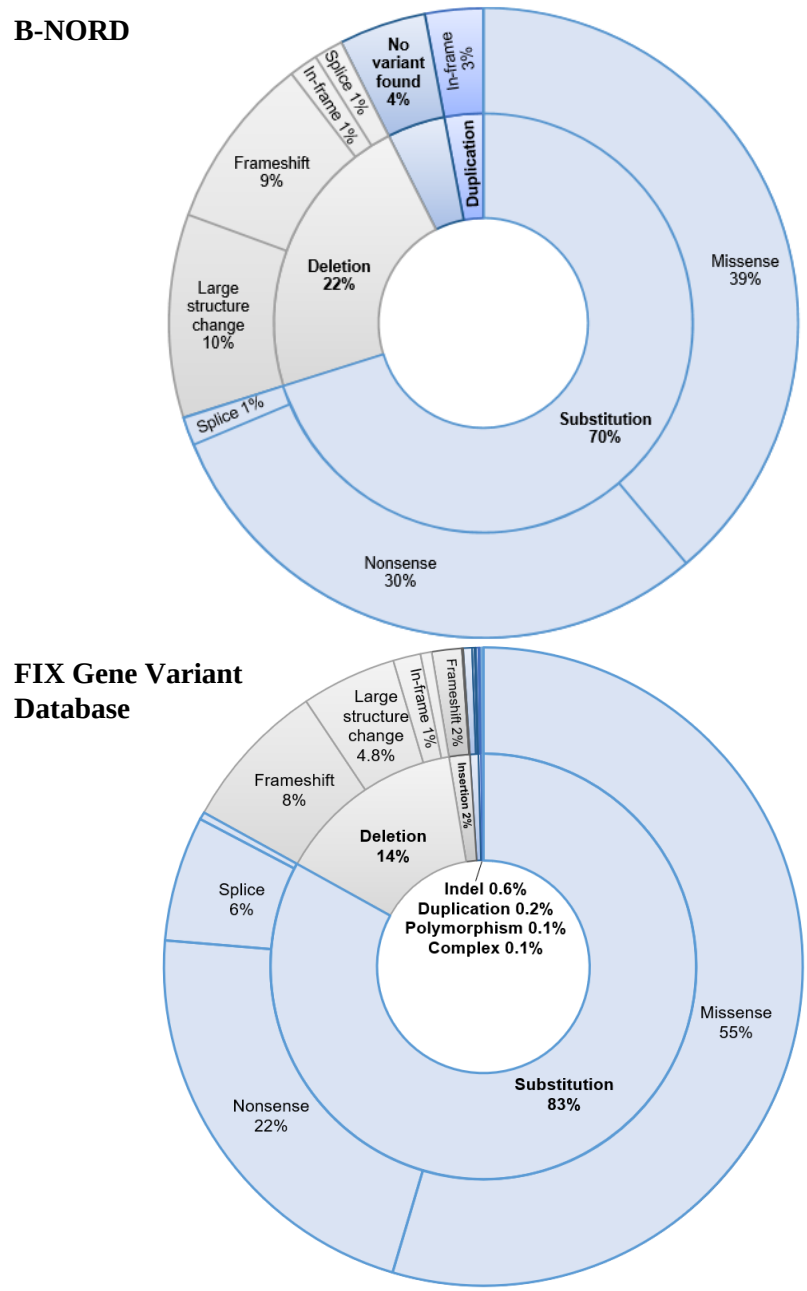


Figure 17. *F9* variants in the B-NORD cohort and in severe haemophilia B in the Factor IX Gene (*F9*) Variant Database. Variant type is presented in the inner circle and variant effect in the outer circle. For comparison, missing data are excluded from the B-NORD cohort.

Treatment characteristics

Seventy-five PwHB (95%) in the B-NORD study were treated with FIX prophylaxis: median age at start of prophylaxis was 3.0 years (Q1-Q3: 1.0-16 years) and this did not differ from the age at start of prophylaxis in the control persons with HA. Recombinant FIX products were given to 70% of PwHB and 27% of these were treated with EHL products. Among the PwHA, 89% were treated with recombinant FVIII products and none with EHL products. As described in the 'Methods' section in this thesis, the period for enrolment of the controls with HA was slightly earlier than that for the PwHB and this is thought to be the explanation for the fact that no PwHA were treated with EHL products. The enrolment of PwHB ended in April 2020. The use of the EHL product Alprolix (eftrenonacog alfa) was approved in Denmark and Norway in 2017, in Sweden in 2018, and in Finland in 2019; Refixia (nonacog beta pegol) was approved in the Nordic countries in 2018; and Idelvion (albutrepenonacog alfa) was approved in 2018, except for in Sweden where it still lacks approval for subsidised use. The majority of HTCs included in the B-NORD study have not implemented a systematic change to EHL products but have, instead, chosen to change the replacement product at the patient's annual routine check-up if deemed to be beneficial to the individual, This strategy may have somewhat delayed the switch to EHL products. The use of EHL products has increased in the Nordic countries since the closure of the B-NORD study and, if the enrolment had begun today, the proportion of both HB and HA patients on EHL products would most likely be greater.

In the B-NORD study, the annual median factor consumption for recombinant SHL products was 3900 IU/kg/year for both PwHA and PwHB. For PwHB on EHL products, this figure was 2000 IU/kg/year (Q1-Q3: 1,500-2400). For PD products, the annual median factor consumption was 2900 IU/kg/year (Q1-Q3: 1600-6000) for FIX products in HB patients and 5000 IU/kg/year (Q1-Q3: 3500-5800) for FVIII products in HA patients. The treatment characteristics are presented further in detail in Table 4.

The WFH defines high-dose prophylaxis to be a median annual factor consumption of >4000 IU/kg per year.[38] Somewhat unexpectedly, the median FIX consumption of the PwHB enrolled in B-NORD on recombinant SHL products was just below this level, meaning that fewer than 50% of these Nordic patients received high-dose prophylaxis. This was also true for PwHB on PD products. However, the very few PwHA on PD products had a factor consumption that was equivalent to that of high-dose prophylaxis. However, the important issue is not the factor consumption *per se*, but whether or not the patient suffered from bleedings and complications to bleedings: this is discussed further and related to factor consumption in the section on 'Bleeding characteristics', below.

Table 4. Treatment characteristics in the B-NORD study.

	HB	HA
Factor concentrate (%)		
Plasma derived	21 (27)	8 (10)
Recombinant	55 (70)	70 (89)
Standard half-life	40	70 (89)
Extended half-life	15	
Bypassing therapy	2 (2.5)	1 (1.3)
Non-factor replacement	1 (1.3)	
Prescribed factor dose IU/kg/dose, median (Q_1-Q_3)		
Plasma derived	28 (22-36)	28 (24-37)
Recombinant		
Standard half-life	38 (27-43)	23 (14-29)
Extended half-life	44 (39-50)	
Annual factor consumption IU/kg/year, median (Q_1-Q_3)		
Plasma derived	2912 (1613-6000)	5005 (3518-5760)
Recombinant		
Standard half-life	3931 (2673-4735)	3910 (2660-4873)
Extended half-life†	2012 (1485-2418)	
Prophylaxis frequency (%)		
Daily	3 (4.0)	11 (15)
Every 2nd day	11 (15)	27 (36)
Every 3-5 days	33 (44)	37 (49)
Weekly	21 (28)	1 (1.3)
Less than weekly	6 (8.0)	

Numbers (%) or median (Q1, first quartile - Q3, third quartile). HB, haemophilia B. HA, haemophilia A.
†In three cases, no further specification than 'less than weekly' was given, so treatment every 10 days was used in the calculation. HB PD products: Immunine, Mononine, NanoFIX, Octanine. HB recombinant SHL products: BeneFIX, Rixubis. HB EHL products: Alprolix, Idelvion, Refixia. HB bypassing therapy: NovoSeven. HB non-factor replacement: Concizumab. HA PD products: Helixate NexGen, Octanate, Wilate. HA recombinant products: Advate, Kogenate, Kovaltry, ReFacto, ReFacto AF. HA bypassing therapy: FEIBA.

The median factor consumption for tolerant PwHB with former inhibitors was 6638 IU/kg/year (Q1-Q3: 4141-10,115). PwHB without inhibitor history had a significantly lower factor consumption of 3406 IU/kg/year (Q1-Q3: 2178-4583) (p = 0.005). This is discussed further below in the section on 'Non-neutralising antibodies'.

The consumption of FIX PD products was 26% lower compared with recombinant SHL FIX concentrates. An explanation for this may be the somewhat different pharmacokinetic properties of the two types of concentrates with recombinant FIX SHL products having a slightly lower recovery than PD products, but with similar

half-lives.[45] Patients treated with EHL FIX products used about half of the amount of FIX concentrate in comparison to those treated with FIX SHL products.

The factor consumption of recombinant SHL products was similar between PwHA and PwHB. However, as discussed previously, the pharmacokinetic properties between replacement therapy with FVIII and FIX products differ in that the recovery is lower for SHL FIX products (0.8-1.0 [IU/dL]/[IU/kg]) compared to that for SHL FVIII products (1.5-2.0 [IU/dl]/[IU/kg]) but, in contrast, the half-life is longer for FIX products (~18 hours) compared to that for FVIII concentrates (~12 hours).[96] The definitions of high- and low-dose prophylaxis by the WFH do not differ between HB and HA, and the lower recovery of FIX products is somewhat compensated for by their longer half-life compared to that of FVIII products; however, the different pharmacokinetic properties between the two products make precise comparisons difficult to make.

Treatment adherence

In evaluating treatment outcomes, it is crucial to also evaluate patient adherence to treatment in order to assess the true results of the therapeutic intervention and relate the outcome to the given treatment. Treatment adherence was evaluated by the questionnaire VERITAS in PwHB. The results indicated good overall adherence, with a median total score for PwHB on prophylaxis of 38 (Q1-Q3: 33-48), and only two PwHB had a total result equal to, or above, the proposed cut-off level that equated to 'non-adherence' (≥ 57).[131] The questionnaire is divided into six subscales and the highest scores, i.e. the least adherent scores, were found in the dimension 'communicate'. When looking only at this category, 36% of the PwHB had a result consistent with 'non-adherence'. This part of the questionnaire evaluates how often the patients contact their HTC for advice, treatment decisions and before surgical procedures. It can be debated as to whether a high score in this category necessarily equates to a lower rate of adherence, since it could also reflect the situation of well-educated patients who are confident in making their own treatment decisions and take responsibility in modifying their treatment themselves in particular situations. The communication with patients, especially younger patients, might benefit from the use of new technologies with information exchange through computers and mobile phone apps for treatment reports, etc. which could make the HTCs more easily accessible by patients.

The median total VERITAS score was somewhat higher in the age group 18-49 years (median score 43, Q1-Q3: 35-50) compared with the younger group <18 years (median score 37, Q1-Q3: 30-39) and older group ≥50 years (median score 33, Q1-Q3: 27-39), see Figure 18. The reason for the lower adherence in this age group is not clear, but it can be speculated that the stress of work and family life might have a negative impact on adherence. A better adherence in children aged <18 years might be the result of parents caring for their child. The results of PwHB on EHL

and SHL products were evaluated and did not show any difference in adherence. A limitation to this evaluation of treatment adherence is that we did not have any comparative VERITAS data from PwHA. However, a report from Miesbach *et al.*, including 397 patients with both HA and HB, shows similar results with a VERITAS-Pro median total score of 34.[152]

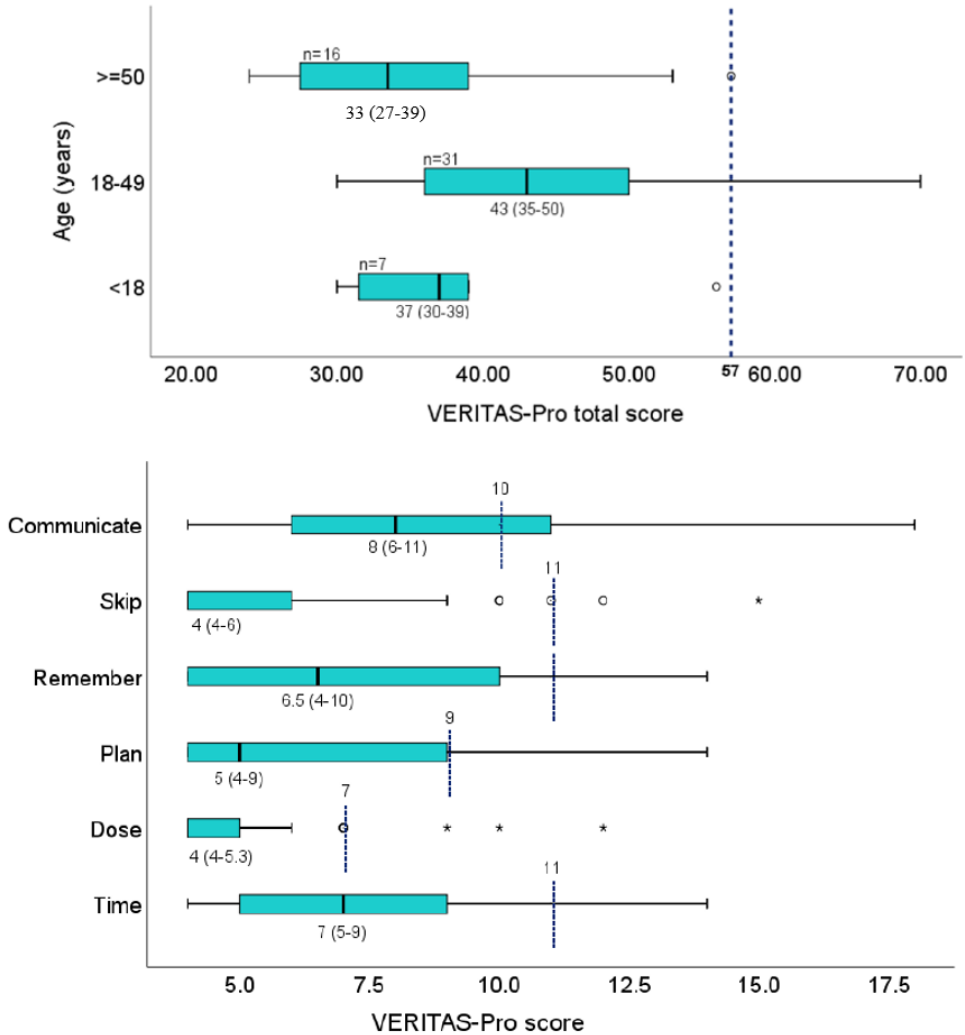


Figure 18. VERITAS scores for haemophilia B patients in the B-NORD study. The numbers represent median scores (Q1-Q3), the vertical dashed lines represent the proposed cut-off values for non-adherence.[131]

Bleeding characteristics

Non-joint bleedings were experienced by 35 (44%) of the PwHB in the prior 12 months before enrolment in the B-NORD study. The median number of joint bleedings for PwHB was 0 (Q1-Q3: 0-1.3, range 0-18); however, 29 PwHB (37%) reported one or more joint bleedings in the prior 12 months. Five of these patients were children under 18 years. This indicates that, despite the majority of PwHB being treated with prophylaxis, the goal of zero bleeds has not been reached in many of the patients.

Five PwHB, all children aged 1-9 years, reported that they had never experienced a joint bleed. Median age at first joint bleed for the remaining PwHB was 2.0 years (Q1-Q3: 1.0-4.0), which is similar to the figure that has been reported previously by the PedNet group (1.2 years) and that by den Uijl *et al.* (2.4 years).[105,106] The joints most affected by bleedings were, as expected, knees, ankles and elbows. In PwHA, it has been reported previously that the knees and ankles are affected more commonly than the elbows. Furthermore, it has been suggested that today, with better and more frequent factor replacement therapy, the ankles have replaced the knees in being the joints most commonly affected by bleeds.[17,153] However, in our small material, we did not see any difference in frequency between bleeding episodes in knees, ankles and elbows.

As described above, the median annual factor consumption for PwHB on recombinant SHL products was just below the definition of high-dose prophylaxis. To evaluate the relationship between factor consumption and bleedings further in these patients, a subgroup analysis on high and low factor consumption was performed and showed no difference in bleeding rate between the groups. We believe this to be the result of successful individualisation of the treatment. Furthermore, PwHB on PD, recombinant SHL and EHL products were evaluated for bleedings, and no difference was seen between the groups. The preserved bleed control but with fewer injections for PwHB on EHL concentrates illustrates the value of these new products to the haemophilia population.

Joint health

HEAD-US

The results from the ultrasound evaluation according to the HEAD-US protocol showed overall low scores in PwHB, with medians of 0 in both elbows (Q1-Q3: 0-5) and knees (Q1-Q3: 0-3) and 1 (Q1-Q3: 0-6) for the ankles. The scores were gathered primarily from observed disease damage (cartilage and bone) and only a minor degree of hypertrophic synovium was seen. The results are presented in Table 5. Unfortunately, no ultrasound examination had been conducted on the controls with HA, and a comparison between results in PwHB and PwHA could not be made.

HJHS

Both PwHB and PwHA were evaluated with the HJHS 2.1. The results showed a significantly lower median total HJHS score among PwHB (median score 4, Q1-Q3: 1.5-21) compared with PwHA (median score 14, Q1-Q3: 2-35) ($p = 0.048$). For a fair comparison, patients with current or former inhibitors, and their matched controls, were excluded from the HJHS calculations. HJHS 2.1 has not been validated for children below 4 years of age and, therefore, they were also excluded from the evaluation (n = 3).

Table 5. Joint outcome assessed by HJHS and HEAD-US in the B-NORD study.

	HJHS, median (Q_1-Q_3)			**HEAD-US, median (Q_1-Q_3)**
	HB n=49	**HA** n=49	***P***	**HB** n=51
Elbow				
Left	0 (0-3)†	0 (0-7.5)¶	0.05	0 (0-3.5) ¶
Right	0 (0-6)†	1 (0-6)	0.14	0 (0-5) ¶
Knee				
Left	1 (0-4)†	1 (0-5.5)	0.47	0 (0-3)*
Right	0.5 (0-2.5)‡	1 (0-6)	0.17	0 (0-4)*
Ankle				
Left	1 (0-4)§	2 (0-6)	0.14	1 (0-6)
Right	1 (0-5)†	1 (0-6)	0.26	1 (0-6)**
Total joint score	4 (1.5-21)	14 (2-35)	0.048	
Global gait score	0 (0-4)†	3 (0-4)	0.34	
Total score	4 (2-26)†	17 (2.5-39)	0.11	
Age (years)***				
<18	1 (0-2.3)	0.5 (0-1.8)	0.65	
18-49	2 (0.3-9.3)	9 (2-22)	0.01	
>50	44 (29-57)	43 (30-50)	0.50	

Median (Q1, first quartile – Q3, third quartile). HB, haemophilia B. HA, haemophilia A. HJHS, Hemophilia Joint Health Score. HEAD-US, Haemophilia Early Arthropathy Detection with Ultrasound.
***HB: Age <18: n=6; 18-49: n=28; >50: n=15. HA: Age <18: n=4; 18-49: n =30; >50: n=15. The number of patients (n) is noted if it deviates from the total number: †n =43, ‡n=42, §n=44, ¶n=49.*n=48, **n= 50.

A subgroup analysis of the cohort divided into age groups of children <18 years, adults 18-49 years, and adults ≥ 50 years, showed that that the difference in HJHS scores between PwHB and PwHA was significant in the group 18-49 years, but not in the groups of persons aged under 18 or above 49 years. The reason for this result is not clear, and data on treatment throughout the life of the study participants has not been taken into account. However, one could speculate that the result indicates earlier development of arthropathy in PwHA, compared with PwHB, and that the difference in arthropathy evens out at older age, or perhaps that prophylaxis is more successful in preventing arthropathy in PwHB. Figure 19 presents the HJHS scores divided by the type of haemophilia and age groups.

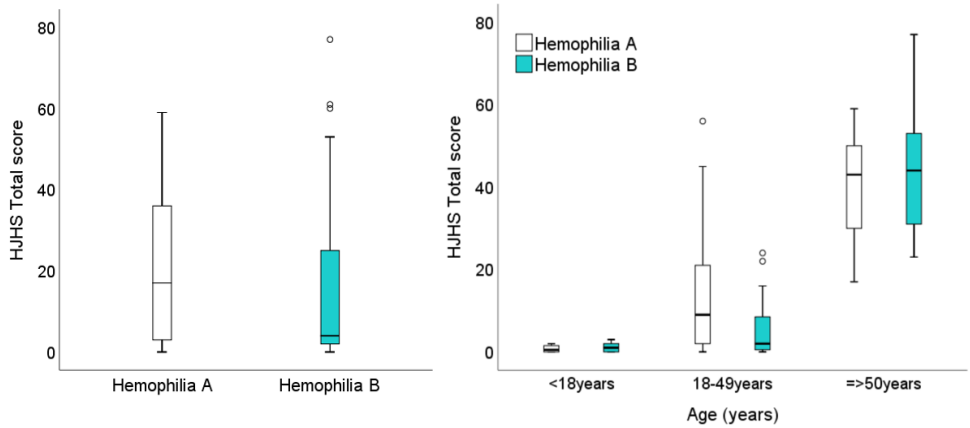


Figure 19. HJHS in haemophilia patients in the B-NORD study divided by type of haemophilia and age group.

Haemophilic arthropathy is a chronic progressive disorder and higher HJHS scores are to be expected with increasing patient age. However, the median scores between the two adult age groups for both HB and HA showed a difference that might be larger than expected. Prophylaxis with replacement therapy was introduced in the 1960s and this method of treatment became more frequent in the Nordic countries during the 1970s. Therefore, it is assumed that PwH in the older age group were treated with on-demand treatment to a greater extent, and started prophylaxis later in life, compared with the younger age group, and this might partly explain the large difference in HJHS scores. An important point is however that the number of PwH enrolled in the older subgroup was small and firm conclusions cannot be drawn.

HJHS is a sensitive instrument for the evaluation of joint health and it can be difficult to interpret small changes in HJHS results and hard to define at what point the score indicates an affected joint. As discussed previously, Sluiter *et al.*[129] reported that 40% of healthy young active adults had HJHS scores of up to 3, so an HJHS score of ≥4 might be a relevant cut-off point to indicate an affected joint. For PwHB in the B-NORD study, a median HJHS score of ≥4 was first reached in the age group ≥50 years whereas, for PwHA, a median score above this level had already been reached in the age group of 18-49 years.

Although we found a difference in HJHS score between PwHB and PwHA, this was explained by a difference in adult PwH and we did not find any difference in HJHS scores between children with HB or those with HA. This is in agreement with a report from the PedNet group in which no difference in bleeding phenotype was observed in young children with severe HA and HB.[105]

The difference we found in HJHS scores indicates better joint health for adult PwHB, compared with PwHA, and is in agreement with several previous reports

suggesting a milder clinical phenotype in PwHB compared to PwHA. This has been discussed above in the 'Introduction' of this thesis under the section '*Haemophilia B – what is the difference to haemophilia A?'*. A difference in joint health between PwHB and PwHA can be speculated to be an effect of lesser treatment intensity for PwHA compared to PwHB. Precise comparisons between treatment in PwHB and PwHA are, as discussed earlier, difficult to make. However, in the B-NORD study, the patients were matched for treatment modality, where the vast majority were on prophylaxis and the factor consumption was, in our estimate, similar between the groups. Therefore, we do not believe the difference in HJHS to be an effect of lesser treatment for PwHA. A limitation to this reasoning is that treatment given previously in life has not been taken into account.

Clinical evidence is limited, but possible explanations for a milder clinical phenotype in PwHB compared to PwHA include:

- *Less severe gene variants.* It has been suggested that the higher prevalence of less severe variants present in PwHB compared to PwHA could be an explanation of a milder bleeding phenotype in HB. A higher frequency of non-null variants in PwHB, with the result of circulating FIX antigen, has been proposed to possibly provide some haemostatic protection.[154]
- *Extravascular FIX*. Unlike FVIII, which resides intravascularly exclusively, FIX also distributes extravascularly, which might provide an explanation for a difference in bleeding phenotype between HA and HB. This is discussed further in this thesis in the section '*Haemophilia B – what is the difference to haemophilia A?'*
- *Presence of thrombophilic variants.* A modulation of the clinical phenotype by the presence of an associated prothrombotic abnormality, such as FV Leiden variants or prothrombin G20210A variants, has been discussed. However, the clinical relevance of this is uncertain and results are conflicting.[95]
- *Anti-inflammatory role of FVIII.* It has been shown that FVIII, independently from the coagulation pathway, has an anti-inflammatory role, but whether this can affect the clinical phenotype in haemophilia patients has not been established.[155]

Joint surgery

A summary of previous joint surgeries in the HB population in the B-NORD study showed that 27 (35%) of PwHB had previously undergone joint surgery, with knee arthroplasty being the most common operation followed by ankle arthrodesis.

FIX neutralising antibodies

Current or former inhibitors to FIX were reported in 12 (15%) of the 79 enrolled PwHB in the B-NORD study. All of these inhibitor patients were registered at the HTCs in Sweden. Clinical characteristics and treatment data of the inhibitor patients are presented in Table 6.

Table 6. Clinical characteristics and treatment data of the inhibitor patients in the B-NORD study.

	Inhibitor patients n=12	**Non-inhibitor patients n=67**
Enrolment country (%)		
Denmark	-	9 (13)
Finland	-	9 (13)
Norway	-	15 (22)
Sweden	12 (100)	34 (51)
Current treatment (%)		
On-demand FIX replacement	-	2 (3.0)
Prophylaxis FIX replacement	8 (67)	65 (97)
Bypass-therapy	2 (17)	-
Non-factor replacement	2 (17)	
Age at start of prophylaxis, years, median (Q_1-Q_3)§	2.7 (1-29)	3.3 (1-16)
Age at inhibitor detection, median (Q_1-Q_3)	2.0 (1.0-8.0)	NA
Allergic manifestation (%)	11 (92)	1 (1.5)
Nephrotic syndrome (%)	3 (25)	-

Numbers (%) or median (Q1, first quartile - Q3, third quartile). NA, not applicable. The number of patients (n) is noted if it deviates from the total number: §n=10(inhibitor), n=60(non-inhibitor)

A prevalence of inhibitors of 15% in PwHB is relatively high, compared to many reports published previously. However, our cohort was restricted to persons with severe HB, and Swedish data published previously have shown similar results to ours,[156] as well as recent results from the subgroup of persons with severe HB in the PedNet Registry.[55] Not all patients with severe HB at the included HTCs were enrolled in our study; however, the prevalence would still be at least 11% if the non-enrolled persons with severe HB were included in the calculations as PwHB not having inhibitors. The occurrence of severe gene defects is relatively high in our study cohort and we believe this to be the main explanation for the relatively high prevalence of inhibitors.

Nine of the 12 inhibitor patients had a null variant (five large deletions, three nonsense, one frameshift) and three had missense variants. Two brothers, both with inhibitors, carried the missense variant *F9* c.316G > A. This is interesting, since this is a missense variant that has been reported 74 times previously in the Factor IX Gene (*F9*) Variant Database, but never before in association with inhibitors. Since both brothers in our study developed inhibitors, this invites us to consider what other factors in their cases could be of importance for inhibitor development. On the other hand, five out of six PwHB in our study with the large structure deletion

g.(?_139530767)_(139562071_?)del developed inhibitors. What factors protected the person with this variant without inhibitors from developing antibodies?

As shown in Figure 20, the frequency of inhibitor development by the *F9* variant effect was 71% in persons with a *F9* large structure change (5/7). Corresponding figures for persons with frameshift variants were 17% (1/6), for nonsense variants 15% (3/20) and for missense variants 12% (3/26). No persons with splice or in-frame *F9* variants developed inhibitors in our cohort.

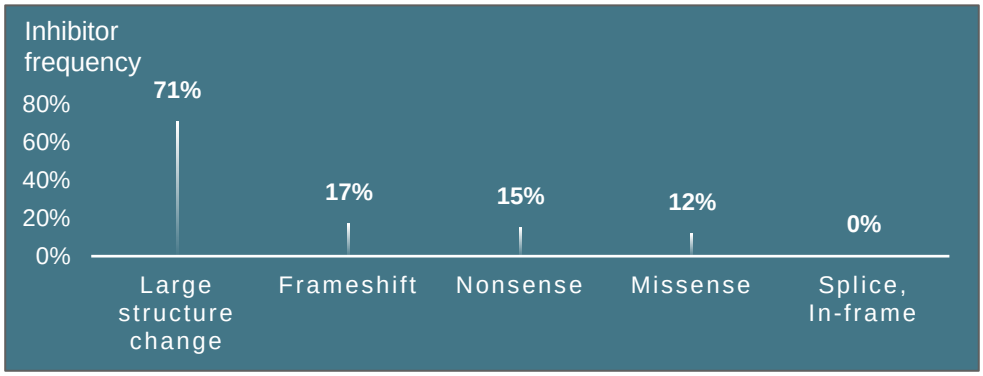


Figure 20. Frequency of inhibitor development by *F9* variant effect in the B-NORD cohort.

The inhibitors in the B-NORD cohort occurred in all cases before 20 exposure days (missing data n = 5) and median age at inhibitor detection was 2.0 years (Q1-Q3: 1.0-8.0). No difference in age at start of prophylaxis was observed between patients with and without inhibitors: median age 2.7 years (Q1-Q3: 1.0-29) and 3.0 years (Q1-Q3: 1.0-16), respectively.

At study enrolment, eight of the 12 inhibitor patients were considered to be tolerant to FIX by their treating physician and they were treated with FIX prophylaxis. One of the remaining four PwHB with inhibitors had on-going ITI treatment, one was treated with prophylactic rFVIIa only, and two were treated with investigational study drugs. All eight PwHB with former inhibitors but now treated with FIX prophylaxis were on SHL products: four were on PD products and four on recombinant concentrates. The median factor dose for these patients was 6638 IU/kg/year (Q1-Q3: 4141-10,115), which was significantly higher than the consumption for those without inhibitor history on SHL products: median dose 3406 IU/kg/year (Q1-Q3: 2178-4583) (p = 0.005).

Allergic reactions and nephrotic syndrome

Eleven out of the 12 (92%) PwHB and inhibitors reported the experience of allergic reactions towards FIX concentrates. The comparative figure in the group of PwHB and no inhibitors was 1 of 67 (1.5%). A figure of 92% is high, compared to previous reports of 60% (the ISTH-SSC International FIX Inhibitor Registry)[54] and 41% (B-

NATURAL study).[157] The *F9* variant profile of our cohort may be an explanation for this higher number of allergic reactions. In five of these inhibitor patients, the allergic reaction was described as anaphylaxis, all of these PwHB had high-titre inhibitors. The remaining PwHB who experienced allergic reactions reported skin rash with/without additional symptoms. The allergic manifestation occurred in six patients after inhibitor detection and in three patients before inhibitor detection. In the remaining two patients, the allergic reaction in relation to inhibitor development was not reported.

Nephrotic syndrome was reported in three (25%) of the PwHB and inhibitors and in none of the patients without inhibitors. In all cases, nephrotic syndrome occurred after inhibitor detection.

Nephrotic syndrome and anaphylaxis were observed mainly in PwHB with null variants; however, they were also reported in one patient with a missense substitution.

Immune tolerance induction

All but one of the inhibitor patients were either treated with ITI treatment at study enrolment or had completed at least one ITI attempt. In total, 22 ITI attempts had been undertaken in these patients over the years. One ITI treatment was ongoing at study enrolment, four (19%) of the completed ITI treatments were considered to be successful, four (19%) were considered to be partially successful and 13 (62%) were considered to be unsuccessful. Eight of 10 PwHB who had completed at least one ITI treatment at study enrolment were considered to be tolerant to FIX by their treating physician and were treated with FIX prophylaxis. This can be translated into a total ITI treatment success rate of 80% in our cohort of PwHB and inhibitors, and indicates that tolerance may be achievable for the majority of HB patients. Four of these tolerant patients had ended their latest ITI treatment with a partially successful result but, over time, with additional long-term FIX treatment, went on to develop tolerance. This is an interesting aspect and indicates that continuous exposure to FIX may induce tolerance with time.

Factors affecting ITI treatment outcome and how they can predict a ‘good’ or ‘bad’ risk for a successful ITI outcome are discussed frequently. Suggested predictors of a ‘good’ risk profile for a successful ITI outcome in PwHA include a historical inhibitor peak titre <200 BU, a titre at ITI treatment start of <10 BU, less than 5 years from the occurrence of the inhibitor to the start of the ITI treatment, and no interruption of the ITI treatment.[158] The type of haemophilia-causing variant has also been shown to have an impact upon ITI treatment outcome. However, experiences from ITI in PwHA cannot be extrapolated to apply to PwHB, and tolerance is harder to achieve in the latter. Data on regimens best applied for a successful ITI treatment outcome in PwHB are also limited.

Factors evaluated in our study that may possibly affect ITI treatment outcome include:

- *Inhibitor titre.* Seven of the inhibitor patients had high-responding inhibitors. Four of these were considered to be tolerant at enrolment, two were not tolerant despite at least one ITI treatment attempt, and one was on on-going ITI therapy. However, all of the successful ITI treatments began with an inhibitor titre of <5 BU/mL. The median inhibitor titre at start for all of the ITI treatment attempts was 2.1 BU/mL (Q1-Q3: 0.93-12). The median titre for 'successful' ITI treatments was 0 BU/mL (Q1-Q3: 0-2.0), that for 'partially successful' ITI treatments was 1.5 BU/mL (Q1-Q3: 0.53-11) and that for 'non successful' ITI treatments was 5.7 BU/mL (Q1-Q3: 1.2-18) ($p = 0.18$).
- *F9 variant.* We could not find any correlation between *F9* variant and ITI treatment outcome in our study cohort and, therefore, could not identify any favourable or unfavourable *F9* variants regarding ITI outcome. The *F9* variants in:
    - Four PwHB who underwent successful ITI were: one large structure deletion, one frameshift deletion, one nonsense substitution, and one missense substitution;
    - Four PwHB for whom ITI was partially successful (but later they were considered to be tolerant) were: one large structure deletion, two nonsense substitutions, and one missense substitution;
    - Two PwHB not tolerant at enrolment were: one large structure deletion, and one missense substitution.
- *Type of factor product.* Only SHL, and no EHL products, were used for ITI treatment in this study population. We could not identify any difference in ITI treatment outcome for PD or recombinant products. Recombinant SHL products were used in two (50%) of the successful, one (25%) of the partially successful and in one (8%) of the unsuccessful ITI attempts.
- *FIX dosing.* We found no difference in dosing of FIX products between successful or non-successful ITI treatment attempts.
- *ITI duration.* No ITI treatment of less than 3 months' duration was considered to be successful. A further indication that ITI treatment should not be stopped too early is the observation that one patient became tolerant after the sixth ITI attempt: this attempt differed from the previous attempts by having a longer duration of 3 months.
- *Immunosuppression.* Immunosuppression was included in seven out of eight (88%) successful or partially successful ITI treatments and in six (46%) of the ITI failures, see Figure 22. Three of the successful/partially

successful ITI attempts included immuno-suppression in line with the Beutel protocol,[59] with a combination of dexamethasone, rituximab, intravenous immunoglobulin (IVIG) and mycophenolate. In the remaining four cases, a combination of cyclophosphamide and IVIG, with the addition of corticosteroids in two cases, was used. In line with our results, several previous reports of ITI treatment in PwHB have shown that the use of immunosuppression can be favourable.[59,62,157,159-164]

- *Allergic reactions.* All but one of the inhibitor patients had reported allergic reactions, but despite this, the majority were considered to be tolerant to FIX at study enrolment. Five PwHB had reported anaphylaxis: of these, three were considered to be tolerant. While allergic manifestations complicate the course of ITI treatment, they are not a definitive predictor of ITI treatment failure.
- *Nephrotic syndrome.* Two out of the three persons with nephrotic syndrome were not considered to be tolerant to FIX at study enrolment.
- *Previous ITI failure.* Out of the four successful ITI treatment attempts, three of the PwHB had previous ITI treatment failures. Out of these, one person had one previous failure, one had two previous failures and one had as many as five previous failures. Out of the four partially successful ITI treatments, two PwHB had previous ITI treatment failures. In total, five PwHB had at least one ITI failure prior to ITI success or partial success. This indicates that tolerance can be attained despite previous ITI treatment failure, which is in concordance with recently published data.[157] These data suggest that it can be valuable to consider more than one ITI treatment attempt in PwHB and inhibitors.

Even though persons with high-titre inhibitors, as well as persons with anaphylaxis and those experiencing nephrotic syndrome, could achieve tolerance, two out of the three persons not tolerant at study enrolment had both high-titre inhibitors and had experienced both anaphylaxis and nephrotic syndrome. The third not tolerant person was receiving on-going ITI treatment. Hence, the combination of high-titre inhibitors, with occurrence of anaphylaxis and nephrotic syndrome, seems to be associated with poor prognosis for tolerance.

Summary of study outcome on factors of potential influence on ITI outcome and tolerance in PwHB:

- PwHB and high-titre inhibitors can achieve tolerance.
- ITI success can be achieved despite previous ITI failures.
- The addition of immunosuppression may enhance the chances of successful ITI treatment.
- The combination of a high-titre inhibitor, occurrence of anaphylaxis and nephrotic syndrome seems to be associated with a poor prognosis for tolerance.
- No favourable or unfavourable *F9* variants for ITI outcome were identified.
- No differences in ITI treatment outcome for PD or recombinant FIX products were identified.
- No ITI treatment with a duration of less than 3 months was considered to be successful.
- Allergic manifestations and nephrotic syndrome complicate the course of an ITI treatment, but are not a definite predictor of ITI failure.

Figure 21. Summary of study outcome on factors of potential influence on ITI outcome and tolerance.

A limitation to our study is that the criteria for ITI treatment success were at the discretion of the treating physician and that no consensus criteria on tolerance were used. In HA, consensus criteria on ITI treatment success have been established and are defined as a negative inhibitor titre (≤ 0.6 BU) and normal FVIII recovery (≥ 66% of predicted), as well as normal FVIII half-life (≥ 6 hours after a 72-hour FVIII washout period).[165] In the same publication, partial success is defined as an inhibitor titre < 5 BU, abnormal FVIII recovery (< 66% of predicted) or FVIII half-life (< 6 hours), but with clinical response to FVIII therapy and no increase in inhibitor titre < 5 BU over 6 months of on-demand or 12 months of prophylactic therapy. Failure is defined as a failure to fulfil the criteria for full or partial success within 33 months, or an inability to achieve a 20% reduction in inhibitor titre during a 6-month period of ITI treatment after the first 3 months. The lack of established criteria and definitions of ITI treatment success and tolerance in PwHB makes comparisons and evaluations of ITI treatment outcome complicated, and there is a need for well-defined established definitions for HB as well, as those that exist for HA.

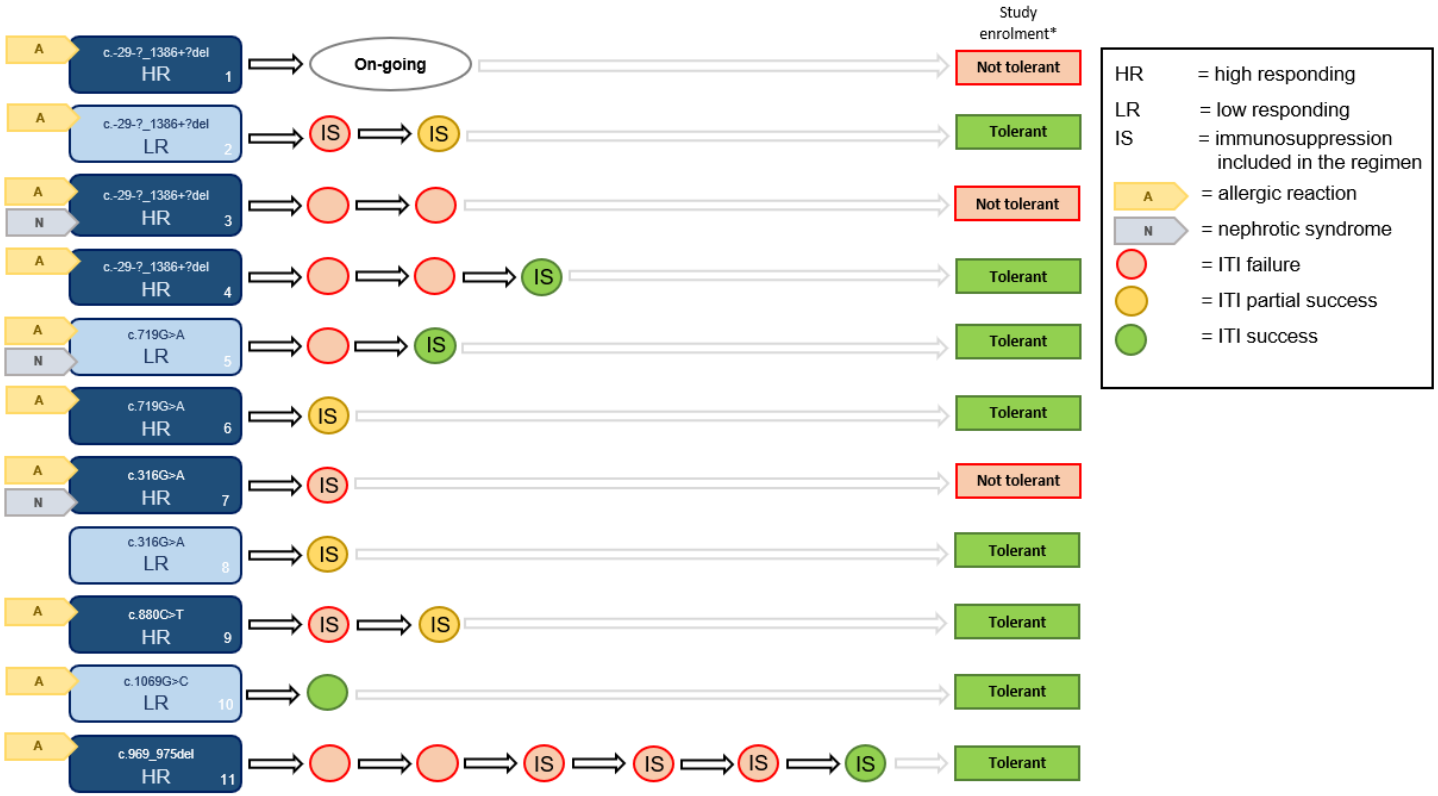


Figure 22. Schematic illustration of ITI attempts in 11 PwHB in the B-NORD study. Each line illustrates the ITI experience of one person and each circle represents an ITI attempt.

The observant reader has noticed that in Paper II 11 PwHB in the B-NORD cohort are reported to have a history of, or current, inhibitors but in Paper III 12 PwHB are reported to be inhibitor patients. When analysing the collected plasma samples for non-neutralising inhibitors for the work in Paper III, a young boy, in the CRF registered as not having inhibitors, with a negative Bethesda result, were found to be positive in both the ELISA and xFLI methods for non-neutralising antibodies as well as in the Bethesda method, conducted at the laboratory in Malmö. The HTC where the child had been enrolled were contacted and it was shown that the plasma samples for the non-inhibitor testing had been collected slightly after enrollment date and that the child two weeks after enrolment had developed inhibitors. We then decided to reclassify the child as an inhibitor patient for the work in Paper III, and collected the additional information on inhibitor circumstances and treatment.

FIX non-neutralising antibodies

The median factor consumption for the tolerised inhibitor patients was observed to be high, well above the level of high-dose prophylaxis and significantly higher than the factor consumption of non-inhibitor patients. This raised the question as to whether this may be the result of NNAs or perhaps small amounts of neutralising antibodies that are undetectable using the Nijmegen-Bethesda assay.

To evaluate the occurrence of NNAs, we gathered plasma samples from 53 (67%) of the PwHB in the B-NORD study, and analysed them using an ELISA method, as well as 48 samples also with an xFLI assay. As seen in Table 7, only two samples were positive in both assays; however, these samples were also positive in the Bethesda assay. In the remaining samples, no consistent findings of NNAs were identified. The concordance between the ELISA and xFLI assays was 87.5%, but some discrepancies were observed. These discrepancies were thought mainly to result from limitations in the ELISA assay with a lack of reproducibility in the low-titre range. The cut-off point in the ELISA method is variable, since it depends on the normal samples in each test run and this is reflected by the high CV (>50%) for the positive control. For this purpose, the ELISA method is in need of further validation, or needs to be replaced with the xFLI method. The clinical significance of NNAs in haemophilia is discussed further in the 'Introduction' section of this thesis.

Table 7. Anti-FIX ELISA and xFLI results.

		ELISA			
		Neg	**Pos**	**MD**	**Total**
xFLI	**Neg**	40	5	0	45
	Pos	1	2†	0	3
	MD	4	1	26	31
	Total	45	8	26	79

MD, missing data.
†Both samples were positive in Bethesda (3 BU/mL and 0.4 BU/mL).

Quality of life

To assess HRQoL, the questionnaire EQ-5D-3L was completed by 126 PwH (63 PwHB and 63 PwHA), between 15 and 76 years of age, without current inhibitors. The results are presented in Paper IV. The degree of employment was similar between the two groups, with 46 (73%) of PwHB and 47 (75%) of PwHA studying or working full or part-time. As reported previously, PwHB had a lower joint score when compared to PwHA in the B-NORD cohort, suggesting a somewhat milder arthropathy. Together with the fact that generally less frequent treatment injections are required in the HB population, we expected a slightly better quality of life in the HB population. However, we found no significant difference in HRQoL between PwHB and PwHA in any of the dimensions in the EQ-5D profile and no difference was seen between the groups in LSS, with a median score of 6 (Q1-Q3: 5-8) in both groups. Furthermore, no difference was observed between the groups in EQ-5D index or EQ VAS values, with mean index scores of 0.80 (SD: 0.17) for PwHB and 0.83 (SD: 0.16) for PwHA (p = 0.24) and mean EQ VAS scores of 70 (SD: 20) for PwHB and 77 (SD: 19) for PwHA (p = 0.061). The profile 11111, corresponding to 'having no problems' in any dimension, was the most frequently occurring profile in both types of haemophilia, being reported by 18 (29%) of PwHB and 24 (38%) of PwHA. However, as many as 46% of PwHB and 44% of PwHA scored either level 2 (some problems) or level 3 (extreme problems) for dimension mobility in the EQ-5D questionnaire. A total of 62% of PwHB and 56% of PwHA reported problems with pain/discomfort, and 33% of PwHB and 17% of PwHA experienced problems with anxiety/depression on the day of the evaluation, see Figure 23. These results are largely in agreement with previously reported data from the B-NATURAL study.[81]

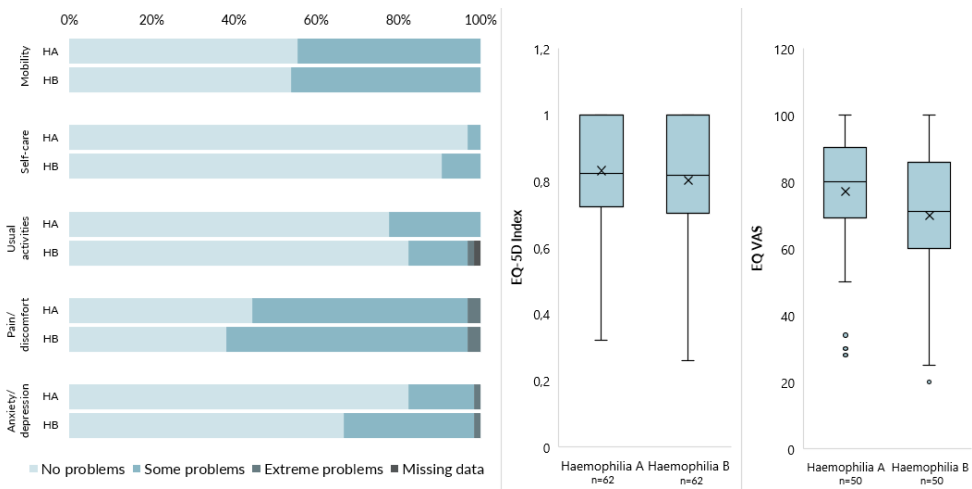


Figure 23. EQ-5D-3L results divided by diagnosis.

In comparison to population norms for EQ-5D-3L from the countries included in the B-NORD study,[166,167] the EQ-5D index values and EQ VAS values were somewhat lower in PwHB; the numbers are presented in Figure 24. The results from the dimensions of mobility, pain and anxiety/depression stood out with a higher frequency of reported problems (levels 2+3) in PwHB compared to those in the general population. However, firm conclusions cannot be drawn from these findings, since different study designs, the use of different value sets and somewhat different mean ages for the cohorts prevent fair comparisons from being made. Altogether, despite the fact that the majority of patients in our study were treated with prophylaxis, impaired quality of life was reported with high frequencies of problems, such as pain, mobility and anxiety/depression, indicating that areas of insufficient care exist.

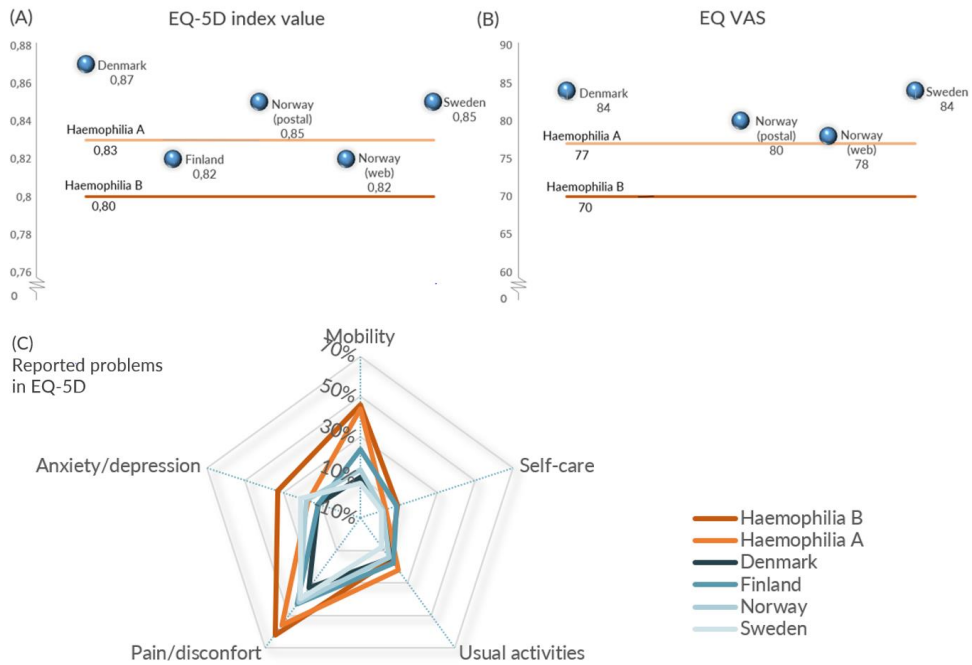


Figure 24. EQ-5D scores for PwH in the B-NORD study in comparison to Nordic population norms.
Population norms from Denmark, Finland and Sweden published by the EuroQol Group,[166] population norms from Norway (postal and web survey) published by Stavem *et al.*[167]. Mean ages (years): Denmark: 47; Finland: 51; Norway: 52 (postal), 51 (web); Sweden: 44.
A) Mean EQ-5D index values.
B) Mean EQ VAS values. No data were found from Finland.
C) Percentages of participants reporting problems (levels 2+3) in the different EQ-5D dimensions. Postal and web survey data from Norway are presented pooled.

The use of analgesics was reported by 56% of the enrolled PwHB. This is in concordance with the number of individuals reporting problems in the dimension of pain. The use of antidepressants or anxiolytics was reported by six (9.5%) PwHB, and only two cases of mental illness – one patient with panic anxiety and one with an unspecified mental illness – were reported. This finding is inconsistent with the fact that 21 (33%) of PwHB reported problems in the dimension anxiety/depression in the EQ-5D questionnaire. Unfortunately, we lack information on non-pharmaceutical treatment for anxiety or depression in our study, but the difference in the self-reported problems in this dimension compared to the use of antidepressants and the reported mental illness in the CRFs/medical records, might reflect the fact that depression and anxiety, to some extent, are unrecognised and undertreated in PwHB at our HTCs. This hypothesis is supported by a publication from the MIND study[85] which included PwHA and PwHB, and showed that out of those who had experienced depression/anxiety, only 24% felt that this was addressed adequately at their HTC. Information on analgesics and antidepressants was incomplete in the KAPPA Registry and was therefore not reported.

In order to be able to interpret changes or differences in HRQoL, the smallest yet clinically meaningful difference or change in the EQ-5D score needs to be identified. Previous studies to define and estimate the minimally important difference (MID) have been conducted, and Luo *et al*. estimated the MID for the UK EQ-5D-3L index score to be 0.082 (SD: 0.032).[168] The sample size in the B-NORD study was not determined, based on a sample size calculation for EQ-5D scores. However, based on the population norm for the UK population,[169] a sample size of 60 in each group would provide a power of 80%, at a significance level of 0.05, to detect a difference in index score equal to the estimated MID of 0.082. We therefore consider a sample size of 63 patients in each group in our study to be acceptable for this evaluation, and for the conclusion to be drawn that no clinical important difference existed between the groups.

Impact of joint health, age, BMI, and extended half-life products on quality of life

Older age was, as to be expected, correlated with a higher HJHS score corresponding to worse joint health (r: 0.76, $p < 0.001$). Both the EQ-5D index and EQ VAS score were significantly associated with the HJHS score when assessed with linear regression adjusted for age. Each increase by one HJHS score point aligned with a 0.003 decrease in the EQ-5D index score (B: -0.003, 95% CI: -0.005 to -0.001, $p = 0.002$) and a 0.37 decrease in the EQ VAS score (B: -0.37, 95% CI: -0.64 to -0.11, $p = 0.007$). This is in agreement with previously published reports suggesting that a decrease in joint health has a negative impact on quality of life in PwH[121,170] and it is not hard to visualise how impaired joint health, with associated mobility limitations and pain, can impact upon a person's HRQoL.

Increasing age was significantly associated with lower EQ-5D index and EQ VAS by the univariate analysis, but did not show significance when adjusted for the HJHS

score, which indicates the importance of joint health in ageing PwH. These results are consistent with the results from Osooli *et al*.,[121] but inconsistent with the data from the B-NATURAL and PROBE-studies.[81,171] However, in these latter studies, no adjustment for joint health was made in the age comparison.

No association was evident between BMI and EQ-5D results (index or VAS) and no difference was observed in the EQ-5D index and EQ VAS values between PwHB using EHL (n = 11) and PwHB treated with SHL products. The less frequent intravenous injections required with EHL products in comparison to SHL products is likely to simplify the everyday lives of PwH and an improvement of quality of life would be imaginable. However, in our study this could not be seen, perhaps as a result of the low number of patients on EHL products included in our cohort.

Conclusions

In this thesis, different aspects of HB have been investigated and evaluated, including diagnostic challenges with a comparison of laboratory assays for FIX analysis, and an evaluation of treatment with replacement therapy, as well as clinical outcomes including bleedings, arthropathy and antibodies to FIX and the assessment of quality of life of PwHB. Joint health and quality of life of PwHB have additionally been compared to those in PwHA.

The main conclusions from the papers included in this thesis are as follows:

Paper I – Diagnostics

- Assay discrepancies between the one-stage and the chromogenic assays, as have been reported previously in HA, also occur in HB. In our cohort, an inverse discrepancy with the chromogenic method presenting with the higher value, was found in one-quarter of the patients with non-severe HB. This discrepancy can be of clinical significance and the use of both the one-stage and the chromogenic methods is of value for the optimal diagnosis and classification of PwHB.
- *F9* variants at the N-terminal site of the activation peptide, as well as in the propeptide, showed assay discrepancies, with a two-fold or greater difference in the results between the one-stage and the chromogenic assays for FIX analysis.

Paper II – Treatment, bleedings and arthropathy

- The Nordic population with severe HB is well managed, with a high frequency of prophylaxis and adherent to individualised treatment regimens but, despite this, the goal of zero bleedings for all has not been achieved.
- Significantly lower HJHS scores in PwHB compared with PwHA suggests that patients with severe HB suffer from milder arthropathy than those with severe HA.

Paper III – Neutralising and non-neutralising antibodies

- A relatively high prevalence of current or former inhibitors – 15% – was seen in the Nordic population of persons with severe HB, but no evidence

of non-neutralising antibodies was found. The high proportion of severe *F9* gene defects found in this PwHB cohort may explain the high prevalence of inhibitors.

- Successful ITI therapy can be achieved in PwHB and inhibitors, despite previous ITI treatment failures, and is independent of the type of *F9* variant.
- Adding immunosuppression to the ITI regimen in PwHB may enhance the chances of therapy success.
- Allergic reactions and nephrotic syndrome complicate ITI treatment, but are not a definite predictor of failure.

Paper IV – Quality of life

- Despite haemophilia being well treated in the Nordic countries, with the majority of patients on prophylactic treatment, quality of life is impaired.
- HRQoL does not differ between persons with severe HA and HB in the Nordic countries.
- Impaired joint health decreases quality of life in persons with haemophilia.
- Depression and anxiety may be unrecognised and undertreated in persons with severe HB.

Clinical implications and future perspectives

Studies focusing on PwHB are limited and, in order to improve our knowledge of the disease and ultimately improve the care of PwHB, there is a need for research studies focused on this population. This thesis is based on studies of PwHB.

Assay discrepancy in HB had, to our knowledge, not been systematically investigated previously. In the Assay Discrepancy study, we found an assay discrepancy in one-quarter of the enrolled patients with non-severe HB. The knowledge of a possible assay discrepancy is critical, since the diagnosis and severity classification of haemophilia are based on the measured factor activity level, and the correct diagnosis and classification are of importance in order not to miss or underestimate a risk of bleeding. The use of only one of the assays in diagnosing or classifying HB might be misleading and the WFH[38] has chosen to mention our findings in their *Guidelines for the Management of Haemophilia*. A recent update on laboratory diagnostics in haemophilia[149] also highlights this issue, not only for HA, but also for HB. However, further studies are needed to investigate assay discrepancy in other study cohorts. At the EAHAD congress in 2021, Kloosterman *et al.*[172] reported their preliminary data on 58 patients with mild or moderate HB, which revealed the occurrence of assay discrepancy in 17% (10/58) of the patients. All discrepant results in this cohort showed, in contrast to our results, a higher factor activity level with the one-stage assay compared to the chromogenic one. Nine of the 10 patients showing discrepant results would have been classified into different disease severities with the different assays. No *F9* variants were reported, but further studies on both the association with phenotype and genotype were reported to be ongoing. Antovic *et al.*[173] reported on 70 tested samples from 40 PwHB, using both the one-stage and the chromogenic assays. Remarkably, in five patients they found an assay discrepancy that was so significant between the two methods that the patients would be classified as having severe disease according to the chromogenic assay, but with moderate or even mild haemophilia B with the one-stage assay. Further, they reported an additional five PwHB with discrepant results, four patients with lower values in the chromogenic assay and one patient with a lower result from the one-stage assay. No *F9* variants were reported. Further evaluations are needed, but these reports support our conclusion that both the one-stage and the chromogenic assays are of value for the correct diagnosis and

classification of HB. We found *F9* variants at the N-terminal site of the activation peptide, as well as in the propeptide, to be associated with assay discrepancy; however, the mechanism by which these variants influence the assays remains unclear and requires further research.

In the B-NORD study, we found the Nordic HB population to be well managed, with the majority of patients using prophylactic replacement therapy, and data supporting the finding that the enrolled persons were adherent to their treatment. However, despite these observations, a large proportion of the PwHB experienced bleedings, arthropathy and an impaired quality of life. These results underline the importance to continue striving towards a better understanding of the disease, and together with improved and novel therapies, find a more optimised treatment for each individual. Furthermore, we found data indicating that depression and anxiety might be unrecognised and undertreated in PwHB. This information is of great value, and an increased awareness of this among the staff working at the HTCs could result in a greater responsiveness and identification of patients in need of support.

Inhibitors in PwHB bring great challenges: the published data are sparse and treatment guidelines are limited. Our hope is that the work in Paper III will help physicians who find themselves facing these difficult situations and need support in deciding how to best treat their patients. Clinical implications of our research, such as the finding that adding immunosuppression to the ITI treatment regimen may enhance the chances of success, and the knowledge that a second ITI treatment attempt can be successful despite previous failures, might be of value in these situations. With the novel non-replacement therapies in the pipeline, the value and future of ITI treatment is being discussed. However, in HB there is still no non-replacement therapy available outside of clinical trials, and in order to be able to offer any treatment and, in the future, maybe even enable gene therapy, the desirable goal in the majority of cases is still a patient who is tolerant to FIX.

The recent advances in the treatment of haemophilia and the novel therapies in the pipeline may bring about major changes and improvements in the future care of haemophilia patients, but this also requires a better understanding of the disease in order to enable improved individualisation of treatment. This thesis contributes to the overall knowledge of diagnostics, the natural history, complications, and treatment of HB.

Populärvetenskaplig sammanfattning

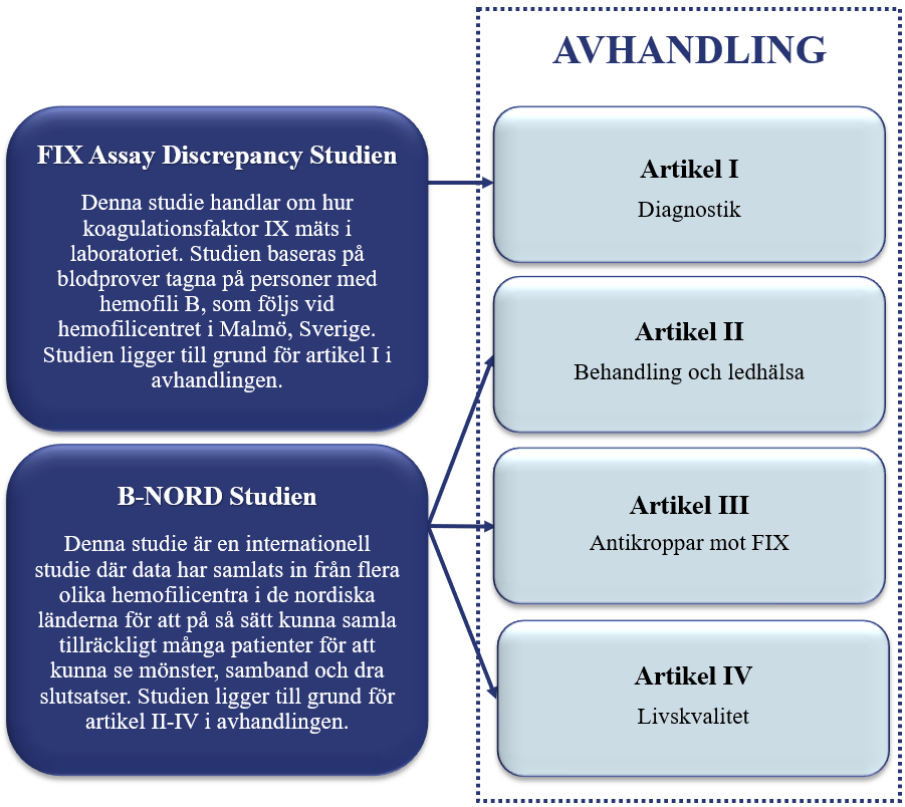
Hemofili B är en sällsynt medfödd blödarsjuka som ärvs via X-kromosomen, det vill säga kvinnor fungerar som bärare av sjukdomen medan i stort sett enbart pojkar drabbas av sjukdomen i dess svåraste former. Uppskattningsvis 1 på 20 000 pojkar föds med sjukdomen. Sjukdomen beror på brist på ett äggviteämne kallat koagulationsfaktor IX (FIX). Bristen uppstår till följd utav en mutation i faktor IX-genen. FIX behövs för blodets levringsförmåga (koagulation) och en brist på äggviteämnet leder till en ökad blödningsbenägenhet. Sjukdomen delas in i tre olika svårighetsgrader: mild, moderat och svår. Indelningen görs beroende på hur stor bristen på äggviteämnet är, där personer med svår hemofili helt saknar äggviteämnet. Blödningar hos obehandlade personer med hemofili är mycket svårstoppade, ett mindre sår kan vara förenligt med livsfara och personerna löper ständig risk för smärtsamma, till synes spontana, inre blödningar där framför allt ledblödningar är vanliga. Upprepade ledblödningar ger skador på lederna vilket kan leda till svårigheter med att gå och röra sig. Idag kan vi behandla hemofili B genom att ge den drabbade en medicin som innehåller det saknade äggviteämnet. Denna medicin finns idag enbart i form av en injektionsvätska, det vill säga personen måste få hjälp med, eller själv injicera medicinen in i ett blodkärl, för att den ska fungera. För att inte ständigt riskera spontana blödningar behöver personer med hemofili i förebyggande syfte regelbundet injicera sig med medicinen. Även om förebyggande medicinering ges krävs i händelse av en operation eller olycka, att ytterligare medicin ges omedelbart, för att en blödning ska upphöra. En del personer med hemofili utvecklar hämmande antikroppar mot sin medicinering. Antikroppar är äggviteämnen som bildas av kroppen som ett försvar mot ämnen som för kroppen ses som främmande och potentiellt skadliga, så som till exempel virus och bakterier, och antikropparna hjälper till att eliminera dessa ämnen från kroppen. När hämmande antikroppar bildas mot medicineringen blir denna i stort verkningslös och leder till en komplicerad situation för den drabbade där andra typer av behandlingar krävs för blödningskontroll och för att bli av med antikropparna.

Hemofili B har mycket gemensamt med den vanligare formen av blödarsjuka, hemofili A, men det finns också viktiga skillnader. Det finns rapporter att hemofili B skulle vara en mildare sjukdom än hemofili A och att förebyggande medicinering globalt används i mindre utsträckning för hemofili B. Andra rapporter menar dock att det inte är någon skillnad i svårighetsgrad mellan de två sjukdomarna. Förekomsten av hämmande antikroppar är vanligare i hemofili A jämfört med

hemofili B men mer svårbehandlat hos personer med hemofili B eftersom sådana antikroppar i hemofili B kan vara förenat med allergiska reaktioner och njurskador. Eftersom hemofili B är en ovanlig sjukdom är det svårt att få ihop tillräckligt med personer med sjukdomen för att samla den mängd information som behövs för att säkert kunna dra slutsatser. Få studier är gjorda med hemofili B patienter i fokus och historiskt sett har många studier inom hemofili främst inkluderat hemofili A-patienter eftersom de är fler till antalet och mycket av vår kunskap och behandlingsstrategier kommer därför från studier där hemofili B patienter varit i minoritet.

Målet med denna avhandling är att med fokus på personer med hemofili B, karaktärisera och belysa hemofili B sjukdomen vad gäller dess diagnostiska utmaningar, behandling, konsekvenser i form utav blödningar, ledskador och antikroppsutveckling, samt de drabbades livskvalitet. Ett ytterligare mål är att jämföra några av dessa karakteristika med hemofili A för att utvärdera eventuella skillnader mellan sjukdomarna.

Avhandlingen bygger på två studier; Assay Discrepancy studien och B-NORD studien.



Artikel I – Diagnostik. Det finns idag två huvudsakliga metoder för att mäta koagulationsfaktor IX-nivån i blodprover; enstegsmetoden och den kromogena metoden. Det har tidigare beskrivits att cirka en tredjedel av personer med hemofili A uppvisar olika faktornivåer i de två analysmetoderna. Detta kan påverka om en patient får diagnosen hemofili, vilken svårighetsgrad av sjukdomen patienten bedöms ha och hur mycket behandling som planeras att ges vid skada eller operation. Om denna skillnad även föreligger vid hemofili B har inte tidigare undersökts. Målet med denna studie var att jämföra analysresultaten av de två metoderna på blodprover tagna på personer med hemofili B för att undersöka om den skillnad i analysresultat som ses vid hemofili A också föreligger inom hemofili B. Femtio blodprover från 36 patienter analyserades med båda metoderna. Ingen skillnad i analysresultat sågs bland personer med den svåra formen av sjukdomen. Bland de 44 prover från personer med mild och moderat sjukdom uppvisade 15 prover från åtta patienter, det vill säga en fjärdedel av de inkluderade patienterna, en dubbelt så stor eller större skillnad mellan resultatet av de två metoderna. Flera av de här patienterna skulle ha diagnostiserats med olika svårighetsgrader av sjukdomen om bara den ena metoden använts. Fjorton av de här proverna kom från sju individer som alla hade sjukdomsorsakande genetiska mutationer på samma ställe på FIX genen. Mutationer på detta ställe sågs inte hos några av patienterna som inte uppvisade en skillnad i analysresultaten. Förekomsten av blödningar hos de här personerna var låg och tyder på att de högre värdena, från den kromogena metoden, var mer rättvisande. Av detta drar vi slutsatsen att skillnader i analysresultat mellan de två metoderna förekommer även vid hemofili B och att båda metoderna behövs för att korrekt diagnostisera och klassificera sjukdomen.

Aritkel II – Behandling och ledhälsa. Denna artikel undersöker behandling och behandlingsutfall med fokus på ledhälsa hos personer med svår hemofili B och jämför detta med kontrollpersoner med hemofili A. Sjuttionio personer med svår hemofili B inkluderades i Sverige, Norge, Danmark och Finland och matchades med lika många kontrollpersoner med svår hemofili A. Information inhämtades från de inkluderade personerna och deras medicinska journaler, en enkätundersökning gjordes för att undersöka hur personerna med hemofili tog sin medicinering i förhållande till sin läkares rekommendation och ledundersökning baserat på ett sjukgymnastiskt poängsystem (Haemophilia Joint Health Score, HJHS) samt en ultraljudsundersökning av lederna enligt ett hemofiliprotokoll (HEAD-US) gjordes. Nästan alla, 95%, av personerna med hemofili B stod på förebyggande faktormedicinering men trots detta rapporterade mer än en tredjedel, 37 %, att de haft minst en ledblödning under det föregående året och 35% hade tidigare genomgått minst en ledoperation. Enbart två personer med hemofili B uppvisade resultat i enkätundersökningen förenligt med att de inte följer sin läkares rekommendationer. Undersökningen med HJHS visade något lägre poäng, förenligt med bättre ledhälsa, för personer med svår hemofili B jämfört med hemofili A. Vi drar av denna studie slutsatsen att personer med hemofili B i Norden är välbehandlade, där den absoluta majoriteten står på förebyggande behandling med

faktormedicin och i stor utsträckning följer sina läkares rekommendationer, men trots detta nås inte målet med blödningsfrihet för alla. Våra studieresultat tyder dessutom på att patienter med svår hemofili B har något mindre ledskador jämfört med personer med svår hemofili A.

Artikel III – Antikroppar mot FIX. Syftet med detta delarbete var att undersöka förekomsten av hämmande, så väl som icke-hämmande antikroppar hos de inkluderade personerna med hemofili B och att utvärdera de behandlingar som gjorts för att bli av med antikropparna. Tolv (15%) av de 79 inkluderade patienterna hade nuvarande eller tidigare haft hämmande antikroppar. Elva, det vill säga 92% av personerna med antikroppar, hade utvecklat allergiska reaktioner i samband med antikroppsutvecklingen och tre (25%) hade utvecklat njurskada. Tio av personerna hade genomgått minst ett behandlingsförsök för att bli av med antikropparna, så kallad immuntoleransinduktion (ITI), och åtta (80%) av dem ansågs fria från antikroppspåverkan vid studiens start. Förutom frekventa doser av faktormedicin var läkemedel med dämpande effekt på kroppens immunförsvar inkluderade i sju av åtta lyckade eller delvis lyckade behandlingsförsök. Fem personer hade minst ett misslyckat behandlingsförsök innan en lyckad eller delvis lyckad behandling. Den sjukdomsorsakande genmutationen hos personerna kartlades och jämfördes med antikroppsutveckling och behandlingsutfall. Undersökningar gjordes även med två olika analysmetoder för att undersöka förekomsten av icke hämmande antikroppar, inga sådana påvisades. Sammanfattningsvis hittade vi en förhållandevis stor andel av patienter med hämmande antikroppar och vår bedömning är att detta beror på en relativt hög andel svåra gendefekter hos de inkluderade patienterna. Ett lyckosamt behandlingsutfall var oberoende av typ av genmutation och kunde uppnås trots allergisk reaktion och tidigare misslyckade behandlingsförsök. Inkludering av immundämpande mediciner i behandlingen kan sannolikt öka chanserna till att behandla bort antikropparna.

Artikel IV – Livskvalitet. Detta delarbete syftar till att undersöka livskvalitet hos personer med svår hemofili B och jämföra detta med livskvalitet hos personer med svår hemofili A. För att undersöka livskvalitet användes ett självskattningsformulär (EQ-5D-3L). Undersökningen visade att 46%, det vill säga nästan hälften av alla inkluderade personer med hemofili B, rapporterade problem med gångförmågan, mer än hälften (62%) rapporterade smärtproblem och en tredjedel (33%) angav besvär med ångest eller nedstämdhet. Vidare visade studien att försämrad ledhälsa, undersökt med HJHS, är associerat med en försämrad livskvalitet. Ingen skillnad sågs i livskvalitet mellan personer med hemofili B och personer med hemofili A.

Sammanfattningsvis bidrar denna avhandling till en ökad kunskap och förståelse för hemofili B sjukdomen och dess innebörd för de personer som lever och kommer att leva med sjukdomen. Ett ökat kunskapsläge ger förutsättningar för en förbättring av vården och tillsammans med nya behandlingsalternativ, som är under utveckling, förhoppningar om en mer individanpassad vård.

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Appendices

- Appendix 1. Haemophilia Joint Health Score 2.1 summary score.
- Appendix 2. HEAD-US scoring method.
- Appendix 3. Sample copy of the VERITAS-Pro questionnaire.
- Appendix 4. Sample copy of the EQ-5D-3L questionnaire.

Appendix 1. Haemophilia Joint Health Score 2.1 summary score.

Reprinted with permission from The Hemophilia Joint Health Score Team, The Hospital for Sick Children, Toronto, Ontario, Canada.

Hemophilia Joint Health Score 2.1 - Summary Score Sheet

	Left Elbow	Right Elbow	Left Knee	Right Knee	Left Ankle	Right Ankle
Swelling	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Duration (swelling)	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Muscle Atrophy	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Crepitus on motion	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Flexion Loss	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Extension Loss	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Joint Pain	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Strength	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE	☐ NE
Joint Total						

Sum of Joint Totals ☐ +

Global Gait Score ☐

(☐ NE included in Gait items)

HJHS Total Score =

NE = Non-Evaluable

Swelling
0 = No swelling
1 = Mild
2 = Moderate
3 = Severe

Duration
0 = No swelling
or < 6 months
1 = ≥ 6 months

Muscle Atrophy
0 = None
1 = Mild
2 = Severe

Crepitus on Motion
0 = None
1 = Mild
2 = Severe

Flexion Loss

	Contralateral:	Normative Tables:
0 =	< 5°	0= within range
1 =	5° - 10°	1 = 1° - 4°
2 =	11° - 20°	2 = 5° - 10°
3 =	> 20°	3 = > 10°

Extension loss (from hyperextension)

	Contralateral:	Normative tables:
0 =	< 5°	0= within range
1 =	5° - 10°	1 = 1° - 4°
2 =	11°- 20°	2 = 5° - 10°
3 =	> 20°	3 = > 10°

Joint Pain
0 = No pain through active range of motion
1 = No pain through active range; only pain on gentle overpressure or palpation
2 = Pain through active range

Strength (Using The Daniels & Worthingham's scale)
Within available ROM
0 = Holds test position against gravity with maximum resistance (gr.5)
1 = Holds test position against gravity with moderate resistance (but breaks with maximal resistance) (gr.4)
2 = Holds test position with minimal resistance (gr. 3+), or holds test position against gravity (gr.3)
3 = Able to partially complete ROM against gravity (gr.3-/2+), or able to move through ROM gravity eliminated (gr.2), or through partial ROM gravity eliminated (gr.2-)
4 = Trace (gr.1) or no muscle contraction (gr.0)
NE = Non-evaluable

Global Gait (walking, stairs, running, hopping on 1 leg)
0 = All skills are within normal limits
1 = One skill is not within normal limits
2 = Two skills are not within normal limits
3 = Three skills are not within normal limits
4 = No skills are within normal limits
NE = Non-evaluable

NOTE: There is an accompanying instruction manual and worksheets that are required when administering the HJHS

General Comments:

Appendix 2. HEAD-US scoring method.
Reprinted from Martinoli *et al*.[44] with permission from Georg Thieme Verlag.

Disease activity (synovitis)	Scale
Hypertrophic synovium	
0. Absent/Minimal	0
1. Mild/Moderate	1
2. Severe	2
Disease damage (articular surfaces)	
Cartilage	
0. Normal	0
1. Echotexture abnormalities, focal partial/full-thickness loss of the articular cartilage involving <25% of the target surface*	1
2. Partial/full-thickness loss of the articular cartilage involving at least ≤50% of the target surface*	2
3. Partial/full-thickness loss of the articular cartilage involving >50% of the target surface*	3
4. Complete cartilage destruction or absent visualization of the articular cartilage on the target bony surface*	4
Bone	
0. Normal	0
1. Mild irregularities of the subchondral bone with/without initial osteophytes around the joint	1
2. Deranged subcondral bone with/without erosions and presence of prominent osteophytes around the joint	2
Note: Elbow: anterior aspect of the distal humeral epiphysis, Knee: femoral trochlea; Ankle: anterior aspect of the talar dome.	

Appendix 3. Sample copy of the VERITAS-Pro questionnaire.

Reproduced by permission of Indiana Hemophilia and Thrombosis Center Inc, Indianapolis, USA.

VERITAS-Pro®

Managing hemophilia is a challenging task. The questions below ask about how you manage hemophilia and prophylaxis. We'd like to get an idea of how often you have done each of these things in the **past three months**. There are no right or wrong answers. The most important thing is for you to answer each question as honestly as possible. Please answer each question using the following scale:

Always – all of the time, 100% of the time
Often – most of the time, at least 75% of the time
Sometimes – occasionally, at least 50% of the time
Rarely – not often, 25% of the time
Never – not at all, 0% of the time

Timing

1. I do prophylaxis infusions on the scheduled days.

   Always Often Sometimes Rarely Never

2. I infuse the recommended number of times per week.

   Always Often Sometimes Rarely Never

3. I do prophylaxis infusions in the morning as recommended.

   Always Often Sometimes Rarely Never

4. I do infusions according to the schedule provided by the treatment center.

   Always Often Sometimes Rarely Never

Dosing

5. I use the doctor-recommended dose for infusions.

   Always Often Sometimes Rarely Never

6. I infuse at a lower dose than prescribed.

   Always Often Sometimes Rarely Never

7. I increase or decrease the dose without calling the treatment center.

   Always Often Sometimes Rarely Never

8. I use the correct number of factor boxes to total my recommended dose.

   Always Often Sometimes Rarely Never

Planning

9. I plan ahead so I have enough factor at home.

   Always Often Sometimes Rarely Never

10. I keep close track of how much factor and how many supplies I have at home.

    Always Often Sometimes Rarely Never

11. I run out of factor and supplies before I order more.

Always Often Sometimes Rarely Never

12. I have a system for keeping track of factor and supplies at home.

Always Often Sometimes Rarely Never

Remembering

13. I forget to do prophylaxis infusions.

Always Often Sometimes Rarely Never

14. Remembering to do prophylaxis is difficult.

Always Often Sometimes Rarely Never

15. I remember to infuse on the schedule prescribed by the treatment center.

Always Often Sometimes Rarely Never

16. I miss recommended infusions because I forget about them.

Always Often Sometimes Rarely Never

Skipping

17. I skip prophylaxis infusions.

Always Often Sometimes Rarely Never

18. I choose to infuse less often than prescribed.

Always Often Sometimes Rarely Never

19. If it is inconvenient to infuse, I skip the infusion that day.

Always Often Sometimes Rarely Never

20. I miss recommended infusions because I skip them.

Always Often Sometimes Rarely Never

Communicating

21. I call the treatment center when I have questions about hemophilia or treatment.

Always Often Sometimes Rarely Never

22. I call the treatment center when I have hemophilia-related health concerns or when changes occur.

Always Often Sometimes Rarely Never

23. I make treatment decisions myself rather than calling the hemophilia center.

Always Often Sometimes Rarely Never

24. I call the treatment center before medical interventions, such as dental extractions, colonoscopies, visits to the emergency room, or hospital stays.

Always Often Sometimes Rarely Never

Appendix 4. Sample copy of the EQ-5D-3L questionnaire.

EQ-5D-3L

Under each heading, please tick the ONE box that best describes your health TODAY.

MOBILITY

- I have no problems in walking about ❑
- I have some problems in walking about ❑
- I am confined to bed ❑

SELF-CARE

- I have no problems with self-care ❑
- I have some problems washing or dressing myself ❑
- I am unable to wash or dress myself ❑

USUAL ACTIVITIES *(e.g. work, study, housework, family or leisure activities)*

- I have no problems with performing my usual activities ❑
- I have some problems with performing my usual activities ❑
- I am unable to perform my usual activities ❑

PAIN / DISCOMFORT

- I have no pain or discomfort ❑
- I have moderate pain or discomfort ❑
- I have extreme pain or discomfort ❑

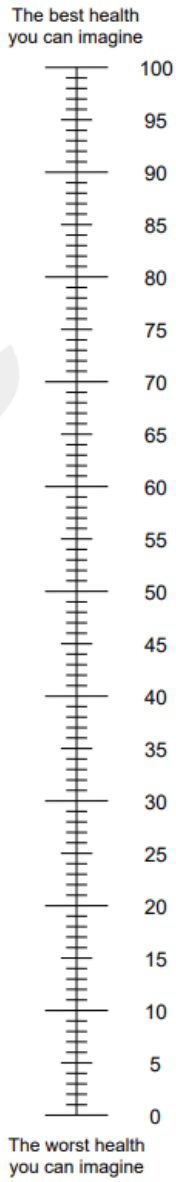
ANXIETY / DEPRESSION

- I am not anxious or depressed ❑
- I am moderately anxious or depressed ❑
- I am extremely anxious or depressed ❑

2

- We would like to know how good or bad your health is TODAY.
- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine.
  0 means the worst health you can imagine.
- Please mark an X on the scale to indicate how your health is TODAY.
- Now, write the number you marked on the scale in the box below.

YOUR HEALTH TODAY = ☐



Paper I

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ORIGINAL ARTICLE

Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B

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Introduction: Assay discrepancy in factor VIII activity between the one-stage and the chromogenic assays has been described in approximately one third of patients with non-severe haemophilia A. Whether assay discrepancy may also occur in patients with haemophilia B remains unknown. **Aim:** This study compared the results from the one-stage and the chromogenic assays in patients with haemophilia B. **Methods:** Plasma samples from patients with haemophilia B attending the haemophilia centre in Malmö, Sweden, were collected after a wash-out period of more than 7 days and analysed with both assays. **Results:** Fifty samples from 36 patients were analysed. No discrepancy was found in patients with severe haemophilia B. Among the 44 plasma samples from patients with non-severe disease, 15 showed a twofold or greater difference between the results of the two methods, with the chromogenic method presenting the higher value (mean FIX:$C_{one\text{-}stage}$ 0.02 vs. FIX:C_{chromo} 0.06 IU mL^{-1}). Of these 15 samples, 14 were from seven individuals from five families with the same mutated amino acid at the N-terminal cleaving site of the activation peptide (FIX: c.572G>A; p.Arg191His or FIX: c.571C>T; p.Arg191Cys). These mutations were not observed in any patients with non-discrepant results. The reported bleeding frequency for these patients was low and indicative of a mild bleeding phenotype. **Conclusion:** Our findings imply that assay discrepancy occurs for factor IX activity and that both type of assays are needed for a correct diagnosis and classification of haemophilia B. The underlying mechanism by which the mutation influences the assays remains to be determined.

Keywords: assay discrepancy, chromogenic assay, coagulation factor IX, haemophilia B, mutations, one-stage assay

Introduction

Haemophilia B is a hereditary recessive X-linked bleeding disorder caused by the deficiency of coagulation factor IX (FIX). FIX is a vitamin K-dependent plasma protein produced in the liver and participates in the blood coagulation by activating factor X. FIX circulates as a zymogen and is activated to a serine protease by factor XIa or factor VIIa in the presence of tissue factor, through cleavage of two peptide bonds at arginine 145 and arginine 180, resulting in the release of an activation peptide [1].

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The diagnosis and severity of haemophilia B are based on the FIX activity (FIX:C) and classified as severe (<0.01 IU mL^{-1}), moderate (0.01–0.05 IU mL^{-1}) and mild (>0.05–<0.40 IU mL^{-1}) [2]. The factor activity can be measured in different ways; the traditional and widely used one-stage clot assay and the more rarely used chromogenic method [3,4].

In approximately one third of patients with non-severe haemophilia A, a discrepancy is seen between the one-stage and the chromogenic assay [4–9]. The one-stage assay most commonly provides a higher result, however, inverse discrepancy has also been reported [3,7,10]. The bleeding phenotype has been shown to better correspond to the two-stage or chromogenic assay than to the one-stage assay in haemophilia A [10,11]. The discrepant assay results have been associated with the causative FVIII gene mutation [5,10].

The chromogenic method for measuring FIX activity has not been available until recently and is therefore

not as widely used as the chromogenic method for factor VIII (FVIII). Signs indicative of assay discrepancy with clinical implications have been seen in haemophilia B but so far not evaluated.

This study assessed and compared the results of the one-stage clotting assay and the chromogenic assay in patients with haemophilia B to investigate whether the discrepancies seen between the methods for FVIII are also present for FIX. An additional aim was to investigate conceivable explanations underlying any observed discrepancies.

Materials and methods

Patient population and data collection

All patients with haemophilia B, registered at the haemophilia treatment centre in Malmö, Sweden, in May 2015, for whom data from the one-stage and the chromogenic assays could be obtained from the same blood sample, were enrolled. Information on treatment, causative mutation and bleeding frequency was retrieved from the study subjects' medical records.

The exclusion criterion was absence of information in the medical records to confirm that the analysed blood sample was taken after a wash-out period of more than 7 days after FIX replacement therapy.

The study has been approved by the regional research ethics committee for southern Sweden.

Factor IX activity assays

Blood was collected in citrated tubes (BD Vacutainer®: Becton Dickinson, Franklin Lakes, New Jersey, US. 4.5 mL, 0.109 M sodium citrate). Samples were immediately centrifuged for 20 min at 2000xg. Plasma was separated and immediately frozen at −70°C. After thawing, each sample was analysed with both the one-stage and chromogenic assay.

FIX:C one-stage assay. The FIX one-stage activity ($FIX:C_{one\text{-}stage}$) was analysed with PTT-Automat-reagent (Diagnostica Stago, Asnieres, France). For five samples, the APTT reagent Actin FSL (Siemens Healthcare AB, Upplands Väsby, Sweden) was also used. The clot reaction was detected in a BCS-XP Coagulation analyser (Siemens Healthcare, Marburg, Germany). FIX-deficient plasma was obtained from George-King (George-King Biomedical Inc, Overland Park, Kansas, US). Detection limit for the method was 0.01 IU mL^{-1}. The imprecision (coefficient of variation, CV%) at level 0.3 IU mL^{-1} was 12% and at 0.06 IU mL^{-1}, 22%. The method is evaluated by participation in ECAT external QA programme. SHP (Siemens Healthcare AB) was used as secondary standard.

FIX:C chromogenic assay. The FIX chromogenic activity ($FIX:C_{chromo}$) was analysed with the chromogenic Rox Factor IX kit (Rossix, Mölndal, Sweden) on the BCS-XP Coagulation analyser (Siemens Healthcare, Marburg, Germany). Detection limit for the method was 0.01 IU mL^{-1}. The imprecision (CV%) at level 0.3 IU mL^{-1} was 10% and at 0.06 IU mL^{-1}, 8%. SHP (Siemens Healthcare AB) was used as secondary standard.

Mutation analysis

The promoter region of the FIX gene and all eight exons with their flanking regions were amplified by PCR using primers as described in Green *et al.* [12]. Mutations were identified by Sanger sequencing. DNA sequencing was performed on a capillary DNA sequencer ABI3130XL or ABI3500XL (Applied Biosystems, Foster City, CA, USA). DNA sequences were aligned to reference sequence from the NCBI-database (NM_000133.3, NG_007994.1) using SEQSCAPE 2.5 or 2.7 software (Applied biosystems, Life Technologies Corporation, Carlsbad, California, USA). Large deletions and duplications were determined by Multiplex Ligation-dependent Probe Amplification (MLPA) using P207-F9 MLPA probe Mix (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's protocol.

FIX activation kinetics

In the one-stage method, the FIX activation kinetics were monitored after the addition of calcium ions as described by Rosén *et al.* [13]. Subsampling was made at different time points and added to a stop solution. Formed FIXa was then determined with the chromogenic kit Rox FIX-A (Rossix). Two independent runs were made on each of the plasmas from three patients with the mutation FIX: c.572G>A; p.Arg191His and on plasma from two patients showing no assay discrepancy (FIX activities of 0.26 and 0.10 IU mL^{-1}). In addition, the FIX activation kinetics were determined on the sample with assigned FIX activity of 0.10 IU mL^{-1} after predilution 1:5 with FIX-deficient plasma to obtain nominally 0.02 IU mL^{-1} and hence an activity similar to the assigned FIX activities with the one-stage method for plasma with the FIX: c.572G>A; p.Arg191His mutation.

In the chromogenic method, FIX activation vs. time was determined using the Rox Factor IX kit. Kit Reagent A (FVIII and FX) and diluted sample were mixed in a deep well plate. At various time points, subsampling of 50 μL was made into different wells of a flat-bottomed microplate preheated to 37°C and containing 150 μL Reagent B (FXIa, prothrombin, phospholipids and $CaCl_2$). FIX activation was allowed

to proceed for 0–12 min followed by simultaneous addition of chromogenic FXa substrate to all wells. The activation was terminated at this step due to inclusion of EDTA in the FXa substrate. The generated FXa activity, reflecting the amount of generated FIXa, was determined through the release of pNA measured at 405 nm. Two independent runs were made on the same plasma samples used for the one-stage method described above.

Statistical analysis

Descriptive statistics are presented. Results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using Microsoft Excel and IBM SPSS Statistics 23 (Armonk, NY, USA). A *P*-value for the disparity between FIX:C outcome was calculated using the Wilcoxon signed-rank test. A *P*-value <0.05 was considered statistically significant.

Results

Eighty-five patients with haemophilia B were enrolled. Of these, 26 were classified as having severe and 59 as non-severe haemophilia B based on the one-stage method. Forty-nine patients were excluded due to insufficient data and wash-out periods (Fig. 1). In total, 50 plasma samples from 36 patients representing 22 families qualified for the study. The samples were collected between 2008 and 2016. Only one of the patients, a patient with severe haemophilia, had a history of inhibitors.

No difference was seen between the results in FIX:C with the one-stage and the chromogenic method in patients with severe disease. These patients were therefore not further evaluated.

Forty-four samples from 32 patients in 18 families with non-severe haemophilia B were analysed with both assays, with mean values of FIX:$C_{one\text{-}stage}$ 0.09 ± 0.09 IU mL^{-1} (range: 0.01–0.35 IU mL^{-1}) and FIX:C_{chromo} 0.11 ± 0.08 IU mL^{-1} (range: <0.01–0.34 IU mL^{-1}), respectively.

The causative mutation was determined in 31 (97%) of the patients with non-severe haemophilia. Twelve different mutations were identified, including eight missense mutations, two splice mutations, one deletion and one silent mutation (Fig. 2). None of the reported mutations have previously been associated with inhibitors. Ten of the mutations were registered in the FIX variant database [14] or the CHBMP F9 Mutation List [15]. Two of the mutations could not be found in the databases (FIX: c.168_169delTCinsA; p.Gln571Lysfs*47 and FIX: c.1105C>G; P.Leu369-Val). The mutation FIX: c.168_169delTCinsA; p.Gln571Lysfs*47 leads to a premature stop codon and would, in a male, cause severe haemophilia. In our material this mutation was present in a female with mild disease. The significance of the mutation FIX: c.1105C>G; P.Leu369Val was evaluated using the prediction software Variant Effect Predictor [16] which reports SIFT and PolyPhen2 scores. The mutation was, respectively, assessed as "deleterious" and "probably damaging".

A detailed description of the patient cohort and individual results is presented in Table 1. The results from the two assays are compared in a Bland–Altman plot as shown in Fig. 3 [17].

To compare the results of the two assays, the FIX:C_{chromo}/FIX:$C_{one\text{-}stage}$ ratio was calculated with a mean of 1.75 ± 1.20 (median 1.15, range: 0–5.00). Results <0.01 were assigned a value of 0 in the calculation. All ratios are presented in Table 1 and Fig. 4. With the exception of one sample (FIX:$C_{one\text{-}stage}$ 0.01 IU mL^{-1} and FIX:C_{chromo} <0.01 IU mL^{-1}), there were no cases of a twofold or greater value from the one-stage assay compared to the chromogenic assay.

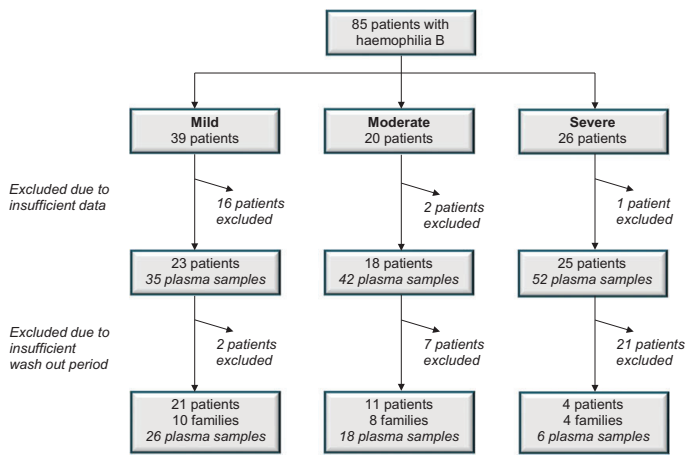

Fig. 1. Patient cohort selection.

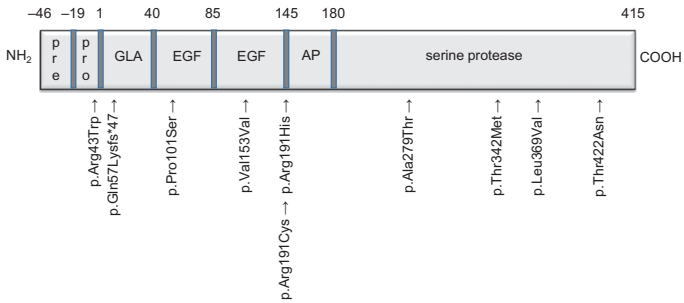

Fig. 2. The domain structure and amino acid positions of the factor IX (FIX) zymogen with the location of the identified mutations in the study population marked. Additional mutations not shown in figure: FIX: c.391+5G>A; p.N/A, FIX: c.88+5G>A; p.N/A.

Fifteen samples from eight patients (25%) showed a twofold or greater difference between the results of the two methods, with the chromogenic method presenting the higher values (mean FIX:$C_{one\text{-}stage}$ 0.02 ± 0.004 and FIX:C_{chromo} 0.06 ± 0.01 IU mL^{-1}). The calculated FIX:C_{chromo}/FIX:$C_{one\text{-}stage}$ ratio is shown in relation to the causative mutation in Fig. 4. Of these 15 discrepant samples, 13 were from six individuals representing four families with the same underlying genotype (FIX: c.572G>A; p.Arg191His), i.e. a mutation located at the N-terminal cleaving site of the activation peptide. This mutation was not observed in any patients with non-discrepant results. All 13 samples showed a result of 0.02 IU mL^{-1} with the one-stage method, whereas the calculated mean with the chromogenic method was 0.06 ± 0.01 IU mL^{-1}, range: 0.05-0.08 IU mL^{-1} (P=0.001). To investigate the six patients' bleeding phenotype, a detailed review of their prior medical records was conducted. Reliable information could be obtained over at least 15 years. The six patients (years of birth 1934, 1962, 1995, 1995, 1999 and 2000) are all, except for one, currently on on-demand treatment with replacement therapy. One of the younger patients is on prophylaxis. He has never had spontaneous bleeding episodes, but has been treated with prophylaxis as a precautionary measure before physical activities. Only one of the six patients has, during the last 15 years, had spontaneous bleeding in need of replacement therapy – one case of haematuria.

One additional discrepant plasma sample was associated with a causative mutation at the N-terminal cleaving site of the activation peptide (FIX: c.571C>T; p.Arg191Cys). The ratio, in this case, was as high as 5.0 (FIX:$C_{one\text{-}stage}$ 0.01 and FIX:C_{chromo} 0.05 IU mL^{-1}). The patient born in 1989 is currently on on-demand treatment, but has been on short-term prophylaxis due to episodes of epistaxis. In the remaining case of assay discordancy, the ratio was 2.0 with FIX levels ranging from 0.01 (FIX:$C_{one\text{-}stage}$) to 0.02 IU mL^{-1} (FIX:C_{chromo}) and the causative mutation located in the propeptide (FIX: c.127C>T; p.Arg43Trp). This mutation has been associated with mild, moderate and severe phenotypes in the FIX variant database.

To determine whether different activators in the one-stage assay had any relevance to the result, the FIX one-stage activity was analysed with both the PTT-Automat- reagent (silica activator) and the Actin-FSL reagent (ellagic acid activator) in five patients, of whom three carry the genotype FIX: c.572G>A; p.Arg191His. The results are shown in Table 2. No significant difference could be identified. In addition, tests for FIX activation kinetics in the one-stage and the chromogenic methods were carried out manually as described in the Methods section. The results are shown in Figs 5 and 6. The discrepant findings were confirmed, but the activation kinetics were similar for all patients.

Discussion and conclusion

Discrepancies between the one-stage and the chromogenic assay, as seen in haemophilia A, appear to also occur in patients with haemophilia B. In our material, patients with non-severe haemophilia B and mutations at the N-terminal site of the activation peptide as well as in the propeptide showed a twofold or greater difference between the results of the two methods, with the chromogenic method presenting the higher values. This corresponded to 25% of our cohort.

The definition of assay discrepancy used for haemophilia A – a twofold or more difference between the results – is common, but not established [10]. Taking the clinical relevance into account, however, we also considered this definition appropriate for haemophilia B. At low levels, i.e. in the range 0.01–0.02 IU mL^{-1}, a ratio of 2.0 can represent an actual difference of only 0.01 IU mL^{-1}. Therefore, the use of a ratio at this level may be misleading. In our case, however, the discrepant findings associated with the same mutated amino acid correspond to higher levels ranging from 0.01 to 0.06 IU mL^{-1}. Regarding the two types of discrepancy, the classic discrepancy in haemophilia A is a lower value for the two-stage or chromogenic assay, whereas in inverse discrepancy, the one-stage assay yields the lower result. Our findings are equivalent with inverse discrepancy. No

Table 1. Description of the cohort and individual results from FIX:$C_{one\text{-}stage}$ and FIX:C_{chromo} for patients with non-severe haemophilia B.

Family	ID	Year of birth	FIX:C one stage (IU mL^{-1})	FIX:C chromogenic (IU mL^{-1})	Ratio (FIX:C chromogenic/ FIX:C one stage)	Mutation		Mutation effect	Domain	No. of patients in the FIX variant database	Severity reported in the FIX variant database
A	1	2004	0.01	<0.01		ND					
B	2	1949	0.09	0.05	0.56	c.1105C>G	p.Leu369Val	Missense	Serine protease	0	–
C	3	1987	0.08	0.06	0.75	c.391+5G>A	p.N/A	Splice		3	Mod
	4	2000	0.09	0.05	0.56						
			0.05	0.06	1.20						
D	5	1961	0.13	0.09	0.69	c.835G>A	p.Ala279Thr	Missense	Serine protease	74	Mi-mod-sev
			0.11	0.11	1.00						
E	6	1951	0.12	0.09	0.75	c.835G>A	p.Ala279Thr	Missense	Serine protease	74	Mi-mod-sev
F	7	1991	0.12	0.11	0.92	c.835G>A	p.Ala279Thr	Missense	Serine protease	74	Mi-mod-sev
			0.10	0.10	1.00						
	8	1997	0.10	0.08	0.80						
			0.06	0.08	1.33						
G	9	1933	0.17	0.22	1.29	c.1265C>A	p.Thr422Asn	Missense	Serine protease	1	Mi
	10	1993	0.11	0.12	1.09						
	11	1940	0.20	0.26	1.30						
	12	1978	0.22	0.22	1.00						
	13	1968	0.33	0.28	0.85						
	14	1970	0.12	0.12	1.00						
	15	2010	0.13	0.13	1.00						
	16	1950	0.13	0.12	0.92						
	17	1967	0.35	0.34	0.97						
			0.26	0.32	1.23						
H	18	1989	0.12	0.12	1.00	c.168_169del (TC)insA	p.Gln57Lysfs *47	Deletion +insertion	GLA	0	–
I	19	1972	0.04	0.04	1.00	c.88+5G>A	p.N/A	Splice		6	Mi-mod-sev
J	20	1989	0.03	0.03	1.00	c.1025C>T	p.Thr342Met	Missense	Serine protease	128	Mod-mi-sev
	21	2000	0.03	0.04	1.33						
K	22	1999	0.06	0.08	1.33	c.301C>T	p.Pro101Ser	Missense	EGF1	1	Mi
L	23	1982	0.19	0.28	1.47	c.459G>A	p.Val153Val	Silent	EGF2	6	Mi
	24	1979	0.27	0.27	1.00						
M	25	1962	0.01	0.02	2.00	c.127C>T	p.Arg43Trp	Missense	Propeptide	65	Mod-sev-mi
N	26	1962	0.02	0.05	2.50	c.572G>A	p.Arg191His	Missense	Linker	85	Mod-mi-sev
			0.02	0.08	4.00						
O	27	1999	0.02	0.05	2.50	c.572G>A	p.Arg191His	Missense	Linker	85	Mod-mi-sev
			0.02	0.06	3.00						
			0.02	0.07	3.50						
	28	1995	0.02	0.06	3.00						
			0.02	0.06	3.00						
			0.02	0.07	3.50						
			0.02	0.07	3.50						
P	29	1995	0.02	0.06	3.00	c.572G>A	p.Arg191His	Missense	Linker	85	Mod-mi-sev
			0.02	0.08	4.00						
	30	2000	0.02*	0.06*	3.00						
Q	31	1934	0.02	0.07	3.50	c.572G>A	p.Arg191His	Missense	Linker	85	Mod-mi-sev
R	32	1989	0.01	0.05	5.00	c.571C>T	p.Arg191Cys	Missense	Linker	58	Mod-sev-mi

Normal range FIX:$C_{one\text{-}stage}$ and FIX:C_{chromo} 0.70–1.30 and 0.80–1.50 IU mL^{-1}, respectively. For subjects with more than one set of results, the data refer to analyses of independently collected plasma samples. Mutations according to the HGVS nomenclature. Severity shown in order of magnitude with the most commonly reported severity first. ND, not determined.

*Plasma from the same venepuncture was analysed on different occasions with the two methods.

consistent findings indicating the presence of a classic discrepancy were observed in our material, and consequently no conclusions as to whether classic discrepancy is of relevance in haemophilia B can be made.

The plasma samples for this study were collected over a period of 8 years. Some of the samples were analysed the same day as they were taken, but some had been frozen and stored over a period of time. A degradation of sample quality is to be considered and cannot fully be excluded. However, the two assays were performed at the same time, and hence the results from each individual should be comparable.

The severities reported among patients previously described with the mutation FIX: c.572G>A; p.Arg191His in the FIX variant database are primarily mild to moderate. The six patients in our material with this genotype would all have been classified with different severities of haemophilia depending on the method used, e.g. mild with the chromogenic method

and moderate with the one-stage method. All six patients appear to have a non-bleeding phenotype, suggesting better prediction by the chromogenic assay.

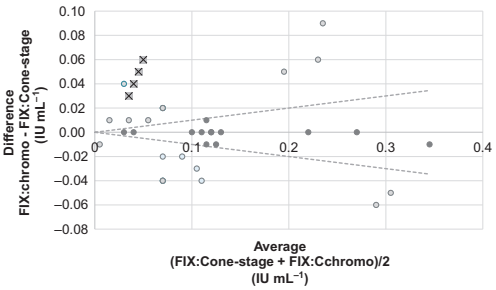

Fig. 3. Bland–Altman plot showing the agreement between FIX:C_{chromo} and FIX:$C_{one\text{-}stage}$ in patients with non-severe haemophilia B. The difference in result between the chromogenic assay and the one-stage assay is shown on the *y*-axis and the average value of the two assays on the *x*-axis. The dotted lines are set at ±10%. Seventy per cent of the samples are located outside of this area and are presented with a lighter colour. The values marked with an *x* are associated with the mutation FIX: c.572G>A; p.Arg191His. The FIX:$C_{one\text{-}stage}$ was analysed with PTT-Automat-reagent.

This is, however, based on retrospective data for a small number of patients and no firm conclusions can be drawn. In addition, it is well-known that the bleeding phenotype among patients with haemophilia with the same factor level and no assay discrepancy may differ.

The one-stage assay measures the ability of plasma to shorten the APTT of haemophilia plasma, whereas the chromogenic method measures the ability of FIX to act as a cofactor for activation of FX. The chromogenic assay has the advantage of high dilution of the clotting factors which limits the interference from lupus anticoagulant (LAC), heparin and other anticoagulants [18]. However, it has not been available until recently and has not been used as widely as the chromogenic method for FVIII. A recent survey in seven countries showed that 68% of the laboratories used the chromogenic assay in haemophilia A compared to only 11% reporting use in haemophilia B [19,20]. The chromogenic method in haemophilia A has, because of its claimed higher cost, been used to a lesser extent worldwide in comparison to the one-stage assay. It is, however, gaining ground because of its specificity and a gradually decreasing cost relative to the one-stage assay.

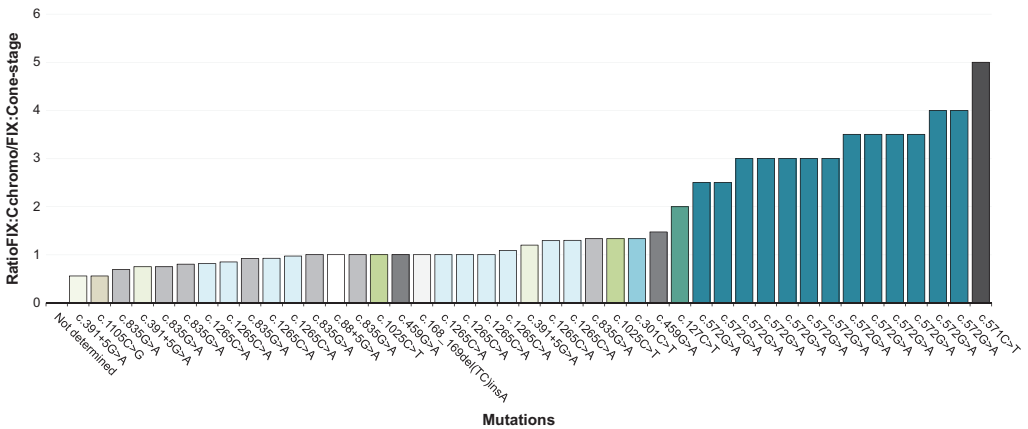

Fig. 4. FIX:C_{chromo}/FIX:$C_{one\text{-}stage}$ ratio for the 44 samples from patients with non-severe haemophilia with the mutations according to the HGVS nomenclature on the *x*-axis. One colour is designated for each separate mutation.

Table 2. FIX one-stage activity analysed with both the PTT-Automat reagent and the Actin-FSL reagent in five patients.

ID	FIX:C one-stage PTT-automat (IU mL^{-1})	FIX:C one-stage Actin FSL (IU mL^{-1})	FIX:C chromogenic (IU mL^{-1})	Ratio (FIX:C chromogenic/FIX:C one-stage) PTT-Automat	Ratio (FIX:C chromogenic/ FIX:C one-stage) Actin FSL	Mutation	
7	0.10	0.11	0.10	1.00	0.91	c.835G>A	p.Ala279Thr
17	0.26	0.21	0.32	1.23	1.52	c.1265C>A	p.Thr422Asn
26	0.02	0.03	0.08	4.00	2.67	c.572G>A	p.Arg191His
28	0.02	0.02	0.07	3.50	3.50	c.572G>A	p.Arg191His
29	0.02	0.03	0.08	4.00	2.67	c.572G>A	p.Arg191His

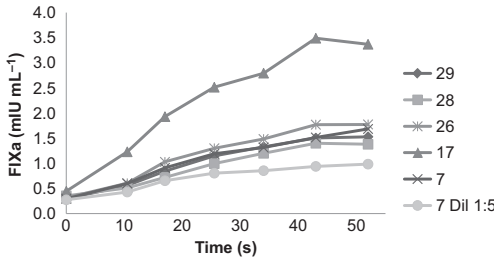

Fig. 5. Factor IX (FIX) activation kinetics in the one-stage method. Mean FIXa from two independent runs in relation to time after addition of calcium ions. Patient numbers 26, 28 and 29 all have the mutation FIX: c.572G>A; p.Arg191His and show assay discrepancy. Numbers 7 and 17 are two control patients showing no assay discrepancy. The activation kinetics were also determined on the sample with assigned FIX activity of 0.10 IU mL^{-1} after predilution of 1:5 with FIX-deficient plasma to obtain nominally 0.02 IU mL^{-1} and hence an activity similar to the assigned FIX activities with the one-stage method for plasma with the FIX: c.572G>A; p.Arg191His mutation.

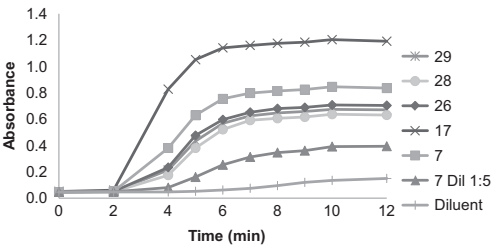

Fig. 6. Factor IX activation kinetics in the chromogenic method. Mean absorbance from two independent runs in relation to time. Patient numbers and samples as described under Fig. 5.

It is likely that certain defects in the FIX molecule may modify assay results in a similar way as for factor VIII. In haemophilia A, several mutations that lead to decreased stability of the active form of FVIII (FVIIIa) have shown to be associated with discrepant results. Inverse discrepancy has been seen in subjects with mutations affecting sites for thrombin cleavage, FIX or VWF binding [6,10,20]. Similarly, our data suggest that a mutation located at the N-terminal cleaving site of the activation peptide of FIX is associated with discrepant results. Interestingly, two different mutations at this location were identified, FIX: c.572G>A; p.Arg191His and FIX: c.571C>T; p.Arg191Cys, both associated with discrepant results. These mutations were not observed in any patients with non-discrepant results. It is reasonable to expect an impact of these mutations on the activation process, but no significant impact on the activation kinetics using different incubation times could be identified. Therefore, the mechanism by which these mutations impede the activation *in vitro* remains unclear and requires further evaluation. This is also true for the potential impact of the mutation in the propeptide.

As various APTT reagents differ in their composition and activity, we evaluated more than one type of reagent with no significant influence on the results. This indicates that the discrepancy is not reagent dependent, which has recently been described to be the case for FIX:C measurement of *N*-glycoPEGylated recombinant FIX [13]. Although several additional reagents with potential impact exist, we consider this an unlikely explanation for our consistent findings. Our findings were consistent in one alternative clotting and a manually performed chromogenic assay, but whether the observed discrepancy will also be consistent in another commercially available automated chromogenic assay has not been settled, as the alternative method has not been used and validated in our laboratory. The presence of LAC is another potential factor that could influence the results. Some of the patients in our study had, however, been tested for LAC with no positive observations. In addition, LAC is an unlikely explanation as the discrepant results were observed in six different individuals all with the same genotype.

Identical mutations carried by apparently independent families may have the same origin, identical by descent (IBD). The Swedish haemophilia B population has previously been subject to investigation with haplotyping [21]. Three of the four families with the mutation FIX: c.572G>A; p.Arg191His (family N, P and Q) were included and have been shown to be IBD. The relevance of this for our findings remains unclear.

In conclusion, assay discrepancy, due to mutations in the N-terminal cleaving site of the activation peptide and in the propeptide, exists in patients with haemophilia B. These findings should be considered in the clinical setting. As is the case for haemophilia A, the use of both the one-stage assay and the chromogenic assay is of value for optimal diagnosis and classification of haemophilia B [22].

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Author contributions

KK, JA and KS designed and performed the research study as well as wrote the paper. SR performed the analyses of the activation kinetics and RL the mutation analyses.

Disclosures

S. Rosén is a consultant for Rossix AB, the manufacturer of the chromogenic assay kits used in this study.

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Paper II

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ORIGINAL ARTICLE

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Treatment outcomes in persons with severe haemophilia B in the Nordic region: The B-NORD study

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Abstract

Introduction: Data on outcome in persons with haemophilia B (PwHB) are limited and mainly extrapolated from studies of haemophilia A (HA).

Aim: To characterize treatment outcomes in persons with severe HB in the Nordic region, with a focus on joint health, compared with matched controls with HA.

Methods: PwHB attending haemophilia centres in Denmark, Finland, Norway and Sweden were enrolled and matched with controls with HA. Joint assessment using Haemophilia Joint Health Score (HJHS) and ultrasound according to Haemophilia Early Arthropathy Detection protocol (HEAD-US) was conducted. Adherence was evaluated using the Validated Haemophilia Regimen Treatment Adherence Scale (VERITAS).

Results: Seventy-nine males with HB, with median age of 30 years (range 1–75), were enrolled. Eleven patients (14%) had a history of or current inhibitor. Twenty-nine PwHB (37%) reported joint bleeds during the prior year, and 35% had previously undergone joint surgery. Ninety-five per cent were on prophylaxis, and 70% used recombinant concentrates, with a median factor consumption of 3,900 IU/kg/year for

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standard half-life products. Only two patients had a VERITAS score corresponding to 'non-adherence'. Joint health, assessed with HJHS, showed a significant lower score among PwHB compared with HA controls, explained by a difference in the 18–49 age group, without observed differences in older or younger subgroups. The HEAD-US scores were overall low.

Conclusion: The Nordic cohort of PwHB is well treated by prophylaxis, but the goal of zero bleeds for all is not reached. Our findings suggest that patients with severe HB suffer from a milder arthropathy than patients with severe HA.

KEYWORDS
adherence, arthropathy, coagulation factor IX, haemophilia B, joint score, phenotype, ultrasound

1 | INTRODUCTION

Haemophilia B (HB) is a rare inherited X-linked bleeding disorder caused by the deficiency of coagulation factor IX (FIX).[1,2] Patients with the severe form of the disease (FIX activity <0.01 IU/mL) suffer from the risk of traumatic and spontaneous bleeding, typically in the joints, causing arthropathy. To prevent bleeding, the use of prophylactic treatment with FIX replacement therapy was introduced in the 1960 s[3] and is still considered the gold standard of care.

There are few reports on treatment and outcome in HB, and when available, HB often constitutes a minor part of a larger cohort, mainly including patients with the more common haemophilia A (HA). Consequently, much of our knowledge and treatment regimens for HB have been extrapolated from studies based on persons with HA (PwHA). HA and HB have historically been considered identical disorders, but there are important differences between the diseases. These include the profile of causative mutations, inhibitor incidence, outcome of immune tolerance induction, treatment complications and differences in clearance and distribution volume of treatment products, with FIX entering the extravascular space.[4-7] It is an ongoing debate whether the phenotypes of HA and HB differ. Reports claiming that the phenotype of HB is milder than that of HA have been published,[8-10] as well as reports of prophylactic treatment being less frequently used in HB.[11,12] However, the data are limited and the findings inconsistent. For example, Clausen et al. found no difference in phenotype in a prospective cohort of children[13] and no difference in bleeding frequency, treatment intensity and/or number of arthroplasties was found at the Van Creveld Clinic.[14]

To better understand HB and improve the care for our patients, studies focusing on persons with HB (PwHB) are of importance, and even more so today with new possibilities of individualized treatment. Extended half-life (EHL) products have recently been introduced, and non-factor products and gene therapy are emerging. Thus, due to the rarity of the disease, multicentre collaborations are needed. The Nordic countries have, through the Nordic Haemophilia Council, a collaborative network aiming to improve and standardize haemophilia care with guidelines and follow-up studies.[15]

The aim of this study was to characterize persons with severe HB in the Nordic countries concerning treatment, bleedings and arthropathy, and to compare their joint health with matched PwHA.

2 | MATERIALS AND METHODS

2.1 | Study design

B-NORD is a multicentre, cross-sectional, observational study conducted in six haemophilia treatment centres (HTCs) in Denmark, Finland, Norway and Sweden. In Norway and Sweden, all haemophilia care is provided by the included centres. The HTC in Copenhagen, caring for approximately half of Denmark's PwHB, was included, as well as the HTC in Helsinki, which covers approximately 60% of Finland's haemophilia population. The data management system was operated at the Center for Thrombosis and Hemostasis Malmö, Sweden.

Ethical approval was obtained from the independent ethics committees in the different countries before enrolment started. The study subject or his legal representative signed an informed consent form before entering the study.

2.2 | Study population

Individuals eligible for inclusion were all males or females, registered at one of the participating centres, with a confirmed diagnosis of congenital severe HB, defined as FIX activity <0.01 IU/mL, in the one-stage or chromogenic assay. Exclusion criteria included concomitant bleeding disorders and the inability to provide informed consent.

Each PwHB was matched by age, gender and treatment modality, to a control person with severe HA from one of the participating Nordic HTCs. The controls were identified in the KAPPA

register,[16] a Web-based international register of PwHA developed by Haemophilia Systems (Munkeby Systems, Malmö, Sweden).

Enrolment of PwHB began in June 2017 and ended in April 2020. The controls were enrolled between October 2013 and December 2017.

2.3 | Study procedures

The study procedure comprised one study visit at enrolment for the PwHB. Data on medical and inhibitor history, including inhibitor response (low-responding <5 BU, high-responding ≥5 BU) and treatment and bleeding episodes over the prior 12 months, were registered. Mainly paper diaries were used. Joint assessment using the Haemophilia Joint Health Score version 2.1 (HJHS)[17] was completed, and ultrasound according to the Haemophilia Early Arthropathy Detection protocol (HEAD-US)[18] was conducted by a physiotherapist or physician within the haemophilia team. The maximum total score for HJHS 2.1 is 124 (worst score possible) with a maximum score of four on global gait and 20 per assessed joint (elbows, knees and ankles). HEAD-US is a validated ultrasound scoring method for elbows, knees and ankles evaluating disease activity (hypertrophic synovium) and disease damage (articular surfaces including cartilage and bone). The maximum score is 8 per joint. Joints with arthroplasties were recorded as missing data. In cases of severe arthropathy and reduced joint mobility preventing optimal ultrasound images, the maximum score was given. If not performed at the study visit, HJHS or HEAD-US results recorded within one year of enrolment were accepted. A target joint was defined as 3 or more bleeding episodes into the same joint in a consecutive three-month period.[19] Since prophylaxis became more frequent in the Nordic countries during the 1970 s, patients above 50 years of age are thought to have been treated with on-demand treatment to a greater extent than younger patients. HJHS was therefore also compared with the cohort divided into three age groups (<18, 18–49 and >49 years).

Treatment adherence was evaluated using the self-/parent-report questionnaire Validated Haemophilia Regimen Treatment Adherence Scale (VERITAS), VERITAS-Pro for patients on prophylaxis and VERITAS-PRN for patients on episodic treatment.[20,21] The questionnaires consist of 24 questions divided into six subscales: time, dose, plan, remember, communicate, and skip (VERITAS-Pro) or treat (VERITAS-PRN). Each answer is assigned a numeric value. The scores are summarized on each subscale and range from 4 ('most adherent'), to 20 ('least adherent'). The subscale scores are summarized to a total score ranging from 24 to 120. A proposed cut-off for 'non-adherence' is set at a score of ≥ 57.[20]

2.4 | Statistical analysis

Descriptive statistics were mainly used. Continuous variables are described using medians and first to third quartiles (Q1-Q3). Categorical data are reported as numbers and percentages. *P*-values for continuous, non-normally distributed variables were calculated using the Mann-Whitney U test when comparing two independent groups and the Kruskal-Wallis test when comparing three or more independent groups. For binary variables, Fisher's exact test and the chi-square test were used. A *p*-value of <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics 25.

3 | RESULTS

3.1 | Patient and treatment characteristics

Out of 108 registered persons with severe HB attending the study centres, 79 (73%) males were enrolled in the study. No females fulfilled the inclusion criteria. Reasons for non-participation were absence from visits at the HTC due to illness, old age or poor compliance (n = 13), a wish not to participate (n = 7), language difficulties or cognitive disabilities (n = 3) or transfer to another HTC (n = 1). Due to local decisions, no ethical approval could be obtained for children in Denmark (n = 5).

The clinical characteristics of the study subjects are provided in Table 1. The median age at enrolment for the PwHB was 30 years (Q1-Q3 19–53, range 1–75). Sixteen patients (20%) were under the age of 18 years. Eleven PwHB (14%) had a history of or current inhibitors, eight with high-responding and three with low-responding inhibitors. All had undergone at least one attempt of immune tolerance induction, and eight were considered tolerant at enrolment. Four patients (5.1%) had human immunodeficiency virus (HIV) infection, and 31 (39%) had a current or recovered hepatitis C infection. Seventy-five subjects (95%) were on prophylactic treatment, and the median age at start of prophylaxis was 3.0 years (Q1-Q3 1.0–16). Seventy per cent of the PwHB were on treatment with recombinant FIX, and 27% of these with EHL. In comparison, 89% of the PwHA were treated with recombinant FVIII. None of the controls were on EHL, explained by the earlier enrolment period. The annual median factor consumption for recombinant products was 3,900 IU/kg/year for both PwHA and PwHB on standard half-life products (SHL), and 2,000 IU/kg/year (Q1-Q3 1,500–2,400) for PwHB on EHL products. The corresponding figure for FIX plasma-derived (PD) products was 2,900 IU/kg/year (Q1-Q3 1,600–6,000) compared with 5,000 IU/kg/year (Q1-Q3 3,500–5,800) for FVIII PD products. Further descriptions of treatment characteristics are provided in Table 2.

3.2 | Bleeding Episodes

Bleeding characteristics are shown in Table 3. Twenty-nine PwHB (37%) reported one or more joint bleeds in the prior 12 months. Of these, five were younger than 18 years. The median number of joint bleeds for the HB cohort was zero (Q1-Q3 0–1.3) and ranged from zero to 18. The number of patients with reported bleeds in the knees, ankles and elbows was similar. Five PwHB (6.4%), one with

a current inhibitor, had a target joint, whereas five (all children between ages 1 and 9) reported no previous joint bleeds. Among those who had experienced a joint bleed, the median age at the first episode was 2.0 years (Q1-Q3 1.0–4.0).

To evaluate the association between bleeding rate and factor consumption, patients on SHL products were divided into three subgroups according to WFH's definition of high-dose (>4,000 IU/kg/year), intermediate-dose (1,500–4,000 IU/kg/year) and low-dose (<1,500 IU/kg/year) prophylaxis.[22] No significant differences in the number of bleeding events were found among these subgroups (Table 4). In addition, patients on PD FIX, recombinant SHL or EHL FIX products showed no significant differences in the occurrence of joint bleeds or other bleeds over the prior 12 months.

TABLE 1 Enrolment data and clinical characteristics

	HB n = 79	HA n = 79
Age at enrolment, years, median (Q_1-Q_3)	30 (19–53)	30 (20–53)
BMI, kg/m^2, median (Q_1-Q_3)	25 (22–28)	24 (21–27)
Age at diagnosis, years, median†(Q_1-Q_3)	0 (0–0.8)	1 (0–2)
Family history of haemophilia (%)	37 (47)	39 (49)
Unknown/missing data	5 (6.3)	34 (43)
History of or current inhibitor (%)	11 (14)	9 (11)‡
Treatment modality (%)		
On-demand*	2 (2.5)	1 (1.3)
Prophylaxis	75 (95)	76 (96)
ITI/Bypass therapy	2 (2.5)	2 (2.5)
Age at start of prophylaxis, years, median§(Q_1-Q_3)	3 (1–16)	3 (2–12)
Previous joint surgery (%)¶	27 (35)	MD
CVAD (%)		
Current CVAD	7 (8.9)	6 (7.6)
Previous CVAD	10 (13)	2 (2.5)
HIV positive (%)	4 (5.1)	3 (3.8)
Unknown/not tested	16 (20)	15 (19)
HCV status (%)		
Never infected (Ab-/PCR-)	37 (47)	29 (37)
HCV positive (Ab+/PCR+)	4 (5.1)	12 (15)
Recovered infection (Ab+/PCR-)	27 (34)	23 (29)
Unknown/not tested	11 (14)	15 (19)

Numbers (%) or median (Q1, first quartile—Q3, third quartile).

BMI, body mass index; CVAD, central venous access device; HA, haemophilia A; HB, haemophilia B; HCV, hepatitis C virus. HIV, human immunodeficiency virus. MD, missing data.

*One child, who had never had a joint bleed, currently on factor IX on-demand treatment had stopped prophylaxis seven months before study enrolment and was matched with a patient with HA on prophylaxis.

The number of patients (n) is noted if it deviates from the total number:†n = 76 (HB), n = 65 (HA), ‡n = 78, §n = 71 (HB), n = 51 (HA), ¶n = 77 (HB).

3.3 | Joint outcome

The HJHS and HEAD-US results are presented in Table 5 and Figure 1. The median total HJHS was significantly lower among PwHB compared with PwHA ($p = 0.048$), having median values of 4 (Q1-Q3 1.5–21) and 14 (Q1-Q3 2–35), respectively. The difference was significant in the age group 18–49 years, but not among those under 18 or above 49 years. Since HJHS 2.1 is not validated for children below four years of age, these patients (n = 3) were not examined. HJHS results were missing in an additional 11 PwHB. The HA controls for PwHB lacking HJHS assessment were excluded

TABLE 2 Treatment characteristics

	HB	HA
Factor concentrate (%)		
Plasma derived	21 (27)	8 (10)
Recombinant	55 (70)	70 (89)
Standard half-life	40	70 (89)
Extended half-life	15	
Bypass therapy	2 (2.5)	1 (1.3)
Non-factor replacement	1 (1.3)	
Prescribed factor dose, IU/kg/dose, median (Q_1-Q_3)		
Plasma derived	28 (22–36)	28 (24–37)
Recombinant		
Standard half-life	38 (27–43)	23 (14–29)
Extended half-life	44 (39–50)	
Annual factor consumption, IU/kg/year, median (Q_1-Q_3)		
Plasma derived	2912 (1613–6000)	5005 (3518–5760)
Recombinant		
Standard half-life	3931 (2673–4735)	3910 (2660–4873)
Extended half-life†	2012 (1485–2418)	
Prophylaxis frequency (%)		
Daily	3 (4.0)	11 (15)
Every 2nd day	11 (15)	27 (36)
Every 3–5 days	33 (44)	37 (49)
Weekly	21 (28)	1 (1.3)
Less than weekly	6 (8.0)	

Numbers (%) or median (Q_1, first quartile—Q_3, third quartile).

HA, haemophilia; HB, haemophilia B

A. †In three cases, no further specification than 'less than weekly' was given, treatment every ten days has been used in the calculation. HB plasma-derived products: Immunine, Mononine, NanoFIX, Octanine. HB recombinant standard half-life products: BENEFIX®, Rixubis. HB recombinant extended half-life products: Alprolix, Idelvion, Refixia. HB bypass Therapy: NovoSeven®. HB non-factor replacement: concizumab. HA plasma-derived products: Helixate NexGen, Octanate, Wilate. HA recombinant products: Advate, Kogenate™, Kovaltry, ReFacto, ReFacto AF. HA bypass therapy; FEIBA™.

from the calculations, as were patients with a history of or current inhibitor.

The HEAD-US results showed overall low scores, with medians of 0 in both elbows (Q1-Q3 0–5) and knees (Q1-Q3 0–3) and 1 (Q1-Q3 0–6) for the ankles. The scores primarily reflected disease damage, equally divided by cartilage and bone, whereas only minor hypertrophic synovium was observed.

Twenty-seven PwHB (35%), with median age of 56 (Q1-Q3 40–66), had undergone joint surgery. Knee arthroplasty was the most common procedure followed by ankle arthrodesis. The detailed data on prior joint surgeries are presented in Appendix 1.

TABLE 3 Bleeding characteristics of the haemophilia B population in B-NORD

Age at first joint bleed, years, median† (Q_1-Q_3)	2.0 (1.0–4.0)
Target joint at visit (%)‡	5 (6.4)¶
Annual joint bleeding rate last 12 months, median‡	0 (Q_1-Q_3 0.0–1.3, range 0–18)
On-demand treatment	5 (range 0–10)
Prophylactic treatment	0 (Q_1-Q_3 0–1, range 0–18)
ITI/bypass therapy[§]	4
Number of patients with at least one joint bleed last 12 months (%)‡	29 (37)
Location of joint bleed, number of patients (%)	
Knee	12 (15)
Ankle	10 (13)
Elbow	10 (13)
Shoulder	6 (7.7)
Hip	4 (5.1)
Wrist	2 (2.6)
Number of patients with at least one non-joint bleed last 12 months (%)	35 (44)

Numbers (%) or median (Q_1, first quartile—Q_3, third quartile). †n = 57. ‡n = 78. [§]n = 1, missing data=1. ¶including one patient with a current inhibitor.

3.4 | Treatment adherence

The median VERITAS-Pro score for PwHB was 38 (Q1-Q3 33–48). Only two patients had a total score of ≥57, the cut-off for 'non-adherence'. As shown in Figure 2, the highest scores (least adherent) were reported in the subscale 'communicate' and the lowest scores (most adherent) in the subscales 'dose' and 'skip'. The median total score was slightly higher, 43 (Q1-Q3 35–50), among the 18–49 years' age group compared with younger and older age groups having scores of 37 (Q1-Q3 30–39) and 33 (Q1-Q3 27–39), respectively. The VERITAS-Pro score did not differ between patients on EHL and patients on SHL products, with median values of 36 (Q1-Q3 28–50) and 38 (Q1-Q3 34–46).

4 | DISCUSSION

This is the first study in the Nordic region to describe treatment and outcome of patients with severe HB, including a comparison to matched controls with HA. The majority (95%) of the patients were on prophylaxis from a young age with no difference in age at start compared with PwHA. Despite the high prophylaxis frequency, 37% of the PwHB reported at least one joint bleed during the prior 12 months and 44% reported non-joint bleeding episode(s).

The median annual joint bleeding rate (AJBR) of zero in our material is at a similar level of reported AJBRs for patients on EHL products[23-25] and lower than that of 3.8 in the cohort from the Van Creveld Clinic.[14] In that cohort, however, only 73% of the patients were on prophylactic treatment. Our finding of 2.0 years as the median age at first joint bleed is similar to that of 1.2 reported by the PedNet group,[13] as well as 2.4 years reported by Uijl et al..[14]

Somewhat unexpected, the median factor consumption among the Nordic PwHB on SHL products was just below 4,000 IU/kg/year, indicating that less than 50% of the population received high-dose prophylaxis as defined by the WFH.[22] However, no difference in bleeding rate was observed in a subgroup analysis of high and low factor consumption and the overall preserved joints indicate successful use of individualized treatment. It is also worth pointing out that PwHB on

TABLE 4 Bleeds and treatment intensity in haemophilia B patients on prophylactic treatment with standard half-life products

	High dose n = 26	Intermediate dose n = 28	Low dose n = 4	p
Number of patients with at least one joint bleed last 12 months (%)	11 (42)	10 (35.7)	1 (25)	0.84
Number of joint bleeds last 12 months, median (Q_1-Q_3)	0 (0–2.3)	0 (0–1)	0 (0–0.75)	0.61
Number of patients with at least one non-joint bleed last 12 months (%)	11 (42)	12 (43)	1 (25)	0.85
Number of non-joint bleeds last 12 months, median (Q_1-Q_3)	0 (0–2)	0 (0–2)	0 (0–1.5)	0.80

Numbers (%) or median (Q_1, first quartile—Q_3, third quartile). High dose: >4,000 IU/kg/year. Intermediate dose: 1,500–4,000 IU/kg/year. Low dose: <1,500 IU/kg/dose.

TABLE 5 Joint outcome

	HJHS, median (Q_1-Q_3)			HEAD-US, median (Q_1-Q_3)
	HB	HA		HB
	n = 49	n = 49	P	n = 51
Elbow				
Left	0 (0–3)†	0 (0–7.5)¶	0.05	0 (0–3.5) ¶
Right	0 (0–6) †	1 (0–6)	0.14	0 (0–5) ¶
Knee				
Left	1 (0–4) †	1 (0–5.5)	0.47	0 (0–3)*
Right	0.5 (0–2.5) ‡	1 (0–6)	0.17	0 (0–4)*
Ankle				
Left	1 (0–4) §	2 (0–6)	0.14	1 (0–6)
Right	1 (0–5) †	1 (0–6)	0.26	1 (0–6)**
Total joint score	4 (1.5–21)	14 (2–35)	0.048	
Global gait score	0 (0–4) †	3 (0–4)	0.34	
Total score	4 (2–26) †	17 (2.5–39)	0.11	
Age (years)***				
<18	1 (0–2.3)	0.5 (0–1.8)	0.65	
18–49	2 (0.3–9.3)	9 (2–22)	0.01	
>50	44 (29–57)	43 (30–50)	0.50	

Median (Q_1, first quartile—Q_3, third quartile).

HA, haemophilia A; HB, haemophilia B; HJHS, Haemophilia Joint Health Score. HEAD-US, Hemophilia Early Arthropathy Detection with Ultrasound.

***HB: Age <18, n = 6; 18–49, n = 24; >50, n = 13. HA: Age <18, n = 4; 18–49, n = 30; >50, n = 15. The number of patients (n) is noted if it deviates from the total number: †n = 43, ‡n = 42, §n = 44, ¶n = 49. *n = 48, **n = 50. Patients with a current or previous inhibitor are excluded from the calculations.

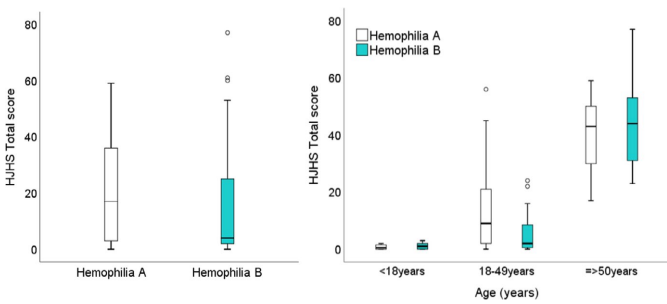

FIGURE 1 HJHS in haemophilia patients divided by type of haemophilia and age group. Patients with a current or previous inhibitor are excluded from the calculations. HJHS, Haemophilia Joint Health Score 2.1

PD products had a 26% lower median factor consumption compared with recombinant SHL FIX, consistent with the differences in pharmacokinetics between these types of concentrates.[26] Moreover, the PwHB on EHL products consumed about half of the amount of factor compared with those receiving SHL products with a preserved bleed protection, emphasizing the value of EHL agents in clinical practice.

Fourteen per cent of the PwHB had a history of or current inhibitor. This is a relatively high number compared with previously published data,[22] and further characterization of these patients will be reported separately.

4.1 | Joint outcome

We found a significantly lower HJHS, indicating better joint health, among PwHB compared with PwHA. This was explained by findings among persons between 18 and 49 years of age, whereas the outcomes for the younger and the older subgroups showed no difference. The reason for this is not clear, and treatment provided over the years needs to be taken into account, but this may indicate that arthropathy develops earlier in PwHA than in PwHB. Arthropathy is a progressive disorder, and the HJHS are, as expected, higher in the

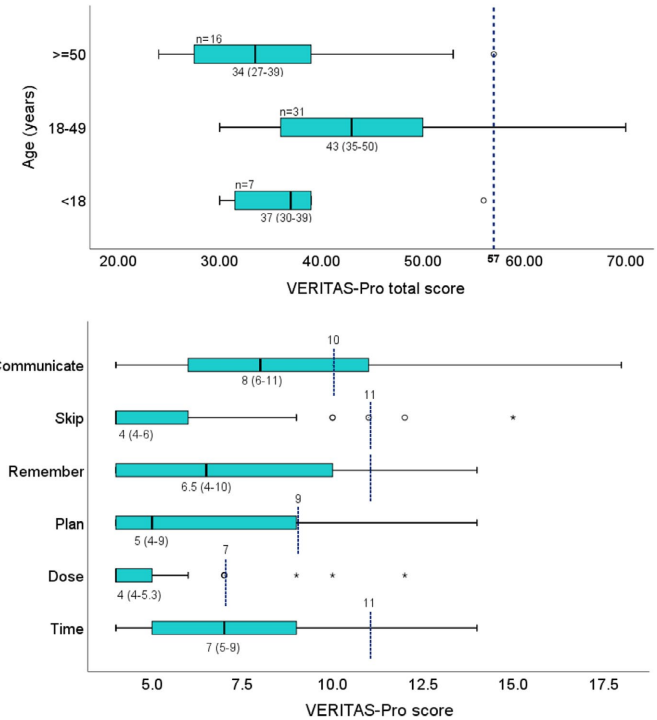

FIGURE 2 VERITAS scores for HB patients in the B-NORD study. n = 54. Median (Q1-Q3). The vertical lines represent the proposed cut-off values for non-adherence.[20] VERITAS, Validated Hemophilia Regimen Treatment Adherence Scale

older age groups of both HA and HB, but without significant difference between the groups. This could indicate that the difference may even out at older age or represents a more successful prophylactic treatment in PwHB compared with PwHA. The difference in median scores between the age groups 18–49 years and ≥50 years may be larger than expected. This might partly be explained by the fact that prophylaxis was introduced later in life in the older age group compared with the younger group. However, the number of study subjects in the older group is relatively small and firm conclusions cannot be drawn. In agreement with our findings for children, the PedNet group reported no difference in bleeding phenotype among young children with severe HA and HB,[13] whereas Melchiorre et al. compared arthropathy in patients with severe HA and HB and concluded that the degree of arthropathy was more severe in PwHA.[8] This conclusion is supported by Nagel et al., who reported more bleeding episodes and surgical procedures in PwHA than in PwHB despite similar factor consumption.[27] Consistent with this, Tagariello et al. found a threefold higher risk for undergoing joint arthroplasty among PwHA compared with PwHB.[9] These studies suggest, in agreement with our findings in persons 18–49 years, a lower risk of developing arthropathy for PwHB than PwHA. We believe it unlikely that the difference in HJHS in our study is an effect of lesser treatment intensity for PwHA, since the factor consumption was similar between the groups, although lifelong consumption has not been taken into account. The potential anti-inflammatory role of FVIII described by Mignot et al.,[28] as well as the role of extravascular FIX in coagulation,[29,30] has been debated, but whether this has an impact on joint outcome and can explain differences between HA and HB is not clear. The same applies for the suggestion that the higher prevalence of missense mutations over null mutations in PwHB compared with PwHA could contribute to a milder clinical phenotype.[10]

4.2 | Treatment adherence

Adherence to treatment is crucial for the risk of developing arthropathy. In our cohort, evaluation by VERITAS indicated overall good adherence. However, it remains to be settled whether these scores reflect the benefits of the structure of haemophilia care in the Nordic region, with centralized care and extensive patient education. Or is it perhaps, the result of bias, as the patients answering the questionnaire (70%) may be the ones with the highest adherence? We found the least adherent scores in the category 'communicate' with 36% of the patients having a score consistent with 'non-adherence'. This category evaluates how often the patients call the HTC for advice and treatment decisions. The use of modern technology for communication might be a way to improve this adherence. The highest adherence was seen in the subgroup of patients ≥50 years and the lowest among patients 18–49 years, potentially indicating the impact of work and family life. It is a limitation of our study that no

VERITAS data were available for the PwHA. However, in support of our findings, Miesbach et al.[31] observed a similar VERITAS-pro median total score of 34 and a significantly higher score among patients aged 20–59 in a cohort of 397 PwHA or PwHB.

4.3 | Strengths and limitations

Despite its international multicentre design, our study has the limitations of a retrospective observational investigation with a limited number of subjects. Furthermore, information on bleedings and joint surgery was incomplete in the KAPPA register; hence, these parameters could not be compared. In addition, the enrolment period for PwHB and PwHA was slightly different. However, our study, in contrast to the majority of previous studies of haemophilia, is focusing on PwHB and includes closely matched controls with HA from the same HTCs. The patients are also from a homogenous geographic area, and the number of included patients is, compared with previously published reports on persons with severe HB, relatively high.

5 | CONCLUSION

Our study indicates that the Nordic cohort of patients with severe HB is well treated and adherent to individualized treatment regimens. Despite this, the goal of zero bleeds for all has not been reached. Hence, in an era of new treatment options, more attention should be given to improve the care for PwHB. Our findings also suggest and support previous findings that patients with severe HB suffer from milder arthropathy than patients with severe HA.

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DISCLOSURES

KK has received research grants from CSL Behring, Stockholm, Sweden. FB has received honoraria as member of advisory board and/or speaker from Sobi, Shire/Takeda, Novo Nordisk, Bayer, Roche, UniQure, Octapharma, BioMarin and Pfizer. MB was supported by funds from Stockholm County Council. EF has received honorarium as speaker for Shire, Roche, Sobi and Takeda. PAH has acted as a paid consultant to Bayer, Shire, Novo Nordisk, Octapharma, CSL Behring, Pfizer and Sobi including lectures. RL has been a member of advisory boards for Sobi, CSL Behring, Takeda, BioMarin, Novo Nordisk, Pfizer, ROCHE and Bayer. MO has received speaker/consultant fees from Novo Nordisk, Shire and Bayer. EB has received research grants and paid consultancy from CSL Behring, Stockholm, Sweden. JA has received research grants from Sobi, CSL Behring, Takeda/Shire and Bayer and speakers' fee and consultant for Octapharma, Novo Nordisk, Pfizer, Bayer, Sobi, CSL Behring, Takeda/Shire, BioMarin, Uniqure and Spark Therapeutics. VN and SR stated that they had no interests, which might be perceived as posing a conflict or bias.

AUTHOR CONTRIBUTIONS

JA, EB and KK designed the research study. KK analysed the data. KK and JA interpreted the data and drafted the paper. KK, JA, FB, MB, EF, PAH, RL, VN and SR enrolled patients and collected the clinical data. MO and EB designed the KAPPA study and developed the KAPPA registry. All authors critically reviewed the manuscript and have read and approved the final version of the manuscript.

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APPENDIX 1

Previous joint surgery in patients with haemophilia B in the B-NORD study.

Joint surgery	Right	Left	Unknown side	Total
Knee	13	20		33
Arthroplasty	12	13		
Synovectomy				
Surgical		3		
Radioactive		2		
Other	1	2		
Ankle	8	5	1	14
Arthrodesis	7	2		
Achillotenotomy		2	1	
Radioactive synovectomy	1			
Arthroplasty		1		
Elbow	4	5	1	10
Resection caput radii	2	2		
Arthroplasty	1	2		
Radioactive synovectomy	1			
Other		1	1	
Hip	1	3	1	5
Arthroplasty	1	3		
Other			1	
Other/unknown joint	1		1	2

Numbers. Knee other: arthroscopic meniscus extirpation, osteotomy. Elbow other: pseudotumor, ulnar nerve transposition. Hip other: septic arthritis. Other/unknown joint: osteomyelitis, carpal tunnel syndrome.

Paper III

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Full Length Article

Factor IX antibodies and tolerance in hemophilia B in the Nordic countries – The impact of *F9* variants and complications

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ABSTRACT

Introduction: The development of inhibitory antibodies (inhibitors) in persons with hemophilia B (PwHB) causes significant morbidity. Data on the impact of the *F9* variant and immune tolerance induction (ITI) outcome are limited.

The aim of this study was to investigate the presence of neutralizing and non-neutralizing antibodies (NNA) in severe hemophilia B (HB) and to evaluate ITI outcome and complications in relation to the pathogenic *F9* variant.

Materials and methods: Persons with severe HB in the Nordic countries were enrolled and information on *F9* variants, inhibitors, ITI and complications were collected. Analyses of anti-FIX antibodies with a fluorescence-immunoassay (xFLI) and an ELISA method were conducted.

Results: Seventy-nine PwHB were enrolled. Null variants were seen in 33 (42 %) PwHB and 12 (15 %) had a current or former inhibitor. Eleven (92 %) of the inhibitor patients had experienced allergic manifestations and three (25 %) nephrotic syndrome. Of 10 PwHB with at least one ITI attempt, eight (80 %) were considered tolerant at enrolment. Immunosuppression was included in seven of eight successful or partially successful attempts. Five PwHB had at least one ITI failure before a successful or partially successful ITI. No NNA could be identified.

Conclusion: A high proportion of severe *F9* gene defects among persons with severe HB in the Nordic countries may explain the observed relatively high prevalence of inhibitors. ITI success was independent of the *F9* variant and attained despite allergic manifestations and previous ITI failures. Inclusion of immunosuppression tentatively enhances the chances of ITI success. No NNA were observed.

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1. Introduction

Hemophilia B (HB) is a rare bleeding disorder occurring in 1 in 30,000 males [1]. The recommended treatment for persons with HB (PwHB) with a severe bleeding phenotype is prophylactic replacement therapy with the deficient factor IX (FIX) protein [2]. A serious complication to the treatment is the development of neutralizing antibodies (inhibitors) against FIX, which can result in the loss of function of infused concentrates. Inhibitors are reported more commonly in individuals with genetic null variants [2–6], i.e. no antigen is being produced, and most often occur before 20 exposures of factor treatment [2,3,7]. Inhibitor development can be complicated further by allergic reactions to replacement therapy, as well as by nephrotic syndrome.

The experience of immune tolerance induction (ITI) to eradicate the inhibitors in PwHB is limited and there is no established consensus on the management of these patients [2]. Different regimens with varied dosing and frequencies of FIX concentrates with or without the addition of immunosuppressive agents have been reported [3,8–12] but the study cohorts are small. Consequently, clinical management is often extrapolated from regimens and studies based on persons with the more common bleeding disorder hemophilia A (HA), i.e. deficiency of coagulation factor VIII (FVIII). However, phenotype and management of inhibitors differ between HA and HB. First, the incidence of inhibitors overall in patients with HB is often reported to be <5 % [2] and is thus much lower than in those with HA. In addition, inhibitors to FIX are mainly observed in patients with the severe form of the disease, i.e. a FIX activity <0.01 IU/mL. In HA, inhibitors are also seen, yet not as frequently, in the non-severe forms [2]. Furthermore, anaphylaxis and nephrotic syndrome are rare in HA, and ITI success rates seem to differ from HB. ITI success rates of 70–80 % are usually reported for HA, compared to only 30–35 % in HB [2,7]. As a conclusion, experience and treatment regimens used for inhibitors in HA cannot be extrapolated easily to manage individuals with HB.

In addition to inhibitory antibodies, the presence and clinical significance of non-neutralizing (non-inhibitory) antibodies (NNA) in HA have been studied and discussed over the years. In a recent meta-analysis, the pooled prevalence of NNA towards FVIII in HA was 25 % [13], and it has been suggested that NNA may predict the development of inhibitors and enhance the clearance of the administered factor concentrates [14–16]. Data on NNA in PwHB are sparse. Boylan et al. [17] assessed the relationship between anti-FIX antibody profiles and inhibitor formation with a fluorescence-based immunoassay (FLI) and found one or more classes of anti-FIX antibodies in 40 % of patient samples which tested negative by the Nijmegen-Bethesda assay. Further studies are, however, warranted to fully appreciate the value of monitoring NNA in routine clinical practice.

The aim of this study was to investigate the presence of neutralizing and non-neutralizing antibodies in patients with severe HB in the Nordic countries and to evaluate ITI outcome and complications in relation to the pathogenic *F9* variant.

2. Methods

2.1. Study design and study population

The B-NORD study is an observational multicenter study conducted in Denmark, Finland, Norway and Sweden and has been described previously [18]. Individuals of all ages with severe congenital HB were enrolled between the years 2017 and 2020. Information on inhibitors, ITI, allergic reactions and nephrotic syndrome was collected. The criteria used for ITI success were at the discretion of the treating physician and included a negative inhibitor titer and the possibility of using replacement therapy. A normal recovery and/or half-life of FIX concentrates were also reported, but not in a systematic manner. The treating physician reported whether the patient was considered tolerant or not at enrollment, but no consensus criteria on tolerance were used.

A positive inhibitor titer was defined according to the cut-off level for inhibitor detection at the local center. The Nijmegen-modified Bethesda assay, described previously [17,19], was performed at the local laboratory and the cut-off levels were 0.4 or 0.5 BU/mL (Bethesda units). The Malmö inhibitor assay was used previously to estimate inhibitors and expressed the inhibitor activity in plasma as the number of units of FIX inactivated by 1 mL of patient plasma [20]. One Malmö inhibitor unit (MIU) corresponds to about 3 BU.

The study was approved by the Regional Ethical Board in Lund, Sweden (Dnr 2016/1089) and by the independent ethics committees in each country. Written informed consent was collected from the study subject or his legally acceptable representative in accordance with the Declaration of Helsinki.

2.2. Variant analysis of F9

Variant analyses from PwHB in Sweden and Finland were performed at the genetic laboratory in association with the hemophilia treatment center (HTC) in Malmö, Sweden. Variant analyses from Norway were performed at the HTC in Oslo, Norway. No variant data were available for the patients from Denmark.

The promoter region of the F9 gene and all eight exons with the flanking intron regions were amplified by polymerase chain reaction (PCR) using primers described by Green et al. [21], modified with M13 tails. Variants were identified by Sanger sequencing as described by Mårtensson et al. [22]. Large deletions and duplications were determined by Multiplex Ligation-dependent Probe amplification (MLPA) using P207-F9 (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. All reports were classified uniformly according to the recommendations of the Human Genome Variation Society (HGVS). The variants were interpreted for clinical significance according to the American College of Medical Genetics and Genomics (ACMG) guidelines applicable in 2021, using the VarSome's ACMG implementation [23] with an automated scoring and a manual review and adjustment of specific criteria. The FIX Gene Variant Database [24,25] was used for comparison.

2.3. Anti-FIX assays for the detection of non-neutralizing antibodies

Two different assays were used to investigate the presence of NNA: one Multi-Analyte Profiling (Luminex xMAP) based fluorescence immunoassay (xFLI) and one enzyme-linked immunosorbent assay (ELISA). Results from the Nijmegen-Bethesda assays were used for comparison to distinguish inhibitors from NNA.

2.3.1. Anti-FIX Luminex xMAP-based fluorescence immunoassay — xFLI method

FIX (nonacog alfa, BeneFIX) was coated to MagPlex microspheres. Citrated plasma samples were diluted in phosphate buffer saline (PBS, Hyclone) supplemented with 0.05 % Tween-20 (PBST, Merck) and 0.1 % ovalbumin (Sigma) (PBST-O), added to wells containing FIX-coupled microspheres and incubated for 2 h, washed with PBST, and incubated with R-phycoerythrin-labeled goat anti-human IgG (Jackson ImmunoResearch, Ely; Cambridgeshire, UK). Readings in a MagPix instrument (Luminex, Corporation, Austin Texas, US) were recorded as median fluorescence intensity (MFI). A general cut-off for positivity was determined from the mean + 3 SDs in healthy individuals (n = 26). The inter-assay CV was 12.2 % for the high and 14.3 % for the low positive control.

2.3.2. Anti-FIX immunological assay — ELISA method

An in-house ELISA was used, in which FIX (nonacog alfa, BeneFIX) was coated overnight. Plasma samples were diluted 50-fold in a Tris-blocking buffer supplemented with 1 mM $CaCl_2$ and incubated for 2 h. The secondary antibody was horseradish-peroxidase conjugated polyclonal rabbit anti-human IgG (Agilent, Santa Clara, CA, US). Absorbance was measured in a microplate reader (Tecan Infinite 200, Männedorf,

Switzerland). The cut-off for each test run was determined by analyzing normal plasma samples (n = 10–12) per test run and given as the mean + 3 SDs. The inter-assay CV was >50 % for the positive control.

2.4. Statistical analysis

Descriptive statistics were used. Continuous variables were described using medians and first-to-third quartiles (Q1-Q3). Categorical data were reported as numbers and percentages. Comparisons of two independent groups of continuous, non-normally distributed variables were performed using the Mann-Whitney *U* test. For binary or categorical data, the Chi-square test or Fisher's exact test was used. A *p*-value of <0.05 was considered to be statistically significant. Statistical analyses were performed using IBM SPSS Statistics 25.

3. Results

3.1. Patient characteristics

Out of 108 persons with severe HB registered at the study centers, 79 (73 %), median age 30 years (Q1-Q3 19–53), were enrolled in the B-NORD study [18]. Patient characteristics are presented in Table 1.

Out of the 79 enrolled PwHB, 12 (15 %) were reported to have current or former inhibitors, all registered at the HTCs in Sweden (Table 1). Two of the inhibitor patients were brothers and two were related more distantly. The age at start of prophylaxis did not differ between PwHB with and without inhibitors, median ages of 2.7 (Q1-Q3 1.0–29) and 3.0 years (Q1-Q3 1.0–16), respectively. The median age at inhibitor detection was 2.0 years (Q1-Q3 1.0–8.0) and in all reported cases occurred before 20 exposure days (missing data n = 5). Eight (67 %) of the 12 patients with inhibitors were considered tolerant at study enrollment by their treating physician and were treated with prophylactic FIX replacement therapy, median dose 6638 IU/kg/year (Q1-Q3 4141–10,115). Four of these tolerant PwHB were on plasma-derived and four on recombinant standard half-life products (SHL). The corresponding consumption for those without inhibitor history was significant lower with a median dose of 3406 IU/kg/year (Q1-Q3 2178–4583) ($p = 0.005$). The four remaining patients with inhibitors had either ongoing ITI, prophylactic treatment with rFVIIa only or were on investigational study drugs (two patients).

Table 1
Study cohort characteristics.

	Inhibitor patients n = 12	Non-inhibitor patients n = 67
Enrollment country (%)		
Denmark	–	9 (13)
Finland	–	9 (13)
Norway	–	15 (22)
Sweden	12 (100)	34 (51)
Age at enrollment, years, median (Q_1-Q_3)	26 (18–42)	31 (19–54)
Age at diagnosis, years, median (Q_1-Q_3)	0 (0–0)	0 (0–1)
Family history of hemophilia (%)†	7 (58)	30 (45)
CVAD, current or previous (%)	5 (42)	12 (18)
BMI, kg/m^2, median (Q_1-Q_3)	23 (19–29)	25 (22–28)
Current treatment (%)		
On-demand FIX-replacement	–	2 (3.0)
Prophylaxis FIX-replacement	8 (67)	65 (97)
Bypass-therapy	2 (17)	–
Non-factor replacement	2 (17)	-
Age at 1st joint bleed, years, median (Q1-Q3) ‡	1.5 (0.71–3.2)	2.1(1.0–4.4)
Age at start of prophylaxis, years, median (Q_1-Q_3) §	1.4 (1–25)	3.3 (1–16)
Previous joint surgery (%)	4 (33)	23 (34)¶
Age at inhibitor detection, median (Q_1-Q_3)	2.0 (1.0–8.0)	NA
Allergic manifestation (%)	11 (92)	1 (1.5)
Nephrotic syndrome (%)	3 (25)	–
HIV positive (%)	1 (8.3)	3 (4.5)
Unknown/not tested	2 (17)	14 (21)
HCV status (%)		
Never infected (Ab-/PCR-)	7 (58)	30 (45)
HCV positive (Ab+/PCR+)	–	4 (6.0)
Recovered infection (Ab+/PCR-)	3 (25)	24 (36)
Unknown/not tested	2 (17)	9 (13)

Numbers (%) or median (Q1, first quartile - Q3, third quartile). BMI, body mass index. CVAD, central venous access device. HCV, hepatitis C virus. HIV, human immunodeficiency virus. NA, not applicable.
The number of patients (n) is noted if it deviates from the total number: †n = 11 (inhibitor), n = 65 (non-inhibitor), ‡n = 9 (inhibitor), n = 48 (non-inhibitor), §n = 11 (inhibitor), n = 60 (non-inhibitor), ¶n = 65.

3.2. F9 variants and comparison to the EAHAD FIX Gene Variant Database

The *F9* variant was identified in 64 patients (81 %). In total, 42 different variants were found (Table 2). Thirty of the variants had been reported previously in the FIX Gene Variant Database. All but one of the *F9* variants identified were classified as 'pathogenic' according to the ACMG classifying system. The remaining variant (c.253-12_253-3delTATTCTTTAT) was classified as 'likely pathogenic'. Null variants defined as nonsense variants, frameshift outside poly-A runs, large structure deletions, and splice-site mutations involving conserved nucleotides were seen in 33 patients (42 %), nine of whom had an inhibitor history. The distribution of variants is presented in Fig. 1 and demonstrates a higher occurrence of large structure deletions of 10 % in the B-NORD cohort (persons with unknown variants are excluded from the calculation), compared to 4.8 % in the FIX Gene Variant Database. Table 3 shows the genetic variants divided by country. As shown in Fig. 2, the frequency of inhibitor development by variant effect was 71 % (5/7) for large structure changes, 17 % (1/6) for frameshift, 15 % (3/20) for nonsense and 12 % (3/26) for missense variants. No PwHB with splice or in-frame variants had an inhibitor history.

Out of the 12 inhibitor patients, nine had a null *F9* variant. Interestingly, two brothers in the study had the *F9* variant c.316G > A and both had developed inhibitors despite the fact that this variant is reported 74 times in the FIX Gene Variant Database without any previously reported inhibitor cases. Six patients had the large structure deletion g.(?_139530767)_(139562071_?)del, and all but one developed inhibitors. The one patient with this large structure deletion but no inhibitors started prophylaxis at the age of 19 years and has since been on prophylaxis with SHL FIX for >40 years.

3.3. Immune tolerance induction

At study enrollment, all but one of the PwHB with inhibitors either were on ongoing ITI or had completed at least one attempt. Detailed information on all 22 ITI attempts performed over the years in the 11 patients is presented in Table 4 and Fig. 3. All but one of the ITI attempts were based on daily administration of factor products with doses of 60–250 IU/kg. No difference could be seen in dosing between successful or non-successful ITI attempts. Out of the 22 attempts, one was ongoing at study start, four (19 %) of the completed attempts were considered successful by the treating physician, four (19 %) were considered partially successful and 13 (62 %) were considered unsuccessful. The shortest time to a successful ITI was 3 months. In total, 10 patients had finished at least one ITI attempt, and eight (80 %) of these were considered tolerant at enrollment. All four patients with partially successful ITI attempts were thus considered tolerant by their treating physician at the time of enrollment in the study and were treated with FIX prophylaxis. However, the definitions used of partial success, normal recovery and half-life differed between the cases. Two of the PwHB considered partially tolerized had a low-titer inhibitor, but were treated successfully with FIX products, and two patients had a negative inhibitor titer, yet without a normal recovery or half-life.

As shown in Table 4, the *F9* variants in the four PwHB having a

Table 2
Genetic variants in the FIX gene found in the B-NORD cohort. No. of inhibitor patients specified in parenthesis.

Variant type	Variant effect	Domain	Coding DNA†	Protein‡	No. (with inhibitors)	No. in the FIX Variant Database* (with inhibitors)
Substitution	Missense	Protease	c.1304G > A	p.(Cys435Tyr)	3	18
			c.1145G > A	p.(Cys382Tyr)	1	8
			c.1237G > A	pGly413Arg	1	7
			c.1052G > A	p.(Gly351Asp)	1	3
			c.1058 T > G	p.(Val353Gly)	1	3
			c.1295G > T	p.(Gly432Val)	1	2
			c.799C > T	p.(His267Tyr)	1	2
			c.1025C > A	p.(Thr342Lys)	1	2
			c.1289G > T	p.(Ser430Ile)	1	1
			c.1069G > C	p.(Gly357Arg)	1 (1)	–
			c.893G > C	p.(Arg298Pro)	1	–
			c.982A > T	p.(Asn328Tyr)	1	–
			c.998C > T	p.(Pro333Leu)	1	–
		EGF1	c.316G > A	p.(Gly106Ser)	2 (2)	74
			c.316G > T	p.(Gly106Cys)	1	2
		EGF2	c.464G > C	p.(Cys155Ser)	1	3
			c.400 T > A	p.(Cys134Ser)	1	–
		Pro-Peptide	c.127C > T	p.(Arg43Trp)	2	65
		Linker	c.533G > T	p.(Cys178Phe)	2	3
		Gla	c.251C > G	p.(Thr84Arg)	1	1
		Act-Peptide	c.676C > T	p.(Arg226Trp)	1	44
	Nonsense	Protease	c.880C > T	p.(Arg294*)	5 (1)	70 (4)
			c.1135C > T	p.(Arg379*)	4	65
			c.892C > T	p.(Arg298*)	2	63 (1)
			c.719G > A	p.(Trp240*)	2 (2)	7 (1)
			c.709C > T	p.(Gln237*)	1	4 (1)
			c.1305 T > A	p.(Cys435*)	1	–
		EGF2	c.484C > T	p.(Arg162*)	3	22
		Linker	c.535G > T	p.(Gly179*)	1	1
		Gla	c.223C > T	p.(Arg75*)	1	73 (8)
	Splice¶	N/A	c.392-1G > C	N/A	1	4
Deletion	Frameshift	Protease	c.969_975del	p.(Pro324Cysfs*2)	1 (1)	–
			c.815delG	p.(Gly272Valfs*25)	1	–
			c.1295delG	p.(Gly432Valfs*6)	1	–
		Gla	c.229delG	p.(Val77Phefs*27)	1	1
			c.161_162del	p.(Glu54Valfs*7)	1	–
		Act-Peptide	c.668delA	p.(Asp223Alafs*22)	1	1
	Large Structure Change (>50 bp)§		g.(?_139530767)_(139562071_?)del	p.0	6 (5)	60 (21)
			g.(?_139530767)_(139551238_139560770)del	p.0	1	2
	Splice¶	N/A	c.253-12_253-3delTATTCTTTAT	N/A	1	1
	In-frame	Protease	c.689_691delGAG	p.(Gly230del)	1	7
Duplication	In-frame	EGF1	c.353_358dup	p.(Cys119_Pro120insArgCys)	2	0
No variant found					3	
Missing data					12	

No., number of patients. *Accessed on 2021-03-05.:†NM_000133.3. ‡NP_000124.1, ¶NG_0079994.1 §NC_000023.11.

successful ITI included one large structure deletion, one frameshift deletion, one nonsense substitution and one missense substitution. The *F9* variants in the four patients with partially successful ITIs, but later tolerant after additional factor IX treatment, included one large structure deletion, two nonsense substitutions and one missense substitution. Finally, the two patients not tolerant at enrollment carried a large deletion and a missense substitution, respectively. In summary, no correlation between ITI outcome and type of underlying *F9* variant was seen in our cohort.

Two of the PwHB with a successful ITI had high-responding inhibitors. However, all of the successful attempts started with a titer <5 BU/mL. The inhibitor titer at the start of ITI was overall low with a median value of 2.1 BU/mL (Q1-Q3 0.93–12). The corresponding figures for 'successful', 'partially successful' and 'not successful' were 0 BU/mL (Q1-Q3 0–2.0), 1.5 BU/mL (Q1-Q3 0.53–11) and 5.7 BU/mL (Q1-Q3 1.2–18), respectively ($p = 0.18$).

The ITI regimens are provided in Table 4. Immunosuppression was included in three of the four successful ITIs and in all of the partially successful attempts. Six (46 %) of the failures included immunosuppression. Among the four successful attempts, one was considered tolerant after the first ITI attempt, one after the second, one after the third and one after the sixth ITI attempt. Recombinant factor products were used in two (50 %) of the successful, one (25 %) of the partially successful and in one (8 %) of the unsuccessful attempts.

3.4. Allergic reactions and nephrotic syndrome

Eleven (92 %) of the PwHB and inhibitors were reported to have experienced allergic manifestations towards FIX compared to only one (1.5 %) of the PwHB without inhibitors (Table 1). In five (42 %) inhibitor patients, the allergic reaction was reported as anaphylaxis. All of these patients had a high-titer inhibitor. In four of these patients, the *F9* variants were null variants (two large deletions, one frameshift deletion, one nonsense substitution) and in one case a missense substitution. The

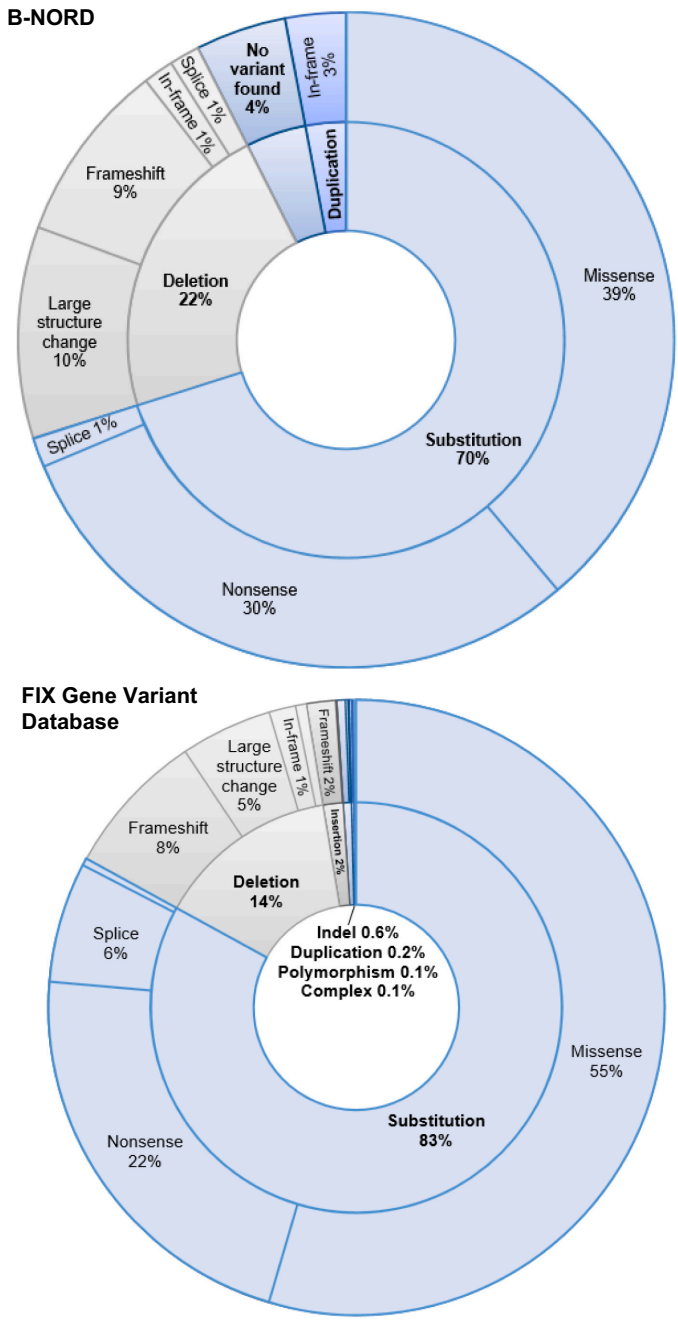

Fig. 1. Genetic variants. Genetic variants by variant type (inner circle) and variant effect (outer circle) in the B-NORD cohort and in severe hemophilia B in the FIX Gene Variant Database. For comparison, missing data is excluded from the B-NORD cohort.

Table 3
Genetic variants divided by country. No. of inhibitor patients specified in parenthesis.

Country	Variant effect No. (with inhibitors)							
	Missense	Nonsense	Large structure change	Frameshift	In-frame	Splice	No variant found	Missing data
Sweden	16 (3)	14 (3)	7 (5)	5 (1)	-	2	-	2
Norway	7	4	-	1	-	-	3	-
Finland	3	2	-	-	3	-	-	1
Denmark	-	-	-	-	-	-	-	9

No., number of patients.

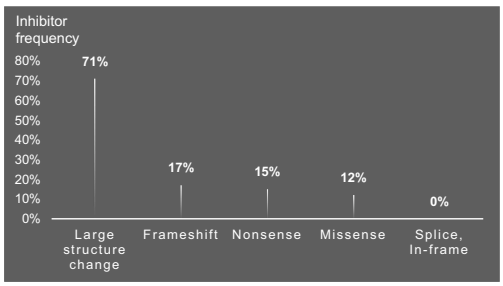

Fig. 2. Frequency of inhibitor development by gene variant effect.

remaining seven inhibitor patients reporting allergic manifestations, but no anaphylaxis, all experienced skin rash with/without additional symptoms. In six patients, the allergic reactions occurred after inhibitor detection, in three cases before and in two patients the onset of allergic reaction in relation to inhibitor development was not reported. The one patient with allergic symptoms in the absence of inhibitor history carried a nonsense substitution (FIX: c.892C > T; p.Arg298*), which is reported 63 times in the FIX Gene Variant Database, one of these with an inhibitor.

Nephrotic syndrome was reported in three (25 %) of the 12 inhibitor patients and in none of the PwHB without inhibitors (Table 1). In all three cases, the nephrotic syndrome was diagnosed after inhibitor detection, and in two cases, the nephrotic syndrome was diagnosed during ITI and contributed to the interruption of the ITI. Two of the PwHB and nephrotic syndrome were not considered tolerant at study enrollment. The genetic *F9* variants in association with nephrotic syndrome include one large structure deletion, one nonsense substitution and one missense substitution (Table 4).

3.5. Non-neutralizing antibodies

Samples from 53 (67 %) of the PwHB were collected and analyzed using the ELISA method and in 48 cases also with the xFLI assay (Table 5).

Samples from all 12 patients with a history of inhibitors were tested with ELISA and 10 of them also with the xFLI assay. The only two samples with a positive Bethesda titer (3 BU/mL and 0.4 BU/mL, respectively) were also positive in both immunoassays. No consistent findings for NNA were obtained in any of the remaining samples. In two cases, however, both negative in the xFLI assay, results were initially positive in the ELISA assay. On retesting, the ELISA assay was negative in one and borderline positive (4.2 SDs above mean) in the other case.

Among the samples from non-inhibitor patients, no consistent findings of NNA were observed. In four cases, the outcome of the two assays was initially discrepant, with the ELISA assay positive in three cases and the xFLI assay positive in one sample. In none of the cases, could retesting confirm the presence of NNA. Altogether, the concordance between the two immunoassays was 87.5 %.

4. Discussion

This Nordic study of persons with severe HB reveals a relatively high proportion of severe *F9* gene defects and a high prevalence of inhibitors. Our study also illustrates the unpredictable challenges, but also possibilities, in the management of PwHB and inhibitors.

A prevalence of 15 % of persons with a history of inhibitors in our Nordic HB population is relatively high compared to many other published reports. Our cohort was, however, restricted to severe HB patients and the inhibitor figure is consistent with the Swedish data previously reported [26], and not dissimilar from that reported recently for the severe subgroup of PwHB in the PedNet Registry [7]. Admittedly, not all persons with severe HB registered at the HTCs were enrolled in our study. The inhibitor prevalence would, however, still be at least 11 %, if the entire severe HB population was included, indicating that inhibitor development in the severe HB population is a significant problem. Importantly, the prevalence of severe gene defects, i.e. large deletions and nonsense variants, is also relatively high, which we believe to be the main explanation for the observed prevalence of inhibitors. The variant distribution in the B-NORD cohort is otherwise largely in agreement with the FIX Variant Database (Fig. 1).

Out of 11 patients having at least one ITI attempt, only one patient had ITI ongoing at study enrollment, with a duration of 2 months. Eight of the remaining 10 patients were considered tolerant at enrollment. This makes a total success rate of 80 % and indicates that tolerance may be achievable for the majority of PwHB and inhibitors. Interestingly, four (40 %) of these patients were considered only partially tolerized after their final ITI, but tolerant with additional long-term FIX replacement. This indicates that tolerance may be achieved with continuous exposure of the deficient factor for bleed prevention. Importantly, the criteria for ITI success and tolerance were determined by the individual physician in our study and the lack of well-defined established definitions of ITI success, and tolerance in HB complicates the comparisons of the outcome of various ITI attempts as well as the evaluation on treatment duration and when tapering of the dose is suitable.

Five patients had at least one ITI failure before an attempt leading to success or partial tolerance, which indicates, in line with recently published data [27], that ITI success can be attained despite previous ITI failures and that more than one ITI attempt can be considered in PwHB. We could not identify any favorable or unfavorable *F9* variant on the ITI outcome and no difference in outcome for plasma-derived or recombinant products. In this context, it is important to highlight that no extended half-life (EHL) products were used. In seven out of eight (88 %) successful or partially successful ITI attempts, immunosuppression was included in the regimen. In three of these attempts a combination of rituximab, intravenous immunoglobulin (IVIG), dexamethasone and mycophenolate, in line with the Beutel protocol [8], was used and in four cases a combination of cyclophosphamide and IVIG. In two of these latter cases, corticosteroids were used in addition. Only one case of ITI success was achieved without immunosuppression. This was in a patient with a missense substitution in the *F9* gene and a low-responding inhibitor and the treating physician reported doubting the clinical

Table 4
Detailed data on immune tolerance induction attempts.

ID	Genetic variant	Age at inhibitor detection (years)	Tolerant at enrollment†	Peak titer (BU)	Allergic symptoms	Nephrotic syndrome	ITI attempt	Age at ITI (years)	Titer at start of ITI (BU)	ITI regimen§	ITI success‡ (time to success or termination)
1	c.-29-?_1386+?del	9	N	129	Y	N	1.	14	129	**PD non-monoclonal antibody purified SHL** (*NanoFIX*)(68 IU/kg once daily) On-going, duration 2 months at study enrollment	On-going
2	c.-29-?_1386+?del	1	Y	2.7	Y	N	1.	3	2.4	**PD non-monoclonal antibody purified SHL** (*Nanotiv)* (88 IU/kg twice daily) ***IVIG, Dexamethasone/ Betamethasone, Mycophenolate***	N (57 months)
							2.	14	1.7	***Recombinant SHL*** (*BeneFIX*) (91 IU/kg twice daily) ***Rituximab, IVIG, Dexamethasone, Mycophenolate***	PT (42 months)
3	c.-29-?_1386+?del	1	N	61	Y	Y	1.	0.5	MD	**PD non-monoclonal antibody purified SHL** (*Nanotiv)* (100 IU/kg daily)	N (1 day)**
							2.	2	MD	**PD non-monoclonal antibody purified SHL** (*Nanotiv)* (80 IU/kg daily) ***IVIG***	N (35 months) ***
4	c.-29-?_1386+?del	1	Y	28	Y	N	1.	1	*1.2**	**PD non-monoclonal antibody purified SHL** (*Nanotiv)* (100–200 IU/kg 2–3 times per week)	N (3 months)
							2.	2	*9.0**	**PD monoclonal antibody purified SHL** (*Mononine*) (35 IU/kg 3 times per week, after 20 months increased dose to 105 IU/kg daily)	N (25 months)
							3.	19	<0.4	***Recombinant SHL*** (*BeneFIX*) (65 IU/kg twice daily, tapering of the dose after 1 month) ***Rituximab, IVIG, Dexamethasone, Mycophenolate*** Simultaneous implantation of venous access catheter	Y (6 months)
5	c.719G > A	2	Y	2.2	Y	Y	1.	1	1.0	***Recombinant SHL*** (*BeneFIX*) (60 IU/kg daily)	N (36 months) ***
							2.	4	<0.4	***Recombinant SHL*** (*BeneFIX*) (86 IU/kg twice daily) ***Rituximab, IVIG, Dexamethasone, Mycophenolate***	Y (3 months)
6	c.719G > A	16	Y	*>300**	Y	N	1.	53	*1.2**	**PD monoclonal antibody purified SHL** (*Mononine*) (35 IU/kg 4 times daily, after 15 days tapering of the dose) ***Cyclophosphamide, Hydrocortisone, IVIG***	PT¶1 (40 days)
7	c.316G > A	2	N	40	Y	Y	1.	2	1.8*	**PD monoclonal antibody purified SHL** (*Mononine*) (110 IU/kg daily) ***IVIG, Cyclophosphamide***	N (15 months)
8	c.316G > A	2	Y	1.9	N	N	1.	4	0.3*	**PD monoclonal antibody purified SHL** (*Mononine*) (93 IU/kg daily) ***IVIG, Cyclophosphamide***	PT (MD)

(*continued on next page*)

Table 4 (*continued*)

ID	Genetic variant	Age at inhibitor detection (years)	Tolerant at enrollment†	Peak titer (BU)	Allergic symptoms	Nephrotic syndrome	ITI attempt	Age at ITI (years)	Titer at start of ITI (BU)	ITI regimen§	ITI success‡ (time to success or termination)
9	c.880C > T	15	Y	*>300**	Y	N	1.	37	*21**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (total dose 69,000 IU during 10 days¤) ***Plasmapheresis, Cyclophosphamide*** Simultaneous surgery of elbow	N (10 days)
							2.	39	*14**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (31 IU/kg/dose 3–4 times daily) ***Plasmapheresis, Cyclophosphamide, IVIG*** Simultaneous extraction of eight teeth	PT (15 days)¶2
10	c.1069G > C	5	Y	0.9	Y	N	1.	1	<0.5	**PD non-monoclonal antibody purified SHL** (*NanoFIX)* (71 IU/kg once daily)	Y (4 months)
11	c.969_975del	5	Y	*>300**	Y	N	1.	10	*0.9**	**PD non-monoclonal antibody purified SHL** (*Preconativ*) (total dose 24,500 IU during 9 days¤) **IVIG** Simultaneous straightening treatment of knee	N (9 days)
							2.	10	*150**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (total dose 30,000 IU during 9 days¤) ***Plasmapheresis, IVIG*** Simultaneous straightening treatment of knee	N (9 days)
							3.	11	*6**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (one dose of 227 IU/kg, hereafter 45 IU/kg three times daily) ***Cyclophosphamide, IVIG*** Simultaneous surgery of knee	N (8 days)
							4.	11	*18**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (total dose 71,000 IU during 8 days¤) ***Plasmapheresis, IVIG, Cyclophosphamide, Hydrocortisone*** Simultaneous treatment of larger bleed	N (8 days)
							5.	12	*5.7**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (total dose 76,000 IU during 11 days¤) ***Plasmapheresis, IVIG, Cyclophosphamide, Hydrocortisone*** Simultaneous surgery of knee, extraction of teeth and injection therapy of elbow	N (11 days)
							6.	13	*2.7**	**PD non-monoclonal antibody purified SHL** (*Preconativ)* (one dose of 125 IU/kg, hereafter 33 IU/kg 2–6 times daily, day 14 tapering of the dose)	Y (3 months)

(*continued on next page*)

Table 4 (*continued*)

ID	Genetic variant	Age at inhibitor detection (years)	Tolerant at enrollment†	Peak titer (BU)	Allergic symptoms	Nephrotic syndrome	ITI attempt	Age at ITI (years)	Titer at start of ITI (BU)	ITI regimen§	ITI success‡ (time to success or termination)
										IVIG, Cyclophosphamide, Hydrocortisone Simultaneous straightening treatment of knee	

Y, yes. N, no. BU, Bethesda units. PT, partial tolerance. PD, plasma-derived FIX. SHL, standard half-life. IVIG, intravenous immunoglobulin. MD, missing data. *Inhibitor titer measured and reported in MIU, Malmö inhibitor units, and recalculated to Bethesda units by multiplying by a factor of three. **Termination due to anaphylaxis. ***Termination due to nephrotic syndrome. §In case of changed doses, the most intensive regimen is presented. †Considered tolerant by the treating physician. ‡Assessed ITI success by the treating physician. ¤No data on dose/kg and frequency could be collected from the medical journals. ¶1After 40 days considered partially tolerant and transition to every other day prophylaxis. ¶2Termination of ITI after 15 days, considered partial tolerant since treatable with FIX-concentrate.

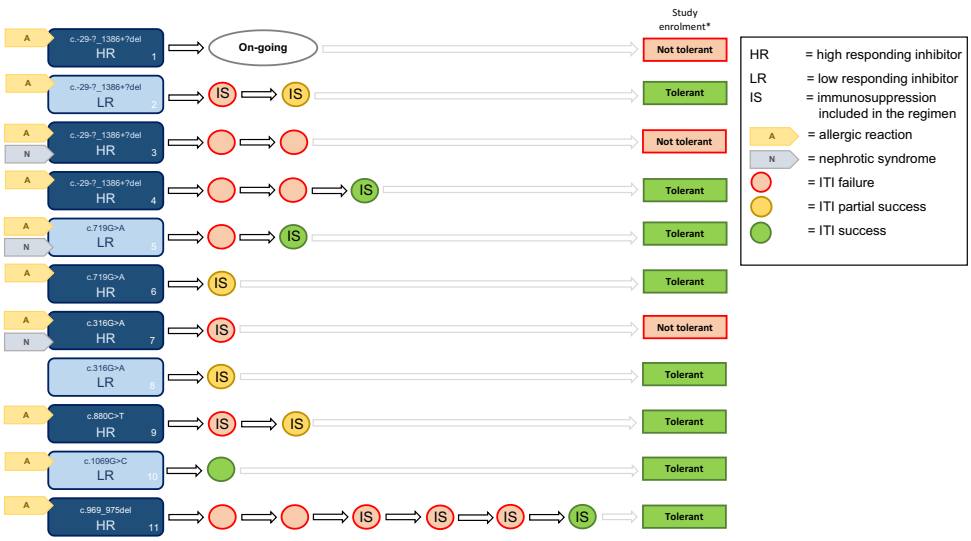

Fig. 3. Schematic illustration of immune tolerance induction attempts (ITI) in eleven persons with hemophilia B and inhibitors. Each line illustrates the ITI experience of one patient and each circle represents an ITI attempt. *The treating physician reported whether the patient was considered tolerant or not at enrolment in the study.

Table 5
Anti-FIX ELISA and xFLI results.

		ELISA			
		Neg	Pos	MD	Total
xFLI	Neg	40	5	0	45
	Pos	1	2†	0	3
	MD	4	1	26	31
	Total	45	8	26	79

MD, missing data.
† Both samples were positive in Bethesda (3 BU/mL and 0.4 BU/mL).

relevance of the inhibitor. A successful use of immunosuppression is in concordance to several previous reports of ITI in HB [8,9,27–33] and our study further supports this approach; adding immunosuppression as a first-line treatment should be considered in these patients. Interestingly, one PwHB had five ITI failures before he became tolerant after the sixth attempt. This attempt was mainly distinguished from the previous attempts by a longer duration of 3 months, indicating that treatment should not be terminated too early. The shortest time to a successful ITI among our patients was 3 months.

All but one of the persons with inhibitors (92 %) had experienced allergic manifestations towards FIX. This figure is high compared to that of 60 % reported by the ISTH-SSC International FIX Inhibitor registry [3], or that of 41 % in the recent B-NATURAL study [27]. This may be due to the underlying *F9* genetic profile in our cohort. Five patients had experienced anaphylaxis; at enrollment, three of these were considered tolerant. Different desensitization protocols have previously been described [34,35] in attempts to overcome the allergic reactions to FIX. Seven of the patients with inhibitors and allergic reactions in our study

underwent some kind of desensitization therapy, four of these with a reported successful or partly successful outcome. Desensitization regimens have however not been the focus of the B-NORD study and therefore no further details can be provided. Accordingly, allergic reactions to FIX complicate an ITI but they are not a definite predictor of failure. The same reasoning applies to the development of nephrotic syndrome. Out of the three PwHB who developed nephrotic syndrome, one was considered tolerant at enrollment. In our, as well as in other published cohorts, however, the combination of a high-titer inhibitor together with the occurrence of both anaphylaxis and nephrotic syndrome seems to be associated with a poor prognosis for achieving tolerance. Importantly, although anaphylaxis and nephrotic syndrome predominantly occurred in patients with null variants, they were also seen in one patient with a missense substitution.

The median factor consumption of 6638 IU/kg/year for the tolerized inhibitor patients in our cohort is significantly higher than that reported for the non-inhibitor patients and well above 4000 IU/kg/year, the level of high-dose prophylaxis, defined by the WFH [2]. This raises the question as to whether the high consumption reported may actually indicate an unfavorable pharmacokinetic profile due to non-neutralizing and/or small amounts of neutralizing antibodies not detectable with the Nijmegen-Bethesda method. However, we did not find any evidence for this when using both the ELISA and xFLI anti-FIX methods. The concordance obtained between the ELISA and xFLI assays was high, but we observed some discrepancies, mainly explained by a lack of reproducibility of the ELISA assay in the low-titer range. The cut-off used in each ELISA test-run is variable, since it is dependent on the normal samples run in each test. The high coefficient of variation (CV) (>50 %) for the positive control in the ELISA assay reflects this issue and indicates the need for further validation of this assay or replacement with the xFLI assay.

4.1. Strengths and limitations

Besides the relatively low number of inhibitor patients, which is a concern in all studies of PwHB, the retrospective study design with the extraction of data from medical records brings further limitations. A key limitation is also the lack of consistent criteria for ITI success in HB. The strengths of the study include the still relatively large study population of PwHB with carefully defined *F9* variants genotyped enrolled at HTCs with a close collaboration and the common Nordic treatment guidelines [36]. In addition, we have evaluated the presence of all types of antibodies using both the Nijmegen-Bethesda assay and two different immunoassays.

5. Conclusions

Our study reveals a high proportion of severe *F9* gene defects among persons with severe HB in the Nordic countries and a relatively high frequency of inhibitors, but no evidence of NNA. Our data also indicate that ITI success can be attained in PwHB despite previous ITI failures independent of the type of *F9* variant and that the addition of immunosuppression to the regimen may enhance the chances of success. Furthermore, our study supports the findings that allergic reactions as well as the development of nephrotic syndrome complicate the clinical management, but do not necessarily correlate with specific *F9* null variants.

Disclosures

KK has received research grants from CSL Behring, Stockholm, Sweden. FB has received honoraria as a member of an advisory board and/or speaker from Sobi, Shire/Takeda, Novo Nordisk, Bayer, Roche, UniQure, Octapharma, BioMarin and Pfizer. MB was supported by funds from Stockholm County Council. EF has received honorarium as a speaker for Shire, Roche, Sobi and Takeda. PAH has acted as a paid consultant to Bayer, Shire, Novo Nordisk, Octapharma, CSL Behring, Pfizer and Sobi including lectures. RL has been a member of advisory boards for Sobi, CSL Behring, Takeda, BioMarin, Novo Nordisk, Pfizer, Roche and Bayer. VN has received research grant from CSL Behring, Stockholm, Sweden. SR has received research grants from the Childhood Cancer Foundation, PedNet and Stockholm County Council, is a member of steering committee for Roche and investigator in clinical trials promoted by Roche, Novo Nordisk and Sobi. KS has received speaker fees from Octapharma, Sobi, Shire and Novo Nordisk and has been scientific advisory board member for Novo Nordisk, Sobi and BioMarin. NGA has served as a speaker and/or on advisory boards for Bayer, CSL Behring, Octapharma and Sobi. EB has received research grants and paid consultancy from CSL Behring, Stockholm, Sweden. JA has received research grants from Sobi, CSL Behring, Takeda/Shire, and Bayer and speaker's fee and consultant's fee for Octapharma, NovoNordisk, Pfizer, Bayer, Sobi, CSL Behring, Takeda/Shire, BioMarin, Uniqure, and Spark Therapeutics. MM stated that she had no interests, which might be perceived as posing a conflict or bias.

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CRediT authorship contribution statement

JA, EB and KK designed the research study. KK analyzed the data. KK, JA and NGA interpreted the data and drafted the paper. KS and MM designed the NNA methods and drafted these parts of the paper. KK, JA, NGA, FB, MB, EF, PAH, RL, VN and SR enrolled patients and collected the clinical data. All authors reviewed the manuscript critically and read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: KK has received research grants from CSL Behring, Stockholm, Sweden.

FB has received honoraria as a member of an advisory board and/or speaker from Sobi, Shire/Takeda, Novo Nordisk, Bayer, Roche, UniQure, Octapharma, BioMarin and Pfizer.

MB was supported by funds from Stockholm County Council.

EF has received honorarium as a speaker for Shire, Roche, Sobi and Takeda.

PAH has acted as a paid consultant to Bayer, Shire, Novo Nordisk, Octapharma, CSL Behring, Pfizer and Sobi including lectures.

RL has been a member of advisory boards for Sobi, CSL Behring, Takeda, BioMarin, Novo Nordisk, Pfizer, Roche and Bayer.

VN has received research grant from CSL Behring, Stockholm, Sweden.

SR has received research grants from the Childhood Cancer Foundation, PedNet and Stockholm County Council, is a member of steering committee for Roche and investigator in clinical trials promoted by Roche, Novo Nordisk and Sobi.

KS has received speaker fees from Octapharma, Sobi, Shire and Novo Nordisk and has been scientific advisory board member for Novo Nordisk, Sobi and BioMarin.

NGA has served as a speaker and/or on advisory boards for Bayer, CSL Behring, Octapharma and Sobi.

EB has received research grants and paid consultancy from CSL Behring, Stockholm, Sweden.

JA has received research grants from Sobi, CSL Behring, Takeda/Shire, and Bayer and speaker's fee and consultant's fee for Octapharma, NovoNordisk, Pfizer, Bayer, Sobi, CSL Behring, Takeda/Shire, BioMarin, Uniqure, and Spark Therapeutics.

MM stated that she had no interests, which might be perceived as posing a conflict or bias.

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ORIGINAL ARTICLE

No difference in quality of life between persons with severe haemophilia A and B

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Abstract

Introduction: Good health-related quality of life (HRQoL) is an important goal in the treatment of persons with haemophilia B (PwHB). Studies focusing on this population are limited, however, and data are insufficient.

Aim: To assess the HRQoL in PwHB and to compare this to data on persons with haemophilia A (PwHA), as well as to evaluate the impact of joint health on HRQoL and to identify areas of insufficient care.

Methods: The B-NORD study enrolled persons with severe haemophilia B and matched controls with haemophilia A. HRQoL was assessed using the EQ-5D-3L questionnaire and joint health using Haemophilia Joint Health Score 2.1 (HJHS).

Results: The EQ-5D-3L was completed by 63 PwHB and 63 PwHA. Mobility problems were reported by 46% of PwHB and 44% of PwHA, pain/discomfort by 62% and 56%, and anxiety/depression by 33% and 17%, respectively. No significant difference was observed between PwHA and PwHB in EQ-5D profiles, level sum score, EQ-5D index (PwHB mean .80, PwHA mean .83, $p = .24$), or EQ VAS score (PwHB: mean 70, PwHA: mean 77, $p = .061$). Linear regression adjusted for age demonstrated that an increase in HJHS score was associated with a significant decrease in both EQ-5D index (B -.003, R_2 .22) and EQ VAS score (B -.37, R_2 .17).

Conclusion: Despite the majority of patients being treated with prophylaxis, impaired HRQoL was reported in both PwHB and PwHA. No differences in HRQoL were found between the two groups. Impaired joint health had a significant negative impact on HRQoL.

KEYWORDS
arthropathy, coagulation factor IX, EQ-5D, haemophilia A, haemophilia B, quality of life

1 | INTRODUCTION

Haemophilia is a rare chronic bleeding disorder caused by the deficiency of coagulation factor VIII (FVIII) (haemophilia A, HA) or coagulation factor IX (FIX) (haemophilia B, HB). The main disease burden of persons with haemophilia (PwH) is an increased risk of traumatic and spontaneous bleedings, causing uncertainty and psychological strain. The development of arthropathy secondary to repeated joint bleeds is a particularly problematic chronic complication and can lead to pain and impaired mobility with impact on the daily lives of PwH.

HA is more common and has been studied more widely than HB. Consequently, much of our knowledge on HB has been extrapolated from studies based on persons with HA (PwHA). The B-NORD study[1] was designed with HB patients in mind, to better understand the disease, its similarities to and differences from HA, and to further improve and individualize the care of HB patients.

FIX replacement therapy was introduced in the 1960s and the gold standard of care in HB is still considered to be prophylactic treatment with either standard half-life (SHL) or extended half-life (EHL) FIX products, but new treatment possibilities are in the pipeline. With the current treatments of today, we believe and hope for patients with HB to have a normal, or close to normal, life expectancy; however, the replacement therapy still needs to be administered intravenously which brings great impact on the everyday lives of persons with HB (PwHB).

Health-related quality of life (HRQoL) can be referred to as how well a person functions in his/her life and his/her perceived well-being in physical, mental, and social domains of health.[2] Significant impairment of HRQoL with high frequencies of pain and functional impairments, as well as an increased prevalence of depression and anxiety in PwH, have been reported.[3–8] A good HRQoL is an important goal in the treatment of PwH, and the use of questionnaires for HRQoL in routine follow-up at haemophilia treatment centres (HTC) might be of value to gain better insight into, and address problems in the everyday lives of PwH appropriately.

The objective of the present study was to assess the HRQoL in persons with severe HB and to compare this to the data on matched individuals with HA. An additional aim was to evaluate the impact of joint health on HRQoL, to identify any areas of insufficient care and to improve our understanding of health in a wider perspective in PwH.

2 | MATERIALS AND METHODS

2.1 | Study design and study population

The observational study B-NORD has been described previously[1] and enrolled persons registered at HTCs in Denmark, Finland, Norway and Sweden with a confirmed diagnosis of congenital severe HB (FIX level < .01 IU/ml). Control persons with severe HA matched by age, gender and treatment modality (on demand/prophylaxis) from one of the Nordic HTCs were identified using the KAPPA register.[9] Seventy-nine PwHB of all ages were enrolled between the years 2017 and 2020. The controls with HA were enrolled between 2013 and 2017. In addition to previously reported data,[1,10] information on socioeconomics was registered as well as data on medical history, joint status, comorbidities, replacement therapy and the use of analgesics and antidepressants/anxiolytics. For a fair comparison of quality-of-life, persons with current inhibitors were excluded.

2.2 | Health-related quality of life

To measure HRQoL, patients 12 years and older[11] were asked to complete the self-administered 3-level version of the questionnaire EQ-5D (EQ-5D-3L).[12] EQ-5D is a standardized measure of HRQoL and

assesses health status in a 'generic' manner since it is not specific to any particular health condition or patient group. The official language versions of the questionnaire corresponding to the spoken language at the HTCs were used.

The questionnaire consists of two pages: the EQ-5D-3L descriptive system and the EQ visual analogue scale (EQ VAS). The descriptive system consists of the five dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has three levels: no problems, some problems, and extreme problems (Levels 1–3). The participants mark the level of each dimension which best describes their experience. The composite result is expressed as the 'EQ-5D profile'. By adding up the levels (1, 2, or 3) of each dimension, treating each level as a number rather than a categorical description, the Level Sum Score (LSS) is calculated and can be used as a crude measurement of severity. The best EQ-5D profile (11111) represents 'having no problems' in all five dimensions and adds up to LSS 5; the LSS for the worst health state (33333) is consequently 15.

The EQ-5D health state can be converted into a single summary number (index value) by attaching values from a value set to each of the levels. The index value hereby reflects health according to the preferences of the general population of a country/region. The index scores range from less than 0 to 1, with higher scores indicating better health and score one representing a health state equivalent to perfect health.[11] Index values were calculated using the Danish value set,[13] which was thought to be the most representative available of the Nordic cohort in the B-NORD study.

The second page of the EQ5D-3L questionnaire includes the EQ VAS, a scale from 0 to 100 where the study subjects are asked to indicate their 'overall health state today' with 0 representing 'worst health imaginable' and 100 'best health imaginable'. The EQ VAS provides additional data reflecting the patient's assessment of their overall health, including dimensions that the descriptive part of the questionnaire may not cover.

2.3 | Assessment of joint health

A physiotherapist or physician at the HTCs used the Haemophilia Joint Health Score version 2.1 (HJHS)[14] to evaluate the joints. The HJHS is a physical examination tool that assesses the patient's ankles, knees and elbows in nine different categories including swelling, duration of swelling, muscle atrophy, crepitus on motion, flexion loss, extension loss, joint pain, strength and global gait. Higher scores indicate more severe joint damage. The maximum total score is 124, with a maximum score of 20 per assessed joint and four on global gait.

2.4 | Statistical analysis

IBM SPSS Statistics 25 was used for the statistical analyses. Descriptive statistics, including means (M) with standard deviations (SD) and medians with first-to-third quartiles (Q1-Q3) were used for continuous variables. Categorical data were reported as numbers and percentages. McNemar's test was used to assess whether the number of persons reporting a problem in EQ-5D differed between PwHB and PwHA. Differences in EQ VAS scores and index values between PwHB and their matched HA controls were analyzed using paired samples *t*-test. The correlation between HJHS score and age was examined with the Pearson correlation coefficient. Linear regression was applied to examine the relationships between EQ-5D results, HJHS, age and body mass index (BMI). Differences in EQ-5D index values and EQ VAS scores between PwHB treated with SHL and EHL were assessed using independent samples *t*-test. *p*-Values $< .05$ were considered to be statistically significant.

3 | RESULTS

In total, 126 PwH (63 PwHB, 63 PwHA), 15–76 years of age, without current inhibitors completed the EQ-5D questionnaire. The mean age was 40 years (SD 18 years) for PwHB and 41 (SD 18 years) for PwHA. Five PwH were children between 15 and 17 years of age. All but one (98%) of PwHB were on prophylaxis with factor products; PwHA were matched accordingly. Of PwHB, 82% used SHL and 11 (18%) used EHL products. All PwHA used SHL products. Six PwHB (9.5%) and five PwHA (7.9%) had developed inhibitors earlier but were considered tolerant at study enrolment. Employment was similar in the two groups with 46 (73%) of PwHB and 47 (75%) of PwHA studying or working (full or part-time). Fifty-six percent of PwHB reported use of analgesics and 9.5% reported taking antidepressants or anxiolytics. Unfortunately, information on the use of analgesics and antidepressants was incomplete in the KAPPA register and was therefore not reported for PwHA. All but four PwHA and 10 PwHB had filled in the EQ VAS: the persons with missing data and their matched study subjects were excluded from the VAS calculations. Fourteen PwHB had a missing HJHS total score and were excluded in this domain. Clinical charateristics and socioeconomics are presented in Table 1. No differences were observed in the reported comorbidities between PwHB and PwHA (Table 2).

3.1 | EQ-5D profiles and EQ VAS

A high proportion of the patients: 46% of PwHB and 44% of PwHA, reported problems (Levels 2+3) with mobility and more than half of the patients with either HB or HA reported problems in the dimension of pain/discomfort (62% HB, 56% HA). As many as 33% of PwHB experienced problems with anxiety/depression, compared to 17% among PwHA. The majority of patients who reported problems, reported 'some problems' (level 2). Only rarely, 'extreme problems' (level 3) was reported (1.6%–3.2% in dimensions usual activities, pain/discomfort, anxiety/depression). No significant difference was detected between PwHB and PwHA in the different dimensions. The profile 11111, that is, 'having no problems', was the most frequent profile in both groups and reported by 18 (29%) of PwHB and 24 (38%) of PwHA. The LSS did not differ between PwHB and PwHA, the median being 6 (Q1-Q3 5–8)

TABLE 1 Clinical characteristics and socioeconomics.

		HA $n = 63$	HB $n = 63$
Age at enrolment, years, mean (SD)		41 (18)	40 (18)
BMI, kg/m^2, mean (SD)		25 (3.3)[b]	26 (4.7)
Former inhibitor (%)		5 (7.9)	6 (9.5)
Haemophilia treatment (%)			
Treatment modality (%)			
	On-demand	1 (1.6)	1 (1.6)
	Prophylaxis	62 (98)	62 (98)
Age at start of prophylaxis, years, median (Q1-Q3)[c]		6 (2-23)	5.5 (1.1-19)
Factor concentrate (%)			
	Plasma derived standard half-life	5 (7.9)	20 (32)
	Recombinant standard half-life	58 (92)	32 (51)
	Extended half-life		11 (18)
HJHS, mean (SD)			
	Total joint score	20 (19)	15 (19)[d]
	Total score	23 (20)	19 (21)[e]
Highest completed education (%)			
	Below high-school diploma	14 (22)	13 (21)
	High-school diploma	25 (40)	40 (64)
	University education	23 (37)	10 (16)
	MD	1 (1.6)	–
Current main employment (%)			
	Student	12 (19)	8 (13)
	Working full time	29 (46)	32 (51)
	Working part time	6 (9.5)	6 (9.5)
	Unemployed	1 (1.6)	2 (3.2)
	Normal retirement	7 (11)	6 (9.5)
	Early retirement	6 (9.5)	9 (14)
	MD	2 (3.2)	–
Smoking (%)			
	Current smoker	11 (18)	8 (13)
	Former smoker	18 (29)	16 (25)
	Never smoked	34 (54)	36 (57)
	MD	–	3 (4.8)
Use of analgesic (%)		MD	35 (56)
	NSAID	MD	23 (37)
	Paracetamol	MD	22 (35)
	Other[a]	MD	10 (16)
Use of antidepressants/anxiolytics (%)			6 (9.5)

Abbreviations: BMI, body mass index; HA, haemophilia A; HB, haemophilia B; HJHS, haemophilia joint health score; MD, missing data.
[a]Other analgesic includes codeine, tramadol, buprenorphine, oxycodone, pregabalin.
Statistics presented: Numbers (%). Mean (SD, standard deviation).
The number of patients (n) is noted if it deviates from the total number:
[b]$n = 62$.
[c]$n = 41$ (HA), $n = 58$ (HB).
[d]$n = 55$.
[e]$n = 49$.

TABLE 2 Summary of reported comorbidities.

	HA $n = 63$	HB $n = 63$	p
Hypertension	13 (21)[a]	14 (22)	.63
HIV positive (%)	3 (4.8)	4 (6.3)	1.0
Unknown/Not tested	5 (7.9)	6 (9.5)	
HCV status (%)			
Never infected (Ab-/PCR-)	23 (37)	30 (48)	.23
HCV positive (Ab+/PCR+)	12 (19)	4 (6.3)	.035
Recovered infection (Ab+/PCR-)	23 (37)	27 (43)	.66
Unknown/ Not tested	5 (7.9)	2 (3.2)	
Other chronic disease reported (%)	16 (25)	19 (30)	
Diseases specified:			
Respiratory and allergic diseases	2	5	
Malignancies	2	3	
Kidney and urological disorders	3	2	
Diabetes	2	2	
Cardiovascular disease	2	1	
Mental illness	1	2	
Disorders of the gastrointestinal tract	1	2	
Disorders of the musculoskeletal system	1	1	
Neurological disease		2	
Other	3	3	

Note: Haemophilia related musculoskeletal disorders excluded.
Statistics presented: Numbers (%). Significance levels from Mc Nemar's test. The number of patients (n) is noted if it deviates from the total number:
[a]$n = 54$.
Malignancies: prostate cancer, cancer papilla vateri, colon cancer, basal cell carcinoma, essential thrombocythemia.
Respiratory and allergic diseases: asthma, sleep apnoea, allergy unspecified.
Kidney and urological disorders: kidney transplantation, medullary sponge kidney, decreased kidney function unspecified, benign prostate hyperplasia.
Cardiovascular disease: coronary disease, cerebrovascular disorder. Mental illness: depression, panic anxiety.
mental illness unspecified. Disorders of the gastrointestinal tract: irritable bowel syndrome, coeliac disease. Disorders of the musculoskeletal system: carpal tunnel syndrome, osteoporosis. Neurological disease: epilepsy, fascial nerve palsy.
Other: obesity, hepatitis A, deafness (one-sided), CVID, vitiligo.
Abbreviations: HA, haemophilia A; HB, haemophilia B; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

in both groups. Detailed information on the different profiles and their frequencies are presented in Supplemental Table 1.

The EQ VAS and the EQ-5D profiles are thought to be supplementary to each other and a moderate-to-strong correlation between EQ VAS and EQ-5D index scores ($r = .64$, $p < .001$) confirmed the concordance of the two evaluations. EQ-5D index or EQ VAS values between PwHB and PwHA did not differ, with mean index scores of .80 (SD .17) for PwHB and .83 (SD .16) for PwHA ($p = .24$) and mean EQ VAS scores of 70 (SD 20) for PwHB and 77 (SD 19) for PwHA ($p = .061$). To assess the potential impact of former inhibitors on the outcome, a similar comparison was performed with these patients and their controls excluded. The results were similar and showed no significant differences between PwHB and PwHA with mean index scores of .82 (SD .16) for HB and .83 (SD .17) for HA ($p = .76$), and the corresponding mean VAS scores of 71 (SD20) and 79 (18 SD) ($p = .073$). Similar results were seen when patients with HIV or HCV infection were excluded. The EQ-5D responses are summarized in Table 3 and in Figures 1 and 2.

3.2 | Impact of joint health, age, BMI, and extended half-life products

Impact of joint health, age and BMI were analyzed with the cohort merged (PwHB and PwHA together). To reduce uncertainty and to search for sources of errors, the combined group was divided into PwHB and PwHA for sensitivity analysis, which provided similar outcomes.

As expected, older age was correlated with higher HJHS score (r .76, $p < .001$). Linear regression adjusted for age demonstrated that both the EQ-5D index and EQ VAS score were significantly associated with the HJHS score. Each increase by one HJHS score point aligned with a .003 decrease in the EQ-5D index score (B -.003, 95% CI -.005 to -.001, $p = .002$), and the regression model explained 22% of the variation in the EQ-5D index. Each increase by one HJHS score point was also associated with a .37 decrease in the EQ VAS score (B -.37, 95% CI -.64 to -.11, $p = .007$), and the regression model explained 17% of the variation in EQ VAS. Increasing age was significantly associated with lower EQ-5D index and EQ VAS by the univariate analysis only and did not show significance when HJHS score was included in the analysis. No association was evident between BMI and EQ-5D index or EQ VAS score. Associations between patient characteristics and EQ-5D indexes and EQ VAS are presented in Table 4 and Figure 3.

Eleven participants with HB were treated with EHL products. EQ-5D index and EQ VAS values did not differ between PwHB treated with EHL and PwHB managed with SHL products: mean EQ-5D index for the EHL group was .81 (SD .15), for the SHL group .80 (SD .17) ($p = .91$). Mean EQ VAS for the EHL group was 73 (SD 16), and for the SHL group 70 (SD 21) ($p = .68$).

4 | DISCUSSION

In this Nordic study we assessed the HRQoL in persons with severe HB and matched HA controls, using EQ-5D profiles and EQ VAS, and further evaluated the impact of age and joint health on HRQoL. This was performed as a follow-up to our previous report of the B-NORD cohort,[1] suggesting a slightly milder arthropathy for PwHB based on a significant lower joint score in PwHB than in PwHA. Bearing in mind a milder arthropathy for PwHB and generally less frequent injections required, a somewhat better HRQoL outcome for PwHB could be

TABLE 3 Summary of EQ5D-3L responses divided by diagnosis

	Mobility *n* (%)		Self-care *n* (%)		Usual activities *n* (%)		Pain/Discomfort *n* (%)		Anxiety/Depression *n* (%)	
	HA	HB	HA	HB	HA	HB	HA	HB	HA	HB
Level 1	35 (56)	34 (54)	61 (97)	57 (91)	49 (78)	52 (83)	28 (44)	24 (38)	52 (83)	42 (67)
Level 2	28 (44)	29 (46)	2 (3.2)	6 (9.5)	14 (22)	9 (14)	33 (52)	37 (59)	10 (16)	20 (32)
Level 3	–	–	–	–	–	1 (1.6)	2 (3.2)	2 (3.2)	1 (1.6)	1 (1.6)
MD	–	–	–	–	–	1 (1.5)	–	–	–	–
Total	63 (100)	63 (100)	63 (100)	63 (100)	63 (100)	63 (100)	63 (100)	63 (100)	63 (100)	63 (100)
Reporting problems (Level 2+3)	28 (44)	29 (46)	2 (3.2)	6 (9.5)	14 (22)	10 (16)	35 (56)	39 (62)	11 (17)	21 (33)
p	1.0		.22		.39		.50		.064	

Level 1, no problems. Level 2, some problems. Level 3, extreme problems.
Statistics presented: Numbers (%). Significance levels from Mc Nemar's test.
Abbreviations: HA, haemophilia A; HB, haemophilia B; MD, missing data.

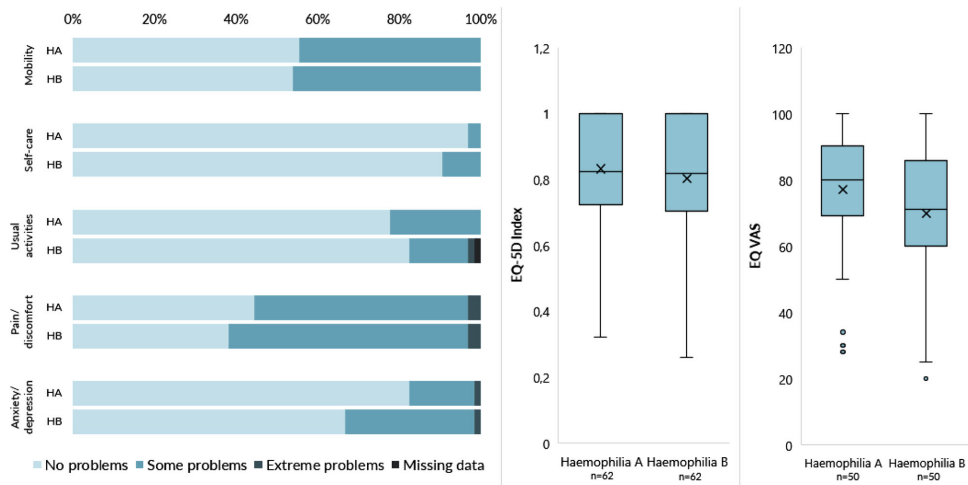

FIGURE 1 EQ5D-3L responses divided by diagnosis. HA, haemophilia A. HB, haemophilia B.

expected. This was, however, not observed since we did not detect any significant differences in the EQ-5D profiles or EQ VAS between PwHB and PwHA. We did not even see a trend towards better HRQoL in PwHB. In agreement with previously reported data,[9,15] we observed an increasing HJHS score, adjusted for age, to be associated with decreasing EQ-5D index and EQ VAS scores, suggesting impaired joint health to be of significant negative impact on HRQoL.

Contrary to Berntorp et al.,[4] we found no association between BMI and HRQoL, and increasing age was associated significantly with lower QoL scores by univariate analysis only, and without significance when adjusted for HJHS results. This indicates the importance of preserved joint health in ageing PwH and is in agreement with the analysis by Osooli et al.,[9] but in contrast to what was reported from the B-NATURAL and PROBE-studies[4,16]; however, these latter studies made no adjustment for joint health in the age comparison.

Population norms for EQ5D-3L values from Denmark, Finland and Sweden have been published by the EuroQol Group,[17] as well as data from Norway by Stavem et al.,[18] As shown in Figure 2, EQ-5D index values and EQ VAS values were slightly lower in PwHB compared to the general population. Frequencies of reported problems in the dimensions mobility, pain and anxiety/depression stood out with higher frequencies of problems (Levels 2+3) in PwHB compared to the general population. The mean age for the population norm groups was slightly older than in our cohort and, together with different study designs, and the use of different value sets, this prevents fair comparisons and firm conclusions. However, the fact remains that, on the day of the

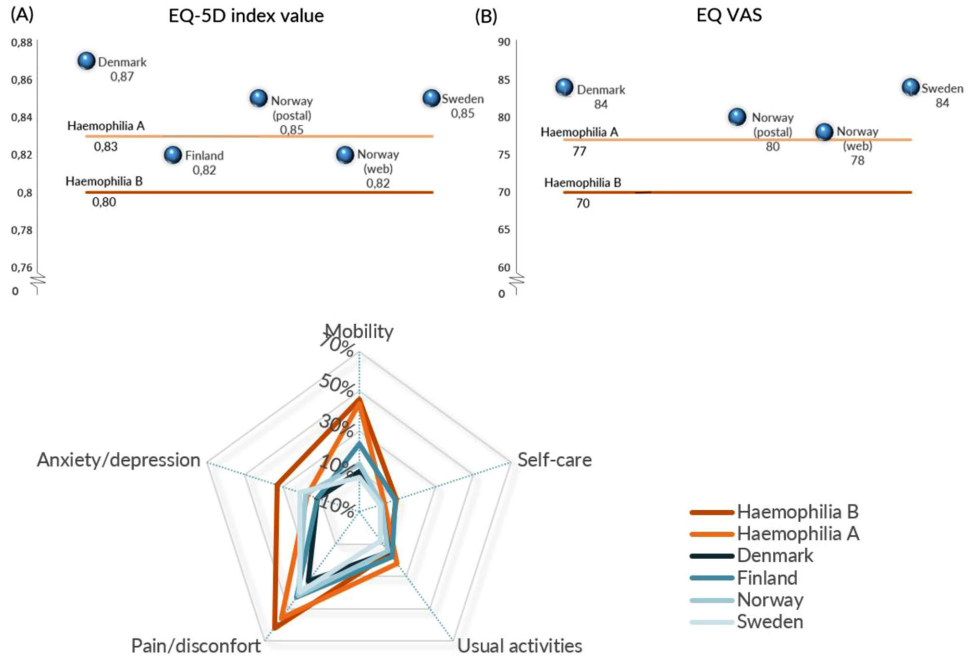

FIGURE 2 EQ-5D for persons with haemophilia in the B-NORD study in comparison to Nordic population norms. Population norms from Denmark, Finland and Sweden published by the EuroQol Group (17), population norms from Norway (postal and web survey) published by Stavem et al. (18). Persons aged ≥18 years (≥30 years for Finland) included in the population norms, mean ages; Denmark 47 years, Finland 51 years, Norway 52 (postal), 51 (web) years, Sweden 44 years. (A) Mean EQ-5D index values for persons with haemophilia in comparison to Nordic population norms. (B) Mean EQ VAS values for persons with haemophilia in comparison to Nordic population norms. No data was found from Finland. (C) Percentages of participants reporting problems (Level 2+3) in the different EQ-5D dimensions. Population norms presented for males. Postal and web survey data from Norway is presented pooled.

TABLE 4 Analysis of predictors of EQ-5D index and EQ VAS

Analysis of predictors of EQ-5D index						
	Univariate			Multivariate*		
	B coefficient	95% CI	*p*	B coefficient	(95% CI)	*p*
HJHS	−.004	(−.005, −.002)	<.001	−.003	(−.005, −.001)	.002
Age	−.004	(−.005, −.002)	<.001	−.001	(−.003, .002)	.59
BMI	.001	(−.006, .009)	.77			
Analysis of predictors of EQ VAS						
	Univariate			Multivariate**		
	B coefficient	95% CI	*p*	B coefficient	(95% CI)	*p*
HJHS	−.41	(−.59, −.23)	<.001	−.37	(−.64, −.11)	.007
Age	−.34	(−.54, −.14)	.001	−.050	(−.35, .25)	.74
BMI	−.36	(−1.4, .72)	.51			

Abbreviations: BMI, body mass index; HJHS, haemophilia joint health score.
*$R_2 = .223$; $F = 15.7$, $p < .001$.
**$R_2 = .17$; $F = 10.1$, $p < .001$.

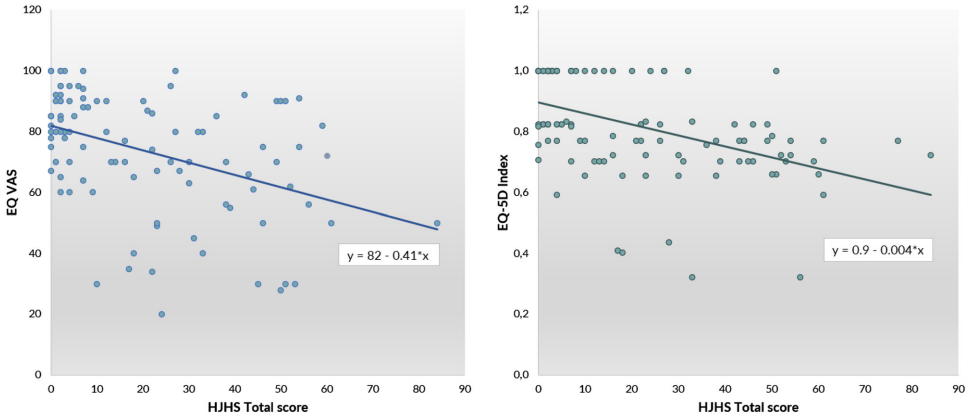

FIGURE 3 Associations between EQ-5D and joint score. HJHS, Haemophilia Joint Health Score.

evaluation, more than half of the PwHB in our study experienced pain, nearly half described difficulty in walking and one-third of PwHB suffered from anxiety or depression.

Largely in agreement with our study, the B-NATURAL study reported a frequency of problems with mobility of 54% and 28% (inhibitor/no inhibitor), pain 58% and 41% (inhibitor/no inhibitor) and anxiety/depression 50% and 21% (inhibitor/no inhibitor), using EQ-5D-3L and included patients with severe HB with (n = 29) and without inhibitors (n = 39). The B-HERO study[3] also found pain, functional impairment and depression/anxiety to be present at higher-than-expected levels. The B-HERO study included 299 PwHB with all severities of the disease and used the 5-level version of the EQ-5D questionnaire for assessing HRQoL. The different inclusion criteria and EQ-5D versions makes precise comparisons to our results uncertain; however, they reported only 22% of the study subjects answering having 'no problems' with mobility, 7% with pain and 19% with the dimension anxiety/depression.

In our study, 56% of PwHB reported use of analgesics, a number concordant with the frequency of individuals reporting pain. However, in only two cases was mental illness (panic anxiety n = 1, mental illness unspecified n = 1) recorded, and only six PwHB reported use of antidepressants/anxiolytics, in contrast to the 21 individuals reporting problems in the EQ-5D dimension anxiety/depression. Information on non-pharmaceutical treatments for anxiety/depression was unfortunately lacking, but despite this, the difference in numbers makes us consider that depression and anxiety might be unrecognized and undertreated in our HB patients. A recent report from the MIND study[8] showed results to support this hypothesis. In the latter study 343 persons with HA or HB, all severities, were enrolled, and the authors reported that only 24% of those who had experienced depression/anxiety felt that this was addressed adequately by their HTC.

No PwHA and only 11 PwHB used EHL in our study, the low number reflects the inclusion period before the wider use of EHL products, and in our small material no significant impact on quality of life could be seen. However, EHL products and new treatment possibilities to come in the future may prove to simplify the everyday lives and be of importance in the pursuit of good health and quality of life for PwH.

4.1 | Strengths and limitations

All studies of PwHB have the concern of a limited number of subjects and, despite its international multicentre design, our study is no exception. However, focusing on patients with severe HB, the enrolled population is still relatively large and representative from a homogenous geographical area with HTCs in close collaboration and with the majority of patients registered at the HTCs enrolled in the study. The inclusion of matched healthy controls without haemophilia would have given the study additional strength, and incomplete information on bleedings in the control group preventing evaluation of impact on HRQoL, is also a limitation to our study. However, in contrast to the majority of previous studies of haemophilia, this is a study with PwHB in focus, which is of particular value, as well as the closely matched controls with HA.

5 | CONCLUSION

In this Nordic study we did not find any significant differences in HRQoL between PwHB and PwHA. Furthermore, we report results confirming that impaired joint health significantly decreases HRQoL. We describe a well-treated haemophilia population with the majority of patients on prophylaxis but, despite this, impaired QoL is reported with a high frequency of pain, mobility problems and anxiety/depression. This indicates that areas of insufficient care exist, and we present data suggesting that depression and anxiety may be unrecognized and undertreated in PwHB. An increased awareness among

the staff at the HTCs as well as attention and responsiveness to signals of ill health are important first steps to improve the QoL of our patients.

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CONFLICT OF INTEREST STATEMENT

KK has received research grants from CSL Behring, Stockholm, Sweden. FB has received honoraria as a member of an advisory board and/or speaker from Sobi, Shire/Takeda, Novo Nordisk, Bayer, Roche, UniQure, Octapharma, BioMarin and Pfizer. MB has been a member on the advisory board for CSL Behringer, Idogen, Novo Nordisk and Sobi, has had consultant assignments for Novo Nordisk and received lecturer honoraria from Pfizer and Sobi. EF has received honorarium as a speaker for Shire, Roche, Sobi, Takeda and BMS. PAH has acted as a paid consultant to Bayer, Shire, Novo Nordisk, Octapharma, CSL Behring, Pfizer and Sobi including lectures. RL has been a member of advisory boards for Sobi, CSL Behring, Takeda, BioMarin, Novo Nordisk, Pfizer, Roche and Bayer. VN has received a research grant from CSL Behring, Stockholm, Sweden. SR has received research grants from the Childhood Cancer Foundation, PedNet Research Foundation and Stockholm County Council, is a member of a steering committee for Roche and investigator in clinical trials promoted by Roche, Novo Nordisk and Sobi. NGA has served as a speaker and/or on advisory boards for Bayer, CSL Behring, Octapharma and Sobi. EB has received research grants and paid consultancy fees from CSL Behring, Stockholm, Sweden. JA has received research grants from Sobi, CSL Behring, Takeda/Shire, and Bayer and speaker's fee and consultant's fee from Octapharma, NovoNordisk, Pfizer, Bayer, Sobi, CSL Behring, Takeda/Shire, BioMarin, Uniqure, and Spark Therapeutics.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

The study was approved by the Swedish Ethical Review Authority (Dnr 2016/1089) and by the independent ethics committees in each participating country. In accordance with the Declaration of Helsinki, written informed consent was collected from the study subject or his legally acceptable representative.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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SUPPLEMENTAL TABLE 1.
EQ5D-3L profiles

EQ5D profile	HA n=63 (%)	HB n=63 (%)
11111	24 (38)	18 (29)
11121	9 (14)	10 (16)
21121	6 (9.5)	10 (16)
21122	6 (9.5)	5 (7.9)
21221	6 (9.5)	-
21222	2 (3.2)	4 (6.3)
11112	-	4 (6.3)
22221	2 (3.2)	1 (1.6)
21111	2 (3.2)	1 (1.6)
11122	1 (1.6)	1 (1.6)
21131	1 (1.6)	1 (1.5)
21211	2 (3.2)	-
21232	1 (1.6)	1 (1.6)
11223	1 (1.6)	-
21123	-	1 (1.6)
21322	-	1 (1.6)
22121	-	1 (1.6)
22122	-	1 (1.6)
22222	-	2 (3.2)
22232	-	1 (1.6)
11?11	-	1 (1.6)

Abbreviations: ?, missing data.
HA, haemophilia A, HB, haemophilia B.
Statistics presented: Numbers (%).

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