Developments in modern hemophilia care

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Contents

Chapter 1
Introduction

General background

Hemophilia is an X-linked hereditary bleeding disorder. Hemophilia A is caused by a defect in the *F8* gene which leads to a deficiency in functional clotting factor VIII (FVIII) while hemophilia B is caused by a defect in the *F9* gene which leads to a deficiency in functional clotting factor IX (FIX). The prevalence at birth is 24.6 per 100,000 persons for hemophilia A and 3.8 per 100,000 for hemophilia B.[1] The severity of the disease is based on an individual's residual clotting factor activity. Severe hemophilia is defined as having ‹ 0.01 international unit (IU)/mL clotting factor activity, while patients with moderate and mild hemophilia have clotting factor levels of 0.01-0.05 IU/mL and › 0.05-0.40 IU/mL, respectively.[2]

In 1-4% of neonates with severe hemophilia, intracranial hemorrhaging can occur during the perinatal period, which can lead to permanent neurological damage. In children and adults with severe hemophilia, spontaneous bleeds in muscles and joints are common. In the long term, joint bleeds cause bleeding-induced arthropathy, leading to long-term disability. In patients with mild hemophilia, the disease primarily manifests as increased bleeding after trauma or surgery.[2]

Throughout history, references can be found to bleeding disorders similar to hemophilia. The earliest reference can be found in the Babylonian Talmud, which was compiled around the 2nd century AD. In these writings, warnings against circumcision in children with brothers that previously died due to excessive bleeding after this intervention can be found.[3] Usage of the actual term "hemophilia" to describe a hereditary bleeding disorder first appeared in 1828 in a text by Friedrich Hopff, a student at the University of Zürich.[3]

Assessment of the health status of the Dutch hemophilia population

Important developments in hemophilia care over time

Until the 1970s, patients suffering from hemophilia were treated with plasma or whole blood. Due to the low amount of clotting factor in these preparations, this was not effective at treating bleeds. Consequently, most patients died due to major bleeding in vital organs in adolescence or early adulthood. The introduction of cryoprecipitate in 1964 and freeze-dried clotting factor concentrates (which contain higher concentrations of FVIII or FIX) in the 1970s made effective treatment of bleeds possible and dramatically reduced mortality. The introduction of regular treatment with clotting factor concentrates to prevent the occurrence of bleeding episodes (also

called prophylactic treatment) during this period improved quality of life immensely as patients suffered from less joint bleeds and consequently, less bleeding-induced arthropathy. The introduction of desmopressin, which works by releasing endogenous FVIII from endothelial cells, added a treatment option for patients with mild hemophilia A that was safe and effective.[3]

This so-called 'golden era' of hemophilia ended when many patients were infected with the human immunodeficiency virus (HIV), or with the hepatitis C virus (HCV) through the infusion of contaminated blood products during the 1980s. This led to many deaths due to AIDS, as well as many cases of HCV-related liver disease. The adoption of new viral inactivation techniques as well as new screening methods have stopped transmission of HIV or HCV through blood products since 1992. In the early 1990s, the first clotting factor products produced through recombinant technology were introduced to the market. The supposed risk of transfusion transmitted diseases was further decreased by these new products (especially infections by as-yet-unknown pathogens), and production could be increased as the supply of blood donors was no longer a limiting factor. The first treatment options for patients infected with HIV and HCV became available in the 1990s which improved the survival of these groups.[3]

Previous studies confirmed that the average life expectancy of patients with hemophilia has been steadily increasing.[4] Consequently, age-related diseases are occurring increasingly among patients with hemophilia. Compared to patients without a bleeding disorder, managing age-related diseases might require a more personalized approach as certain treatment options might be contra-indicated in patients with an increased bleeding tendency. Furthermore, bleeding-induced arthropathy, which is cumulative and increases with age, may become even more of an issue as the population gets older.

Knowledge gap & aim

It is unknown how treatment- and non-treatment related factors (e.g. the higher uptake of prophylactic treatment, the introduction of more efficacious HCV-treatment options, demographic changes etc.) have impacted the current Dutch hemophilia population in terms of clinical- and psychosocial outcomes.

Furthermore, new treatment options for hemophilia have recently been introduced in the Netherlands or are in the process of obtaining market approval. About 28% of patients with severe hemophilia currently receive prophylactic treatment with emici-

zumab.[5] Since its introduction, emicizumab has been regarded as the treatment of choice by many physicians due to the ease of administration and its long half-life.[6] An accurate overview of the current health status of the Dutch hemophilia population will enable the assessment of the added value of emicizumab and other novel treatment modalities (such as gene therapy) in the coming years. Therefore, the first aim of this thesis was to describe the current health status of the Dutch hemophilia population.

In order to achieve this aim, we initiated the 6th Hemophilia in the Netherlands study (the HiN-6 study) that followed a series of nationwide studies that were held in 1972, 1978, 1985, 1992 and 2001. Broadly speaking, the previous studies explored important medical and psychosocial research questions in the Dutch hemophilia population. The HiN studies have always been organized in close collaboration with patients with hemophilia (represented by the Netherlands Hemophilia Patient Society) and physicians who are specialized in treating patients with hemophilia (represented by the Dutch Society for Hemophilia treaters), which has led to high study response rates for all studies. The previous HiN studies consisted of questionnaires that were sent out to all patients in the Netherlands known to have hemophilia at the time. In the current HiN-6 study, patients were asked to fill out a similar questionnaire, as well as provide a blood- and urine sample. In addition, clinical information was obtained from each patient's medical record. By combining information from previous HiN studies with the current HiN-6 study, it was possible to perform longitudinal evaluation of the health status of the Dutch hemophilia population over a span of almost 50 years.

Identifying patients at a high risk of inhibitor development and presenting an overview of anti-drug antibody prevention strategies used in other diseases

Inhibitor development

A major complication of replacement therapy with FVIII, is the development of anti-drug antibodies in response to infused FVIII. These polyclonal high-affinity IgG anti-FVIII antibodies (also called inhibitors) neutralize FVIII, rendering it ineffective. The incidence of inhibitor development is highest in patients with severe hemophilia A. In this group, 25%-30% of patients develop inhibitors.[7] In general, inhibitors tend to develop early in treatment, after a median of 10-15 days of exposure to FVIII treatment.[7] Furthermore, inhibitors almost always arise within the first 75 days of exposure to FVIII. The incidence of inhibitor development in patients with at least 150 days of exposure to FVIII is very low, about 2 per 1000 person-years, but increases with age.[8, 9]

Several characteristics have been identified that are strongly associated with inhibitor development. An important risk factor for inhibitor development is the type of *F8* mutation.[7] For example, the risk of inhibitor development in patients with a large deletion is around 38% while the risk associated with missense mutations is roughly 20%.[10] Other gene variants in genes that are involved in immune regulation such as the *IL-10* gene, the *CTLA-4* gene, and genes in the HLA locus may also play a role.[11]

There are also several important treatment-related risk factors for inhibitor development. Intensive treatment with FVIII for at least 5 consecutive days to treat major bleeding or after surgical interventions at the first moment of exposure to FVIII was associated with a twofold increased risk of inhibitor development.[12] Furthermore, recombinant FVIII products also seem to be more immunogenic, as patients using these products have almost double the risk of inhibitor development, compared to patients on plasma-derived FVIII products.[13]

In patients with inhibitor development, FVIII bypassing agents such as recombinant activated FVII (rFVIIa) or activated prothrombin complex concentrate (aPCC) are used[14] Unfortunately, both products have a lower efficacy than FVIII with regards to controlling bleeding. Frequent administration of FVIII over a long period of time, also known as immune tolerance induction (ITI) is currently the standard method to eradicate inhibitors. ITI protocols that are often used are the Bonn protocol (which consists of infusing 100–150 IU/kg FVIII twice daily)[15] and the "van Creveld" protocol (which starts with infusing FVIII at a dose of 25 IU/kg FVIII every other day, the dosage is then decreased when FVIII recovery exceeds 30%)[16]. The time needed to fully eradicate inhibitors using these protocols can vary anywhere from months to years and the treatment fails in about one-third of patients.[17] Patients with a persistent inhibitor that is refractory to ITI have higher mortality rates than patients without an inhibitor (which is mostly attributable to more deaths to bleeding-related complications).[18]

Knowledge gap & aim

Although hemophilia treatment has improved in many ways, inhibitor development continues to be a significant problem in patients treated with clotting factor products. Overall, much progress has been made in unraveling the pathophysiological mechanisms underlying inhibitor development. Despite this, accurately predicting the individual probability of inhibitor development is currently not possible for many patients. Furthermore, strategies to prevent inhibitor development in patients at high risk of inhibitor development are also lacking. Therefore, the second aim of this thesis was to identify patients at a high risk of inhibitor development and to present an

overview of anti-drug antibody strategies that could potentially be applied to these patients.

Thesis outline

In the first section of this thesis (***Chapters 2 and 3***), we analyzed the HiN-6 study to describe the current health status of the Dutch hemophilia population, focusing on the most important clinical and psychosocial outcomes:

- In ***Chapter 2***, we describe how treatment changes have influenced major clinical outcomes among patients with hemophilia from 1972 to 2019.
- Overall mortality and causes of death among patients with hemophilia from 1972 to 2018 are described in ***Chapter 3***.

Although hemophilia treatment has improved in many ways, inhibitor development continues to be a significant problem in patients treated with clotting factor products. Therefore, in the second section of this thesis (***Chapters 4-7***), we evaluated different strategies to identify patients at a high risk of inhibitor development and present an overview of anti-drug antibody strategies that could potentially be applied to these patients:

- In ***Chapter 4,*** we assessed the immunogenicity of several recombinant-derived FVIII products in patients with severe or moderately severe hemophilia A who were exposed to FVIII for at least 50 days.
- In ***Chapter 5***, we developed and evaluated a new clinical risk prediction tool for inhibitor development that incorporated several novel predictors.
- In ***Chapter 6***, we assessed if a novel high-throughput epitope mapping technique could be used to accurately assess the FVIII-specific IgG epitope repertoire of patients with severe hemophilia A and predict future inhibitor development.
- In ***Chapter 7*** strategies to prevent anti-drug antibodies in disorders other than hemophilia were reviewed and assessed with regards to their possible application in patients with hemophilia.

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Chapter 2

Health and treatment outcomes of patients with hemophilia in the Netherlands, 1972-2019

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Summary

Introduction

We conducted six cross-sectional nationwide questionnaire studies among all patients with hemophilia in the Netherlands from 1972 until 2019 to assess how health outcomes have changed, with a special focus on patients › 50 years of age.

Methods

Data were collected on patient characteristics, treatment, (joint) bleeding, joint impairment, hospitalizations, human immunodeficiency virus and hepatitis C infections, and general health status (RAND-36).

Results

In 2019, 1009 patients participated of whom 48% had mild, 15% moderate and 37% severe hemophilia. From 1972 to 2019, the use of prophylaxis among patients with severe hemophilia increased from 30% to 89%. Their median annual bleeding rate decreased from 25 to 2 bleeds. Patients with severe hemophilia aged ‹ 16 years reported joint impairment less often over time, but in those aged › 40 years joint status did not improve. In 2019, 5% of all 1009 patients were positive for the human immunodeficiency virus. The proportion of patients with an active hepatitis C infection drastically decreased from 45% in 2001 to 2% in 2019 due to new anti-hepatitis C treatment options. Twenty-five percent had significant liver fibrosis even after successful therapy. Compared with the general male population, patients aged › 50 years reported much lower scores on the RAND-36, especially on physical functioning.

Discussion & Conclusion

Our study shows that increased use of prophylactic treatment and effective hepatitis C treatment have improved joint health and nearly eradicated hepatitis C infection in patients with hemophilia in the Netherlands. However, patients still suffer from hemophilia-related complications, especially patients aged › 50 years.

Introduction

Hemophilia is a hereditary X-linked bleeding disorder, characterized by a lack of functional coagulation factor VIII (hemophilia A) or IX (hemophilia B). Patients with severe hemophilia suffer from spontaneous bleeds in joints/muscles, leading to disability. Patients with moderate/mild hemophilia mainly develop bleeds after trauma or surgery.[1]

Effective treatment was lacking before the 1970s, and most patients with severe hemophilia lived with severe physical disabilities, and only survived until childhood or early adulthood due to bleeding in vital organs (with intracranial bleeds being especially common).[2, 3] The introduction of cryoprecipitate and subsequently coagulation factor concentrates greatly improved survival.

Transmission of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) through contaminated coagulation factor products during the 1980s led to many deaths.[4] New viral inactivation techniques were introduced from 1985 onwards that eliminated the contamination risk after 1990. During this time, the first treatment for HIV and HCV became widely available[3]. Also, the first national consensus-based treatment guidelines were established.[5]

Around the 2000s, hemophilia treatment in the Netherlands was gradually centralized. From 2013 onwards, a standard set of quality criteria was introduced for comprehensive hemophilia treatment centers.[6] Additionally, the national consensus-based treatment guidelines from 1987 were revised in 2009 to harmonize treatment practices.[7] Lastly, treatment with direct-acting antivirals became available for all hepatitis C infected patients in 2014.[8]

Along with these developments, the life expectancy of patients with hemophilia is increasing.[9] Elderly patients are now increasingly experiencing age-related diseases which require a more tailored approach. Additionally, as elderly patients age, the effect of bleeding-induced arthropathy on daily life may worsen despite adequate treatment.

From 1972 until 2019, six nation-wide surveys have been performed to assess the health status of the Dutch hemophilia population.[10-12] In this study we evaluated health outcomes of patients during the past five decades of hemophilia treatment,

with a special focus on the health status of aging patients with hemophilia › 50 years of age.

Methods

Study design

In 2019, a cross-sectional study was performed among all patients with congenital hemophilia in the Netherlands. The current study was preceded by 5 surveys in 1972, 1978, 1985, 1992 and 2001.[10-12] All patients registered at one of the six national hemophilia treatment centers were invited to participate. The first 5 surveys consisted of a questionnaire. The current study consisted of a questionnaire, as well as clinical data collection from medical records and sampling of blood and urine. For the current analysis, only data derived from the questionnaires and medical records were used. From June 2018 until July 2019, questionnaires were sent to patients by e-mail or regular mail, followed by 2 reminders. The study was approved in 2018 by the Medical Ethics committee at Leiden University Medical Center. Informed consent was obtained from all patients.

Measurements

Of the 2019 study participants, information on age, severity of hemophilia, HIV status, HCV status and inhibitor status was obtained from electronic health records. When electronic health record data were missing, answers from the questionnaire were used if available. In case of discrepancies between the electronic health records and questionnaire, data from the electronic health records were used. All other 2019 data were obtained from the questionnaire only.

The following patient characteristics were collected; age, type and severity of hemophilia, and family history of hemophilia. Hemophilia severity was categorized as severe (‹ 0.01 IU/mL), moderate (0.01-0.05 IU/mL) or mild (› 0.05-–0.40 IU/mL). The following treatment characteristics were collected: treatment modality (prophylactic treatment or on-demand treatment), the annual coagulation factor consumption and the type of coagulation factor product.

The questionnaires contained the following self-reported outcomes: annual (joint) bleeding rate, level of joint impairment, orthopedic interventions, hospital admission rate and duration of stay, HIV status, HCV status, age-related co-morbidities and general health status.

Definition of outcome variables

Prophylaxis was defined as periodic infusion of coagulation factor products to prevent bleeding. Annual coagulation factor consumption was defined as the total number of units of coagulation factor used divided by bodyweight per year (IU/kg/year). The annual (joint) bleeding rate was defined as the number of self-reported (joint) bleeds in the preceding 12 months. In children, the annual (joint) bleeding rate was based on the results of the last 3 months, which was then multiplied by 4.

Joint impairment was calculated using a point system; no joint impairment (0 points), mild impairment (no daily problems, 1 point), moderate impairment (daily problems, 2 points) or severe impairment (no movement in joint, 3 points). This information was reported for the knee, elbow, ankle and wrist joints. Hospital admission was defined as having been admitted to the hospital in the preceding 12 months for at least 1 day (day admissions were included) Hospital duration was calculated as the number of nights spent in the hospital (day admissions were excluded). Age-related co-morbidities were defined as being treated by a medical specialist or a general practitioner for a set of age-related conditions (see Supplemental Table 1 for full list).

Inhibitor status was based on the Bethesda assay, using each center's own cut-off level, which varied from › 0.6 BU to › 1.0 BU. A current inhibitor was defined as being currently inhibitor-positive. A past inhibitor was defined as having been inhibitor-positive in the past but currently inhibitor-negative. HIV status was reported for patients treated with coagulation factor before 1985 and was defined as positive if the patient had a confirmed clinical diagnosis of HIV. HCV status was reported for patients treated with coagulation factor before 1992. Patients were classified as having a "past infection" when they had a confirmed clinical diagnosis of HCV infection in the past and "current infection" if they were currently HCV-RNA positive.

General health status was assessed in adults using the RAND 36-Item Health Survey (RAND-36).[13] The RAND-36 is a 36-item questionnaire that measures perceived health status across 8 different domains: physical functioning, social functioning, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, energy/fatigue, bodily pain, and general health perceptions. Domain scores were calculated when a patient had completed at least half of the items of a domain according to RAND-36 scoring instructions.[14] Domain scores were converted to a 100-point scale. Based on a review of the literature, a difference of 4 points on any RAND-36 domain between groups was regarded as clin-

ically significant.[15] Scores were compared with RAND-36 scores of the Dutch general male population.[16]

Statistical analysis
Descriptive statistics were reported as mean/SD, median/IQR, or as proportions. Treatment characteristics and health outcomes were summarized and compared over all 6 surveys stratified by age or severity of hemophilia. Patients with missing data for a given analysis were excluded.

To measure the response rate, the total number of unique patients registered at each hemophilia treatment center was retrieved. This was done by anonymizing and then merging patient data of all registered patients. A trusted third party (ZorgTTP, Houten, the Netherlands) was responsible for the process of anonymization and merging of data.

Data sharing statement
For original data, please contact S.C.Gouw@lumc.nl.

Results

Response and patient characteristics
From 1972-2019 the number of participants in the questionnaire varied from 447 to 1009 patients. (Table 1) In the latest study 2192 patients were invited to participate of whom 33% had severe hemophilia, 13% had moderate hemophilia and 54% had mild hemophilia (Table 2). Of these, 1312 patients participated in at least one part of the study (by filling in the questionnaire, consenting to the use of their clinical data, or both). 1009 patients completed the questionnaire (a response rate of 46%). Of these 1009, 729 patients also consented to the use of their clinical data. Response rates of the previous questionnaires were 84% in 1972, 70% in 1978, 81% in 1985, 78% in 1992, 68% in 2001.[10-12]

Table 1 shows the patient characteristics of each survey. Of the 1009 patients, 378 (37%) had severe hemophilia, 149 (15%) moderate hemophilia and 482 (48%) mild hemophilia. The mean age of participants increased from 21 years in 1972 to 40 years in 2019. During this period the mean age of the Dutch male population increased from 32 years to a similar mean age of 41 years.[17]

Table 1. Characteristics of participants in the Hemophilia in the Netherlands studies obtained from questionnaire data.

	1972 (N = 447)	1978 (N = 560)	1985 (N = 935)	1992 (N = 980)	2001 (N = 1066)	2019 (N = 1009)
Age in years*						
Mean (range)	21 (0-47)	23 (0-70)	27 (0-85)	30 (0-84)	35 (0-90)	40 (0-88)
Severity of hemophilia (%)						
Severe	159 (36)	245 (44)	384 (41)	387 (39)	420 (39)	378 (37)
Moderate	83 (19)	106 (19)	175 (19)	173 (18)	176 (17)	149 (15)
Mild	172 (38)	138 (25)	376 (40)	420 (43)	470 (44)	482 (48)
Type of hemophilia (%)†						
Hemophilia A	377 (84)	481 (86)	801 (86)	853 (87)	925 (87)	867 (87)
Hemophilia B	70 (16)	79 (14)	134 (14)	127 (13)	141 (13)	129 (13)
Family history of hemophilia (%)‡						
Negative	112 (25)	128 (23)	237 (25)	195 (20)	246 (23)	168 (18)
Positive	335 (75)	432 (77)	698 (75)	785 (80)	820 (77)	753 (82)
HIV infection (%)§						
Current infection	-	-	36 (4)	55 (8)	29 (5)	22/412 (5)¶
Hepatitis C infection (%)**						
Current infection	-	-	-	-	344 (45)	8/412 (2)††
Past infection	-	-	-	-	97 (13)	226/412 (55)††
Inhibitory antibodies (%)‡‡						
Ever inhibitors	-	-	31/384 (8)	51/388 (13)	52/420 (13)	66/361 (19)§§
Current inhibitors	-	-	19 (5)	29 (7)	15 (4)	6/361 (2)§§
Past inhibitors	-	-	12 (3)	22 (6)	37 (9)	60/361 (17)§§

* Age was unknown for 8 patients.
† Type of hemophilia was unknown for 13 patients.
‡ Family history of hemophilia was unknown for 88 patients.
§ Reported for patients treated with coagulation factor before 1985.
¶ HIV status was unknown for 4 patients.
** Reported for patients treated with coagulation factor before 1992.
†† HCV status was unknown for 84 patients.
‡‡ Reported for patients with severe hemophilia.
§§ Inhibitor status was unknown for 17 patients.

Treatment characteristics

From 1972-2019, the proportion of patients with severe hemophilia receiving prophylactic treatment increased from 30% to 89%. In 2019, almost all (98%) patients aged 0-16 years were on prophylaxis. (Table 3, Fig 1a) Also, 25% of patients aged 0-16 years with moderate hemophilia were on prophylactic treatment. As expected only 3% of patients aged 0-16 years with mild hemophilia were treated with prophylaxis. The median age at initiation of prophylaxis in patients with severe hemophilia decreased from 8 years (range: 0-15) in 1978 to 3 years (range: 0-79) in 2019. (Table 3) Median annual coagulation factor consumption (in IU/kg) for patients with severe hemophilia on prophylaxis increased from 886 IU/kg (IQR: 632-1259) in the 1970s[18] to 2535 IU/kg (IQR: 1885-3614) in 2019.

In 2019, only 5 out of 827 patients (1%) were treated with a plasma-derived coagulation factor product. In patients with hemophilia A, 48 out of 724 (7%) were treated with extended half-life FVIII products. Among patients with hemophilia B, 30 out of 103 (29%) used extended half-life FIX products. Six out of 724 patients with hemophilia A (1%) were treated with emicizumab, three of which were patients with an active inhibitor.

Treatment outcomes, 1972-2019

Annual bleeding rates

Since 1972, the median annual bleeding rate (ABR) of patients with severe hemophilia decreased from 25 to 2 bleeds. (Fig 1b) In 2019, the highest ABR (4 bleeds) was reported by patients in the youngest age group aged 0-16 years (Table 3 and Fig 1b). The same ABR was reported in 0-16 year-olds with moderate and mild hemophilia. The vast majority were nosebleeds (55%). For comparison, only 6% of bleeds were classified as nosebleeds in patients › 25 years.

In patients with severe hemophilia on prophylaxis, 125 out of 285 patients (44%, 95%CI: 38-50%) had at least one joint bleed in the past year. (Table 4) The median annual joint bleeding rate (AJBR) in 2019 for patients with severe hemophilia ‹ 25 years was 0 (n 118, IQR 0-0), in both patients treated on-demand (n 4) or on prophylactic treatment (n 113) (Table 4). In patients with mild hemophilia (n 417) and moderate hemophilia (n 128), the AJBR in 2019 was 0 (IQR 0-0) for all age groups (Table 4). In patients with severe hemophilia with an active inhibitor the AJBR was 6 (n 5, IQR 0-12) vs. 0 (n 52, IQR 0-3) in patients with a previously cleared inhibitor and 0 (n 259, IQR 0-3) in non-inhibitor patients (Table 4). The median AJBR was the same

(zero) for both patients with severe hemophilia A and patients with severe hemophilia B. (Supplemental Table 6).

Table 2. Comparison of age distribution and severity hemophilia of the 2019 HiN-6 study with the Dutch hemophilia population.

	Dutch hemophilia population* (N = 2192)	**2019 HiN-6 study (N = 1009)**
Age category (%)		
0-17 years	446 (21)	196 (20)
18-25 years	254 (12)	108 (11)
26 years or older	1436 (67)	697 (70)
Missing	56†	8
Severity of hemophilia (%)		
Severe	704 (33)	378 (37)
Moderate	282 (13)	149 (15)
Mild	1148 (54)	482 (48)
Missing	58†	0

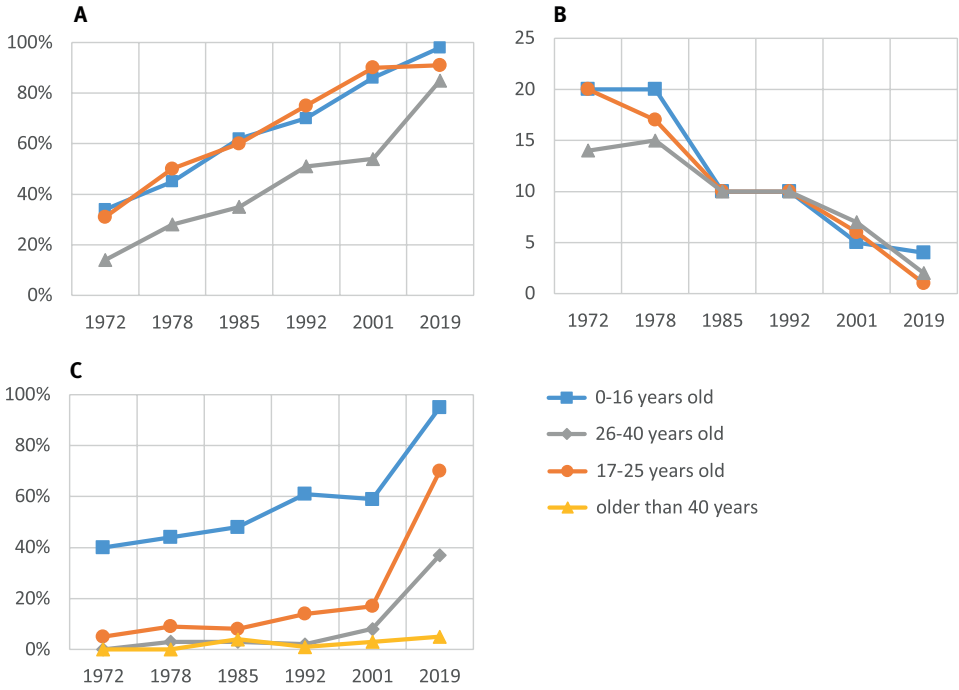
* All patients who were registered at a hemophilia treatment center in the Netherlands. †56 patients from one treatment center had missing data for age and severity of hemophilia. Furthermore, two patients from another treatment center had evaluable data for age but not for severity of hemophilia.

Joint impairment

Between 1972-2019, there was an increase in patients with severe hemophilia with no joint impairment in the ankles, elbows, and knees. (Fig 1c, Supplemental Table 2) The proportion of patients reporting no joint impairment changed between 1972-2019 from 40% to 95% in patients aged 0-16 years, from 5% to 70% in patients aged 17-25 years old and from 3% to 37% in patients 25-40 years old. In patients › 40 years, there were none without joint impairment in 1972, and this percentage did not improve much, only 5% in 2019. In patients with moderate hemophilia, a similar, but less pronounced trend was seen over time (Supplemental Table 2). In 2019, the proportion of patients with mild hemophilia with an absence of joint impairment ranged from 98% among the 0-16 year olds to 87% in the 40+ group (Supplemental Table 2). Patients with severe hemophilia B had similar joint impairment and instances of joint replacement surgery as patients with severe hemophilia A. (Supplemental Table 6).

Figure 1. Health- and treatment outcomes over time.

Legend:
Graph **A** shows the proportion of patients with severe hemophilia on prophylactic treatment, from 1972 to 2019, stratified by age.
Graph **B** shows the median annual bleeding rate of patients with severe hemophilia, from 1972 to 2019, stratified by age.
Graph **C** shows the self-reported absence of joint impairment in ankles, knees and elbows in patients with severe hemophilia, from 1972 to 2019, stratified by age.



Hospital admissions

The proportion of patients with severe hemophilia requiring hospitalization in the previous year decreased from 51% in 1972 to 22% in 2019. (Table 3) The hospital admission rate in patients with mild hemophilia (25%) and severe hemophilia (22%) was similar. (Table 3) However, hospitalization for a non-hemophilia-related problem was more common in patients with mild hemophilia (29%) than in patients with severe hemophilia (17%).

Inhibitor development, HIV status and HCV status

The percentage of patients with severe hemophilia A or B with a past or current inhibitor increased from 8% in 1985 to 19% in 2019. (Table 1) In 2019, 21% and 7% of patients with severe and mild hemophilia A respectively reported having a past or current inhibitor.

Table 3. Prophylaxis usage, annual bleeding rates and hospital admission.

	1972 (N = 447)	**1978 (N = 560)**	**1985 (N = 935)**	**1992 (N = 980)**	**2001 (N = 1066)**	**2019 (N = 1009)**
Severe hemophilia	159	245	384	387	420	378
Patients on prophylaxis (%)						
Children, 0-16y	22/65 (34)	41/91 (45)	69/111 (62)	64/92 (70)	112/130 (86)	93/95 (98)
Young adults, 17-25y	12/39 (31)	27/54 (50)	43/72 (60)	NR	38/42 (90)	42/46 (91)
Adults, older than 25y	8/57 (14)	28/99 (28)	71/201 (35)	119/232 (51)	134/248 (54)	193/228 (85)
Median age at first prophylaxis, years (range)	NR	8 (0-15)	5 (1-15)	NR	2 (0 -11)	3 (0-79)
Median ABR* by age (range, IQR†)						
Children, 0-16y	20 (0-98)	20 (0-70)	10 (0-65)	10 (0-98)	5 (0-51)	4 (0-228, 0-12)
Young adults, 17-25y	20 (0-98)	17 (0-100)	10 (0-90)	10 (0-98)	6 (0-75)	1 (0-12, 0-2)
Adults, older than 25y	14 (0-97)	15 (0-100)	10 (0-90)	10 (0-82)	7 (0-75)	2 (0-100, 0-6)
Hospital admissions						
Hemophilia patients (%)	51	38	25	22	22	73/330 (22)
Median duration, (range)	28 (2-252)	20 (1-180)	11 (1-100)	5 (1-330)	7 (1-89)	7 (1-125)
Moderate hemophilia	23	106	175	173	176	149
Patients on prophylaxis (%)						
Children, 0-16y	6/41 (15)	9/41 (22)	7/59 (12)	7/41 (17)	7/46 (15)	6/24 (25)
Young adults, 17-25y	4/14 (29)	7/26 (27)	1/19 (5)	NR	4/23 (17)	4/19 (21)
Adults, older than 25y	1/27 (4)	4/39 (10)	10/97 (10)	11/98 (11)	10/107 (9)	14/104 (13)
Median ABR* by age (range, IQR†)						
Children, 0-16y	4 (0-40)	10 (0-104)	3 (0-66)	7 (0-33)	2 (0-57)	4 (0-32, 0-8)
Adults, older than 17y	4 (0-50)	5 (0-100)	2 (0-40)	3 (0-52)	1 (0-71)	1 (0-100, 0-2)

Hospital admissions						
Hemophilia patients (%)	51	27	23	22	15	21/136 (15)
Median duration (range)	17 (2-180)	10 (1-50)	7 (1-50)	5 (1-72)	6 (1-31)	6 (1-120)
Mild hemophilia	NR	NR	NR	NR	NR	482
Patients on prophylaxis (%)						
Children, 0-16y	NR	NR	NR	NR	NR	2/68 (3)
Young adults, 17-25y	NR	NR	NR	NR	NR	1/43 (2)
Adults, older than 25y	NR	NR	NR	NR	NR	7/346 (2)
Median ABR* by age (range, IQR†)						
Children, 0-16y	NR	NR	NR	NR	NR	4 (0-100, 0-14)
Young adults, 17-25y	NR	NR	NR	NR	NR	0 (0-88, 0-1)
Adults, older than 25y	NR	NR	NR	NR	NR	0 (0-40, 0-0.5)
Hospital admissions						
Hemophilia patients (%)	NR	NR	NR	NR	NR	103/415 (25)
Median duration (range)	NR	NR	NR	NR	NR	5 (1-175)

* Annual bleeding rate. †IQR: Interquartile range. NR: Not reported.

HIV was first reported in 1985 when 4% of patients were HIV infected. Among still-living patients treated with coagulation factor before 1985, the prevalence of HIV increased to 8% in 1992 and afterwards decreased to 5% in 2019. Currently, out of 412 patients that were treated with coagulation factor before 1985, 22 are HIV-positive. (Table 1) HCV infections among patients treated with coagulation factor before 1992 were common in the year 2001 with 45% of patients reporting to have an active HCV infection. In 2019, 8 (2%) patients had an active HCV infection. (Table 1)

Self-reported general health status

There were no clinically relevant differences in reported general health status measured using the RAND-36 between the 2001 cohort and the 2019 cohort. (Table 5) Compared with the Dutch general population, the 2019 cohort scored lower on all domains, except for emotional well-being (2019 cohort score: 77.1, general population score: 77.9) and role limitations due to personal or emotional problems (2019 cohort score: 85.0, general population score: 85.8). (Table 5) Patients under 50 years

of age had scores similar to the general population, except for the domain of energy/fatigue (2019 cohort ‹ 50 score: 65.6, general population score: 71.6).

Current health status of patients older than 50 years

Bleeding rate and joint impairment
Only 4% of older patients with severe hemophilia had no joint impairment in the ankles, elbows and/or knees vs. 75% of patients with non-severe hemophilia. (Table 6) In addition, 75% of older patients with severe hemophilia had undergone orthopedic surgery and the mean number of life-time orthopedic interventions was 1.9. (Table 6) Twenty percent of older patients had joint impairment in their wrists, this number increased to 48% in patients with some knee impairment.

HCV status
Among older patients who were treated with coagulation factor products before 1992, 62% were currently or previously infected with HCV. Among patients with severe hemophilia, 97% were currently or previously infected (Table 6). Overall, only 2% of older patients were currently HCV-positive (Table 6).

Eighty-five percent of older patients had received antiviral treatment in the past. Half of these were treated with older treatment methods (interferon, peg-interferon and/or ribavirin), while the other half were treated with direct-acting antiviral drugs (Table 6). Among patients who were or had been HCV-positive, 25% had clinically significant liver fibrosis or cirrhosis. (Table 6)

Self-reported general health status
Patients › 50 years scored substantially lower on the RAND-36 than the Dutch general population and younger patients with hemophilia. (Table 5) Patients with severe hemophilia reported even more pronounced limitations, especially on the domains of physical functioning and role limitations due to physical health problems (Table 5). Emotional well-being scores of older patients were similar to those of the general population (Table 5).

Table 4. Self-reported annualized joint bleeding rates in 2019.

	N of patients	Median annual joint bleeding rate (range, IQR*)
Severe hemophilia	378†	
Children, 0-16y	78	0 (0-12, 0-0)
Young adults, 17-25y	40	0 (0-6, 0-1)
Adults, older than 25y	204	2 (0-70, 0-4)
Patients on prophylactic treatment		
Children, 0-16y	77	0 (0-12, 0-0)
Young adults, 17-25y	36	0 (0-6, 0-1)
Adults, older than 25y	172	2 (0-70, 0-4)
Patients on prophylactic treatment with at last one joint bleed		
0 bleeds	160	NA
≥ 1 bleeds	125	NA
Patients treated on-demand		
Children, 0-16y	1	0 (0-0, 0-0)
Young adults, 17-25y	3	0 (0-0, 0-0)
Adults, older than 25y	32	1.5 (0-50, 0-6)
Inhibitory antibodies		
Never inhibitor-positive	259	0 (0-50, 0-3)
Currently inhibitor-positive	5	6 (0-15, 0-12)
Previously inhibitor-positive	52	0 (0-70, 0-3)
Moderate hemophilia	149‡	
Children, 0-16y	23	0 (0-4, 0-0)
Adults, older than 17y	105	0 (0-20, 0-1)
Mild hemophilia	482§	
Children, 0-16y	59	0 (0-0, 0-0)
Young adults, 17-25y	40	0 (0-4, 0-0)
Adults, older than 25y	313	0 (0-25, 0-0)

* IQR: Interquartile range.
† Annualized joint bleeding rate was unknown for 56 patients with severe hemophilia.
‡ Annualized joint bleeding rate was unknown for 21 patients with moderate hemophilia.
§ Annualized joint bleeding rate was unknown for 65 patients with mild hemophilia. NA: not applicable.

Age-related co-morbidities

Among 367 patients › 50 years, the most common age-related co-morbidities were hypertension (37%), hypercholesterolemia (17%), malignancies (13%) and type 2 diabetes (10%). (see Supplemental Table 1) The prevalence of hypertension was even higher in patients with severe hemophilia (47%).

Table 5. General health status of patients in HiN-5 cohort, overall HiN-6 cohort, HiN-6 cohort › 50 years and Dutch general male population.

Domains of the RAND 36-Item Health Survey	HiN-5 (2001)	HiN-6 (2019)	HiN-6 (≤50)	HiN-6 (› 50)	HiN-6 (› 50) severe hemophilia	HiN-6 (› 50) non-severe hemophilia	General Male Population (all ages) [16]
N	623	706-757*	368-398*	339-358*	101-108*	236-250*	-
Physical functioning, mean (SD)	75.8 (29)	77.9 (27.5)	87.5 (20.1)	67.1 (30.5)	43.0 (27.3)	77.5 (25.6)	88.3 (21)
Social functioning, mean (SD)	82.0 (24)	83.3 (20.8)	86.1 (20.2)	80.3 (21.2)	77.0 (20.8)	81.7 (21.2)	87.5 (20)
Role limitations (physical health problems) , mean (SD)	73.3 (40)	76.4 (37.5)	83.6 (32.6)	68.4 (40.9)	52.9 (43.7)	75.1 (37.9)	83.3 (32)
Role limitations (personal/emotional problems) , mean (SD)	83.4 (34)	85.0 (31.4)	87.7 (28.3)	82.1 (34.3)	77.3 (36.5)	84.1 (33.2)	85.8 (30)
Emotional well-being, mean (SD)	76.9 (18)	77.1 (15.6)	77.7 (14.5)	76.4 (16.7)	75.5 (17.7)	76.8 (16.3)	77.9 (17)
Energy/fatigue, mean (SD)	67.1 (20)	64.7 (17.7)	65.6 (17.0)	63.7 (18.3)	60.0 (18.3)	65.3 (18.2)	71.6 (18)
Bodily pain, mean (SD)	78.8 (24)	77.4 (22.6)	82.0 (21.0)	72.3 (23.3)	64.9 (21.8)	75.5 (23.2)	83.5 (23)
General health perception, mean (SD)	67.0 (23)	64.5 (22.3)	69.1 (21.8)	59.6 (21.7)	54.7 (21.5)	61.7 (21.4)	72.9 (20)

* Scores for each domain were calculated if a participant had completed at least half of the items of that domain. Therefore, the total of number of participants for which a score was calculated differs per domain.

Discussion

We evaluated clinical outcomes in patients with hemophilia in the Netherlands from 1972-2019 using a series of 6 national questionnaires. The same outcome definitions were used for all questionnaires, enabling direct comparison of different cohorts over

time. Bleeding rate and joint impairment decreased dramatically. Furthermore, HCV has almost been eradicated.
The prevalence of hemophilia in the Netherlands was 25.5 cases per 100,000 males, which is higher than reported previously[6,19] but similar to a recent estimate of the birth prevalence (29.6 cases per 100,000 live male births).[20] The higher prevalence is most likely due to the high level of care increasing survival[9], as well as improved diagnosis and registration of patients with previously undetected mild hemophilia over time (Table 1). Although, our reported prevalence is high, it is still lower than the reported birth prevalence[20], indicating the presence of unregistered patients with mild hemophilia and/or excess mortality due to hemophilia.

Change in health outcomes, 1972 to 2019
The annual bleeding rate has decreased due to more prophylaxis usage and higher dosing schemes, enabling children with hemophilia to participate safely in sports. (which improves muscle function and quality of life[21]) Over time, factor consumption in patients on prophylaxis has increased, from 886 IU/kg/year in the 1970s[18], 1514 IU/kg/year in the 1980s[18], 1880 IU/kg/year in the 1990s[18], and finally 2534 IU/kg in the 2010s. Despite coagulation factor accounting for › 90% of total treatment costs[22,23], direct comparisons of prophylactic dosing schemes are scarce. A previous study showed that a high-dose protocol (4000 IU/kg per year) only marginally improved outcomes compared with an intermediatedose protocol (2100 IU/kg per year), while being 66% more expensive.[24] Our results seem to confirm that intermediate-dose prophylaxis can lead to good joint outcomes.

The median ABR was highest in the 0-16 group (4 bleeds). However, joint bleeds were rare and most bleeds were nosebleeds, which were far less common in adults. Among non-hemophilic males, the prevalence of epistaxis is also highest in children[25], and is commonly caused by irritation due to digital trauma.[26]

The median AJBR for patients with severe hemophilia on prophylaxis was zero. Still, 44% of patients (95%CI: 38%-50%) had at least one joint bleed, leaving room for improvement. This is similar to a report from the UK (another high-income country), which found that in 2018, between 32.5% to 59.9% of patients on prophylaxis still reported at least one joint bleed per year.[27] Details on the cause/severity of joint bleeds were not available.

The hospital admission rate in patients with severe hemophilia after 1985 was 22%, which is higher than for Dutch men (9.8% in 1986 to 8.6% in 2017).[28] The hospital

admission rate in patients with mild hemophilia was similarly high (25%). Interestingly, the proportion of hospitalizations for non-hemophilic problems was higher in mild hemophilia (29%) than in severe hemophilia (17%). The reason for hospitalization was not included in the questionnaire and similar studies to compare our results with were not available.

Table 6. Health outcomes in patients with hemophilia over 50 years old.

	< 50 (N = 613)	50+, overall (N = 388)	50+, severe hemophilia (N = 115)	50+, non-severe hemophilia (N = 273)
Median annual bleeding rate	613	388	115	273
Rate (IQR)	1 (0-228)	0 (0-100)	3 (0-100)	0 (0-100)
Missing	109	45	14	31
Median annual joint bleeding rate	613	388	115	273
Rate (IQR)	0 (0-70)	0 (0-70)	2 (0-70)	0 (0-25)
Missing	95	44	14	30
Hospital admissions (%)	613	388	115	273
No	419 (82)	261 (72)	86 (77)	175 (69)
Yes	93 (18)	103 (28)	26 (23)	77 (31)
Missing	101	24	3	21
¶ Duration of hospital stay in days	66	83	25	58
Median (range)	5 (1-80)	6 (1-175)	7 (1-125)	5 (1-175)
Missing	5	2	0	2
Joint impairment (%)	613	388	115	273
Some impairment	123 (25)	153 (47)	96 (96)	57 (25)
No impairment	376 (75)	175 (53)	4 (4)	171 (75)
Missing	114	60	15	45
Orthopedic surgery in the past, any type (%)	613	388	115	273
No	280 (82)	219 (60)	28 (25)	191 (76)
Yes	63 (18)	145 (40)	84 (75)	61 (24)
Missing	270	24	3	21
Orthopedic surgery in the past, joint replacement surgery (%)	343	364	112	252
No	325 (95)	274 (75)	46 (41)	228 (90)
Yes	18 (5)	90 (25)	66 (59)	24 (10)

Orthopedic surgery in the past, arthrodesis (%)	343	364	112	252
No	327 (95)	311 (85)	72 (64)	239 (95)
Yes	16 (5)	53 (15)	40 (36)	13 (5)
Orthopedic surgery in the past, synovectomy (%)	343	364	112	252
No	334 (97)	346 (95)	100 (89)	246 (98)
Yes	9 (3)	18 (5)	12 (11)	6 (2)
Number of orthopedic interventions	343	364	112	252
Mean (SD)	0.4 (0.9)	0.9 (1.4)	1.9 (1.5)	0.5 (1.1)
Missing	2	4	1	3
§ HIV status (%)	136	280	108	172
Negative	126 (93%)	264 (95%)	95 (88%)	169 (100%)
Positive	9 (7%)	13 (5%)	13 (12%)	0 (0%)
Missing	1	3	0	3
* HCV status (%)	198	298	108	190
Always HCV-negative	85 (51)	93 (38)	3 (3)	90 (62)
Past infection	80 (48)	146 (60)	92 (93)	54 (37)
Current infection	2 (1)	6 (2)	4 (4)	2 (1)
Missing	31	53	9	44
HCV treatment among HCV-positive patients (%)	82	152	96	56
No	12 (15)	23 (15)	11 (12)	12 (22)
Yes	67 (85)	126 (85)	83 (88)	43 (78)
Missing	3	3	2	1
† Last treatment (%)	167	126	83	43
DAA	15 (28)	28 (25)	19 (26)	9 (24)
DAA + RBV	2 (4)	24 (21)	13 (18)	11 (29)
DAA + RBV + PEG-IFN	3 (6)	5 (4)	4 (5)	1 (3)
PEG-IFN + RBV	19 (35)	24 (21)	16 (22)	8 (21)
IFN + RBV	11 (20)	21 (19)	13 (18)	8 (21)
IFN	4 (7)	10 (9)	9 (12)	1 (3)
Missing	13	14	19	5

‡ Liver fibrosis/cirrhosis (%)	82	152	96	56
No significant fibrosis (‹ 9.5 kPa)	32 (91)	56 (75)	37 (76)	19 (73)
Significant fibrosis (9.5 -12.4 kPa)	1 (3)	7 (9)	4 (8)	3 (12)
Cirrhosis (› 12.4 kPa)	2 (6)	12 (16)	8 (16)	4 (15)
Missing	47	77	47	30

* Reported for 298 patients › 50 years treated with coagulation factor before 1992.
† DAA: direct acting antivirals, RBV: ribavirin, PEG-IFN: pegylated-interferon, IFN: interferon.
‡ Based on FibroScan measurements.
§ Reported for 280 patients › 50 years treated with coagulation factor before 1985
¶ Reported for patients that stayed at least one night in the hospital (day admissions were excluded).

Unlike patients ‹ 40 years, patients › 40 years did not improve in joint function over time. This is due to accrued irreversible joint damage (in a period of time when there was no treatment or when it was still suboptimal). There is some evidence that hemophilia A and B differ in their clinical phenotype.[29] In the 2019 cohort, patients with severe hemophilia and A and B reported roughly similar bleeding- and joint outcomes. However, given the small sample size, no conclusion can be drawn from these results.

The proportion of patients with severe hemophilia (A or B) with a past or current inhibitor increased from 8% in 1985 to 19% in 2019. Among patients with severe hemophilia A the percentage is 20%, which is low when compared to most clinical trials.[1] In contrast, a US study reported that between 1998-2011, 11.5%-17.0% of patients with severe hemophilia (A or B) had a past or current inhibitor.[30] The increasing prevalence over time may be due to more low-titer inhibitors being detected.[31] Also, due to lower sensitivity tests and less testing in the past, some low-titer inhibitors in patients in the 1980s/1990s would have been missed. (this probably also explains the similarly low inhibitor prevalence in the US study)

General health status did not change meaningfully from 2001-2019. The probability of not detecting a meaningful improvement is unlikely as the RAND-36 questionnaire is reported to be sensitive to changes in health over time.[32] Similar results have been reported by several European studies.[33-35] A possible explanation for this may be response shift, which is defined as a change in the meaning of one's self-evaluation of quality of life as a result of changes in internal standards, values and the conceptualization of quality of life.[36] Persons with hemophilia may have changed their internal standards over time: while their health has deteriorated (e.g. as a result of recurrent bleeding), their previous idea of a bad health status may have been lower than what they currently experience.

Current health status of older patients

The prevalence of joint replacement surgery among patients with severe hemophilia of all ages was high (30%), which is in line with an earlier Dutch study (31%).[37] For comparison, a UK study reported a prevalence of 5% for joint replacement surgery among males › 60 years.[38] Eighty-four percent of patients with knee impairment also reported having wrist problems. This most likely due to the fact that these patients put more weight on their hands when standing up, in order to alleviate their knees.

Mental health status among 50+ patients appeared to be similar to that of the Dutch general male population, both in the 2001 survey[39], and in the 2019 survey, which is in agreement with several other studies.[40-42] The high level of mental health might be due to adequate hemophilia treatment in a multidisciplinary care setting.

Although HCV has almost been eradicated, 25% of cured patients still have moderate-severe liver fibrosis. Follow-up of these patients is warranted as they remain at increased risk for complications.[43]

Limitations

Reported study response rates have decreased over time (from 84% in 1972, to 46% in 2019). The burden of participating in multiple studies (which is becoming more common), as well as the requirement of a hospital visit may have dissuaded some. However, participation rates in previous studies may have been overestimated, as evidenced by the high prevalence of hemophilia in 2019 (25.5 cases per 100,000 males). Despite lower participation, the 2019 cohort was similar to the Dutch hemophilia population with regards to age distribution and severity of hemophilia. (Table 2) Therefore, the results are likely to be highly generalizable. Nevertheless, non-response bias cannot be ruled out. Patients who participated in the questionnaire might have been more adherent to treatment, which would have skewed results towards a more positive direction.

Differentiating between joint bleeds and flare-ups of chronic arthropathy is difficult.[44] Therefore, the bleeding rate in patients with significant hemophilic arthropathy is probably slightly overestimated. The annual bleeding rate in children was based on the results of the last 3 months, multiplied by 4. This may have artificially increased bleeding rates due to recall bias.

The RAND-36 reference values were obtained from a validation study from 1992-1996[16] and may not be representative of the current Dutch population. Yet, RAND-36

domain scores were shown to remain relatively stable over a time-period of almost 20 years.[45] In addition, age-specific domain scores were not available, so domain scores of the overall population (mean age: 43.1) were used for comparisons with the hemophilia cohort.

Lastly, patients tend to underreport co-morbidities.[46] This might explain the higher prevalence of hypertension reported by other studies.[47,48]

Conclusion

Even though the increase in prophylactic treatment, coagulation factor dosage and centralization of care has improved outcomes, many patients with severe hemophilia still experience joint bleeds and report decreased physical health. Many older patients with severe hemophilia suffer from severe painful joint impairment, which greatly decreases quality of life. This emphasizes the need for personalized treatment focusing on bleed control, adequate pain management and timely reference to an orthopedic surgeon or physiatrist.[49] With the increased use of novel treatment options and expected further health gains, regular measurements of patient-relevant outcomes may identify areas for improvement and directions for further research.

In conclusion, our study shows that bleeding rates, joint health and HCV cure rates have strongly improved over the past five decades. However, there are still opportunities for improvement.

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Supplemental Table 1. Age-related co-morbidities in patients › 50 years old (self-reported information from questionnaire).

* Disorder (%)	Overall (N = 367)	Severe hemophilia (N = 109)	Non-severe hemophilia (N = 258)
Hypertension	135 (37)	51 (47)	84 (33)
Hypercholesterolemia	62 (17)	20 (18)	42 (16)
Ischemic heart disease	7 (2)	1 (1)	6 (2)
Ischemic stroke	5 (1)	1 (1)	4 (2)
Hemorrhagic stroke	3 (1)	2 (2)	1 (0)
Diabetes type 2	35 (10)	13 (12)	22 (9)
Osteoporosis	5 (1)	0 (0)	5 (2)
Chronic kidney disease	6 (2)	2 (2)	4 (2)
Malignancy†	42/322 (13)	13/98 (13)	29/224 (13)

* Self-reported treatment by a medical specialist or physician for a disorder. †Information on malignancies is missing in 45 patients.

Supplemental Table 2. Self-reported absence of impairment in ankles, knees, and elbows in patients with mild, severe or moderate hemophilia.

	1972	1978	1985	1992	2001	2019
Severe hemophilia						
No joint impairment (%)						
0-16 years old	26 (40)	40 (44)	53 (48)	56 (61)	76 (59)	76/80 (95)
Aged 17-25 years old	2 (5)	5 (9)	7 (8)	9 (14)	7 (17)	23/33 (70)
Aged 25-40 years old	0 (0)	2 (3)	3 (3)	2 (2)	7 (8)	18/49 (37)
Older than 40 years of age	0(0)	0(0)	3 (4)	1 (1)	4 (3)	7/147 (5)
Moderate hemophilia						
No joint impairment (%)						
0-16 years old	23 (55)	28 (68)	42 (71)	29 (71)	37 (80)	22/22 (100)
Aged 17-25 years old	5 (36)	13 (50)	9 (41)	17 (50)	11 (48)	15/18 (83)
Aged 25-40 years old	2 (11)	3 (13)	14 (24)	14 (31)	13 (37)	20/29 (69)
Older than 40 years of age	3 (33)	5 (33)	11 (31)	15 (28)	17 (24)	21/60 (35)
Mild hemophilia						
No joint impairment (%)						
0-16 years old	NR	NR	NR	NR	NR	58/59 (98)
Aged 17-25 years old	NR	NR	NR	NR	NR	36/37 (97)
Aged 25-40 years old	NR	NR	NR	NR	NR	46/52 (88)
Older than 40 years of age	NR	NR	NR	NR	NR	209/241 (87)

NR: Not reported in previous publications.

Supplemental Table 3. HCV status and treatment in all patients who were treated with clotting factor before 1992.

	N = 496
HCV status (%)	
Always HCV-negative	178 (43)
Past infection	226 (55)
Current infection	8 (2)
Missing	84
HCV genotype (%)	234
genotype 1, subtype unknown	19 (12%)
genotype 1a	39 (25%)
genotype 1b	59 (37%)
genotype 2, subtype unknown	8 (5%)
genotype 2a	9 (6%)
genotype 2b	5 (3%)
genotype 3	16 (10%)
genotype 4	3 (2%)
missing	76
Hepatitis C treatment (%)	234
No	35 (15)
Yes	193 (85)
Missing	6
* Last treatment (%)	193
DAA	43 (26)
DAA + RBV	26 (16)
DAA + RBV + PEG-IFN	8 (5)
PEG-IFN + RBV	43 (26)
IFN + RBV	32 (19)
IFN	14 (8)
Missing	27
† Liver fibrosis/cirrhosis (%)	234
no significant fibrosis (< 9.5 kPa)	88 (80)
significant fibrosis (9.5 -12.4 kPa)	8 (7)
cirrhosis (> 12.4 kPa)	14 (13)

* DAA: direct acting antivirals, RBV: ribavirin, PEG-IFN: pegylated-interferon, IFN: interferon.

† Based on FibroScan measurements.

Supplemental Table 4. Characteristics of participants in the Hemophilia in the Netherlands studies obtained from questionnaire data, according to type of hemophilia (A or B).

	2019 (N = 1009)	**2019, HA (N = 867)**	**2019, HB (N = 129)**
Mean age in years (range) *	40 (0-88)	41 (0-88)	36 (1-77)
Severity of hemophilia (%)			
Severe	378 (37)	325 (38)	53 (41)
Moderate	149 (15)	124 (14)	23 (18)
Mild	482 (48)	418 (48)	53 (41)
Type of hemophilia (%)†			
Hemophilia A	867 (87)	867 (100)	0 (0)
Hemophilia B	129 (13)	0 (0)	129 (100)
Family history of hemophilia (%)‡			
Negative	168 (18)	145 (18)	22 (20)
Positive	753 (82)	654 (82)	89 (80)
HIV infection (%)§			
Current infection	22/412 (5)‖	20/362 (6)	2/47 (4)
Hepatitis C infection (%)¶			
Current infection	8/412 (2)#	7/365 (2)	1/45 (2)
Past infection	226/412 (55)#	187/365 (51)	37/45 (82)
Inhibitory antibodies (%)**			
Ever inhibitors	66/361 (19)††	64/312	2/49 (4)
Current inhibitors	6/361 (2)††	6/312	0/49 (0)
Past inhibitors	60/361 (17)††	58/312	2/49 (4)

* Age was unknown for 8 patients.
† Type of hemophilia was unknown for 13 patients.
‡ Family history of hemophilia was unknown for 88 patients.
§ Reported for patients treated with coagulation factor before 1985.
‖ HIV status was unknown for 4 patients.
¶ Reported for patients treated with coagulation factor before 1992.
# HCV status was unknown for 84 patients.
** Reported for patients with severe hemophilia.
†† Inhibitor status was unknown for 17 patients.

Supplemental Table 5. Prophylaxis usage, annual bleeding rates and hospital admission, according to type of hemophilia (A or B).

	2019 (N = 1009)	2019, HA (N = 867)	2019, HB (N = 129)
Severe hemophilia	378	325	53
Patients on prophylaxis (%)			
Children, 0-16y	93/95 (98)	74/76 (97)	19/19 (100)
Young adults, 17-25y	42/46 (91)	38/42 (90)	4/4 (100)
Adults, older than 25y	193/228 (85)	171/199 (86)	22/29 (76)
Median age at first prophylaxis, years (range)	3 (0-79)	4 (0-79)	2 (0-58)
Median ABR* by age (range, IQR†)			
Children, 0-16y	4 (0-228, 0-12)	4 (0-228, 0-12)	4 (0-64, 0-22)
Young adults, 17-25y	1 (0-12, 0-2)	1 (0-12, 0-2)	1 (0-4, 0.5-2.5)
Adults, older than 25y	2 (0-100, 0-6)	2 (0-100, 0-6)	1 (0-9, 0-3)
Hospital admissions			
Hemophilia patients (%)	73/330 (22)	65/285 (23)	8/45 (18)
Median duration, (range)	7 (1-125)	7 (1-125)	4 (1-15)
Moderate hemophilia	149	124	23
Patients on prophylaxis (%)			
Children, 0-16y	6/24 (25)	6/21 (29%)	3/3 (100)
Young adults, 17-25y	4/19 (21)	2/14 (14)	2/5 (40)
Adults, older than 25y	14/104 (13)	12/89 (13)	2/13 (15)
Median ABR* by age (range, IQR†)			
Children, 0-16y	4 (0-32, 0-8)	4 (0-20, 0-8)	16 (0-32, 0-32)
Adults, older than 17y	1 (0-100, 0-2)	1 (0-100, 0-2)	0 (0-6, 0-1)
Hospital admissions			
Hemophilia patients (%)	21/136 (15)	20/115 (17)	1/19 (5)
Median duration (range)	6 (1-120)	6 (1-120)	21 (21-21)
Mild hemophilia	482	418	53
Patients on prophylaxis (%)			
Children, 0-16y	2/68 (3)	1/60 (2)	1/5 (20)
Young adults, 17-25y	1/43 (2)	1/36 (3)	0/7 (0)
Adults, older than 25y	7/346 (2)	6/306 (2)	1/35 (3)
Median ABR* by age (range, IQR†)			
Children, 0-16y	4 (0-100, 0-14)	6 (0-100, 0-14)	0 (0-36, 0-36)
Young adults, 17-25y	0 (0-88, 0-1)	0 (0-88, 0-1)	1 (0-1, 0-1)
Adults, older than 25y	0 (0-40, 0-0.5)	0 (0-40, 0-1)	0 (0-2, 0-0)

Hospital admissions			
Hemophilia patients (%)	103/415 (25)	92/368 (25)	10/43 (23)
Median duration (range)	5 (1-175)	5 (1-175)	4.5 (1-7)

* Annual bleeding rate. †IQR: Interquartile range. NR: Not reported.

Suplemental Table 6. Bleed rates and joint outcomes of the 2019 cohort, according to type of hemophilia (A or B).

	HA (N = 867)	**HB (N = 129)**	**severe HA (N = 325)**	**Severe HB (N = 53)**
Mean age in years (range)	41 (0-88)	36 (1-77)	36 (1-82)	32 (1-75)
Severity of hemophilia (%)				
Severe	325 (38)	53 (41)	NA	NA
Moderate	124 (14)	23 (18)	NA	NA
Mild	418 (48)	53 (41)	NA	NA
Prophylaxis (%)				
No	536 (63)	71 (58)	34 (11)	7 (13)
Yes	311 (37)	51 (42)	283 (89)	45 (87)
Missing	20	7	8	1
Median age at first prophylaxis, years (range)	4 (0-79)	2 (0-58)	4 (0.79)	2 (0-58)
Missing	605	89	98	20
Annual clotting factor consumption for patients on prophylaxis (IQR)	2482 (1862-3564)	2777 (1962-3583)	2491 (1862-3614)	2819 (1968-3659)
Missing (no info on prophylaxis)	621	89	97	16
Median annual bleeding rate				
Rate (IQR)	1 (0-4)	0 (0-2)	2 (0-7)	2 (0-5)
Missing	122	29	53	12
Median annual joint bleeding rate				
Rate (IQR)	0 (0-1)	0 (0-0)	0 (0-4)	0 (0-0)
Missing	112	24	47	9
Joint impairment (%)				
Some impairment	237 (33)	37 (35)	160 (61)	25 (54)
No impairment	484 (67)	69 (65)	203 (39)	21 (46)
Missing	146	23	62	7
Orthopedic surgery in the past, joint replacement surgery (%)				
No	530 (85)	69 (85)	146 (67)	21 (70)
Yes	95 (15)	12 (15)	73 (33)	9 (30)
Missing	242	48	106	23

Chapter 3

Mortality, life expectancy and causes of death of persons with hemophilia in the Netherlands 2001-2018

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Summary

Background
Treatment of patients with hemophilia has advanced over the past decades, but it is unknown whether this has resulted in a normal life expectancy in the Netherlands.

Objective
This observational cohort study aimed to assess all-cause and cause-specific mortality in patients with hemophilia in the Netherlands between 2001-2018 and to compare mortality and life-expectancy with previous survival assessments from 1973 onwards.

Methods
All 1066 patients with hemophilia who participated in a nationwide survey in 2001 were followed until July 2018.

Results
Information on 1031 individuals (97%) was available, of whom 142 (14%) deceased during follow-up. Compared with the general Dutch male population, mortality of patients with hemophilia was still increased (standardized mortality ratio: 1.4, 95% confidence interval: 1.2-1.7). Intracranial bleeding and malignancies were the most common causes of death. Estimated median life expectancy of patients with hemophilia was 77 years, six years lower than the median life expectancy of the general Dutch male population (83 years). Over the past 45 years, death rates of patients with hemophilia have consistently decreased, approaching the survival experience of the general population. Over the past decades, mortality due to human immunodeficiency virus and hepatitis C virus infections has decreased, death due to intracranial hemorrhages has increased and death due to ischemic heart disease has remained consistently low over time.

Conclusions
Survival in patients with hemophilia in the Netherlands has improved over time but is still lower than that of the general population.

Introduction

Hemophilia is a hereditary disease caused by a deficiency of clotting factor VIII or IX. The introduction of clotting factor concentrates in the 1970s[1-3] and other improvements such as prophylactic treatment, home treatment and low dose immune tolerance drastically improved life expectancy of patients.[2]

Unfortunately, exposure to human immunodeficiency virus (HIV) and hepatitis C virus (HCV) through contaminated blood products in the 1980s led to a sharp increase in mortality.[4-10] Through the use of viral inactivation techniques, the transmission of both HIV and HCV has been halted since 1992.[2] From 1999 onwards, hemophilia treatment in the Netherlands was gradually centralized and quality criteria were introduced for the comprehensive hemophilia treatment centers.[11] In addition, dosages for prophylactic treatment have steadily increased since the 1970s.[12] It is still insufficiently known whether these treatment advances have resulted in a completely normalized life expectancy in the Netherlands.

This observational cohort study aimed to evaluate all-cause and cause-specific mortality in patients with hemophilia in the Netherlands from 2001 to 2018 and to compare mortality and life-expectancy over the past 45 years. In addition, we investigated potential determinants of mortality of patients with hemophilia during this period.

Methods

Study design

This was an observational cohort study following patients from 1973-2018. From 1973-2001, three cohort studies evaluated mortality of patients with hemophilia in the Netherlands.[9, 10, 13] The studies were performed from Jan 1st 1973 to Jan 1st 1986 (the 1973-1986 cohort); Jan 2nd 1986 to May 31st 1992 (the 1986-1992 cohort); and June 1st 1992 to June 30th 2001 (the 1992-2001 cohort). The current study followed patients who participated in a nationwide survey from July 1st 2001 to July 1st 2018. An invitation to participate in the survey was sent to all 1567 known patients with hemophilia via their hemophilia physician or the Netherlands Hemophilia Patient Society. In total, 1066 patients (68%) completed the survey.[9] The present study was approved in 2018 by the Committee of Medical Ethics of the Leiden University Medical Center.

Patient characteristics at inclusion

All male patients with mild, moderately severe and severe hemophilia A and B who participated in the 2001 survey were included. The following self-reported information, obtained from the 2001 patient survey, was collected at baseline; date of birth, hemophilia severity, HIV status and HCV status. If the self-reported data on HIV or HCV was missing, it was obtained from the medical files.

Hemophilia severity was categorized as severe (‹ 0.01 IU/mL), moderate (0.01– 0.05 IU/mL) or mild (› 0.05–0.40 IU/mL). Information on hemophilia severity and type were verified from the medical files. Patients born after 1985 or who reported no treatment with clotting factor between 1979-1985 were considered to be HIV-negative. Patients born after 1992 or who reported no treatment with clotting factor before 1992 were considered to be HCV-negative. If HIV/HCV status was missing, the information was obtained from the medical files. HCV status was categorized as "never infected with HCV", "HCV infection cleared" and "chronic hepatitis C". As a double-check, HCV status as reported by the patients was compared with HCV status from the medical files for a random sample of patients (N = 92).

Follow-up and outcomes

Patients were followed from July 1st 2001 until July 1st 2018 or until their last known hospital visit. Date of death and cause of death were obtained from the medical files. The primary cause of death, as written on the death certificate was not directly available, but the same information was also reported in the patients' medical file at the time of death. Causes of death were then manually classified according to the 10th revision of the International Classification of Injuries, Diseases and causes of Death-10 (ICD-10).[14] All-cause mortality, cause-specific mortality and life expectancy of the general male population of the Netherlands for the years 1973-2017 retrieved from the Dutch Central Bureau of Statistics (CBS).[15]

All-cause mortality, cause-specific mortality and life expectancy

The standardized mortality ratio (SMR) is a measure of the change in mortality in a given population, with respect to a reference population. SMRs were calculated for the years 2001-2018 to estimate the risk of all-cause and cause-specific death among patients compared to the general male population, while adjusting for the age distributions of the two populations. The SMR is calculated by dividing the observed number of deaths in a study population, by the expected number of deaths in the study population (which is based on the age distribution of the study population and the age-specific death rates of the general male population). When calculating

cause-specific SMRs, other causes of death were censored. To assess all-cause and cause-specific death rates over time, we calculated crude death rates for the current and previous cohort studies, stratified by 15-year age categories.

The median life expectancy at birth for the 2001-2018 cohort was calculated from a life-time survival curve using age as the time scale. To calculate median life expectancy, patients had to survive until the start of the study observation period (2001). A standard analysis of the data would have induced a type of bias, which is sometimes called length bias.[16] To correct for this problem, we adjusted for left truncation, i.e. patients were included in the analysis from the start of the study observation period instead of their date of birth. Median life expectancy was defined as the age at which cumulative survival was 50%. To assess changes in median life expectancy over time, information on median life expectancy for the previous cohort studies was obtained from previously published data.[9, 10, 13]

Potential determinants of mortality

Crude and adjusted associations between hemophilia severity/HIV status/HCV status and mortality were assessed with the Cox proportional hazards model, in the standard way, using time on study as the time-scale and with patients being included in the risk-set of the model from the time of study entry (2001). Participants with missing values were excluded from the analysis.

Sensitivity analysis

Severely ill patients may not have participated in the 2001 survey, which could have led to an underestimation of death rates during follow-up. We examined a possible 'healthy cohort effect' by performing a sensitivity analysis where we excluded the first three years of follow-up.

Data sharing statement

For original data, please contact S.C.Gouw@lumc.nl.

Results

Patient characteristics

In the current cohort study we included 1031 out of 1066 (97%) patients with available data. Thirty-five patients with missing follow up data were excluded. Eighty-seven percent of patients had hemophilia A and 13% had hemophilia B (*Table 1)*. In total, 412 patients (40%) suffered from severe hemophilia, 175 (17%) from moderate

Table 1. Patient characteristics at baseline (2001)

Baseline variable	**N = 1031**
Age in years	
Mean (SD, min-max)	33.9 (20.5, 0.1-89.3)
Severity of disease (n, %)	
Severe	412 (40)
Moderate	175 (17)
Mild	444 (43)
Type of hemophilia (n, %)	
Hemophilia A	893 (87)
Hemophilia B	138 (13)
Severity of disease, hemophilia A (n, %)	
Severe	351 (39)
Moderate	154 (17)
Mild	388 (43)
Severity of disease, hemophilia B (n, %)	
Severe	61 (44)
Moderate	21 (15)
Mild	56 (41)
HIV infection (n, %)	
No (in 2001)	984 (95)
Yes (in 2001)	29 (3)
Unknown	18 (2)
HCV infection (n, %)	
Never infected with HCV (in 2001)	581 (56)
HCV infection cleared (in 2001)	96 (9)
Chronic hepatitis C (in 2001)	336 (33)
Unknown	18 (2)

hemophilia and 444 (43%) from mild hemophilia. In 2001, 29 patients were known to be infected with HIV (3%) and 336 patients had chronic hepatitis C (33%).
In the random sample of 92 patients of whom HCV status was verified from medical records, 92% (85/92) accurately reported their hepatitis C status. Stratified by HCV status, 93%, 75% and 100% correctly reported their HCV status in patients with chronic HCV, patients who previously cleared the virus, and patients who were never infected, respectively. Median follow-up time was 17.0 years (min-max: 0.3-17.4) and

the mean age at baseline was 33.9 years (SD: 20.5, min-max: 0.1-89.3) in 2001. For comparison, the mean age of the Dutch male population in 2001 was 37.1 years. The age distribution of the study population and the general male population in the Netherlands in 2001 is presented in *Supplemental Figure 1*. The total number of deaths per age group in the study population is presented in *Supplemental Figure 2*.

All-cause mortality

In total, 142 patients died during follow-up (14%) at a median age of 69.8 years (min-max: 16.4-98.0). A life-time survival curve for the study cohort is presented in

Figure 1. Life-time survival curve for 2001-2018 cohort.

Legend: The figure shows the survival curve for patients with mild, moderate and severe hemophilia, using age as the timescale. The cumulative probability of survival is shown on the Y-axis, the age in years is shown on the X-axis.

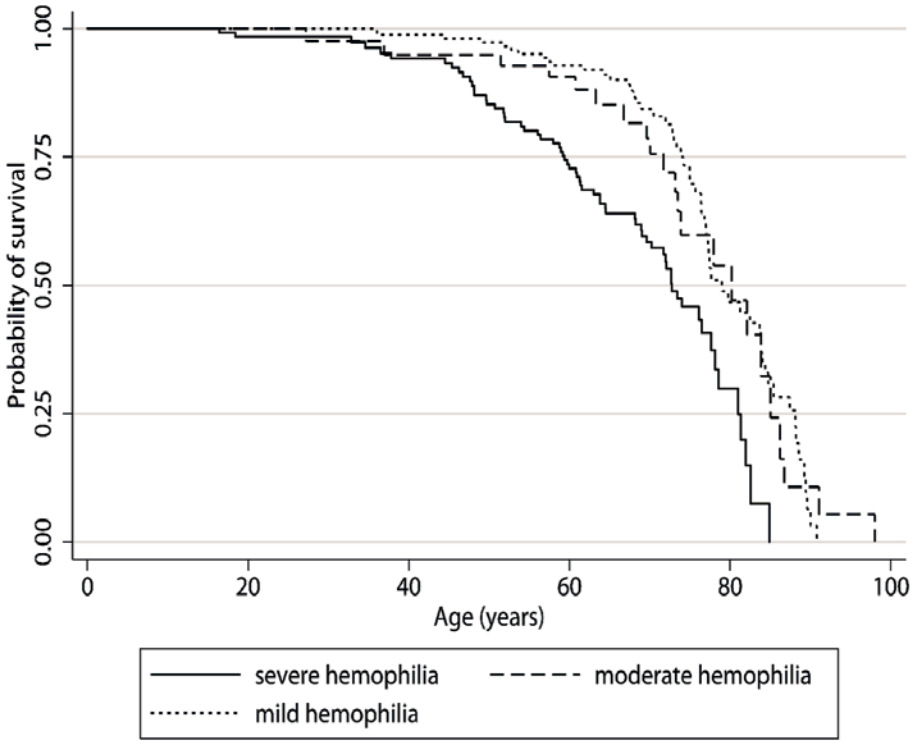


Figure 1. The overall crude death rate was 8.9 deaths per 1000 person-years. The crude death rate in the general male population was 8.2 per 1000 person-years in the period 2001-2017. Overall, age-standardized mortality in patients with hemophilia was 40% higher compared with the general male population (SMR 1.4, 95% CI 1.2-1.7) (*Table 2)*. Crude death rates for the period 1973-2018, stratified by age are presented as a graph (*Figure 2*) and as a table (*Table 3*). Overall, crude death rates followed a decreasing trend for all age-categories. For the period 1973-2018, crude death rates changed from 0.7 to 0 deaths per 1000 person years for persons aged 0-14 years, 2.4 to 0.8 deaths per 1000 person years for persons aged 15-29 years, 4.7 to 2.3 deaths per 1000 person years for persons aged 30-44 years, 13.8 to 19.4 and then to 8.1 deaths per 1000 person years for persons aged 45-59 years and 57.9 to 33.7 deaths per 1000 person years for persons aged 60 years and older.

Cause-specific mortality

From 2001-2018, frequent causes of death were non-hepatic malignancies (26%) and intracranial bleeding (14%). AIDS (2%), chronic liver disease (7%), and hepatocellular carcinoma (7%) were less frequent causes of death. *(Table 2)*

Table 2. All-cause and cause-specific Standardized Mortality Ratios (SMR) in patients with hemophilia from the Netherlands between 2001-2018.

Cause of death	**N*(%)**	**SMR (95% CI)†**
All-cause mortality	142 (100)	1.4 (1.2 - 1.7)
AIDS	3 (2)	27.9 (5.8 - 81.6)
Hepatocellular carcinoma	10 (7)	13.2 (6.3 - 24.2)
Chronic liver disease	10 (7)	9.1 (4.3 - 16.6)
Ischemic heart disease	3 (2)	0.3 (0.1 - 0.9)
Ischemic stroke	1 (1)	1.1 (0.03 - 6.0)
Intracranial bleeding	20 (14)	12.8 (7.8 - 19.8)
Malignancies (non-hepatic)	37 (26)	1.0 (0.7 - 1.4)
All other causes‡	33 (23)	not applicable
Unknown	25 (18)	not applicable

* Number of deaths.

† Standardized mortality ratio, ratio of the observed and expected number of deaths and 95% confidence intervals.

‡ bacterial infections (11), gastrointestinal bleeding, intra-abdominal bleeding or pericardial bleeding (6), sudden cardiac death (5), trauma (3), chronic heart failure (2), suicide/substance abuse (3) or other causes (3).

In comparison with the general male population, mortality due to AIDS (SMR 27.9, 95% CI 5.8-81.6) and due to HCV infection (SMR 13.2, 95% CI 6.3-24.2 for hepatocellular carcinoma, SMR 9.1, 95% CI 4.3-16.6 for chronic liver disease) were increased (*Table 2*). In addition, mortality due to intracranial bleeding was increased (SMR 12.8, 95% CI 7.8-19.8). Mortality due to ischemic heart disease was decreased (SMR 0.3, 95% CI 0.1-0.9) while mortality due to ischemic stroke was similar (SMR 1.1, 95%CI 0.03-6.0) compared with the general male population. Lastly, mortality due to non-hepatic malignancies was also similar to that of the general male population (SMR 1.0, 95%CI 0.7-1.4).

Cause-specific crude death rates over the past 45 years are presented in *Table 4*. Mortality due to AIDS was first reported in the period 1986-1992, reaching its peak in the period 1992-2001, and decreased thereafter. A similar pattern is seen for chronic liver disease, although the decrease in mortality after 2001 is less pronounced. Mortality due to ischemic heart disease was low in all cohorts between 1973-2018.

Table 3. Deaths, person-time and death rates for different age categories, stratified by study cohort.

Age category	1973-1986			1986-1992			1992-2001			2001-2018		
	n*	PY†	death rate‡	n*	PY†	death rate‡	n*	PY†	death rate‡	n*	PY†	death rate‡
0-14	1	1530	0.7	0	873	0	0	452	0	0	1920	0
15-29	7	2978	2.4	6	1887	3.2	1	995	1.0	3	3630	0.8
30-44	9	1906	4.7	10	1797	5.6	8	1246	6.4	8	3424	2.3
45-59	11	799	13.8	8	839	9.5	18	929	19.4	33	4075	8.1
60+	15	259	57.9	21	372	56.5	16	466	34.3	98	2906	33.7

* N: deaths; †PY: person-years; ‡crude death rate per 1000 person-years

Table 4. Cause-specific crude death rates (per 1000 person-years) over time.

Cause of death	1973-1986 (N = 717) (PY* = 7788)		1986-1992 (N = 919) (PY* = 5753)		1992-2001 (N = 967) (PY* = 8868)		2001-2018 (N = 1066) (PY* = 15909)	
	n†	death rate‡	n†	death rate‡	n†	death rate‡	n†	death rate‡
AIDS	0	0	12	2.09	24	2.71	3	0.19
Hepatocellular carcinoma	NR	NR	NR	NR	5	0.56	10	0.63
Chronic liver disease	0	0	5	0.87	10	1.13	10	0.63
Ischemic heart disease	1	0.13	0	0	6	0.68	3	0.19
Ischemic stroke	3	0.39	0	0	0	0	1	0.06
Intracranial bleed	3	0.39	9	1.56	4	0.45	20	1.26
Non-hepatic malignancies	13	1.67	7	1.22	12	1.35	37	2.33

* PY: person-years, †n: number of deaths, ‡death rate per 1000 person-years, NR: not reported in original publication

Median life-expectancy

Median life-expectancy from 1973-2018 is presented in *Figure 3* and additional information on the number of deaths/total person-time is presented in *Table 5*. Median life expectancy of the cohort increased from 66 years in 1973-1986 to 77 years in 2001-2018, a gain of 11 years. In comparison, median life expectancy of the general male population increased from 79 years to 83 years during the same timeframe. (a gain of 4 years) For the 2001-2018 cohort, median life expectancy of patients with severe hemophilia was 73 years (SMR 2.4, 95% CI 1.8-3.0), whereas median life expectancy was 80 years for patients with moderate hemophilia (SMR 1.1, 95%CI 0.7-1.7) and 79 years for patients with mild hemophilia (SMR 1.0, 95%CI 0.8-1.4). Although patients with severe hemophilia had the lowest median life expectancy they also showed the biggest gains after 2001 (59 years in the 1992-2001 cohort vs 73 years in the 2001-2018 cohort).

Potential determinants of mortality

Compared with patients with mild hemophilia, mortality in patients with severe hemophilia was 80% higher (adjusted HR 1.78, 95%CI: 1.08-2.94) (*table 6*). Twenty-nine patients were HIV positive in 2001, 8 patients died during follow-up. Three patients died due to AIDS-related complications, 2 patients died due to HCV-related complications and 3 patients died due to other causes. Compared with HIV-negative patients, mortality among HIV-positive patients was increased (adjusted HR 2.65, 95%CI:

1.26-5.58). (*Table 6*) In total, 336 patients were HCV positive in 2001. Compared with HCV-negative patients, mortality among HCV-positive patients was slightly increased (adjusted HR 1.25, 95%CI: 0.85-1.83). (*Table 6*)

Sensitivity analysis

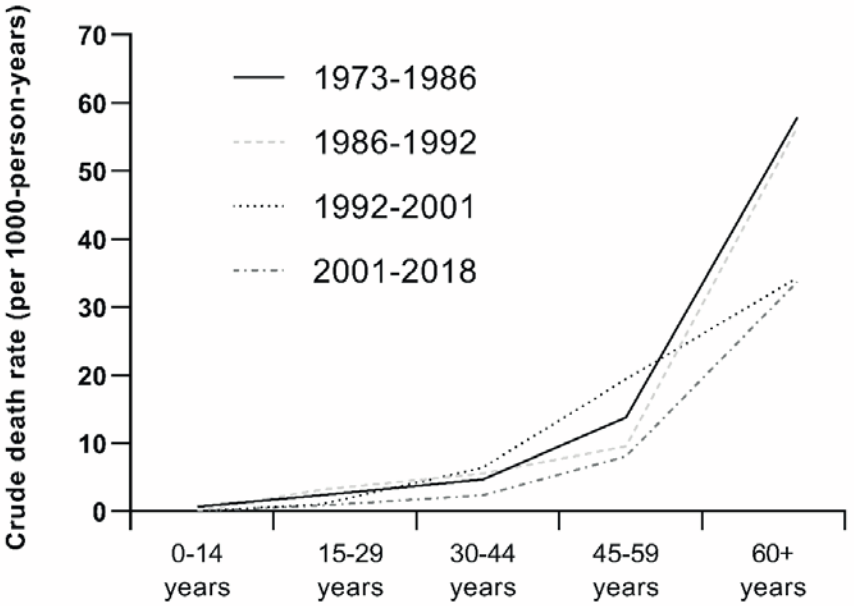
The sensitivity analysis excluding the first three years of follow-up yielded similar results as the overall analysis (*Supplementary table 1*).

Discussion

From 1973-2019, life expectancy slowly increased, except for the period 1986-2001 (which coincides with the HIV/HCV epidemic). This temporary dip in life expectancy

Figure 2. Crude death rates over time.

Legend: The line-chart shows the crude death rates (per 1000 person-years) for different age categories, stratified by study cohort. The accompanying table (Table 3) shows the total number of deaths, total person-time, and resulting death rate for different age categories, stratified by study cohort.



was, as expected, strongest in patients with severe hemophilia (who were most exposed to clotting factor products). Overall, the life expectancy of patients with hemophilia increased by 11 years vs. 4 years for the general male population. The differences in mortality rates between time-periods were most pronounced in the older age groups. (*Figure 2*) This was most likely simply due to the high baseline risk of dying in older patients. However, mortality is still increased when compared with the general male population.

The cause of the decrease in mortality is most likely a combination of two main factors; namely the increased prophylactic dosages and the decrease in HCV- and HIV-related deaths over time. Evidence from the literature shows that integrated care most likely also decreases mortality.[17] We hypothesized that this would also be the case in our study, however, it was impossible to estimate the effect of integrated care on mortality directly as there were no patients that were treated outside of this care model in our study. (i.e. there was no control group to compare against our care model)

Our findings are in line with a study among Italian patients with hemophilia, which showed a similar decrease over time (SMR 2.0 95%CI 1.5-2.5 from 1990-1999, SMR 1.1 95%CI 0.8-1.4 from 2000-2007).[18] A study among Brazilian patients of the period 2000-2014 reported a SMR of 1.13 (95% CI: 1.01-1.16), the same outcome as that of the 2000-2007 Italian cohort.[19] However, it should be noted, that SMRs from different populations cannot be compared directly when the reference populations are not the same.[20] Compared to the Italian cohort, a similar HCV-related death rate (1.12 deaths per 1000 person-years) and a higher HIV-related death rate (0.84 deaths per 1000 person-years) than in our study was reported.[18]

In our study, 20 out of 142 patients died due to intracranial bleeding (14% of total deaths), a thirteen-fold increase compared to the general male population. The proportion of patients that died due to intracranial bleeding was similar for patients with severe hemophilia (2.4%, 95%CI 1.2-4.5) and mild hemophilia (2.0%, 95%CI 1.0-3.9). A European/Australian cohort study that followed 2709 non-severe hemophilia A patients who were treated with factor VIII from 1996-2010 reported a 3.5 fold increased risk (95%CI 2.0-5.8), compared with the general population.[21] As only patients with non-severe hemophilia were included, the lower mortality risk seems plausible. A recent retrospective study from Brazil based on mortality data from the entire male population for the period 2001-2014 found more similar results, as 137 out of 784 deaths in this cohort (17.5%, 95%CI 14.9-20.3) were due to intracranial bleeding.[19] Intracranial hemorrhages in neonates are a known complication of

hemophilia[22-24], but these patients were not included in our study. In our population, median age at death from intracranial bleed was similar to other causes (67 vs. 68 years). Further studies are needed to mitigate the risk for intracranial bleeding.

Hemophilia potentially complicates treatment of age-related conditions such as malignancies and ischemic heart disease, which are becoming more common as the population ages.[25-29] For example, patients with a malignancy may need additional hemostatic replacement during surgical interventions or after chemotherapy-induced thrombocytopenia. Bleeding symptoms will also develop at an earlier stage in patients with gastro-intestinal malignancies.[30] In our cohort, the incidence of deaths due to non-hepatic malignancies is now similar to that of the Dutch male population. This is most likely due to the fact that patients are living longer, and due to less hemophilia-related deaths like bleeding-related complications or HCV/HCV.

Based on the literature, it seems that patients with hemophilia have an unfavorable cardiovascular risk profile, compared to the general population.[31, 32] In our cohort, the mortality due to ischemic heart disease was lower than in the general population, which is also in line with literature.[30] The low clotting factor levels may hinder

Figure 3. Median life expectancy in patients with hemophilia in the Netherlands between 1973-2018.

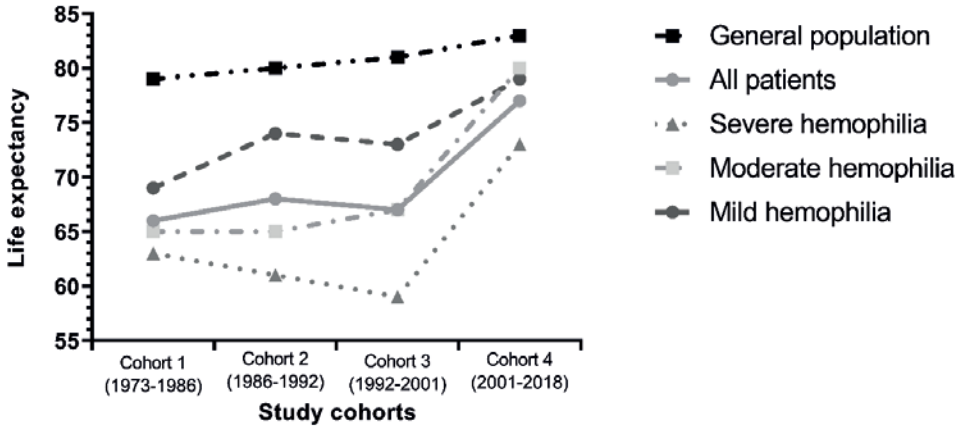


Table 5. The number of deaths and total person-time for each study cohort, stratified by severity.

	1973-1986		1986-1992		1992-2001		2001-2018	
	deaths	PY*	deaths	PY*	deaths	PY*	deaths	PY*
All patients	43	7776	45	5753	94	8314	142	15909
Severe hemophilia	20	3649	19	2396	47	3259	65	6406
Moderate hemophilia	10	1861	11	1070	15	1454	22	2683
Mild hemophilia	13	2266	15	2287	32	3600	55	6819

* PY: person-years

thrombus formation at the site of plaque rupture.[33] The optimal evidence-based anti-platelet/anti-coagulant therapy to reduce thrombotic risk as well as bleeding risk has not yet been identified.[30]

Hemophilia severity, HIV status and HCV status were independently associated with mortality. Compared to HCV-negative patients, the increased risk of mortality for HCV-positive patients was relatively minor. This is probably because the vast majority of patients were successfully treated with either (peg)interferon-based treatment and/or the newer direct acting antivirals. Pre-treatment severity of fibrosis is strongly associated with mortality after successful treatment.[34] As information on the extent of liver damage was not available, we were not able to assess this in our dataset.

Our study has several strengths. Firstly, due to the availability of data from 1972 onwards we could study trends in mortality during the last 45 years. Secondly, this study included the majority of patients with hemophilia in the Netherlands, with little loss-to-follow-up (3%) during the period 2001-2018. Our study also has limitations. Other important determinants of mortality such as inhibitor status, immune tolerance induction, bleeding phenotype, joint status and prophylaxis were not assessed. Lastly, as we were not able to collect information on all possible confounding factors (such as bleeding phenotype, treatment adherence, the level of cirrhosis, inhibitor status, prophylaxis etc.) some residual confounding may still exist. In our cohort, we only reported the combined results for hemophilia A and B. However, some retrospective studies have reported that patients with hemophilia B have a milder phenotype, but this has not been confirmed in subsequent studies.[35]

Table 6. Mortality rates and hazard ratios, according to HIV status and HCV status in 2001-2018.

Group	N	Deaths (%)	PY*	Crude rate (per 1000 PY*)	Crude hazard ratios (95%CI)	Adjusted hazard ratios (95%CI)
Overall	1031	142 (13.8)	15909	8.9	-	-
Mild	444	55 (12.4)	6819	8.1	ref	ref
Moderate	175	22 (12.6)	2683	8.2	1.00 (0.61-1.64)	1.24 (0.63-2.44)†
Severe	412	65 (15.8)	6406	10.2	1.28 (0.89-1.83)	1.78 (1.08-2.94)†
¶ HIV -	984	127 (12.9)	15260	8.3	ref	ref
¶ HIV +	29	8 (27.6)	415	19.3	2.33 (1.14-4.76)	2.65 (1.26-5.58)‡
# HCV -	677	61 (9.0)	10652	5.7	ref	ref
# HCV +	336	74 (22.0)	4997	14.8	2.58 (1.84-3.62)	1.25 (0.85-1.83)§

* Person years.
† Adjusted for age.
‡ Adjusted for age, HCV status and hemophilia severity.
§ Adjusted for age, HIV status and hemophilia severity.
¶ HIV status was unknown in 18 patients.
# HCV status was unknown in 18 patients.

Also, there were only 29 patients with HIV, with only 3 deaths due to AIDS. However, the incidence of AIDS in the general population was far lower, resulting in a very high SMR of 27.9 (95CI: 5.8-81.6). Due to the small sample size, the estimate of the SMR is not very precise (hence the wide confidence intervals around the estimate). Furthermore, as some information was self-reported, there was a risk of misclassification bias. To reduce this bias, hemophilia type, severity and HCV status were checked from the medical files. In the case of HCV status, this was done for a random sample of patients (N = 92). Results showed that the self-reported HCV status was correct in most cases (92%). Furthermore, life expectancy estimates should be interpreted with caution as these are always future projections based on current trends. It is expected that age-specific mortality rates will further decrease over time (despite the current transient effect of the COVID-19 epidemic on mortality) due to improvements in health care and novel treatment options. For example, direct-acting antivirals that are used to treat HCV have an almost 100% success rate.[36] Furthermore, compared

to treatment with clotting factor products, novel non-gene therapy options such as emicizumab offer better bleeding management (especially for patients with an inhibitor) and possibly a better safety profile as well.[37]

Conclusion

A decrease in mortality and an increase in life expectancy in patients with hemophilia in the Netherlands over the period 1973-2018 was seen. However, survival is still lower than that of the general population and warrants further improvements in hemophilia care.

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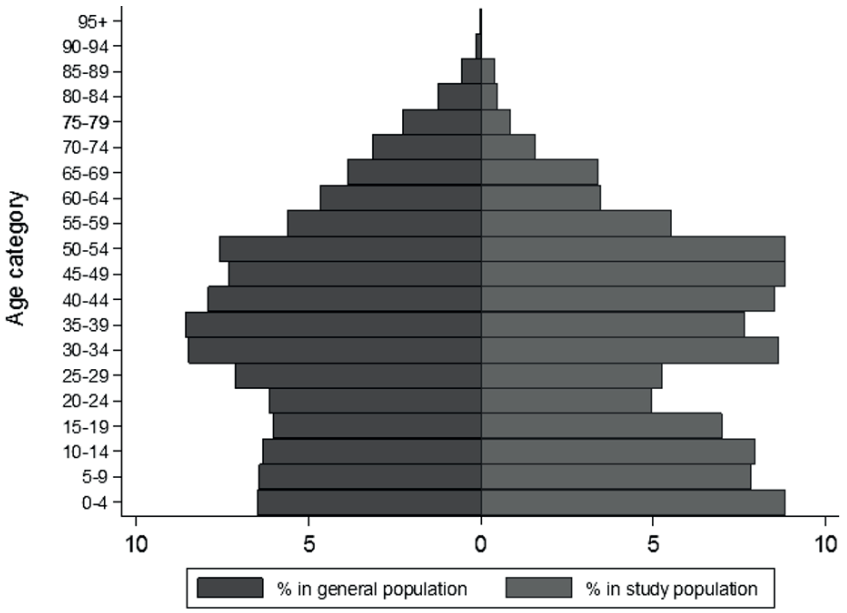
Supplemental Table 1. Sensitivity analysis for healthy cohort effect, excluding the first three years of follow-up (i.e. follow-up starts from 2004 instead of 2001).

N	PY*	Deaths	SMR±	Life expectancy
First three years of follow-up included				
1031	15910	142	1.4 (1.2-1.7)	77
First three years of follow-up excluded				
999	12861	116	1.3 (1.1- 1.6)	78

* Person-years.
± Standardized mortality ratio.

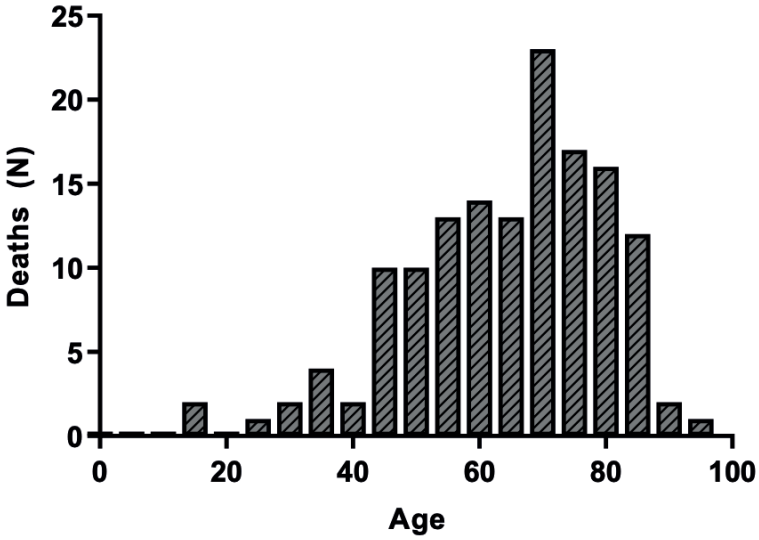
Supplemental Figure 1. Population pyramid of study population and general male population.

Legend: The graph shows the population age structure of the study population (on the right side) and the Dutch general male population (on the left side) in 2001. On the Y-axis, the age categories are shown. For each age category, the number of people as a percentage of the total population is shown.



Supplemental figure 2. Total number of deaths per age group.

Legend: This bar chart shows the number of deaths per age group in the 2001-2018 study cohort.



Chapter 4

Factor VIII products and inhibitor development in previously treated patients with severe or moderately severe haemophilia A: a systematic review

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Summary

Background

Patients with severe haemophilia A who have been treated extensively with factor VIII (FVIII) products face a low but potentially serious risk of inhibitor development. It is unknown why these patients break immunological tolerance and data on product-related immunogenicity is scarce.

Aims

To summarize the currently available evidence on the relationship between inhibitor development and recombinant FVIII product type in previously treated patients with severe haemophilia A.

Methods

Longitudinal studies were included that reported on de novo inhibitor formation in patients with baseline FVIII activity levels less than 0.02 IU/ml who had been treated with FVIII for at least 50 days. Pooled incidence rates of inhibitor development according to product types were calculated using a random intercept Poisson regression model.

Results

Forty-one independent cohorts were included, 39 patients developed de novo inhibitors during 19,157 person-years of observation. The overall incidence rate was 2.06 per 1000 person-years (p-y) with a 95% confidence interval (CI95) of 1.06-4.01. According to product type, the pooled incidence rate was 0.99 (CI95: 0.37-2.70) per 1000 p-y for patients treated with Advate, 5.86 (CI95: 0.25-134.92) per 1000 p-y for those treated with Kogenate/Helixate, 1.35 (CI95: 0.66-2.77) per 1000 p-y for Kogenate FS/Helixate NexGen, 12.05 (CI95: 1.53-94.78) per 1000 p-y for Refacto and 4.64 (CI95: 0.82-26.43) per 1000 p-y for Refacto AF.

Conclusion

These results suggest that some products may be associated with increased immunogenicity. However, the low incidence of inhibitors in PTPs and the differences in study design may cause significant variation in estimates of risk.

Introduction

The development of factor VIII (FVIII)-specific neutralizing antibodies (inhibitors) remains the most important treatment complication in patients with congenital haemophilia A. Inhibitor development is associated with increased morbidity and mortality[1-3] and occurs primarily during the first 50 days of treatment with FVIII[4,5] after a median of 14.5 days of exposure to FVIII (IQR: 9.75-20.0)[6]. Patients who have been treated with FVIII for more than 50 days, also termed previously treated patients (PTPs), are relatively tolerant to FVIII and inhibitor development is rare[7], with a reported rate of 2.14 per 1000 person-years[8]. It has been suggested that inhibitor incidence follows a bimodal distribution and that at older age the risk of developing inhibitors increases again[9].

Knowledge about immunogenicity of recombinant FVIII (rFVIII) products in PTPs is scarce, which is largely due to the rarity of inhibitor development during this phase of replacement therapy. In addition, findings on a differential inhibitor rate among rFVIII products in PTPs might seem conflicting[7, 10]. The observed differences in immunogenicity between rFVIII products may be explained by product characteristics such as the specific amino acid sequence, culture conditions, stabilizing agents and/or post-translational modifications.[11]

Two previous meta-analyses have assessed product-related immunogenicity in previously treated haemophilia A patients.[7, 10] Several new studies have been published since the latest review (published in 2013), which is one of the reasons to perform a new meta-analysis. Moreover, a new meta-analysis is needed with methods that can appropriately handle rare event situations and differences in follow-up time among included studies.

The objective of this systematic review and meta-analysis was to quantify and compare the current knowledge on incidences of inhibitor formation according to rFVIII product type among PTPs affected with severe or moderately severe haemophilia A.

Methods

A systematic literature review was performed to identify studies that assessed de novo inhibitor development in PTPs with severe or moderately severe haemophilia A who were treated exclusively with one brand of rFVIII. The Meta-analysis of Observational Studies in Epidemiology (MOOSE)[12] and Strengthening of Reporting of Observational Studies in Epidemiology (STROBE)[13] guidelines were followed.

Inclusion/exclusion criteria

Types of studies
All longitudinal studies that assessed de novo inhibitor development and that reported total, mean or median follow-up time in person-years were eligible. Original articles, letters published in peer-reviewed journals and meeting abstracts were eligible for inclusion. There was no restriction on date of publication or language. We excluded case-control studies, case-series, cross-sectional studies, studies with a follow-up time of less than 3 months, studies with fewer than 10 patients, studies in which treatment for surgery was the main goal, pharmacokinetic studies and studies with duplicate data. Authors of studies in which inhibitor incidences were not reported separately for PTPs were asked to provide these data. In case these data were not provided, these studies were excluded.

Type of patients
All patients with severe moderately severe haemophilia A (baseline FVIII activity ‹ 0.02 IU/ml) with at least 50 days of prior exposure to FVIII, were eligible. Furthermore, only patients that were exclusively treated with one brand of rFVIII during the observation period were eligible. Studies that also included patients with fewer than 50 days of exposure to FVIII were only included when separate results were available for the subset of patients with more than 50 days of exposure to FVIII.

Types of rFVIII products
rFVIII product type (analysed according to brand) was the determinant in the primary analysis. The following brands were included; Advate (Shire), Kogenate (Bayer), Kogenate FS/Bayer (Bayer), Helixate (Bayer), Helixate FS/NexGen (CSL Behring), Refacto (Wyeth), Refacto AF (Pfizer). Also included were GreenGene F (Green Cross), Kovaltry/Iblias (Bayer), NovoEight (Novo Nordisk), Nuwiq (Octapharma) and Recombinate (Baxter). Kogenate and Helixate users were grouped into one category. Similarly, Kogenate FS/Bayer and Helixate FS/NexGen users were grouped together.

For the secondary analyses, rFVIII products were also categorised according to length (full-length vs B-domain deleted) and the cell line used for production (Chinese hamster ovary cells, baby hamster kidney cells or human embryonic kidney cells). Lastly, rFVIII products were also categorised according to generation; first-generation products (human/animal proteins in production and final formulation), second-generation products (human/animal proteins in production but not in final formulation), third-generation products (no human/animal proteins used in production or final

formulation) and fourth-generation products (no human/animal proteins used in production or final formulation and human embryonic kidney cells used as cell line). Studies performed with extended half-life rFVIII products were excluded, mainly since there were not enough studies done with these products.

Type of endpoints

The primary endpoint was de novo inhibitor development defined as the first occurrence of an inhibitor according to the cut-off used by the investigators of the original studies. The secondary outcome was high titre de novo inhibitor formation, defined as a peak inhibitor titre of at least 5 Bethesda Units (BU)/mL.

Search strategy

We searched the following databases; PubMed, Embase, Web of Science, Cochrane database and CINAHL. The search strategy was designed and supervised by an experienced librarian (J.W. Schoones, MA, Walaeus Library, Leiden University Medical Center). The initial search was performed in February 2016. Additional studies were included by monthly searches in PubMed up to November 2017. (search terms are reported in supplemental figure S1)

Study selection and data extraction

Two reviewers (S. Hassan and A. Cannavò) independently scanned all titles and abstracts to select articles for further scrutiny. Full text versions of each selected article were reviewed to assess eligibility. Inclusion of an article was determined by consensus between the two reviewers. Consultation of a third reviewer (J.G. van der Bom) was carried out in case of disagreement. To avoid multiple counting of patients included in more than one study, recruitment periods and catchment areas were recorded and, if needed, authors were contacted for clarification. Data were extracted independently by two investigators (S. Hassan and A. Cannavò). A structured electronic data extraction form was used. When the required data were missing, the original investigator(s) were contacted for further information.

Quality assessment

The methodological quality of each article was assessed using the Downs and Black checklist[14]. For the non-comparative studies in our systematic review, only items relevant to this study design were scored (18 of the 27 items from the original checklist[14]). The modified Downs and Black checklist contained 8 items about reporting accuracy, 3 items about external validity, 6 items concerning internal validity and 1 item about study power. Eight items that were only applicable to comparative studies

(i.e. all items about randomisation, blinding, concealment of treatment allocation and confounding) and one item about the use of p-values were removed. The wording of some questions was modified to provide clearer scoring criteria to improve consistency among raters. (supplemental table S2) Each item could be scored as "no" or "unknown" which yielded 0 points or "yes" which yielded 1 point. The overall score was derived by adding up each item score, each study could score between 0-18 points. Two reviewers (A. Cannavò and S. Hassan) evaluated each article independently and a third reviewer (J.G. van der Bom) was consulted in case of any discrepancy.

Data analysis

Statistical analysis

The total inhibitor incidence rate and high titre inhibitor incidence rate in PTPs was estimated for each study as the number of de novo inhibitors divided by the number of person-years on a given rFVIII product. Conventional random effects meta-analysis methods (such as the DerSimonian-Laird random-effects method) are biased when the outcome of interest is rare, also when continuity corrections are applied[15]. Therefore, we pooled the incidence rates of the individual studies and calculated the pooled incidence rate ratio (IRR) of inhibitor development according to product type using a random intercept Poisson regression model[16]. Heterogeneity was explored by estimating the between-study variance (^{2}) as well as visually assessing the extent to which the confidence intervals of the individual studies overlapped. As the most frequently used product, we used Advate as the reference category in the analysis according to product type.

Sensitivity analysis

To verify whether the results were robust to changes in methodology two sensitivity analyses were conducted. In the first sensitivity analysis, we restricted the main analysis to studies that only reported information for severe patients (baseline FVIII activity ‹ 0.01 IU/ml). In the second sensitivity analysis, we restricted the main analysis to large studies (i.e. studies with › 150 person-years of follow-up time).

Summary of findings

The main results of the product comparisons (including an overall quality assessment) are also summarized in a "summary of findings" table (table 3), according to the GRADE approach.[17]

Results

Included studies

A flowchart of the literature search is reported in figure 1 and the search terms are reported in supplemental figure S1 (see appendix). In total, 1605 articles were screened on their title and abstract. Eighty-two unique articles were reviewed in full, of these, 52 articles were excluded. Thirty articles[18-47] were selected for the analysis, four additional articles[48-51] were included after monthly searches on PubMed. Most articles reported on a single cohort of patients using one brand of rFVIII product, whereas three articles[23, 25, 26] provided information on multiple cohorts. Fischer et al[23] reported on five cohorts using different rFVIII products, Recht et al[26] reported on 2 cohorts with slightly different inclusion criteria and Hay et al[25] reported on three cohorts using different rFVIII products. In total, 34 articles reporting on 41 cohorts were included[18-51]. Characteristics of the 52 excluded papers are reported in supplemental table S1, references to the 52 excluded papers (labelled S1-S52) are also reported in supplemental table S1. Eighteen articles did not separately report inhibitor incidence and follow-up time for severe or moderately severe PTPs (but were otherwise eligible for inclusion). The corresponding authors were contacted but did not provide additional data. Consequently, these 18 articles were excluded from the meta-analysis. (supplemental table S1)

Study characteristics

Overall, 39 patients developed inhibitors during 19,157 person-years of observation. (table 1) One study did not provide information on the total number of patients[23], therefore, the overall number of patients included in this meta-analysis is unknown. Seven studies evaluated Advate (6043 person-years, 6 inhibitors), four studies evaluated Kogenate or Helixate (537 person-years, 5 inhibitors), ten studies evaluated Kogenate FS/Bayer or Helixate FS/NexGen (7386 person-years, 10 inhibitors), three studies evaluated Refacto (609 person-years, 7 inhibitors) and four studies (containing 5 cohorts) evaluated Refacto AF (3226 person-years, 10 inhibitors).

Furthermore, one study used GreenGene F (56 person-years, 1 inhibitor), three studies used Kovaltry/Iblias (165 person-years, 0 inhibitors), three studies used NovoEight (551 person-years, 0 inhibitors), three studies used Nuwiq (85 person-years, 0 inhibitors) and two studies evaluated Recombinate (499 person-years, 0 inhibitors). Because of the small sample sizes, studies evaluating GreenGene F, Kovaltry/Iblias, NovoEight, Nuwiq and Recombinate were only included when calculating the overall incidence rate but were excluded from product-specific analyses. In total, 12 studies were excluded (1356 person-years, 1 inhibitor).

Table 1. Study characteristics.

Advate						
Author	**Year**	**Study design**	**Country**	**Inclusion criteria**	**INH testing**	
Blanchette [33]	2008	Clinical trial	US, Europe	≤ 2%, EDs ≥ 50	3 months	
Den Uijl [36]	2009	Registry	The Netherlands	Any severity, EDs ≥ 50	12 months	
Valentino [38]	2012	Clinical trial	US, Europe	≤ 2%, EDs ≥ 150	3 months	
Fukutake [19]	2014	Surveillance	Japan	Any severity, EDs ≥ 4	Unknown	
Hay (cohort 2) [25*]	2015	Surveillance	UK	≤ 1%, 12 months of prior treatment	6 months	
Oldenburg [21**]	2010	Surveillance	US, Europe	Any severity, All previous EDs	routine detection	
Fischer (cohort 1) [23]	2015	Registry	Europe	‹1%, EDs› 50	routine detection	
Kogenate, Helixate						
Aygören-Pürsün [8]	1997	Clinical trial	Germany	‹ 15%, EDs› 100	3 months	
Seremetis[20]	1999	Clinical trial	US, Europe	‹5%, EDs › 50	Monthly (at beginning), every 6 months (at end)	
Yoshioka[30]	2006	Clinical trial	Japan	Any, EDs › 50	At months 0-3-6-9-12-18-24	
Singleton[32]	2007	Retrospective survey	Ireland	Any severity, All previous EDs	routine detection	
Kogenate FS/Bayer, Helixate FS/Nexgen						
Abshire[22]	2000	Clinical trial	North America, Europe	‹2%, EDs≥ 100	week 0-4-12-24, months 12-18-24	
Musso[34]	2008	Surveillance	Europe	‹2%, EDs› 0	routine detection	
Delumeau[35]	2008	Surveillance	Japan	Any severity, All previous EDs	routine detection	
Youn [18]	2009	Surveillance	Taiwan	Any severity, All previous EDs	routine detection	
Collins[37]	2010	Clinical trial	US, Europe	‹1%, EDs› 100	baseline and 13 months	
Manco-Johnson[41*]	2013	Clinical trial	Worldwide	‹2%, EDs≥ 150	0 and 3 months, 1, 2 and 3 years	
Lalezari [27*]	2014	Clinical trial	Worldwide	‹1%, EDs≥ 150	Week 1-2-3-7-12-26-38-52	
Gouider [45]	2015	Surveillance	Worldwide	‹4%, all previous EDs	routine detection	
Hay (cohort 3) [25*]	2015	Surveillance	UK	≤ 1%, 12 months of prior treatment	6 months	
Fischer (cohort 2) [23]	2015	Registry	Europe	‹1%, EDs› 50	routine detection	

	Sample size	Follow-up (person-years)	Follow-up (exposure days)	Inhibitors	Age
	53	56	8268	0/0	Mean 3.1 years (SD, 1.5)
	71	213	-	0/0	Median 25 years (range, 0.5-67)
	73	97	-	0/0	Median 26 years (range, 7-59)
	271	542	.	0/0	Median 24 years (range, 0-81)
	118	118	-	0/0	Switchers: mean 25 years (IQR 13-44) Non-switchers: mean 22 years (IQR 14-33)
	348	361	30972	1/0	29.9% ‹ 12 years 10.4% 12-16 years 59.3% ≥ 16 years
	-	4656	-	5/-	-
	22	22	1507	0/0	Median 27 years (range:2-62)
	54	254	12204	1/1	Median 25 years (range:1-72)
	74	121	7134	4/0	Mean 24 years (range:1-73)
	84	140	-	0/0	51.1%: › 18 years 11.7%: 13-18 years 37.2%: ≤ 12 years
	71	119	11867	0/0	NA: mean 22.6 years (SD: 10.2) EU: mean 32.6 years (SD: 13.3)
	181	352	33847	0/0	Mean 23.6 years (range:0.1-71)
	323	409	-	1/0	Mean 23.7 years (SD, 16.6)
	38	34	-	0/0	Mean 20.3 years (SD, 15.6)
	20	22	2231	0/0	Mean 36.4 years (SD, 3.5)
	84	143	11676	0/0	Median 30.6 years (range, 15-50)
	72	56	8834	0/0	Mean 34.4 years (range, 13-64)
	118	236	-	1/0	Mean 13.8 years (SD, 13.6)
	509	509	-	1/1	Switchers: mean 25 years (IQR 13-44) Non-switchers: mean 22 years (IQR 14-33)
		5506	-	7/-	-

Refacto						
Author	**Year**	**Study design**	**Country**	**Inclusion criteria**	**INH testing**	
Gringeri [24]	2004	Cohort study	Italy	‹1%, EDs≥ 50	3 months	
Pollmann [31]	2007	Surveillance	Germany, Austria	Any severity, All previous EDs	routine detection	
Fischer (cohort 3) [23]	2015	Registry	Europe	‹1%, EDs› 50	routine detection	
Refacto AF						
Recht (cohort 1) [26]	2009	Clinical trial	Worldwide	≤ 2%, EDs≥ 150	Months 0-1-3-6	
Recht (cohort 2) [26]	2009	Clinical trial	Worldwide	≤ 2%, EDs≥ 250	Months 0-1-3-6	
Lopez [43*]	2015	Clinical trial	Europe	‹1%, EDs› 150	At 1, 10-15, 50 EDs and then every 6 months	
Hay (cohort 1) [25*]	2015	Registry	UK	≤ 1%, EDs› 50 or 12 months of prior treatment	6 months	
Fischer (cohort 4) [23]	2015	Registry	Europe	‹1%, EDs› 50	routine detection	
GreenGene F						
Hyun [44]	2015	Clinical trial	Korea	≤ 2%, EDs› 150	3 months	
Kovaltry, Iblias						
Kavakli [46]	2015	Clinical trial	Worldwide	‹1%, EDs≥ 150	-	
Ljung [47]	2016	Clinical trial	Worldwide	‹1%, EDs≥ 50	Months 0-1-2-6	
Saxena [50]	2016	Clinical trial	Worlwide	‹1%, EDs≥ 150	-	
NovoEight						
Kulkarni [39]	2013	Clinical trial	Worldwide	≤ 1%, EDs› 50	At 6/8 study visits	
Lentz [40]	2013	Clinical trial	Worldwide	≤ 1%, EDs› 150	At 8/9 study visits	
Lentz [49]	2016	Clinical trial	Worldwide	≤ 1%, EDs› 50	Every 6 months	
Nuwiq						
Lissitchkov [42]	2015	Clinical trial	Europe	≤ 1%, EDs› 150	EDs 1, 2, 10–15, months 3 and 6.	
Tiede [48]	2016	Clinical trial	Europe	≤ 1%, EDs› 150	-	
Lissitchkov [51]	2017	Clinical trial	Europe	≤ 1%, EDs› 150	At baseline and study completion	

	Sample size	Follow-up (person-years)	Follow-up (exposure days)	Inhibitors	Age
	25	12.5	610	1/1	Median 31 years (range:6-60)
	188	387	55259	2/1	Mean 26.3 years (range:0-67)
	-	209	-	4/-	-
	94	62	6741	2/0	Median 24 years (range: 12-60)
	110	48	6860	1/0	Median 19 years (range: 7-70)
	208	207	19552	0/0	Mean 30.5 years (SD:13)
	571	571	-	4/1	Switchers: mean 25 years (IQR 13-44) Non-switchers: mean 22 years (IQR 14-33)
	-	2338	-	3/-	-
	70	56	6397	1/-	Mean 31.9 years (SD, 9.6)
	79	79	-	0/0	Median 28.5 years (range: 14-59)
	50	25	3650	0/0	Mean 6.4 years (SD, 3.0)
	61	61	-	0/0	Mean 31.5 years (SD, 12.7)
	63	24	3780	0/0	Mean 6.1 years (SD, 2.9)
	150	75	12750	0/0	Mean 28 years (SD, 11.8)
	199	452	72320	0/0	
	32	16	2723	0/0	Mean 37.3 years (SD, 13.6)
	22	20	1030	0/0	Mean 39.6 years (SD, 14.1)
	66	49	6612	0/0	Mean 33.6 years (SD, 9.89)

Recombinate						
Author		**Study design**	**Country**	**Inclusion criteria**	**INH testing**	
White [29]		Clinical trial	Worldwide	≤ 5%, EDs›200	-	
Fischer (cohort 5) [23]		Registry	Europe	‹1%, EDs› 50	routine detection	

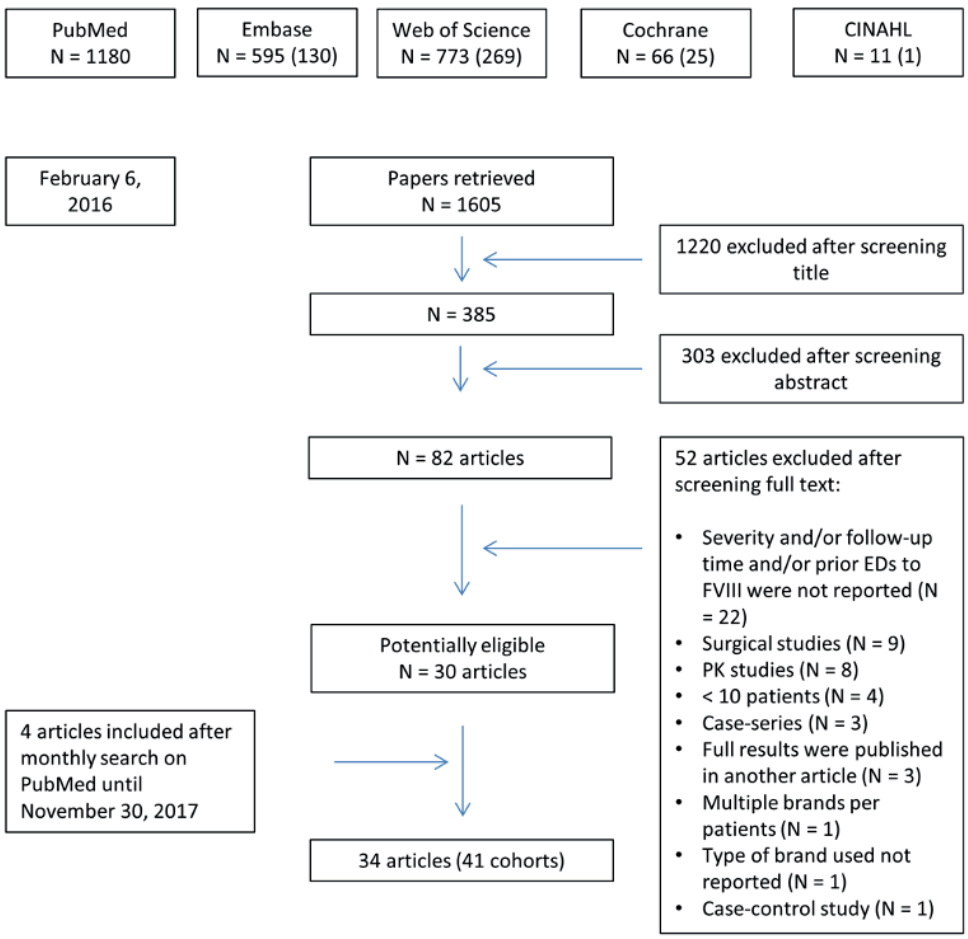
* Possible overlap with EUHASS registry.[21]

** Patient recruitment period not reported, unclear if there is any overlap with EUHASS registry.[21]

	Sample size	Follow-up (person-years)	Follow-up (exposure days)	All Inhibitors / High-titre inhibitors	Age
	67	248	-	0/0	33% › 18 years 67% ≥ 18 years
		251	-	0/-	-

Figure 1. Flowchart of the search strategy (number of unique reports are indicated in parentheses).

The search was run on February 6, 2016. Two additional studies were included by performing monthly searches on Pubmed until November 30, 2017.



We found similar methodological quality across studies with the modified Downs and Black checklist (median score: 11, range: 6-16), except for two studies with a high risk of bias which were published as a letter to the editor[18] (score: 6) and a conference poster[19] (score: 8). (supplemental table S2) The majority of studies were similar in quality, therefore, we did not perform a sensitivity analysis based on methodological quality.

Risk of inhibitor formation according to recombinant rFVIII product

Overall incidence rate and incidence rate per rFVIII product

The overall inhibitor incidence rate among previously treated patients was 2.06 per 1000 person-years with a 95% confidence interval (CI95) of 1.06-4.01). The incidence rate of inhibitor formation was 0.99 (CI95: 0.37-2.70) per 1000 person-years for Advate, 5.86 (CI95: 0.25-134.92) per 1000 person-years for Kogenate/Helixate, 1.35 (CI95: 0.66-2.77) per 1000 person-years for Kogenate FS/Helixate NexGen, 12.05 (CI95: 1.53-94.78) per 1000 person-years for Refacto and 4.64 (CI95: 0.82-26.43) per 1000 person-years for Refacto AF (figure 2).

Inhibitor formation by product

Compared with Advate, the pooled incidence rate ratio (IRR) was 9.77 (95%CI: 1.97-48.41) for Kogenate/Helixate, 1.51 (95%CI: 0.34-6.69) for Kogenate FS/Helixate NexGen, 14.40 (95%CI: 2.84-72.94) for Refacto and 4.81 (95%CI: 0.99-23.34) for Refacto AF. (table 2). Compared with full-length rFVIII, the pooled IRR for B-domain-deleted rFVIII was 4.80 (CI95: 1.32-17.40). Compared to rFVIII products derived from Chinese hamster ovary (CHO) cells, the pooled IRR was 0.62 (CI95: 0.17-2.34) for rFVIII products derived from baby hamster kidney (BHK) cells. Compared to second generation rFVIII products, the pooled IRR was 2.54 (CI95: 0.45-14.27) for first generation rFVIII products and 0.75 (CI95: 0.21-2.66) for third-generation rFVIII products. (table 2)

Sensitivity analysis

The sensitivity analyses showed that the results for each rFVIII brand varied significantly with changes to methodology. (supplemental table S4 and S5) However, this can be partly explained by the low number of studies per brand. Furthermore, the results of the sensitivity analyses were roughly in line with the results of the main analysis with regards to the overall incidence rate and when rFVIII products were analysed according to length, cell line and generation. Nevertheless, this shows that that the most important results of the main analysis are not very robust to changes in methodology. (supplemental table S4 and S5)

Discussion

This meta-analysis comprehensively reviews published reports of rFVIII products in relation to immunogenicity among previously treated patients with haemophilia. In total, 34 studies reporting on 41 cohorts were included with 39 inhibitor events and

19,157 person-years of observation. The incidence rate among PTPs was 2.06 per 1000 person-years (CI95: 1.06-4.01).

Formal comparisons of products yielded a statistically significant higher incidence of inhibitors among patients using Kogenate/Helixate and Refacto when compared with Advate, but not Kogenate FS/Helixate NexGen or Refacto AF. Taken as a whole, B-domain deleted rFVIII products were associated with an increased risk of inhibitor formation when compared to full-length rFVIII products. However, the overall quality of evidence was low, mainly due to the high risk of bias and confounding, lack of power to detect an effect in most studies (given the rare outcome) and the lack of consistency among studies evaluating the same rFVIII product. Therefore, the aforementioned results have to be interpreted with caution (Table 3).

Comparison with previous reviews

The overall incidence of inhibitors in PTPs in our study corroborates earlier findings[8, 52-55]. Recently, two previous systematic reviews have evaluated the association between rFVIII product type and inhibitor formation in PTPs[7, 10].

In 2011, the first of the two meta-analyses was published, its focus was mainly on the risk of inhibitor formation with B-domain deleted rFVIII products compared to full-length rFVIII products[10]. This meta-analysis included prospective studies of patients who were treated for more than 50 exposure days at baseline. A mixed effects Cox proportional hazards model with study as a random effect was used to pool and compare studies. Due to incomplete reporting, individual follow-up time was estimated for most non-inhibitor patients. Fourteen out of 29 studies in the previous meta-analysis were also included in our current meta-analysis. The following 9 studies were included in the previous meta-analysis but excluded from the current meta-analysis; 3 surgical studies [S7, S27, S28], 1 case-series [S2], 2 studies that did not adequately report prior exposure to FVIII [S49, S51] and 3 studies that did not adequately report follow-up time [S39, S41, S46] (see supplemental table S1 for references of excluded studies). Similar to our study, this meta-analysis found a statistically significantly higher risk of inhibitor formation in previously treated patients using B-domain deleted rFVIII, compared to previously treated patients using full-length rFVIII (HR: 7.26, CI95: 2.12–24.9).

A more recent meta-analysis from 2013 did not report any differences in immunogenicity[7]. Thirteen out of 33 studies in this previous meta-analysis were also included in the current meta-analysis. The following 11 studies were included in the previous

Figure 2. Incidence rates of inhibitor development per study.

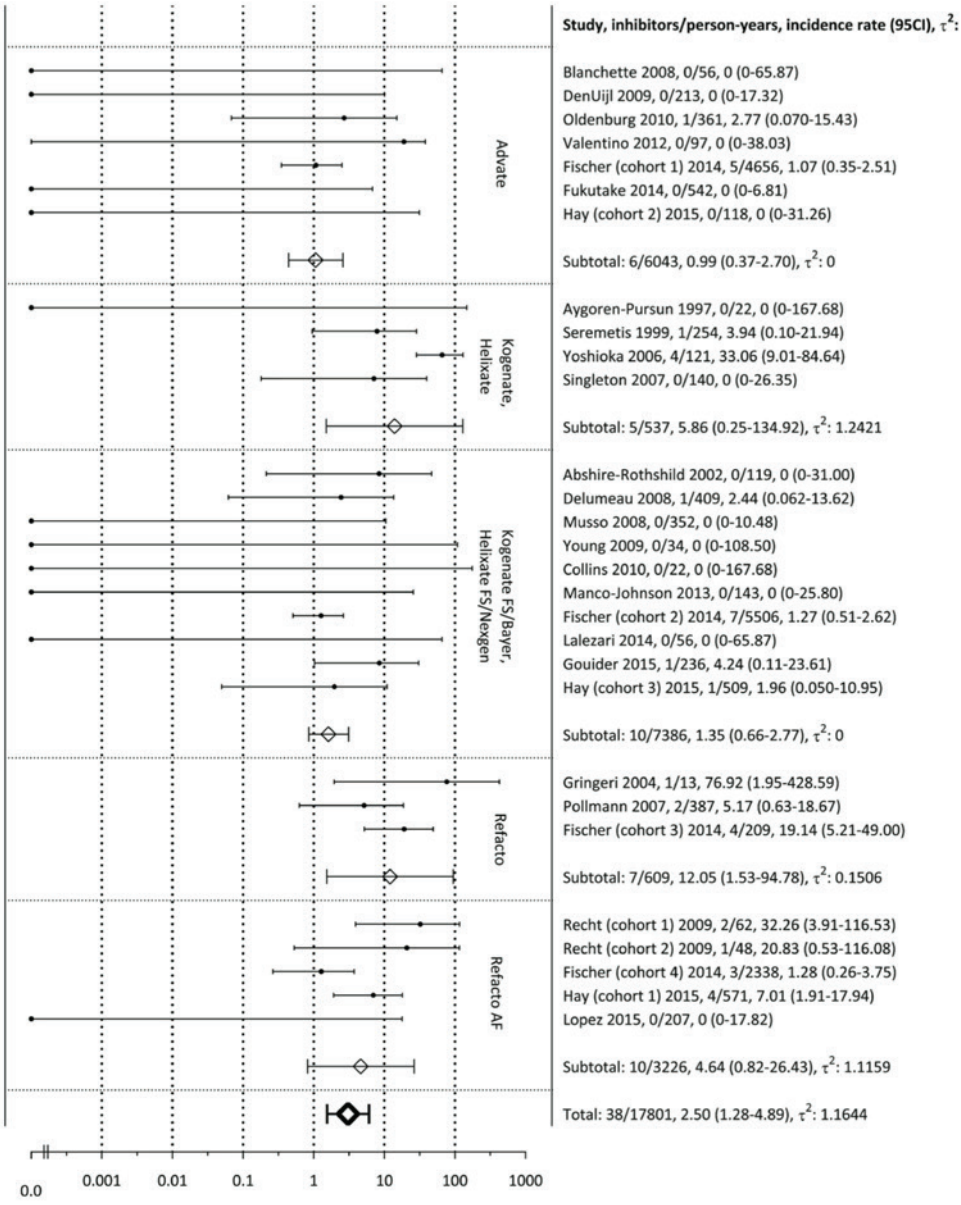


Table 2. Pooled incidence rates and incidence rate ratios of inhibitor development by product type.

Variable	N	Inhibitors/ p-y	Pooled inhibitor incidence rate per 1000 p-y (CI95)	between-study variance (^{2})	Incidence rate ratio (CI95)
Overall (main products only):	29	38/17801	2.50 (CI95: 1.28-4.89)	1.1644	
Product					
Advate	7	6/6043	0.99 (CI95: 0.37-2.70)	0	Ref
Kogenate/Helixate	4	5/537	5.86 (CI95: 0.25-134.92)	1.2421	9.77 (CI95: 1.97-48.41)
Kogenate FS/Helixate NexGen	10	10/7386	1.35 (CI95: 0.66-2.77)	0	1.51 (CI95: 0.34-6.69)
Refacto	3	7/609	12.05 (CI95: 1.53-94.78)	0.1506	14.40 (CI95: 2.84-72.94)
Refacto AF	5	10/3226	4.64 (CI95: 0.82-26.43)	1.1159	4.81 (CI95: 0.99-23.34)
rFVIII length[1]					
Full-length rFVIII	21	21/13966	1.46 (CI95: 0.59-3.59)	0.8967	Ref
B-domain deleted rFVIII	8	17/3835	6.93 (CI95: 2.28-21.08)	0.9980	4.80 (CI95: 1.32-17.40)
Cell line[2]					
CHO-cells	15	23/9878	3.01 (CI95: 1.20-7.54)	1.3115	Ref
BHK-cells	14	15/7923	1.96 (CI95: 0.63-6.15)	1.0564	0.62 (CI95: 0.17-2.34)
rFVIII generation[3]					
Second-generation rFVIII	13	17/7995	2.66 (CI95: 1.06-6.66)	0.7128	Ref
First-generation rFVIII	4	5/537	5.86 (CI95: 0.25-134.92)	1.2421	2.54 (CI95: 0.45-14.27)
Third-generation rFVIII	12	16/9269	1.95 (CI95: 0.70-5.40)	0.9157	0.75 (CI95: 0.21-2.66)

[1] Full-length rFVIII (Kogenate/Helixate, Kogenate FS/Helixate NexGen and Advate) is compared with B-domain deleted rFVIII (Refacto and Refacto AF).

[2] rFVIII derived from CHO-cells (Refacto, Refacto AF and Advate) is compared with rFVIII derived from BHK-cells (Kogenate/Helixate and Kogenate FS/Helixate NexGen).

[3] First Generation rFVIII (Kogenate/Helixate) is compared with second generation rFVIII (Refacto and Kogenate FS/Helixate NexGen) and third generation rFVIII (Advate and Refacto AF).

meta-analysis but excluded from the current meta-analysis; 3 surgical studies [S7, S27, S28], 3 studies that did not report haemophilia severity and/or prior EDs to FVIII [S43, S49, S9], 4 studies that did not report follow-up time [S40, S41, S46, S47] and 1 study in which the type of FVIII brand used was not specified [S10] (see supplemental table S1 for references of excluded studies). The method of Laird and Mosteller was used to pool study results. Crude proportions of inhibitor development for each FVIII product were indirectly compared by evaluating whether statistically significant between-groups heterogeneity existed according to the Cochran's Q statistic. The crude proportion of inhibitor development was 1.0% (CI95: 0.5%-1.8%) for Advate, 2.6% (CI95: 1.6%-4.4%) for Kogenate (first generation) and 1.9% (CI95: 1.1%-3.4%) for Refacto (first generation).

No statistically significant Q-statistic was found based on the type of FVIII concentrate (Q statistic = 6.854, P = 0.077), this was confirmed by a univariate meta-regression analysis (these results were not shown). Cochran's Q, however, is not a sensitive tool for assessing heterogeneity as it has low power to detect heterogeneity if the event rate is very low[56], and hence this meta-analysis at most indicated the absence of gross differences by product.

In this meta-analysis Kogenate/Helixate and Kogenate FS/Helixate NexGen were categorized and analysed as one product group, complicating comparisons between individual rFVIII products. Further, only information on the cumulative incidence of inhibitor formation (i.e., the numbers of events per persons) per product was provided without correcting for study follow-up time. It is mentioned in the article that "similar results were obtained when the incidence rate was calculated as events per person-years" (however, these data were not shown). As development of inhibitors to FVIII is dependent on exposure to FVIII and therefore follow-up time, the reporting of incidence rates is preferred over proportions of inhibitor patients. In addition, conventional data pooling methods (such as the one used in the aforementioned meta-analysis) are based on large sample approximations which produce biased estimates when applied to studies with very low event rates[56], which is the case in inhibitor development in PTPs.

Study strengths and limitations

Study strengths

The last review included studies up to January 2013. Of the 41 cohorts included in this analysis, 14 cohorts were published after this date.

In contrast to previous reviews, the inhibitor incidence rate was the main study outcome. This was preferred over the cumulative inhibitor incidence as the main outcome because the study duration was not identical across studies and over the hazard rate as the main outcome because most studies did not report the follow-up time of non-inhibitor patients. Unlike earlier reviews, we also directly compared the pooled inhibitor incidence rates of all major rFVIII products with each other.

Standard meta-analysis methods (e.g. the DerSimonian-Laird random effects method) can give biased results when applied inappropriately. Firstly, the effect estimate and standard error of each study are usually correlated. Secondly, pooling studies with zero events leads to computational errors, this is often avoided by applying a continuity correction. Lastly, the within-study distribution of the effect estimate is assumed to be normal, this assumption is often violated when the event rate is very rare. The meta-analysis model used in this review, a random intercept Poisson regression model, avoids the aforementioned problems[16].

Table 3. Summary of findings.

Main recombinant FVIII products compared to Advate in previously treated patients with severe haemophilia A					
Intervention: Kogenate/Helixate					
Outcomes	**Absolute effects*** (95% CI)		Relative effect (95% CI)	Nº of person-years (studies)	Certainty of the evidence (GRADE)
	Risk with Advate	**Risk with Kogenate/ Helixate**			
Inhibitor incidence assessed with: Bethesda assay	0.99 per 1,000	**5.86 per 1,000** (0.25 to 134.92)	**RR 9.77** (1.97 to 48.41)	6580 (11 non-comparative observational studies)	⨁◯◯◯ VERY LOW
Intervention: Kogenate FS/Helixate NexGen					
Outcomes	**Absolute effects*** (95% CI)		Relative effect (95% CI)	Nº of person-years (studies)	Certainty of the evidence (GRADE)
	Risk with Advate	**Risk with Kogenate FS/Helixate NexGen**			
Inhibitor incidence assessed with: Bethesda assay	0.99 per 1,000	**1.35 per 1,000** (0.66 to 2.77)	**RR 1.51** (0.34 to 6.69)	13429 (17 non-comparative observational studies)	⨁◯◯◯ VERY LOW

Intervention: Refacto					
Outcomes	**Absolute effects*** (95% CI)		Relative effect (95% CI)	Nº of person-years (studies)	Certainty of the evidence (GRADE)
	Risk with Advate	**Risk with Refacto**			
Inhibitor incidence assessed with: Bethesda assay	0.99 per 1,000	**12.05 per 1,000** (1.53 to 94.78)	**RR 14.40** (2.84 to 72.94)	6652 (10 non-comparative observational studies)	⨁◯◯◯ VERY LOW
Intervention: Refacto AF					
Outcomes	**Absolute effects*** (95% CI)		Relative effect (95% CI)	Nº of person-years (studies)	Certainty of the evidence (GRADE)
	Risk with Advate	**Risk with Refacto AF**			
Inhibitor incidence assessed with: Bethesda assay	0.99 per 1,000	**4.64 per 1,000** (0.82 to 26.43)	**RR 4.81** (0.99 to 23.34)	9269 (12 non-comparative observational studies)	⨁◯◯◯ VERY LOW

* **The risk in the intervention group** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

CI: Confidence interval; **RR:** Risk ratio

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Limitations - Random variation

The pooled results have to be interpreted with caution due to the low number of inhibitors within each product type, which give rise to significant random variation as indicated by the broad confidence intervals. Furthermore, haemophilia severity, follow-up time and the prior number of exposure days to FVIII were not accurately reported in several studies (supplemental table S1), these studies were excluded (after attempts to retrieve this information by contacting the corresponding authors). Due to the low event rate overall, the absence of these studies in the meta-analysis may have significantly impacted our results.

Limitations - Confounding

As no comparative studies were found, we could only compare single-arm trials in our analysis of inhibitor formation by product type. Due to differences in the distri-

bution of genetic/treatment-related risk factors, comparing single-arm trials may be misleading.

Many studies also included moderately severe patients (the exact proportion varied per study). If moderately severe patients are at a significantly lower risk of inhibitor formation, then this could have confounded our results.

Compared to on-demand treatment, patients on prophylactic treatment are exposed to more units of FVIII over a given time period and are therefore at a higher risk of inhibitor formation. Correcting for this problem by using exposure days to FVIII instead of person-years as the unit of time in the main analysis was not feasible due to the low number of studies that accurately reported the total number of exposure days to FVIII.

Adjustment for other potential confounders such as F8 genotype, ethnicity, family history and surgery was not possible due to incomplete reporting (supplemental table S3). Overall, there is a moderate chance of confounding, mainly due to variables that may have influenced the physician's choice of rFVIII product (F8 genotype, family history of inhibitors).

Limitations - Bias
The cut-off level and screening frequency of the inhibitor assays, which could have influenced the reported number of low-titre inhibitors, varied across studies. This could have introduced misclassification bias and consequently over- or underestimation of inhibitor incidences. Patients in market approval studies undergo more intensive screening for inhibitors. (Transient) low-titre inhibitors that were not detected before the study or at study baseline may be detected after inclusion. Due to this, newer products for which data is mainly available from market approval studies may seem more immunogenic than older products which have also been evaluated in post-approval studies.

Over time, the screening intensity has increased, possibly leading to an increased detection of low-titre inhibitors in newer studies. However, screening intensity was slightly higher among older products (Kogenate/Helixate and Refacto) when compared to newer products (Kogenate FS/Helixate NexGen and Refacto AF). (table 1) This observation is in line with our results, as Kogenate/Helixate and Refacto were also the most immunogenic products in our analysis. Correcting for this problem by only analysing high-titre inhibitors was not feasible due to the very low number of high-titre inhibitors overall.

In addition, there could have been some overlap between 5 studies (that evaluated Advate, Kogenate FS/Helixate NexGen or Refacto AF) and the EUHASS registry[23] (table 1) Double counting could have led to over- or underestimating inhibitor incidences and producing overly narrow confidence intervals. Because Advate was used as the reference product, reported incidence rate ratios for all product types would also be biased. Overall, double counting could have influenced the main results.

Many patients were treated with a different FVIII product before study inclusion (especially in market approval trials). Consequently, increased immunogenicity due to product switching could have biased the results. However, there have been several national product switches and there was no evidence of increased immunogenicity.[57]

Biological explanation of a causal effect

Several differences between rFVIII products could explain the reported results. Second- and third generation full length rFVIII products vary slightly in their FVIII amino acid sequence. Furthermore, differences in product formulation such as culture conditions and stabilizing agents could also be relevant. Lastly, the type of cell culture used for production such as CHO cells, BHK cells or, more recently HEK 293 cells, leads to rFVIII products with different post-translational modifications that may influence immunogenic potential[11].

Implications of these results for future research

Comparing single-arm trials may be misleading due to bias and confounding. Single-arm trials are useful for identifying extremely immunogenic products but less suitable for detecting smaller effects (e.g. the difference in inhibitor risk found in the studies by Peyvandi et al[2] or Gouw et al[58]). Nevertheless, these studies could be used more effectively if a standardized data reporting system was used. This system should include all relevant variables such as known genetic/treatment-related confounders.[59] Lastly, future research should focus on using study designs that are appropriate for evaluating rare outcomes (i.e. case control studies).

Conclusion

These results suggest that some products may be associated with increased immunogenicity. However, these findings should be interpreted with caution, both the low incidence of inhibitors in PTPs and the differences in study design may cause significant variation in estimates of risk.

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Supplemental Table S1. List of excluded papers (including references).

First author	Year	Reason for exclusion	Product	Patients	Inhibitors
Sennet [S1]	2004	Case series	Refacto	2	0
Keeling [S2]	2006	Case series	Refacto	3	3
Ishaku [S3]	2015	Case-control study	-	48	3
Kocher [S4]	2012	Multiple brands per patient	rFVIII and pdFVIII	119	0
Von Auer [S5]	2005	Case series	-	10	10
Giles [S6]	1998	Severity and prior exposure to FVIII were not reported	rFVIII	-	-
Auerswald [S7]	2013	Surgery	rFVIII and pdFVIII	29	0
Batorova [S8]	2012	Surgery, cross-sectional study	-	742	9
Siegmund [S9]	2010	Prior exposure to FVIII was not reported	rFVIII and pdFVIII	118	0
Aznar [S10]	2014	Type of brand used not accurately reported	rFVIII and pdFVIII	97	9
Xuan [S11]	2014	Prior exposure to FVIII and follow-up were not reported.	-	926	40
Martinowitz [S12]	2011	Study on pharmacokinetics	Novoeight, Advate	23	-
Lambert [S13]	2007	Study on pharmacokinetics	Refacto	14	-
Di Paola [S14]	2007	Study on pharmacokinetics	Refacto, Advate	18	0
Kelly [S15]	1997	Study on pharmacokinetics	rFVIII	10	-
Barnes [S16]	2006	Study on pharmacokinetics	Kogenate-FS	20	-
Kessler [S17]	2005	Study on pharmacokinetics	BDD rFVIII, pdFVIII	18	-
Mulcahy [S18]	2005	Only treatment during surgery and/or severe bleeding	rFVIII	12	2
Mannucci [S19]	1994	Prior exposure to FVIII was not reported	Kogenate	51	0
Lalezari [S20]	2013	Follow-up and inhibitor information not reported	Kogenate FS	68	-
Shah [S21]	2015	Study on pharmacokinetics	Kovaltry	45	-
Tuddenham [S22]	2010	Early findings of a study, results of full study were published later	-	-	-
Oldenburg [S23]	1995	Prior exposure to FVIII, severity and follow-up were not reported	Kogenate, Recombinate	112	-
Ewenstein [S24]	2004	Prior exposure to FVIII and follow-up were not accurately reported	Recombinate/ Bioclate	-	-
Lusher [S25]	2005	Follow-up was not reported	Refacto	218	33
Jiménez-Yuste [S26]	2015	Study on pharmacokinetics	NovoEight	76	0

Windyga [S27]	2010	Surgery	Refacto AF	30	1
Négrier [S28]	2008	Surgery	Advate	58	0
Meijer [S29]	2015	Surgery	Kogenate FS	25	0
Santagostino [S30]	2015	Surgery	Novoeight	33	0
Scharrer [S31]	2000	Surgery	Kogenate FS	15	1
Martinowitz [S32]	2009	Surgery	Kogenate FS	14	0
Shirahata [S33]	2000	< 10 patients	Kogenate FS	5	0
Zanon [S34]	1999	< 10 patients treated with rFVIII products	rFVIII and pdFVIII	62	7
Fukui [S35]	1991	< 10 patients	Kogenate	5	0
Prezotti [S36]	2015	Follow-up and prior exposure to FVIII were not reported	Advate	346	5
Chen [S37]	2012	Prior exposure to FVIII was not reported	Advate	40	0
Rubinger [S38]	2008	Prior exposure to FVIII was not reported	Kogenate-FS	274	0
Roussel-Robert [S39]	2003	Follow-up was not reported	Refacto	70	4
Tarantino [S40]	2004	Follow-up was not reported	Advate	108	1
Shi [S41]	2007	Follow-up was not reported	Kogenate-FS	49	0
Rea [S42]	2009	Prior exposure to FVIII was not reported	Refacto	33	1
Bacon [S43]	2011	Prior exposure to FVIII was not reported	Advate	96	1
Zhang [S44]	2011	Prior exposure to FVIII was not reported	Advate	58	1
Chang [S45]	2015	< 10 patients	Refacto AF	8	4
Smith [S46]	2005	Follow-up was not reported	Refacto	60	3
Vidovic [S47]	2010	Follow-up was not reported	Kogenate-FS	306	0
Pollmann [S48]	2013	Subanalysis of earlier report (duplicate data)	-	-	-
Schwartz [S49]	1990	Prior exposure to FVIII was not reported accurately, long-term results are published in later report	Kogenate	107	8
Rothschild [S50]	2002	Subanalysis of earlier report (duplicate data)	-	-	-
Petrini [S51]	2009	Prior exposure to FVIII was not reported	Refacto	57	0
Klukowska [S52]	2015	Follow-up not reported	Nuwiq	59	0

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Supplemental Table S2. Quality assessment score. The methodological quality of each article was assessed using a modified Downs and Black checklist.

Study	REPORTING	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8
		Is the aim clearly described?	Are the main outcomes clearly described in the methods?	Are the characteristic of the patients clearly described?	Are the interventions of interest clearly described?	Are the main study findings clearly described?	Does the study provide estimates of the random variability in the data for the main outcomes?	Have all important adverse events that may be a consequence of the intervention been reported?	Have the characteristics of patients lost to follow-up been described?
Abshire [22]		*	*	*	*	*		*	
Aygören-Pürsün [28]		*	*	*	*	*		*	*
Blanchette [33]		*	*	*	*	*		*	*
Collins [37]		*	*	*	*	*		*	*
Delumeau [35]		*	*	*	*	*		*	
Den Uijl [36]		*	*	*	*	*		*	
Fischer [23]		*	*	*	*	*	*		*
Fukutake [19]		*	*	*		*	*		
Gouider [45]		*	*	*	*	*		*	*
Gringeri [24]		*	*	*	*	*			
Hay [25]		*	*	*	*	*	*	*	
Hyun [44]		*	*	*	*	*		*	*
Kavakli [46]		*	*	*	*	*		*	*
Kulkarni [39]		*	*	*	*	*		*	*
Lalezari [27]		*	*	*	*	*		*	
Lentz [40]		*	*	*	*	*	*	*	*
Lentz [49]		*	*		*	*		*	

	EXTERNAL VALIDITY				INTERNAL VALIDITY						POWER		
	Q9	Q10	Q11		Q12	Q13	Q14	Q15	Q16	Q17		Q18	
	Were the subjects asked to participate representative of the entire population?	Were those subjects who were prepared to participate representative of the entire population from which they were recruited ?	Were the staff, places and facilities representative of the treatment the majority of patients receive?		If any of the results of the study were based on *data dredging*, was this made clear?	Do the analyses adjust for different lengths of folluw-up of patients?	Were the statistical tests used to assess the main outcomes appropriate?	Was compliance with the interventions reliable?	Were the main outcome measures used accurate (valid and reliable)?	Were losses of patients to follow-up taken into account?		Did the study have sufficient power? (>100 person-years)	SCORE
	*		*		*				*			*	11
	*		*		*				*				11
	*		*		*				*	*			12
	*		*		*				*				11
			*		*				*			*	10
	*		*		*			*	*	*		*	13
	*	*	*		*	*	*		*	*		*	16
	*		*									*	8
	*		*		*			*	*			*	13
	*		*		*				*				9
	*		*		*	*	*	*	*			*	15
			*		*			*	*				10
	*		*		*			*	*				12
	*		*		*	*	*	*	*	*			15
	*		*		*			*	*				11
	*		*		*			*	*	*		*	15
	*		*		*			*	*			*	11

Study	REPORTING	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8
		Is the aim clearly described?	Are the main outcomes clearly described in the methods?	Are the characteristic of the patients clearly described?	Are the interventions of interest clearly described?	Are the main study findings clearly described?	Does the study provide estimates of the random variability in the data for the main outcomes?	Have all important adverse events that may be a consequence of the intervention been reported?	Have the characteristics of patients lost to follow-up been described?
Lissitchkov (2015) [42]		*	*	*	*	*		*	*
Lissitchkov (2017) [51]		*	*	*	*	*		*	*
Ljung [47]		*	*	*	*	*		*	
Lopez [43]		*	*	*	*	*		*	
Manco-Johnson [41]		*	*	*	*	*		*	*
Musso [34]		*	*	*	*	*		*	
Oldenburg [21]		*	*	*	*	*	*	*	*
Pollmann [31]		*	*	*	*	*		*	
Recht [26]		*	*	*	*	*		*	
Saxena [50]		*	*	*	*	*		*	*
Seremetis [20]		*	*	*	*	*		*	
Singleton [32]		*	*	*	*	*			
Tiede [48]		*	*	*	*	*		*	*
Valentino [38]		*	*	*	*	*		*	*
White [29]		*	*	*	*	*		*	
Yoshioka [30]		*	*	*	*	*		*	
Young [18]		*	*	*					

	EXTERNAL VALIDITY				INTERNAL VALIDITY							POWER		
	Q9	Q10	Q11		Q12	Q13	Q14	Q15	Q16	Q17		Q18	
	Were the subjects asked to participate representative of the entire population?	Were those subjects who were prepared to participate representative of the entire population from which they were recruited ?	Were the staff, places and facilities representative of the treatment the majority of patients receive?		If any of the results of the study were based on *data dredging*, was this made clear?	Do the analyses adjust for different lengths of folluw-up of patients?	Were the statistical tests used to assess the main outcomes appropriate?	Was compliance with the interventions reliable?	Were the main outcome measures used accurate (valid and reliable)?	Were losses of patients to follow-up taken into account?		Did the study have sufficient power? (>100 person-years)	SCORE
	*		*		*				*				11
	*		*		*			*	*				12
	*		*		*			*	*				10
	*		*		*				*			*	11
	*		*		*			*	*			*	13
	*		*		*				*			*	11
	*		*		*			*	*			*	14
	*		*		*			*	*			*	12
	*		*		*				*			*	11
	*		*		*			*	*				12
	*		*		*				*			*	11
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Supplemental table S3. information on the distribution of potential confounders in the studies that were included in the main analysis.

Author	Family history of inhibitors	Treatment type (prophylaxis/on-demand)
Advate		
Blanchette [33]	-	90.6% prophylaxis 3.8% on-demand 5.7% on-demand/prophylaxis
Den Uijl [36]	-	65.9% prophylaxis, 34.1% on-demand
Valentino [38]	-	Patients were treated with on-demand regimen during the first 6 months, and then with a prophylactic regimen for the following 6 months
Fukutake [19]	-	53.4% Prophylaxis 30.7% On-demand 15.9% Mixed
Hay (cohort 2) [25]	-	-
Oldenburg [21]	-	57.0% Prophylaxis 43.0% On-demand
Fischer (cohort 1) [23]	-	-
Kogenate, Helixate		
Aygören-Pürsün [28]	-	-
Seremetis [20]	-	-
Yoshioka [30]	-	-
Singleton [32]	-	52.1% Prophylaxis 38.3% On-demand 9.6 % Unknown
Kogenate FS/Bayer, Helixate FS/NexGen		
Abshire [22]	-	Prophylaxis: 43.8% N. America, 60.7% EU On-demand: 12.1% N. America, 12.9% EU
Musso [34]	-	31.8% Prophylaxis

	F8 genotype	Ethnicity	Surgery during follow-up
	40% intron 22 inversion 27% missense mutation 13% nonsense mutation 11% frameshift mutation 5% deletion 2% intron 1 inversion 2% splice defect	90.6% Caucasian 5.6% African-American 3.8% Unspecified	5 patients underwent a surgical procedure
	-	-	27 surgical procedures
	-	87.7% White 5.5% Hispanic 4.1% Black/African-American 1.4% Asian 1.4% Other	-
	-	100% Asian	-
	-	-	-
	-	90.8% Caucasian 3.3% Black 1.5% Asian 3.6% Other	16 surgical procedures (15 patients)
	-	-	-
	-	-	-
	-	-	25 surgical procedures (22 patients)
	-	100% Asian	10 patients underwent at least one surgical procedure (not included in analysis)
	-	-	-
	-	-	22 surgical procedures (15 patients)
	-	81.8% White 1.4% Black 0.9% Asian 3.6% other	46 surgical procedures (37 patients)

Author	Family history of inhibitors	Treatment type (prophylaxis/on-demand)
Delumeau [35]	-	17.6% prophylaxis
Young [18]	-	12.9% regular prophylaxis 87.1% Other
Collins [37]	-	Patients were treated on-demand for 6 months, followed by 7 months prophylaxis
Manco-Johnson [41]	-	50% prophylaxis 50% on-demand
Lalezari [27]	-	100% prophylaxis
Gouider [45]	-	60.2% prophylaxis
Hay (cohort 3) [25]	-	-
Fischer (cohort 2) [23]	-	-
Refacto		
Gringeri [24]	-	100% on demand
Pollmann [31]	-	81 patients treated on prophylaxis for at least one treatment-year 39 patients treated on-demand for at least one treatment-year
Fischer (cohort 3) [23]	-	-
Refacto AF		
Recht (cohort 1) [26]	-	100% prophylaxis
Recht (cohort 2) [26]	-	100% prophylaxis
Lopez [43]	-	74% prophylaxis, 25% on-demand, 1% Other
Hay (cohort 1) [25]	-	-
Fischer (cohort 4) [23]	-	-

	F8 genotype	Ethnicity	Surgery during follow-up
	-	100% Asian	-
	-	98.6% Asian 1.4% Caucasian	-
	-	95% White 5% Hispanic	-
	-	90.5% White 2.4% Asian 7.1% Hispanic	-
	-	-	-
	-	81.2% Caucasian 2.2% Black 5.4% Asian 5.9% Other	18 surgical procedures (15 patients)
	-	-	-
	-	-	-
	-	-	-
	47.0% Intron 22 inversion 16.7% Missense mutation 10.8% Small deletion or insertion	100% Caucasian	-
	-	-	-
	-	94.7% White 5.3% Other	Surgery during study was not permitted
	-	86.4% White 13.6% Other	9 patients underwent at least one surgical procedure
	-	96.6% White 1.0% Asian 0.5% Black 1.9% Other	-
	-	-	-
	-	-	-

Supplemental Table S4. Sensitivity analysis. Main analysis restricted to studies that only reported information for severe patients (baseline FVIII activity <0.01 IU/ml).

Variable	N	Inhibitors/ p-y	Pooled inhibitor incidence rate per 1000 p-y (CI95)	Between-study variance (τ^2)	Incidence rate ratio (CI95)
Overall (main products only)	20	30/16181	1.88 (CI95: 0.72-4.92)	1.6120	
Product					
Advate	4	5/5529	0.62 (CI95: 0.11-3.46)	0	Ref
Kogenate/Helixate	3	4/283	6.34 (CI95: 0.01-7819.34)	1.8502	15.63 (CI95: 3.84-63.63)
Kogenate FS/Helixate NexGen	8	9/7031	1.28 (CI95: 0.58-2.82)	0	1.42 (CI95: 0.44-4.55)
Refacto*	2	5/222	-	-	-
Refacto AF	3	7/3116	2.30 (CI95: 0.19-28.48)	0.4149	2.48 (CI95: 0.73-8.45)
rFVIII length[1]					
Full-length rFVIII	15	18/12843	1.14 (CI95: 0.30-4.33)	1.4962	Ref
B-domain deleted rFVIII	5	12/3338	5.19 (CI95: 0.85-31.77)	1.3310	4.49 (CI95: 0.70-28.58)
Cell line[2]					
CHO-cells	9	17/8867	2.13 (CI95: 0.52-8.67)	1.7264	Ref
BHK-cells	11	13/7314	1.62 (CI95: 0.32-8.09)	1.5944	0.74 (CI95: 0.12-4.53)
rFVIII generation[3]					
Second-generation rFVIII	10	14/7253	2.40 (CI95: 0.65-8.83)	1.1894	Ref
First-generation rFVIII	3	4/283	6.34 (CI95: 0.01-7819.34)	1.8502	3.56 (CI95: 0.44-28.77)
Third-generation rFVIII	7	12/8645	1.35 (CI95: 0.44-4.12)	0.3568	0.47 (CI95: 0.10-2.23)

[1] Full-length rFVIII (Kogenate/Helixate, Kogenate FS/Helixate NexGen and Advate) is compared with B-domain deleted rFVIII (Refacto and Refacto AF).

[2] rFVIII derived from CHO-cells (Refacto, Refacto AF and Advate) is compared with rFVIII derived from BHK-cells (Kogenate/Helixate and Kogenate FS/Helixate NexGen).

[3] First Generation rFVIII (Kogenate/Helixate) is compared with second generation rFVIII (Refacto and Kogenate FS/Helixate NexGen) and third generation rFVIII (Advate and Refacto AF).

* Not enough studies for analysis.

Supplemental Table S5. Sensitivity analysis. Main analysis restricted to large studies (i.e. studies with › 150 person-years of follow-up time).

Variable	N	Inhibitors/ p-y	Pooled inhibitor incidence rate per 1000 p-y (CI95)	Between-study variance (τ^2)	Incidence rate ratio (CI95)
Overall (main products only)	15	30/16750	2.06 (CI95: 1.09-3.91)	0.5248	
Product					
Advate	4	6/5772	1.04 (CI95: 0.28-3.81)	0	Ref
Kogenate/Helixate*	1	1/254	-	-	-
Kogenate FS/Helixate NexGen	5	10/7012	0.88 (CI95: 0.29-2.69)	0	1.37 (CI95: 0.33-5.75)
Refacto*	2	6/596	-	-	-
Refacto AF	3	7/3116	2.30 (CI95: 0.19-28.48)	0.4149	2.16 (CI95: 0.47-9.86)
rFVIII length[1]					
Full-length rFVIII	10	17/13038	1.83 (CI95: 1.15-2.91)	0	Ref
B-domain deleted rFVIII	5	13/3712	4.07 (CI95: 1.01-16.39)	0.7253	3.20 (CI95: 1.09-9.40)
Cell line[2]					
CHO-cells	9	19/9484	2.23 (CI95: 0.79-6.28)	0.9128	Ref
BHK-cells	6	11/7266	1.51 (CI95: 0.70-3.29)	0	0.68 (CI95: 0.18-2.51)
rFVIII generation[3]					
Second-generation rFVIII	7	16/7608	2.77 (CI95: 0.94-8.18)	0.6663	Ref
First-generation rFVIII	1	1/254	-	-	-
Third-generation rFVIII	7	13/8888	1.49 (CI95: 0.58-3.86)	0.2716	0.51 (CI95: 0.14-1.83)

[1] Full-length rFVIII (Kogenate/Helixate, Kogenate FS/Helixate NexGen and Advate) is compared with B-domain deleted rFVIII (Refacto and Refacto AF).

[2] rFVIII derived from CHO-cells (Refacto, Refacto AF and Advate) is compared with rFVIII derived from BHK-cells (Kogenate/Helixate and Kogenate FS/Helixate NexGen).

[3] First Generation rFVIII (Kogenate/Helixate) is compared with second generation rFVIII (Refacto and Kogenate FS/Helixate NexGen) and third generation rFVIII (Advate and Refacto AF).

* Not enough studies for analysis.

Supplemental Figure S1. Search strategy

Pubmed

(((“Factor VIII”[Mesh] OR “Factor VIII”[tw] OR “Factor 8”[tw] OR “Thromboplastinogen”[tw] OR “Hyate-C”[tw] OR “Hyate C”[tw] OR “Factor VIIIC”[tw] OR “F VIII-C”[tw] OR “F VIII C”[tw] OR “FVIII”[tw] OR antihemophilic factor*[tw] OR anti-hemophilic factor*[tw] OR antihaemophilic factor*[tw] OR anti-haemophilic factor*[tw] OR “Factor VIIIa”[tw] OR “Coagulation Factor VIIIa”[tw]) AND (“recombinant”[tw] OR “Recombinant Proteins”[Mesh]) AND (“INH”[tw] OR “inhibitor development”[tw] OR “inhibitors development”[tw] OR (inhibitor*[tw] AND (develop*[tw] OR occurence*[tw])) OR inhibitor*[tw] OR “inhibitory”[tw])) **OR** (“Factor VIII/antagonists and inhibitors”[Mesh] AND (“recombinant”[tw] OR “Recombinant Proteins”[Mesh])) **OR** ((“Advate”[tw] OR “rAHF-PFM”[tw] OR “Refacto”[tw] OR “Refacto AF”[tw] OR “Kogenate-FS”[tw] OR “Kogenate”[tw] OR “Helixate”[tw] OR “Helixate-FS”[tw] OR “Recombinate”[tw] OR “Xyntha”[tw]) AND (“INH”[tw] OR “inhibitor development”[tw] OR “inhibitors development”[tw] OR (inhibitor*[tw] AND develop*[tw]) OR inhibitor*[tw] OR “inhibitory”[tw] OR “antagonists and inhibitors”[Subheading])) **OR** ((“Advate”[ti] OR “rAHF-PFM”[ti] OR “Refacto”[ti] OR “Refacto AF”[ti] OR “Kogenate-FS”[ti] OR “Kogenate”[ti] OR Helixat*[ti] OR “Recombinate”[ti] OR “Xyntha”[ti] OR recombinant factor VIII*[ti])) **OR** ((“Factor VIII”[majr] OR “Factor VIII”[ti] OR “Factor 8”[ti] OR “Thromboplastinogen”[ti] OR “Hyate-C”[ti] OR “Hyate C”[ti] OR “Factor VIIIC”[ti] OR “F VIII-C”[ti] OR “F VIII C”[ti] OR “FVIII”[ti] OR antihemophilic factor*[ti] OR anti-hemophilic factor*[ti] OR antihaemophilic factor*[ti] OR anti-haemophilic factor*[ti] OR “Factor VIIIa”[ti] OR “Coagulation Factor VIIIa”[ti]) AND (“concentrates”[tw] OR “concentrate”[tw]) AND (“INH”[ti] OR “inhibitor development”[ti] OR “inhibitors development”[ti] OR (inhibitor*[ti] AND (develop*[ti] OR occurence*[ti])) OR inhibitor*[ti] OR “inhibitory”[ti]) AND (“Clinical Study”[Publication Type] OR “Epidemiologic Studies”[Mesh] OR “Support of Research”[Publication Type]))) AND (“Hemophilia A”[Mesh] OR “hemophilia”[tw] OR “haemophilia”[tw] OR hemophil*[tw] OR haemophil*[tw]) NOT (“Animals”[mesh] NOT “Humans”[mesh]) NOT ((acquired haemophil*[ti] OR acquired haemophil*[ti]) NOT Congenital*[ti])

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binant".ti,ab OR exp *"Recombinant Protein"/) AND ("INH".ti,ab OR "inhibitor development".ti,ab OR "inhibitors development".ti,ab OR (inhibitor*.ti,ab ADJ5 (develop*.ti,ab OR occurence*.ti,ab)) OR inhibitor*.ti OR "inhibitory".ti OR *"blood clotting factor 8 inhibitor"/)) OR ((*"recombinant blood clotting factor 8"/ OR "Advate".ti,ab OR "rAHF-PFM".ti,ab OR "Refacto".ti,ab OR "Refacto AF".ti,ab OR "Kogenate-FS".ti,ab OR "Kogenate".ti,ab OR "Helixate".ti,ab OR "Helixate-FS".ti,ab OR "Recombinate".ti,ab OR "Xyntha".ti,ab) AND ("INH".ti,ab OR "inhibitor development".ti,ab OR "inhibitors development".ti,ab OR (inhibitor*.ti,ab ADJ5 develop*.ti,ab) OR inhibitor*.ti OR "inhibitory".ti OR *"blood clotting factor 8 inhibitor"/))) AND ("Hemophilia A"/ OR "Hemophilia"/ OR "hemophilia".ti,ab OR "haemophilia".ti,ab OR hemophil*.ti,ab OR haemophil*.ti,ab) AND exp "Humans"/ NOT ((acquired haemophil*.ti OR acquired haemophil*.ti) NOT Congenital*.ti) NOT "conference review".pt NOT "conference abstract".pt

Web of Science

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(((("blood clotting factor 8" OR "Factor VIII" OR "Factor 8" OR "Thromboplastinogen" OR "Hyate-C" OR "Hyate C" OR "Factor VIIIC" OR "F VIII-C" OR "F VIII C" OR "FVIII" OR antihemophilic factor* OR anti-hemophilic factor* OR antihaemophilic factor* OR anti-haemophilic factor* OR "Factor VIIIa" OR "Coagulation Factor VIIIa") AND ("recombinant" OR "Recombinant Protein") AND ("INH" OR "inhibitor development" OR "inhibitors development" OR (inhibitor* ADJ5 (develop* OR occurence*)) OR inhibitor* OR "inhibitory" OR "blood clotting factor 8 inhibitor")) **OR** (("recombinant blood clotting factor 8" OR "Advate" OR "rAHF-PFM" OR "Refacto" OR "Refacto AF" OR "Kogenate-FS" OR "Kogenate" OR "Helixate" OR "Helixate-FS" OR "Recombinate" OR "Xyntha") AND ("INH" OR "inhibitor development" OR "inhibitors development" OR (inhibitor* ADJ5 develop*) OR inhibitor* OR "inhibitory" OR "blood clotting factor 8 inhibitor"))) AND ("Hemophilia A" OR "Hemophilia" OR "hemophilia" OR "haemophilia" OR hemophil* OR haemophil*)

CINAHL
(((("blood clotting factor 8" OR "Factor VIII" OR "Factor 8" OR "Thromboplastinogen" OR "Hyate-C" OR "Hyate C" OR "Factor VIIIC" OR "F VIII-C" OR "F VIII C" OR "FVIII" OR antihemophilic factor* OR anti-hemophilic factor* OR antihaemophilic factor* OR anti-haemophilic factor* OR "Factor VIIIa" OR "Coagulation Factor VIIIa") AND ("recombinant" OR "Recombinant Protein") AND ("INH" OR "inhibitor development" OR "inhibitors development" OR (inhibitor* ADJ5 (develop* OR occurence*)) OR inhibitor* OR "inhibitory" OR "blood clotting factor 8 inhibitor")) **OR** (("recombinant blood clotting factor 8" OR "Advate" OR "rAHF-PFM" OR "Refacto" OR "Refacto AF" OR "Kogenate-FS" OR "Kogenate" OR "Helixate" OR "Helixate-FS" OR "Recombinate" OR "Xyntha") AND ("INH" OR "inhibitor development" OR "inhibitors development" OR (inhibitor* ADJ5 develop*) OR inhibitor* OR "inhibitory" OR "blood clotting factor 8 inhibitor"))) AND ("Hemophilia A" OR "Hemophilia" OR "hemophilia" OR "haemophilia" OR hemophil* OR haemophil*)

Chapter 5

Performance of a clinical risk prediction model for inhibitor formation in severe hemophilia A

Shermarke Hassan, Roberta Palla, Carla Valsecchi, Isabella Garagiola, Amal El-Beshlawy, Mohsen Elalfy, Vijay Ramanan, Peyman Eshghi, Mehran Karimi, Samantha C. Gouw, Pier M. Mannucci, Frits R. Rosendaal, Flora Peyvandi

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Abstract

Background

There is a need to identify patients with hemophilia who have a very low or high risk of developing inhibitors. These patients could be candidates for personalized treatment-strategies.

Aims

The aim of this study was to externally validate a previously published prediction model for inhibitor development and to develop a new prediction model that incorporates novel predictors.

Methods

The population consisted of 251 previously untreated or minimally treated patients with severe hemophilia A enrolled in the SIPPET study. The outcome was inhibitor formation. Model discrimination was measured using the C-statistic and model calibration was assessed with a calibration plot. The new model was internally validated using bootstrap resampling.

Results

Firstly, the previously published prediction model was validated. It consisted of three variables: family history of inhibitor development, *F8* gene mutation and intensity of first treatment with factor VIII (FVIII). The C-statistic was 0.53 (95%CI: 0.46-0.60) and calibration was limited.

Furthermore, a new prediction model was developed that consisted of four predictors: *F8* gene mutation, intensity of first treatment with FVIII, the presence of factor VIII non-neutralizing antibodies before treatment initiation and lastly, FVIII product type (recombinant vs. plasma-derived). The C-statistic was 0.66 (95CI: 0.57-0.75) and calibration was moderate. Using a model cut-off point of 10%, positive- and negative predictive values were 0.22 and 0.95, respectively.

Conclusion

Performance of all prediction models was limited. However, the new model with all predictors may be useful for identifying a small number of patients with a low risk of inhibitor formation.

Background

A major treatment complication in hemophilia A is the formation of neutralizing antibodies against factor VIII (also called inhibitors) which render subsequent treatment with factor VIII (FVIII) ineffective and are associated with increased morbidity/mortality.[1] There is a need to identify patients with a very low/high risk of developing inhibitors as these patients could be candidates for personalized treatment-strategies.[2]

Two published prediction models for inhibitor formation have been suggested for clinical use.[3, 4] The second model[4] (a modified version of the earlier model[3]), was developed using data from the CANAL study and PedNet registry. The study population consisted of 825 previously untreated patients (PUPs) with severe hemophilia A, followed from 1-50 days of exposure to FVIII (EDs). The model contained three predictors: family history of inhibitors, *F8* gene mutation and intensity of the first FVIII treatment episode. The model C-statistic was 0.69 (95% CI 0.65–0.73). The calibration plot overestimated the inhibitor risk in the higher ranges of inhibitor incidences (› 0.55). This model urgently needs to be externally validated in another dataset.

New risk factors for inhibitor formation have been identified using the SIPPET study cohort.[5-7] Firstly, the use of recombinant FVIII (rFVIII) was associated with a higher inhibitor risk than plasma-derived FVIII (pdFVIII) (hazard ratio: 1.87, 95CI: 1.17-2.96).[5]

Furthermore, the presence of non-neutralizing anti-FVIII antibodies (NNAs) before FVIII exposure was associated with an increased risk of inhibitor formation in previously untreated and minimally treated patients with severe hemophilia A (HR: 1.83, CI95: 0.84-3.99).[7] Studies have also shown that NNAs are detectable in non-hemophilic subjects. (most of whom were never exposed to blood components such as fresh-frozen plasma).[8] This suggests that some autoreactivity against endogenous FVIII is relatively common.[9]

Lastly, a genetic analysis showed that inhibitor prediction based on FVIII mutation could be improved by also accounting for FVIII antigen production.[6] A new model incorporating these new data could be useful for clinical practice.

The first aim of this study was to externally validate the latest published prediction model for inhibitor development.[4] The second aim was to develop a new clinical prediction model that incorporates novel predictors.

Methods

Study design and population

Data from the SIPPET study were used.[5] The SIPPET study enrolled 251 severe (FVIII:C ‹ 1%) hemophilia A patients without previous treatment with FVIII or only minimal treatment with blood components. Patients were followed-up for 50 EDs or 3 years of observation (whichever came first). The cumulative number of EDs to FVIII was used as the timescale.

Defining outcome and predictor variables

Validation of 2015 model

The outcome, inhibitor formation, was defined as any inhibitor higher than 0.4 Bethesda Units (BU), measured using the Bethesda assay with Nijmegen modification. The 2015 prediction model consisted of three predictors; family history of inhibitors, *F8* gene mutation and intensity of the first treatment with FVIII.[4]

Family history of inhibitors was analyzed as a categorical variable (not applicable/negative, positive, unknown). Family history of inhibitors was classified as 'not applicable' when the patient had a negative family history of hemophilia.

F8 gene mutation was defined as a categorical variable (missense mutations, null mutations, other, unknown). The category 'null mutations' consisted of deletions of › 200 base pairs, nonsense mutations, intron 22 inversions and intron 1 inversions. The category 'other mutations' consisted of small deletions of ‹ 200 base pairs, insertions and splice site defects.

Intensity of first treatment was a continuous variable defined as the product of the number of consecutive EDs at first treatment (ranging from the first ED up to the 10th consecutive ED) and the mean daily dose in IU/kg of FVIII used during this period. The result was expressed as a fraction of 50 IU/kg. (As an example, an individual who was treated for 5 consecutive EDs with a mean daily dose of 75 IU/kg would have a value of 5 EDs x (75 IU/kg ÷ 50 IU/kg) = 5 x 1.5 = 7.5.)

Development of new model

To improve clinical applicability, high-titer inhibitor formation, defined as a peak inhibitor titer of at least 5 Bethesda units, was used as the outcome.

On the basis of literature and subject-matter knowledge, four predictors were considered: intensity of the first treatment with FVIII, *F8* gene mutation, NNA status before treatment initiation and treatment with pdFVIII or rFVIII.

Treatment intensity was defined as being treated for at least 2 consecutive EDs at first treatment. For *F8* gene mutation, we used the classification by Spena et al.[6] In this classification, in silico predicted null mutations were reclassified as non-null if there were detectable FVIII antigen levels. Missing values were encoded as a separate category labeled 'unknown'. NNA status before treatment initiation was analyzed as a dichotomous variable (negative or positive), according to cut-off values of the NNA assay (≥ 1.64mg/mL of specific anti-FVIII IgG[7]). Treatment type was defined as treatment with either plasma-derived FVIII (pdFVIII) or recombinant-derived FVIII (rFVIII).[5]

Statistical analysis

Validation of 2015 model
The predicted risk of inhibitor formation was calculated for each individual in the SIPPET study, using the formula described in the original paper.[4]

Development of new model
Three different models were fit using logistic regression. The first two models were developed to be used before any FVIII exposure; the first model contained only *F8* gene mutation as a predictor, the second model also included NNA status.

The third model was developed to predict inhibitor risk just after the first treatment episode and consisted of *F8* gene mutation, NNA status, treatment intensity and treatment type. Variable selection was based on the strength of the predictors as well as subject-matter knowledge. Family history was difficult to ascertain correctly and was therefore not included as a predictor. For treatment intensity, we chose 2 ED's instead of 5 ED's because the aim was to develop a model that could be implemented almost immediately after the start of treatment. Consequently, patients with an inhibitor event in the first 2 EDs were excluded from the analysis of the full model.

Internal validation of the new model using a bootstrapping procedure
To correct for overfitting, a uniform shrinkage factor was estimated using the bootstrap resampling method.[10] Next, model coefficients were multiplied by the shrinkage factor and the model constant was re-estimated with the shrunken coefficients.

Evaluating model performance
Discrimination is the level to which a model can distinguish between patients developing and not developing the outcome. Discriminative power of each model was assessed with the C-statistic. The C-statistic can be calculated by taking all possible pairs in which one subject developed the outcome and the other did not. Pairs in which the patient with the outcome also had a higher predicted risk of the outcome are called concordant pairs. The higher the proportion of concordant pairs among all pairs, the higher the C-statistic. The C-statistic can range from 0.5 (no discrimination) to 1 (perfect discrimination).

Calibration refers to the degree to which predicted and observed outcomes are similar. Calibration of each model was reported visually in a calibration plot, with expected outcome probabilities plotted against observed outcome frequencies, for each quintile of predicted risk. Furthermore, a LOWESS (Locally Weighted Scatterplot Smoothing) line was estimated to examine calibration across the whole range.

Sensitivity, specificity, positive and negative predictive values were calculated for different cut-off values of the new model.

Handling missing values
Missing values for any of the predictors or outcome variable in the SIPPET dataset were imputed using multivariate imputation by chained equations. Model coefficients of each imputed dataset, their C-statistics and corresponding standard errors were pooled using Rubin's rules to obtain the final estimates.[11] Internal validation using bootstrap resampling was performed within each imputed dataset. The results (i.e. the calibration intercept, slope, shrinkage factor and optimism corrected C-statistic) were also pooled using Rubin's rules. The calibration plot was constructed by combining the imputed datasets and fitting the shrunken model to this pooled dataset.

Statistical packages
The data was prepared for analysis using IBM SPSS statistics version 25. Analysis were performed using R version 3.1.0.

Results

General information

Characteristics of the validation cohort are shown in Table 1. Overall, 76/251 patients developed an inhibitor, 50/76 inhibitor patients had a high-titer inhibitor. Further-

Table 1: Patient characteristics.

Predictors of 2015 model	**All patients (N = 251)**	**inhibitor-negative (N = 175)**	**inhibitor-positive (N = 76)**
F8 gene mutation type (Hashemi 2015)			
Missense	22 (8.8%)	18 (10.3%)	4 (5.3%)
Null	166 (66.1%)	111 (63.4%)	55 (72.4%)
Other	46 (18.3)	33 (18.9%)	13 (17.1%)
Unknown	17 (6.8%)	13 (7.4%)	4 (5.3%)
Family history			
Negative/not applicable	205 (81.7%)	140 (80.0%)	65 (85.5%)
Positive	24 (9.6%)	19 (10.9%)	5 (6.6%)
Unknown	22 (8.8%)	16 (9.1%)	6 (7.9%)
Intensive treatment†			
Mean (SD)	0.82 (SD: 5.9)	0.96 (SD: 6.6)	0.48 (SD: 0.9)
Predictors of new model	**All patients (N = 251)**	**High-titer inhibitor-negative (N = 201)**	**High-titer inhibitor-positive (N = 50)**
Study treatment			
pd-FVIII	125 (49.8%)	105 (52.2%)	20 (40.0%)
rec-FVIII	126 (50.2%)	96 (47.8%)	30 (60.0%)
Pre-treatment NNA status‡			
Negative	219 (92.4%)	178 (94.2%)	41 (85.4%)
Positive	18 (7.6%)	11 (5.8%)	7 (14.6%)
At least 2 consecutive EDs at first treatment			
No	210 (83.7%)	175 (87.1%)	35 (70.0%)
Yes	41 (16.3%)	26 (12.9%)	15 (30.0%)
F8 gene mutation type (Spena 2018)			
Missense	42 (16.7%)	39 (19.4%)	3 (6.0%)
Null	189 (75.3%)	144 (71.6%)	45 (90.0%)
Unknown	20 (8.0%)	18 (9.0%)	2 (4.0%)

† Mean intensive treatment was a continuous variable defined as the product of the number of consecutive EDs at first treatment (ranging from the first ED up to the 10th consecutive ED) and the mean daily dose in IU/kg of FVIII used during this period. The result was expressed as a fraction of 50 IU/kg.

‡ Pre-treatment NNA status: 14 missing values overall (5.6%).

more, 75% of patients had a *F8* null mutation, 9.6% had a positive family history, 7.6% were NNA-positive and 16.3% were treated for at least 2 consecutive days at first treatment. NNA status was unknown in 14 patients and *F8* gene mutation was unknown in 20 patients.

External validation of the 2015 prediction model

Baseline characteristics of the 825 patients in the development cohort compared to the 251 patients in the validation cohort are shown in Table 2. In the development cohort, 228/825 (27.6%) of patients developed an inhibitor. The C-statistic in this cohort was 0.69 (95% CI 0.65 - 0.73). In our cohort, we found a C-statistic of 0.53 (0.46 - 0.60). Figure 1A shows the calibration plot of the risk score, as applied to the validation cohort. Overall calibration was limited, as the model highly overpredicts in the higher risk ranges.

Development of new prediction models; association between predictors and inhibitor formation

Table 3 shows the unadjusted and adjusted associations (of the full model) between each predictor and high-titer inhibitor formation. In the multivariable model, the strongest predictors were *F8* gene mutation type (odds ratio: 3.94) and NNA status (odds ratio: 3.38).

Development of prediction models before exposure to FVIII products

The C-statistic of the model with only *F8* gene mutation was 0.59 (95CI: 0.54 - 0.64). The C-statistic of the model with only F8 gene mutation and NNA status at treatment initiation was 0.61 (95CI: 0.52 - 0.71).

Development of full prediction model

The C-statistic of the full model was 0.66 (95CI: 0.57-0.75). The shrunken regression coefficients of the final logistic model are shown in Table 4. Figure 1B shows the optimism-corrected calibration plot of the new model. Overall calibration was low to moderate, as the model underpredicted in the higher risk ranges. The predicted inhibitor risk for an individual in the SIPPET cohort ranged from 6% to 62%. Table 5 shows the incidence of inhibitor development across different categories of predicted risk. Table 6 shows the sensitivity, specificity, positive and negative predictive values of the model for different model cut-off points. The positive predictive value was very low when using the low- and medium cut-off values and slightly higher but still low for the high cut-off value. Conversely, the negative predictive value was high for all three model cut-off points.

Discussion

Main findings

A published inhibitor prediction model showed limited performance in our cohort. Furthermore, the performance of a new model that included novel predictors was also limited.

External validation of 2015 model

The limited performance of the old model may partly be explained by differences in patient characteristics between development- and validation cohorts. Curiously, a positive family history of inhibitors was more common among non-inhibitor patients in our cohort (which reduced model performance). Family history was often difficult to ascertain, which could explain the aforementioned results. However, we were able to include the *F8* gene mutation in our model. (which explains a large of part of familial inhibitor risk) Similarly, mean treatment intensity (which is consistently reported to be associated with inhibitor development) was also higher in non-inhibitor patients.

Compared to the observational development cohort, some patients may have been underrepresented as the SIPPET trial was interventional. For example, obtaining informed consent for participation before any FVIII exposure might have been more difficult for patients with a negative family history of hemophilia presenting with acute severe trauma at the emergency department. Similarly, neonates with an intracranial bleed would have been more difficult to enroll if family history of hemophilia was unknown. Unfortunately, patients with a negative family history of hemophilia and patients with a positive family history of hemophilia/negative family history of inhibitors were combined into one category (family history 'Negative/not applicable') in the 2015 model. (Table 2) It was therefore not possible to directly compare the proportion of patients with a negative family history of hemophilia in the SIPPET cohort vs. the development cohort.

Furthermore, the 2015 model used a stepwise predictor selection procedure, which is known to produce overfitted models.[12] However, the study partially corrected for this by shrinking the final model coefficients through bootstrapping.

Lastly, the poor calibration in the higher risk range (over 50%) was mostly due to the very low number of patients in this area.

Table 2. Comparison of participant characteristics in the development cohort and the validation cohort.

Characteristic	2015 model development cohort (n = 825)†	SIPPET cohort (n = 251)‡
Age in months		
Median (IQR)	10 (6-14)	15 (9-29)
Inhibitor status		
Negative (%)	587 (72.4)	175 (69.7)
Positive (%)	228 (27.6)	76 (30.3)
F8 gene mutation type		
% Missense	12	9
% Null	59	66
% Other	17	18
% Unknown	13	7
Family history		
% Negative/not applicable	83	82
% Positive	9	10
% Unknown	8	9
Treatment intensity		
Mean (SD)§	NR	0.82 (5.9)

† CANAL study/PedNet registry.
‡ SIPPET study. NR: not reported in the original article.
§ Intensity of first treatment was a continuous variable defined as the product of the number of consecutive EDs at first treatment (ranging from the first ED up to the 10th consecutive ED) and the mean daily dose in IU/kg of FVIII used during this period. The result was expressed as a fraction of 50 IU/kg.

Overall, whether the 2015 model underperforms in general, or is merely poorly generalizable to the type of patients enrolled in the SIPPET cohort remains an open question.

Development of pre-FVIII exposure prediction models

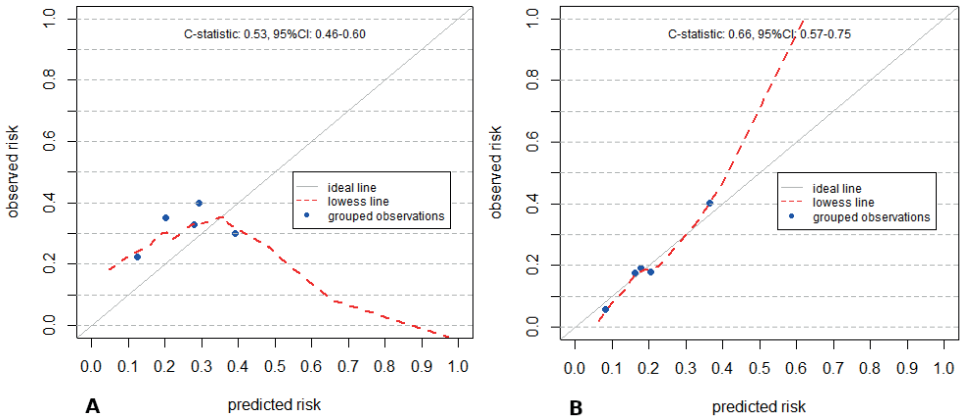
The two simple prediction models were chosen to contain only predictors measurable before FVIII exposure. Both models performed poorly. To construct an accurate pre-FVIII exposure prediction model, additional predictors that can be measured before treatment are necessary. (e.g. certain gene variants)

Development of full prediction model

The full model performed similarly to the 2015 model. The model included treatment intensity, which is consistently associated with inhibitor development.[13] However,

Figure 1. Calibration plot of 2015 model & new model.

Legend: The figure shows the calibration plot of the 2015 model (A), and of the new model (B). On the X-axis, the predicted probability of inhibitor formation according to the model is plotted against the observed risk on the Y-axis. (0 = no inhibitor, 1 = developed an inhibitor) The blue dots represent the proportion of patients experiencing an event, stratified by quintiles of increasing predicted risk. Quintiles with a higher predicted inhibitor risk should have a higher proportion of patients who develop the outcome. (i.e. a higher observed risk) Alternatively, a LOWESS (Locally Weighted Scatterplot Smoothing) line was estimated to examine calibration across the whole range. (shown here as a red dotted line) The grey line represents perfect prediction, meaning that the predicted risk is exactly the same as the observed risk across the whole range. Ideally, both the quintiles and the LOWESS line should lie exactly on top of the grey line.



our definition of treatment intensity (two consecutive EDs) has some limitations, as the second dose might have been a prophylactic dose. Also, instead of receiving one dose, some patients may have gotten two half doses over two days.

The association between FVIII product type and inhibitor development was not statistically significant due to a lack of power caused by not having enough high-titer inhibitor events. This predictor was still included based on previous literature and subject matter-knowledge, as models with predictors selected solely using significance levels perform poorly when externally validated.[14]

However, model performance was still very limited. The maximum predicted inhibitor risk was 62% and, except for one outlier, no patients had a predicted inhibitor risk

over 40%. Therefore, prediction in the higher risk ranges was not possible. However, calibration in the lower risk ranges was acceptable, and the negative predictive value of the model using the lowest model cut-off of 10% was 95%. (i.e. of the 41 patients with a predicted risk below 10% only two developed an inhibitor) Therefore, we can conclude that the model is useful for identifying low-risk patients. However, only 16% of patients fell into this low-risk category. These were all patients with a *F8* non-null mutation or an unknown *F8* mutation, no detectable NNAs before treatment initiation, and who were not intensively treated at first treatment.

The model did not include genetic risk factors other than *F8* gene mutation, and this could have impacted performance. Furthermore, we found no association between family history and inhibitor development in the SIPPET cohort. This result was probably biased, as family history was difficult to ascertain correctly in our cohort (which mostly consisted of patient from the developing world). Therefore, we decided to exclude this predictor from the model.

NNAs are not routinely measured in clinical practice which limits practical implementation of this model.

Information on ethnicity was not included in the model, as most research on ethnicity and inhibitor formation has focused on African-American/Latino populations, and these ethnicities are very uncommon within the SIPPET cohort. Furthermore, many patients within the SIPPET cohort self-identified as "white" (e.g. patients from Egypt, Iran, Saudi-Arabia etc.), while the original studies on ethnicity mostly enrolled "white" patients from a predominantly European background (i.e. from Europe or North-America), which complicates between-study comparisons.

Lastly, the performance of the new model after external validation in a different population remains unknown.

Implications for clinical practice

The overall performance of the original prediction model, as well as the newly developed models was limited. However, the newly developed full model performed relatively well when identifying patients with a low risk of inhibitor formation.

Currently, pre-authorization trials evaluating FVIII therapeutics often enroll previously treated patients (PTPs) who have been exposed to FVIII for more than 50 EDs. Enrolling previously untreated patients (PUPs) with a low predicted risk of inhibitor formation

Table 3. Unadjusted and adjusted association between each predictor and outcome.

Characteristic	All patients (N = 251)	High-titer inhibitor-positive (N = 50)	Univariate Odds Ratio (95%CI)	Multivariable Odds Ratio (95%CI)‡
Study treatment				
pdFVIII	125 (49.8%)	20 (40.0%)	Ref	Ref
rFVIII	126 (50.2%)	30 (60.0%)	1.64 (0.88-3.12)	1.46 (0.75-2.84)
Pre-treatment NNA status†				
Negative	219 (92.4%)	41 (85.4%)	Ref	Ref
Positive	18 (7.6%)	7 (14.6%)	2.76 (0.97-7.46)	3.38 (1.17-9.80)
At least 2 consecutive EDs at first treatment§				
No	209 (83.6%)	34 (69.4%)	Ref	Ref
Yes	41 (16.4%)	15 (30.6%)	2.96 (1.41-6.15)	3.20 (1.47-6.97)
F8 gene mutation type (Spena 2018)				
Missense	42 (16.7%)	3 (6.0%)	Ref	Ref
Null	189 (75.3%)	45 (90.0%)	4.06 (1.39-17.36)	3.94 (1.13-13.73)
Unknown	20 (8.0%)	2 (4.0%)	1.44 (0.18-9.5)	1.38 (0.20-9.37)

† Pre-treatment NNA status: 14 missing values overall (5.6%) ‡: For the multivariable model, missing values were imputed using multiple imputation, one patient with an inhibitor event in the first 2 EDs was excluded from the analysis, so the total sample size for this analysis was 250. §: 1 missing value, due to one patient being excluded from the analysis due to experiencing an inhibitor event in the first 2 EDs of treatment.

might be considered as an alternative, as the study population is a better match for the target population that will actually use the treatment after market approval (not just PTPs but also PUPs). However, due to the difficulty of enrolling such a rare group of patients (only 16% of PUPs), this approach is not practically feasible. For non-factor replacement therapy, this score would not be useful, as these drugs don't elicit anti-FVIII antibodies. The most important use-case for this prediction model would be after market approval. Novel therapeutics are relatively expensive compared to FVIII, and many patients will continue to be treated with FVIII. A score such as this could be used to select low-risk patients who can be safely treated with regular FVIII concentrates (which are relatively cheap).

Table 4. Final logistic regression model.

Regression coefficients	
Intercept	-2.71
Treatment with rec-FVIII (TRT)	0.29
Positive for NNAs (NNA)	0.95
At least 2 consecutive EDs at treatment initiation (ED)	0.90
F8 gene null mutation (F8-null)	1.07
F8 gene mutation unknown (F8-unknown)	0.25

To calculate the individual risk of inhibitor formation, first calculate the linear predictor: (-2.71 + TRT * 0.29 + NNA * 0.95 + ED * 0.90 + F8-null * 1.07 + F8-unknown * 0.25). The formula is then as follows: 1 / (1+ exp(-(linear predictor))). As an example, the risk of inhibitor formation within 50 EDs for a patient treated with plasma-derived FVIII, who was positive at baseline for NNAs, who was treated for at least 2 consecutive EDs at treatment initiation, and whose *F8* mutation is unknown is 1 / (1+ exp(-(-2.71 + 0 * 0.29 + 1 * 0.95 + 1 * 0.90 + 0 * 1.07 + 1 * 0.25))) = 35%.

Table 5. Incidence of inhibitor development across different risk categories.

Predicted risk	**No of inhibitor-negative patients***	**Inhibitor events**	**Observed risk**
‹ 10%	39	2	4.9%
10-25%	134	29	17.8%
25-40%	24	13	35.1%
≥ 40%	4	5	55.6%

* For the construction of the new model, patients with an inhibitor event in the first 2 EDs were excluded. (also mentioned in the Methods section) This was the case for one out of 251 patients, the total number of patients used to construct the new model therefore equals 250.

Table 6. Sensitivity, specificity, positive and negative predictive values of the model for different model cut-off values.

Categories of predicted risk according to model	**Sensitivity**	**Specificity**	**Positive predictive value**	**Negative predictive value**
Low cut-off (10%)	0.96	0.19	0.22	0.95
Medium cut-off (25%)	0.37	0.86	0.39	0.85
High cut-off (›40%)	0.10	0.98	0.56	0.82

These results could be the first step in developing a model for this aim. However, these tools should not be used in clinical practice to select high-risk patients, as all models perform very poorly in this regard. For this reason, the new prediction model was not converted into a tool that could be used by clinicians. (for example, a nomogram or a score chart)

Implications for future research

All prediction models incorporated the most important pre-treatment risk factors. But even so, performance of these models was still unsatisfactory. However, these models did not incorporate time-varying predictors (e.g. the cumulative number of EDs, FVIII exposure frequency, on-demand vs. prophylactic treatment, exposure to FVIII during trauma or during surgery etc.). For example, much information could be gained by measuring the antibody response over time[15], as was done in a recent study by Reipert et al.[16] Interestingly, this study found that during treatment, the appearance of IgG1 antibodies, followed by IgG3 antibodies, was a strong biomarker of future inhibitor development. A different approach would be to incorporate genomic information at baseline, such as HLA class II haplotypes[17, 18] and/or gene variants of other genes previously associated with inhibitor formation (e.g. IL-10 and CTLA-4)[19].

Conclusion

Performance of old and new prediction models for inhibitor formation after external validation is limited. However, the new model with all predictors may be useful for identifying patients with a low risk of inhibitor formation. Further research is needed to obtain more precise prediction models for clinical use.

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Chapter 6

Factor VIII epitope mapping using a random peptide phage-display library approach

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* contributed equally

Manuscript in preparation

Abstract

Background
Inhibitor development is the most severe complication of hemophilia A care, and is associated with increased morbidity and mortality.

Aims
The aim of this study was to use a novel IgG epitope mapping method to explore the factor VIII (FVIII)-specific epitope profile in the SIPPET cohort population.

Methods
The population consisted of 122 previously untreated patients with severe hemophilia A that were followed-up for 50 days of exposure to FVIII. Sampling was performed before FVIII treatment and at the end of the follow-up. The outcome was inhibitor development. The FVIII-specific IgG epitope repertoire was assessed by means of a novel high-throughput epitope mapping technique using a random peptide phage-display library and the resulting peptide sequences were clustered on the basis of sequence similarity. For each cluster, a consensus motif was generated which was then aligned to the linear sequence of FVIII. The degree to which these clusters of peptide sequences could be used to discriminate between patients with and without an inhibitor was assessed by ROC analysis.

Results
The FVIII-specific antibody response is polyclonal with several clusters. The most predominant clusters in inhibitor-positive patients were mapped to the heavy chain of the FVIII molecule. Using plasma samples taken before exposure to FVIII, three clusters (with the consensus motifs “pxyNw”, “PSLxWK” and “sWphxxxxk”) were identified that predicted inhibitor development (with a C-statistic of 0.73, 0.80 and 0.76 respectively).

Conclusion
Information on immunodominant epitopes could be used to generate novel, less immunogenic FVIII proteins and set up diagnostic tests that predict the risk of inhibitor development before starting treatment with FVIII.

Introduction

Recent advances in the treatment of patients with hemophilia A (HA) have greatly improved clinical outcomes and quality of life. Nevertheless, one of the greatest treatment complications in severe hemophilia A is still the development of anti-factor VIII (FVIII) alloantibodies that neutralize FVIII (also called inhibitors). At least one third of patients treated with FVIII replacement therapy develop an inhibitor during the first 20-30 days of exposure to FVIII (EDs)[1], making treatment with FVIII ineffective. This in turn leads to increased morbidity and mortality among these patients.[1]

This complication is the result of a multi-causal immune response involving both patient- and treatment-related factors.[1] The type of FVIII product is one of the most important risk factors for inhibitor development, with the SIPPET randomized clinical trial showing that patients treated with recombinant FVIII (rFVIII) have an almost twofold higher risk of developing an inhibitor than those treated with plasma-derived FVIII (pdFVIII) products.[2] The pathophysiological mechanisms behind this increased immunogenicity remains unknown. Some plausible biological explanations have been postulated, such as the different post-translational modifications caused by the use of different cell lines during the manufacturing process of rFVIII products and the protective role played by Von Willebrand factor (VWF) in pdFVIII products.[3]

Mature FVIII consists of six major domains (A1, A2, B, A3, C1 and C2) and three acidic linking regions (a1, a2, a3); A1-a1-A2-a2-B-a3-A3-C1-C2. The VWF-FVIII complex forms through a high-affinity interaction between the FVIII light chain and the VWF D-D3 domains.[4] FVIII is activated by limited proteolysis through thrombin cleavage of three peptide bonds at Arg391 (a1-A2 junction), Arg759 (a2-B junction) and Arg1708 (a3-A3 junction).[5] After thrombin cleavage, activated factor VIII (sans B-domain) is released from VWF and binds to phosphatidylserine PS on the extracellular surface of activated platelets.[6,7]

The anti-FVIII humoral immune response is highly polyclonal and consists primarily of IgG antibodies, with variable multiple epitopes among patients and even in the same patient over time.[8] Several studies have examined the immunogenicity of FVIII and the mechanisms underlying inhibitor development during treatment with FVIII.[3, 9, 10] The role of FVIII epitopes in inhibitor development has been previously investigated using different techniques. Specific regions in the A2 (region encompassing Arg484-Ile508)[11], A3 (Gln1778-Asp1840)[12], C1 and C2 (residues Glu2181-Val2243) FVIII domains[13] were shown to be target domains for FVIII alloantibody interaction by

several methods including low resolution immunoprecipitation, western blotting and antibody neutralization assays[8,14], as well as high resolution methods such as the phage display technique[15-18].

In recent years, quantitative immunoproteomics has developed rapidly, offering high throughput analyses at relatively low cost. The aim of this study was to use a novel high-throughput epitope mapping technique based on a random peptide phage-display method in order to explore the overall antibody response before and after exposure to either plasma-derived or recombinant FVIII products and to identify specific immunoprofiles that could be predictive for inhibitor development.

Methods

Patient population

Study samples were obtained from patients enrolled in the SIPPET trial, which was designed to investigate the immunogenicity of different FVIII products in patients with severe hemophilia A who were previously untreated with any FVIII concentrates (PUPs) or minimally treated with blood components.[2] Samples from 122 patients were used for this study. These patients were treated with 8 different FVIII products (4 pdFVIII products and 4 rFVIII products). Inhibitor development was measured using the Bethesda assay with Nijmegen modification.[19] Thirty-nine out of 122 individuals developed an inhibitor.

One sample of citrated plasma was collected at baseline (T0) and two samples at the end of the study (EOS). As previously described[2], in inhibitor-positive patients the end of the study was the time of inhibitor development. In inhibitor-negative patients the study ended when the patient reached 50 EDs or after three years of follow-up (whichever came first).

Approval for this study was obtained from the medical ethics committee at each study center and informed consent was obtained from all parents/guardians of patients.

Mimotope-variation analysis

Assay set-up

The total IgG epitope repertoire was assessed using mimotope-variation analysis (MVA), a next generation phage display method. (Protobios, Tallinn).[20] MVA was conducted as previously described. Briefly, 2 µl of plasma was incubated

with 5 µl of phage library (~5 × 10^{10} phage particles, derivative of Ph.D.-12, NEB, UK) overnight at +4 °C. The human immunoglobulin G (IgG)-captured phages were pulled down by protein G-coated magnetic beads (NEB, S1506S). Phage DNA was extracted, enriched and samples were barcoded by PCR amplification. Pooled samples were analyzed by Illumina sequencing (50-bp single end read, Genohub, USA). The resulting DNA sequences were *in silico* translated to 12 amino acid (aa) long peptide sequences. To correct for differences in sequencing depth among the samples, the total count of each unique peptide sequence per sample was normalized in its counts per three million. The resulting output consisted of a database of 12-mer peptide sequences with varying degrees of affinity for IgG antibodies. These peptide sequences are often referred to in the literature as "mimotopes", due to the fact that they may mimic the true epitope of an antibody.

Two versions of the assay were performed, the standard MVA assay (described above) and a competition assay. In the MVA competition assay, different factor VIII products (Alphanate (Grifols), Fanhdi (Grifols), Emoclot (Kedrion Biopharma), Factane (LFB), Advate (Baxalta), Kogenate FS (Bayer AG), ReFacto AF (Pfizer), Recombinate (Baxalta)) were used to precondition study samples before competition analyses. In detail, respective FVIII products (final concentration: 3 uM) were incubated with 2 µl of plasma for 2 hours at room temperature before proceeding with the MVA assay as described above.

Removal of target unrelated peptides (TUPs)

One issue in conducting phage display experiments is the presence of so-called target-unrelated peptides (TUPs). These are false-positive results caused by selection-related TUPs which are peptide sequences binding to materials and reagents used in the assay (for example, plastic surfaces, albumin), or propagation-related TUPs caused by faster propagation of some phage clones, resulting in a higher peptide count for some peptide sequences. To minimize the effect of these TUPs, we removed all peptide sequences that were predicted to be TUPs using the SAROTUP software.[21] Briefly, known TUPs were filtered out exploiting the TUPscan and the mimosearch algorithms. Peptides with a high likelihood ($P > 0.8$) to bind to polystyrene, as assessed by the PSBinder algorithm, were also filtered out.

Quality control using intra- and inter-assay replicates

To increase assay reliability, all peptide sequences with a count lower than a certain threshold were removed from the dataset. To establish the level of the threshold, a healthy control sample was compared with all its intra- or inter-assay replicates,

and the percentage of unreplicated peptide sequences according to each possible count threshold was calculated. (Figure S1) Below a peptide count threshold of 250, a strong increase in the percentage of unreplicated sequences was seen (Figure S1) For the following analyses, we only kept sequences retrieved at least 250 times in at least one patient.

Identification of peptide sequences with high affinity for FVIII

FVIII-specific peptide sequences were defined as present in the EOS sample in which the standard MVA assay was performed but not in the EOS sample in which the MVA competition assay was performed (which was depleted of FVIII-specific antibodies). Thus, the count of each peptide sequence in the two EOS samples (standard MVA assay vs. MVA competition assay) was compared using the Fisher's exact test. We corrected for multiple testing using the Bonferroni method. Only peptide sequences significantly underrepresented in the MVA competition assay samples when compared to the standard MVA assay samples were considered to be FVIII-specific peptide sequences and used for further analyses.

Clustering workflow

Each FVIII epitope can be conceptualized as being represented by multiple peptide sequences, each containing the epitope binding motif. Therefore, the Hammock algorithm, a hierarchical clustering algorithm, was used to cluster peptides sequences based on sequence similarity before further analyses.[22] Applying the algorithm resulted in clusters of highly similar peptide sequences. For each cluster, a consensus motif was generated based on the multiple sequence alignment of the sequences. Highly conserved residues (› 60%) were denoted with an uppercase symbol while moderately conserved residues (30%-60%) were denoted with a lower case symbol. Columns in the multiple sequence alignment where no single residue had a prevalence of › 30% were denoted with "x". The total peptide count of each cluster was calculated as the sum of the count of each peptide sequence included in a cluster. The clustering algorithm was performed firstly on the whole dataset and then separately for data from patients using pdFVIII and patients using rFVIII.

Alignment of consensus motifs to FVIII

The consensus motif derived from each cluster of peptide sequences was then aligned to the linear sequence of FVIII.

Statistical analyses

For the descriptive analyses, a PCA plot of all the clusters identified after the clustering step were generated. To find clusters with a significantly higher count among inhibitor-positive patients compared to inhibitor-negative patients, a Wilcoxon Rank Sum test was performed. Correction for multiple testing was done using the Bonferroni method[23], and an adjusted p-value ‹ 0.05 was considered statistically significant.

To find biomarkers that were able to predict inhibitor development before the start of FVIII therapy, clusters showing a significant association with inhibitor development in the samples taken at the end of the study (the EOS samples) were also evaluated in samples taken before FVIII treatment (the T0 samples). Correction for multiple testing was done using the Bonferroni method[23] and an adjusted p-value ‹ 0.05 was considered statistically significant.

To assess the discriminative performance of the clusters that were also significantly associated with inhibitor development in the T0 samples, we calculated the C-statistic and plotted a receiver operating characteristic (ROC) curve. In addition, a cut-off was selected using Youden's index for each cluster, and based on this cut-off we calculated the sensitivity and specificity of each cluster for inhibitor development.

Results

The MVA assay methodology was applied to 124 previously untreated patients with hemophilia. Of this group, thirty-nine patients were inhibitor-positive. The mean number of unique peptide sequences generated for each patient was 356,365. After removing potential target-unrelated peptides, the mean number of unique peptides generated for each patient decreased to 313,340. From this dataset, we kept only the peptide sequences with a count of at least 250 in at least one patient and used this dataset to identify FVIII-specific peptide sequences as described in the Methods section. This yielded 286 unique peptide sequences per patient.

FVIII-specific epitope profile of patients that developed an inhibitor after replacement therapy with pdFVIII or rFVIII

As shown in Table 1, we found 17 clusters with a significantly higher count in patients who developed an inhibitor as compared with patients who did not. The PCA plot showed a clear difference between patients with or without inhibitors (Figure 1). Clusters that were more common in inhibitor-positive patients were predominantly mapped to the heavy chain of the FVIII molecule (Table 2).

Clusters associated with an inhibitory response against rFVIII

The clustering workflow was then applied to FVIII-specific peptide sequences retrieved in the rFVIII group. Eleven clusters had a significantly higher count among inhibitor-positive patients when compared to inhibitor-negative patients at the end of the study (Table 3). Of these 11 clusters, one cluster (with the consensus motif "pxyNw") was also significantly associated with inhibitor development in the baseline (T0) samples (Figure 2). This cluster was mapped to the A2 domain. The C-statistic of this cluster was 0.73 (95%CI: 0.60-0.86), sensitivity was 86% and specificity was 59%. (Figure 3A)

Clusters associated with an inhibitory response against pdFVIII

Next, the clustering workflow was then applied to the FVIII-specific peptide sequences retrieved in the pdFVIII group. In this group, we found 14 clusters with a significantly higher count among inhibitor-positive patients when compared to inhibitor-negative patients at the end of the study (Table 4). Of these 14 clusters, two were also significantly associated with inhibitor development in the baseline (T0) samples. (Figure 2) The C-statistic of the first cluster (with consensus motif "PSLxWK") was 0.80 (95%CI: 0.66 – 0.93), sensitivity was 76% and specificity was 77%. (Figure 3B) This cluster was mapped to the B domain. The C-statistic of the second cluster (with consensus motif "sWphxxxxk") was 0.76 (95%CI: 0.63 – 0.89), sensitivity was 88% and specificity was 59%. (Figure 3B) This cluster was mapped to the C2 domain.

Discussion

Summary

We assessed the FVIII-specific epitope profile of 122 previously untreated patients with hemophilia A, using a novel random peptide phage-display assay. Our results show that the FVIII-specific antibody response is

highly polyclonal, as our analysis generated many different clusters. In our cohort, we saw an overall slightly stronger response against the A1-A2-B domains than the C1-C2 domains. Using samples obtained before exposure to FVIII, we identified three clusters of peptide sequences (with the consensus motifs "pxyNw", "PSLxWK" and "sWphxxxxk"), that were predictive for inhibitor development (with an C-statistic of 0.73, 0.80 and 0.76 respectively).

Table 1. Consensus motifs of clusters of peptide sequences with a significantly higher count in either inhibitor-negative (INH-) patients or inhibitor-positive (INH+) patients in all patients.

Consensus motif	Mean peptide count* in IHN- Group	Mean peptide count* in IHN+ Group	Adjusted P-value	FVIII Domain(s)	Number of unique peptide sequences in cluster (%)	Peptide count of cluster (%)
kxPxstw	6.10	8.50	2.4e-05	A2	31 (0.11%)	62423 (0.18%)
Yvntxxxt	5.70	8.20	8.0e-04	A1	41 (0.15%)	48730 (0.14%)
pxxWxKp	6.40	8.80	8.2e-04	C1	66 (0.24%)	93414 (0.26%)
kxxTgpq	5.60	7.60	2.2e-03	A2	35 (0.13%)	37907 (0.11%)
KnxHxxxxp	5.50	7.90	2.5e-03	A3	73 (0.26%)	154368 (0.44%)
QxxlPf	4.80	7.20	3.5e-03	A2	73 (0.26%)	89871 (0.25%)
WDrxxxxt	4.60	6.90	8.4e-03	A1	16 (0.06%)	35821 (0.1%)
lsxpK	6.40	8.40	2.3e-02	A1	46 (0.17%)	85081 (0.24%)
QPxxPf	7.60	9.40	4.0e-02	A1	275 (0.99%)	385819 (1.09%)
snHk	6.70	3.90	1.4e-03	B	38 (0.14%)	42742 (0.12%)
pxPtxn	7.30	5.30	3.0e-03	B	49 (0.18%)	43449 (0.12%)
kxtPxnIS	6.70	4.20	3.3e-03	A2	37 (0.13%)	48498 (0.14%)
pskT	8.10	6.50	5.1e-03	B	47 (0.17%)	64470 (0.18%)
kxRPtxxt	8.20	6.40	1.4e-02	A1	86 (0.31%)	122694 (0.35%)
YxDxxLN	9.20	7.50	1.6e-02	A2	224 (0.81%)	334062 (0.94%)
rxxDTxxs	10.40	9.30	2.0e-02	B	421 (1.52%)	532983 (1.5%)
pqNtk	9.20	7.50	3.1e-02	B	130 (0.47%)	216865 (0.61%)

* Mean peptide count is reported as the mean 2log.Total number of unique peptide sequences: 27775. Total peptide count: 35452858.

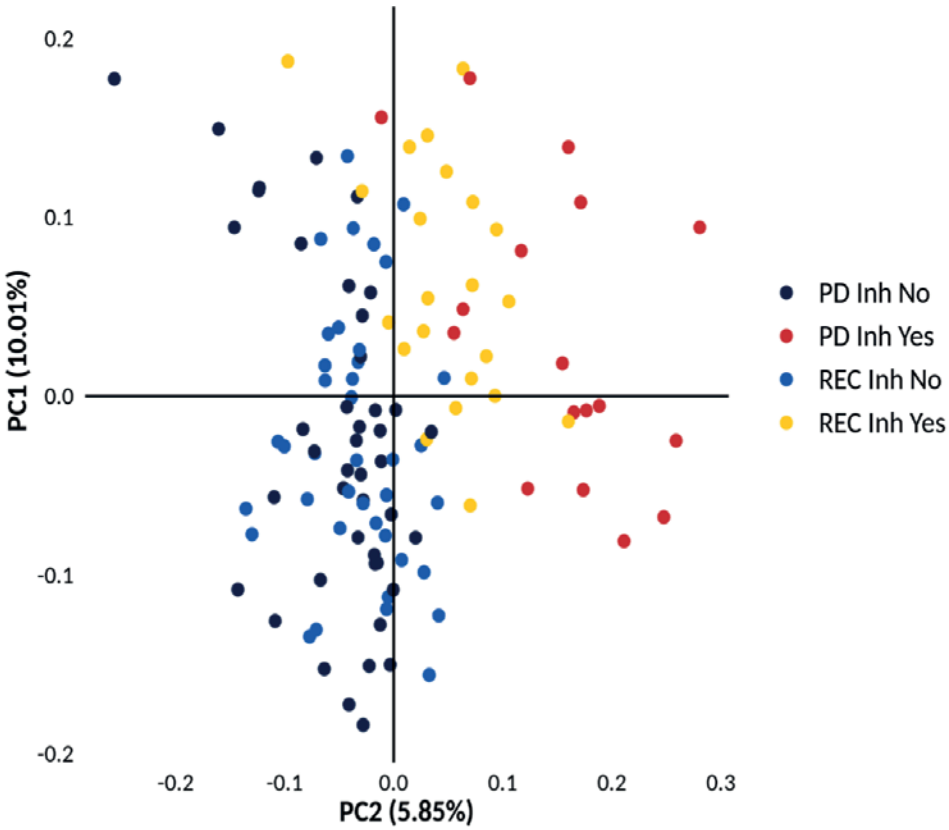
Comparison with the literature

The consensus motif "pxyNw" was mapped to the A2 domain, on residues Phe528 to Trp532 of FVIII. It has been previously reported that this region is part of a binding site for FIXa.[24] To our knowledge, there have been no previous publications of an epitope targeting this region of FVIII. Interestingly, this epitope motif was more common in inhibitor-negative patients than in inhibitor-positive patients.

The consensus motif "sWphxxxxk" was mapped to the C2 domain, on residues Ser2331 to Arg2339, which have been reported to be involved in binding to von Wille-

Figure 1. Principal component analysis performed on clusters of peptide sequences found at the end of the study, for the total cohort.

PD Inh No: inhibitor-negative patient using pdFVIII.
PD Inh Yes: inhibitor-positive patient using pdFVIII. REC Inh No: inhibitor-negative patient using rFVIII. REC Inh No: inhibitor-positive patient using rFVIII.



brand factor and phospholipids. Furthermore, there have been reports describing anti-FVIII antibodies targeting this region.[25]

The consensus motif "PslxWk" was mapped to the B domain, on residues Glu1037 to Phe1042. To our knowledge, there have been no previous publications of an epitope targeting this region of FVIII.

Table 2. Distribution of consensus motifs on domains of FVIII.

FVIII domains	Count (total group)	Count (pdFVIII group)	Count (rFVIII group)
INH+ clusters	9	13	7
A1	4 (44%)	3 (23%)	1 (14%)
A2	3 (33%)	2 (15%)	2 (29%)
B	0 (0%)	6 (46%)	3 (43%)
A3	1 (11%)	1 (8%)	1 (14%)
C1	1 (11%)	0 (0%)	0 (0%)
C2	0 (0%)	1 (8%)	0 (0%)
INH- clusters	8	1	4
A1	1 (12%)	1 (100%)	1 (25%)
A2	2 (25%)	0 (0%)	1 (25%)
B	5 (62%)	0 (0%)	2 (50%)
A3	0 (0%)	0 (0%)	0 (0%)
C1	0 (0%)	0 (0%)	0 (0%)
C2	0 (0%)	0 (0%)	0 (0%)

INH+ clusters: clusters that were more common in inhibitor-positive patients. INH- clusters: clusters that were more common in inhibitor-negative patients

Interestingly, this epitope motif (that was mapped to the B-domain) was more common in inhibitor-positive patients than in inhibitor-negative patients. This is in contrast with previous studies that have suggested that antibodies against the B-domain might be predominantly of the non-neutralizing type[26-28], as the B-domain is not essential for the role of FVIII in blood clotting and is cleaved off after FVIII is activated.

Overall, two out of three consensus motifs that were predictive for inhibitor development were directed against the A2 and C2 domains respectively, which is in line with the results of previous studies that suggest that most antibodies are directed against the A2 and C2 domains.[8, 29-31]

Previous studies have also shown that the peptide presentation profile of monocyte-derived dendritic cells changes when exposed to the FVIII-VWF complex[32,33]. In

our study, the overall epitope profile in the rFVIII group was similar to that of the pdFVIII group in terms of the distribution across FVIII domains of the epitope motifs. However, due to the very small number of consensus motifs, no definitive conclusions can be drawn from these results.

Table 3. Consensus motifs of clusters of peptide sequences with a significantly higher count in either inhibitor-negative (INH-) patients or inhibitor-positive (INH+) patients in the recombinant-derived FVIII treatment group.

Consensus motif	Mean peptide count* in IHN- Group	Mean peptide count* in IHN+ Group	Adjusted P-value	FVIII Domain(s)	Number of unique peptide sequences in cluster (%)	Peptide count of cluster (%)
PTNlxk	7.50	10.00	2.2e-04	B	40 (0.16%)	98681 (0.67%)
sxPxfT	5.00	7.80	3.7e-03	A3	32 (0.13%)	35683 (0.24%)
kyQqlsxxlp	5.20	7.60	1.2e-02	A2	11 (0.04%)	23800 (0.16%)
Qqyxp	7.10	8.90	1.4e-02	A2	39 (0.15%)	60093 (0.41%)
tyvEPxqxxr	5.90	7.80	2.0e-02	A1	8 (0.03%)	32077 (0.22%)
ppxxnxs	5.80	8.30	2.3e-02	B	56 (0.22%)	53923 (0.36%)
pSdsVxs	4.30	7.00	4.3e-02	B	14 (0.06%)	28539 (0.19%)
pWsk	10.40	8.40	3.6e-03	B	147 (0.58%)	276673 (1.87%)
pSNp	6.80	3.80	7.4e-03	A1	42 (0.17%)	31351 (0.21%)
qxixNsK	7.70	4.50	2.8e-02	B	119 (0.47%)	194793 (1.31%)
pxyNw	8.40	5.40	4.7e-02	A2	63 (0.25%)	73568 (0.5%)

* Mean peptide count is reported as the mean 2log. Total number of unique peptide sequences: 25235. Total peptide count: 14820947.

The presence of peptide sequences with high affinity for anti-FVIII antibodies in samples taken before treatment with FVIII might seem unexpected at first glance. However, several studies have reported the presence of non-neutralizing anti-FVIII antibodies in healthy controls.[34] In addition, a previous study using pre-treatment samples of the current cohort reported that roughly 10% of patients had measurable anti-FVIII antibodies.[35] This suggests that natural autoreactivity against endogenous FVIII is relatively common in patients as well as healthy controls. Another hypothesis could be that the detected antibodies were not initially directed against FVIII, but were the result of previous exposure to a pathogen (e.g. a bacteria or virus) that contained a similar epitope. This cross-reactivity of the antibody response has been previously reported in several auto-immune disorders.[36]

Figure 2. Figure showing the location on the FVIII molecule and the mean peptide count in the pre-treatment samples of the three clusters (with motifs "pxyNw", "PslxWk" and "sWphxxxxk") that were able to predict inhibitor development before exposure to FVIII.

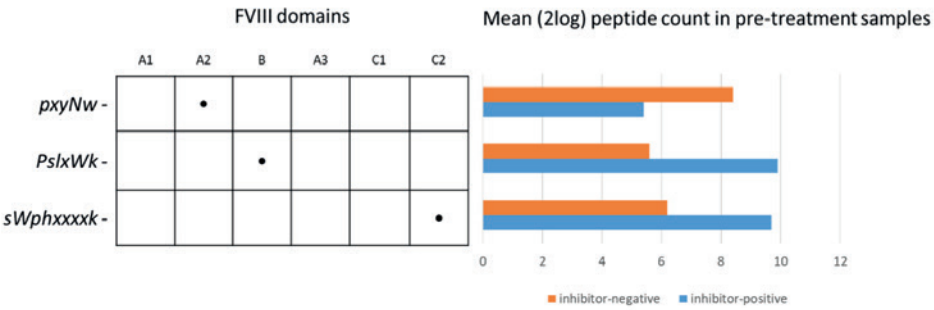
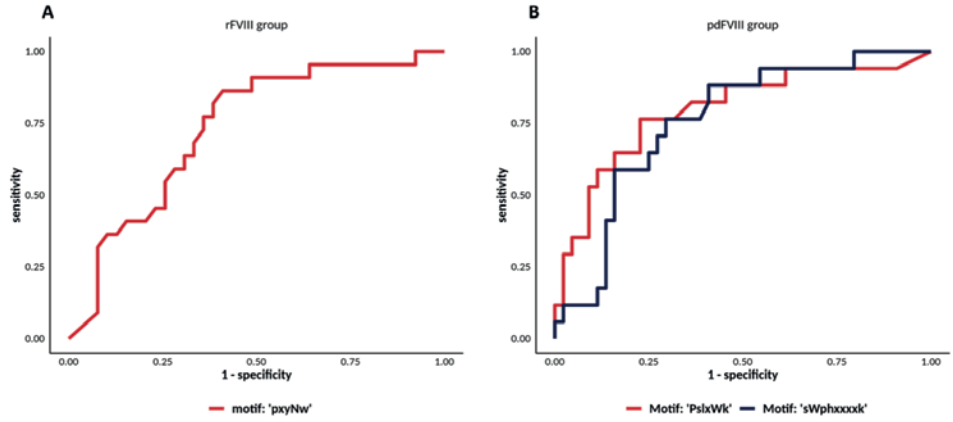


Figure 3. ROC curves showing the degree to which the three clusters (with motifs "pxyNw", "PslxWk" and "sWphxxxxk") were able to predict inhibitor development before exposure to FVIII.



Limitations

This approach has some major limitations. Firstly, it has been shown that only a handful of contact residues within an epitope make a significant contribution to antibody binding.[37] In this study, we tried to identify these residues by clustering highly similar peptide sequences and generating a consensus motif. Using alanine walk mutational analysis, the study by Kahle et al.[18] showed that there was reasonable agreement between a given consensus motif and the crucial binding residues of an epitope. Therefore, the consensus motifs derived from the multiple sequence align-

ment of each cluster of peptide sequences can, in theory, be considered to be potential epitope motifs. However, the accuracy of this approach is unknown and further verification is needed to identify the exact residues involved in binding to an antibody.

Table 4. Consensus motifs of clusters of peptide sequences with a significantly higher count in either inhibitor-negative (INH-) patients or inhibitor-positive (INH+) patients in the plasma-derived FVIII treatment group.

Consensus motif	Mean peptide count* in IHN- Group	Mean peptide count* in IHN+ Group	Adjusted P-value	FVIII Domain(s)	Number of unique peptide sequences in cluster (%)	Peptide count of cluster (%)
PslxWk	5.60	9.90	1.3e-04	B	90 (0.34%)	83908 (0.41%)
qxNxStk	4.10	8.20	7.1e-04	B	52 (0.2%)	59042 (0.29%)
SqnK	8.40	11.40	7.9e-04	B	128 (0.48%)	314257 (1.52%)
Wskp	4.20	8.30	1.2e-03	B	39 (0.15%)	41553 (0.2%)
PHtxk	6.10	10.10	1.3e-03	A2	91 (0.34%)	100570 (0.49%)
pwwp	5.20	8.80	2.4e-03	A1	26 (0.1%)	32816 (0.16%)
PxtFxKp	5.20	8.80	4.2e-03	A1	52 (0.2%)	40083 (0.19%)
iKPxl	4.30	8.40	5.0e-03	B	22 (0.08%)	37313 (0.18%)
sWphxxxxk	6.20	9.70	6.1e-03	C2	41 (0.15%)	94409 (0.46%)
txpmMss	3.70	8.10	1.0e-02	A3	26 (0.1%)	37165 (0.18%)
sGPQ	3.60	7.80	1.0e-02	A2	24 (0.09%)	32688 (0.16%)
nqnK	5.80	10.00	1.2e-02	B	92 (0.35%)	195716 (0.95%)
pdxTpwp	5.00	8.80	1.4e-02	A1	45 (0.17%)	51658 (0.25%)
KxxNexY	7.30	3.70	2.5e-02	A1	57 (0.21%)	81886 (0.4%)

* Mean peptide count is reported as the mean 2log. Total number of unique peptide sequences: 26641. Total peptide count: 20631911.

Furthermore, peptide sequences were clustered based on sequence similarity. However, peptide sequences targeted by the same antibody could have similar physicochemical properties despite not being similar in terms of their amino acid sequence. In this case, clustering based on sequence similarity will not yield optimal results and alternative approaches that take the physicochemical properties of peptide sequences into account might prove more useful.

Each cluster of peptide sequences contained only a small proportion (0.03-1.52%) of the total number of unique peptide sequences available for the clustering step.

Ideally, each cluster would have contained a large proportion of the total number of unique peptide sequences as this would have provided us with stronger evidence for a cluster being related to an epitope.

In addition, the final epitope motifs were mapped to FVIII by aligning the motifs to the linear sequence of FVIII. However, it has been reported that the majority of B-cell epitopes are conformational.[38, 39] (although the exact proportion of B-cell epitopes purported to be conformational is unknown) Therefore, the accuracy of this approach is most likely not high. An alternative approach would involve mapping the epitope motifs to the three-dimensional structure of FVIII, using an in-silico approach. However, a recent study that assessed a set of B-cell epitope prediction algorithms against a benchmark dataset reported that all algorithms performed relatively poorly at mapping a potential epitope to the right location on an antigen.[40]

We removed all peptide sequences that were predicted to be target-unrelated (based on software exploiting publicly available repositories[21]) from the final peptide sequence database. However, the residual impact of target-unrelated peptide sequences that were not removed from the database on the results is difficult to quantify. In addition, some peptide sequences have affinity to both elements of assay as well as an IgG antibody. (i.e. they can be classified as both target-unrelated and target-related peptides) By removing these peptide sequences, we might have inadvertently also removed some important peptide sequences from the initial database.

From the output of the assay, only peptide sequences with a count higher than 250 were selected, this resulted in a much smaller dataset. The cut-off was based on the intra- and inter-assay replicability (Figure S1). It is possible that many peptide sequences that were the target of a FVIII-specific antibody were removed in this step.

Lastly, our analysis of the immune response did not include non-peptidic epitopes (such as the glycans present on the surface of FVIII). One difference between rFVIII and pdFVIII is in their respective glycosylation patterns.[41] Unfortunately, our approach does not allow assessment of the impact of differing glycosylation patterns on immunogenicity.

Conclusion

The reported information on immunodominant epitopes may aid the development of novel, less immunogenic FVIII proteins. In addition, we found several clusters of

peptide sequences that were detectable in patients without any exposure to exogenous FVIII. Information on these clusters could be used to set up diagnostic tests that predict the risk of inhibitor development before starting treatment with FVIII.

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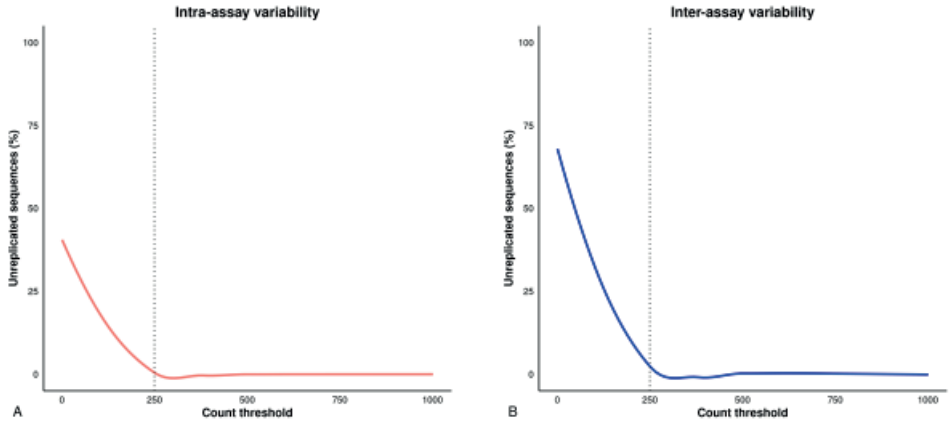
Figure S1. Data quality control. Experimental reproducibility was assessed by comparing technical replicates. Intra-assay reproducibility **(A)** and inter-assay reproducibility **(B)** by minimum count threshold was assessed by calculating the percentage of sequences not present in both technical replicates. A threshold of 250 was chosen to ensure maximum experimental reproducibility.



Chapter 7

Preventing or eradicating factor VIII antibody formation in patients with hemophilia A; what can we learn from other disorders?

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Abstract

Eradication of factor VIII-specific neutralizing antibodies (also known as inhibitors) by the traditional method of immune tolerance induction (ITI) is costly and unsuccessful in one out of three patients. Furthermore, effective inhibitor prevention strategies are presently lacking. An overview is given in this narrative review of anti-drug antibody prevention or eradication strategies that have been used in disorders beyond hemophilia A with the aim of analyzing what we can learn from these strategies for hemophilia A.

Prevention of anti-drug antibody formation using rituximab, methotrexate and intravenous immunoglobulins in patients with Pompe disease seems effective but carries a high risk of adverse events. Based on studies in patients with rheumatoid arthritis and inflammatory bowel disease, it seems likely that treatment with methotrexate alone would also be able to prevent inhibitor formation in hemophilia A patients. Besides side effects, it is unclear whether immune tolerance to FVIII would persist after cessation of immunomodulatory therapy with methotrexate. A combination of cyclophosphamide and corticosteroids, used to treat antibody-mediated pure red cell aplasia, could be further investigated to eradicate inhibitors in hemophilia A patients who are refractory to ITI.

In summary, insights gained from research on anti-drug antibody formation in other diseases could be helpful in devising alternative treatment strategies for inhibitor development.

Hemophilia A

Hemophilia A is a hereditary X-linked hemorrhagic disorder that is caused by genetic mutations in the *f8* gene. These mutations lead to a deficiency of functional clotting factor VIII (FVIII) which is associated with frequent bleeds, especially in joints and muscles. In the long term, repeated joint bleeds cause bleeding-induced joint damage with concomitant disability and reduced quality of life. The disease can be treated by intravenous administration of the deficient factor with FVIII concentrates. The severity of the disease is based on the plasma concentration of clotting factor and is usually classified as severe (‹ 0.01 IU/ml), moderate (0.01-0.05 IU/ml) or mild (› 0.05–0.40 IU/ml). Compared to mild and moderate patients, patients with severe hemophilia A experience more frequent bleeding episodes. In addition, most bleeds in patients with mild or moderate hemophilia A are due to trauma or surgery whereas the majority of bleeds in severe hemophilia A occur spontaneously (i.e., are non-traumatic bleeds).[1]

Inhibitor formation

A major treatment complication in hemophilia A is the formation of neutralizing antibodies against FVIII, also known as inhibitors (because they inhibit the function of FVIII), which renders subsequent treatment with FVIII ineffective. Inhibitor formation is most common in patients with severe hemophilia A, as roughly one in three of these patients develop clinically relevant inhibitors.[2] There is a strong relationship between the incidence of inhibitor formation and the number of days that a patient is exposed to treatment with FVIII (also referred to as the number of exposure days). In patients with severe hemophilia A, inhibitors develop after a median of 15 exposure days[3] and almost all inhibitors occur within the first 75 exposure days[4, 5]. Due to the relatively severe bleeding phenotype of these patients, they are exposed to FVIII at a very young age, especially if prophylactic treatment with FVIII is initiated. Consequently, most severe hemophilia A patients develop inhibitors at a very young age. The median age at which inhibitors were detected was 1.3 years (IQR: 1.0-2.0) in a European registry of 108 severe hemophilia A patients.[6] In patients with neutralizing antibodies, normal doses of FVIII concentrates are no longer effective as prevention or treatment for bleeding. Therefore, these patients need to be treated with FVIII bypassing agents such as recombinant activated factor VII (rFVIIa) or activated prothrombin complex concentrate (aPCC).[7]

Immune tolerance induction

To eradicate inhibitors in hemophilia A patients, frequent administration of high doses of FVIII over a long period of time is needed. This is commonly known as "immune tolerance induction" (ITI).[8] Well known ITI protocols include the "Bonn" protocol (basic protocol: 100-150 IU/kg FVIII twice daily)[9] and the "van Creveld" protocol (starting dose: 25 IU/kg FVIII every other day, dosage is decreased when FVIII recovery exceeds 30%)[10]. The required duration of ITI to obtain tolerance to factor VIII varies per patient. In a large multicenter randomized clinical trial comparing a high (200 IU/kg/day) and a low dose of ITI (50 IU/kg three times/week), the time until complete recovery was 15.5 months (IQR: 10.8-22.0) in the low-dose group and 10.6 months (IQR: 6.3-20.5) in the high-dose group.[11] Inhibitors are successfully eradicated in roughly two-thirds of patients.[12] The inhibitor relapse rate after successful ITI varies between 2.3-10% in most studies.[13-15] As treatment and prophylaxis with rFVIIa or aPCC is less effective and more expensive than treatment with FVIII[7], morbidity among patients with inhibitors is higher and their quality of life is lower than that of patients without inhibitors.[16, 17] In patients with moderate or mild hemophilia A, the inhibitor titer may spontaneously decrease and become unmeasurable due to the continuing production of endogenous FVIII. However, when treatment with a (wild type) FVIII concentrate is indicated, the inhibitor titer may rise again due to an anamnestic response, reflecting lack of sustained tolerance.[18]

Drawbacks of immune tolerance induction

As of now, ITI is the standard treatment for patients with inhibitors. Although ITI is a safe and relatively successful inhibitor eradication strategy, the long duration and high intensity of treatment is very demanding for the usually young patients and their families and it is very costly. In addition, effective treatment options for inhibitor patients who are refractory to ITI are lacking. New inhibitor prevention/eradication strategies are therefore urgently needed to improve patient outcomes and reduce ITI cost.

Preventing or eradicating anti-drug antibody formation: what can we learn from other research disciplines?

The problem of anti-drug antibody formation is not confined to hemophilia A.[19] Many biopharmaceuticals are immunogenic to a certain degree, ranging from a limited immune reaction to a major clinically relevant antibody response. For example, clinically relevant anti-drug antibody formation is (or used to be) a significant problem in patients using tumor necrosis factor (TNF) inhibitors (used in rheumatic diseases)[20], epoetin (used for anemia in chronic renal failure)[21], interferon beta (used in multiple

sclerosis)[22], alglucosidase alfa (used in Pompe disease)[23] and peglocitase (used to treat gout).[24, 25] Most anti-drug antibody research is disease-specific and knowledge is not shared easily across research disciplines.

Promising new therapies to treat or bypass inhibitor development are also underway (e.g. engineered FVIII-specific regulatory T-cells).[26] However, these novel therapies are still out of reach for the near future. There is a need for alternative treatment strategies that can be implemented today, rather than sometime in the future (i.e. that make use of therapeutics that are currently on the market).

Over the last years, many different anti-drug antibody prevention or eradication strategies (mainly using immunomodulatory agents) have been investigated in patients with disorders other than hemophilia A. This review therefore aims to compile information on the efficacy and safety of these different strategies and to contemplate whether knowledge from these other fields might inspire novel treatment strategies for hemophilia A patients.

Prevention of anti-drug antibody formation

What is already known in hemophilia A

In general, risk factors for inhibitor development can be divided into genetic risk factors such as FVIII genotype, ethnicity, HLA-type and genetic polymorphisms that encode proteins involved in the immune system such as IL-10 and TNF-alfa.[27] In addition, there are treatment-related risk factors such as the intensity of FVIII treatment, the frequency of FVIII exposure, FVIII dose, exposure to FVIII during surgery, prophylactic vs. on-demand treatment and the specific type of FVIII product used.[28] As genetic risk factors for inhibitor formation (such as FVIII genotype) are immutable, strategies to prevent inhibitor formation have focused on influencing treatment-related risk factors.

A single-arm study published in 2009 evaluated the effect of a treatment regimen that aimed to minimize the risk of inhibitor formation.[29] The treatment regimen consisted of early initiation of prophylaxis and minimizing exposure to “danger signals” (due to trauma, surgery, infection, vaccination etc.) during FVIII infusion. Surprisingly, only 1/26 (3.8%) patients on this modified treatment regimen developed inhibitors compared to 14/30 (47%) patients in the control group. These results were not replicated in a follow-up study (the EPIC study) as 8/19 (42.1%) patients on the same protocol developed an inhibitor.[30]

As mentioned earlier, there are several novel therapeutics that could be used for preventing inhibitor formation in high-risk patient groups, some of the therapeutics are currently being investigated in patients with hemophilia A. These novel therapeutics include an anti-tissue factor pathway inhibitor antibody (concizumab), a bispecific antibody against FIXa/FX that mimics the function of FVIII (emicizumab), a rFVIIa product with enhanced half-life due to fusion with albumin (rFVIIa-FP) and a short interfering RNA molecule that inhibits production of antithrombin (fitusiran). As of yet, none of these therapeutics have received market approval by the FDA or EMA.[31]

Several animal studies using FVIII-deficient mice have found that a short course of treatment with rapamycin[32], anti-CD20 therapy[33], anti-CD3 therapy[34] or dexamethasone[35] significantly prevented inhibitor formation, even after cessation of the immunomodulatory agent. As of yet, no human studies have evaluated inhibitor prevention with these immunomodulatory agents in patients with hemophilia A.

Prevention of anti-drug antibody formation: what is known from other diseases

Most evidence on the prevention of anti-drug antibody formation comes from patients with Pompe disease and patients with rheumatoid arthritis or inflammatory bowel disease. The following paragraphs will review the available evidence on the efficacy and safety of anti-drug antibody prevention strategies in these patient groups.

Antibodies against recombinant human acid alpha-glucosidase in patients with Pompe disease

There is very limited experience with immunomodulatory therapy to prevent anti-drug antibody formation in very young pediatric patients. Pompe disease (also known as glycogen storage disease type II) is an autosomal recessive lysosomal storage disease caused by a deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA), which leads to accumulation of glycogen in the lysosome. The clinical phenotype is a spectrum that ranges from the classic infantile phenotype (the most severe form in which progressive cardiac hypertrophy is always present) to the late onset "childhood" and "adult" phenotypes.[36]

The overall incidence of Pompe disease is roughly 1:40,000 for all types[37, 38] and 1:138,000 for patients with the classic infantile phenotype[37]. Overall, anti-drug antibody formation is especially problematic in patients with the classic infantile phenotype and less so in patients with the adult-onset phenotype.[39, 40]

The classic infantile form has a more severe and rapidly progressing clinical course than the late-onset childhood and adult forms and is associated with hypertrophic cardiomyopathy, muscle weakness and respiratory distress. In general, untreated patients with classic infantile Pompe disease die within the first year of life.[41, 42]

Enzyme replacement therapy with recombinant human acid alpha-glucosidase (rhGAA) is the only available treatment option. In patients with classic infantile Pompe disease enzyme, replacement therapy is initiated as soon as patients are diagnosed to prevent further clinical deterioration. Roughly, 66-75% of patients with classic infantile Pompe disease have some residual GAA production (CRIM-positive patients) whereas 25-33% produce no GAA at all (CRIM-negative patients).[23, 43, 44] Being CRIM-negative is strongly associated with a low therapeutic response to enzyme replacement therapy.[23] Several studies have shown that the majority (› 90%) of patients with infantile Pompe disease develop anti-drug antibodies, regardless of CRIM-status.[23, 44, 45] Antibody testing is usually performed using an enzyme-linked immunosorbent assay. High-titer antibodies against rhGAA occur more commonly in CRIM-negative patients[44] and are associated with a poor therapeutic response to enzyme replacement therapy. Compared to patients with low-titer antibodies, patients with high-titer antibodies have worse clinical outcomes in terms of overall survival, ventilator-free survival, left ventricular mass index and the Alberta Infant Motor Scale.[46]

Anti-drug antibody formation in other lysosomal storage disorders

Anti-drug antibody formation also occurs in other lysosomal storage disorders, such as Gaucher disease and Fabry disease. Gaucher disease is an autosomal recessive disorder in which the enzyme glucocerebrosidase is deficient, leading to accumulation of glucocerebroside in the lysosomes of cells (mainly macrophages). The most common clinical manifestations are anemia, thrombocytopenia, hepatosplenomegaly and various manifestations of bone disease.[47] Roughly 15% of patients with Gaucher disease develop IgG-antibodies against glucocerebrosidase replacement therapy.[48] Over time, most patients (90%) are tolerized to glucocerebrosidase.[49] Cases of patients with sustained neutralizing antibody activity that impacts clinical efficacy are extremely rare.[50]

Fabry disease is an X-linked disorder in which the enzyme alpha-galactosidase A is deficient, leading to accumulation of globotriaosylceramide in cells. Clinical manifestations during childhood include neuropathic pain and angiokeratoma. In later life, renal, cardiac and cerebral manifestations of the disease become more prominent.[51] Around 73% of men and 12% of women with Fabry disease develop IgG-anti-

bodies against alpha-galactosidase A replacement therapy. Males with Fabry disease have less residual enzyme activity compared to females (because Fabry disease is X-linked) which leads to higher rates of anti-drug antibody formation in males. Anti-drug antibody formation seems to negatively influence biochemical parameters in the blood and urine.[52, 53] The association between anti-drug antibody formation and clinical outcomes is less clear.[53, 54] This is in part caused by the lack of a uniform assay methodology to detect anti-drug antibodies and the limited effectiveness of enzyme replacement therapy in this progressive disorder.[53] Overall, anti-drug antibody prevention/eradication strategies are very rarely applied in patients with Gaucher disease or Fabry disease. The next paragraph will focus on anti-drug antibody prevention strategies in patients with Pompe disease.

Overview of anti-drug antibody prevention strategies in Pompe disease

Several small studies (mostly case-reports or case-series) have evaluated anti-drug antibody prevention strategies; the mostly CRIM-negative patients with Pompe disease in these studies were treated with immunomodulatory agents at the start of enzyme replacement therapy. Because Pompe disease is a progressive disorder, anti-drug antibody formation (that renders enzyme replacement therapy ineffective) leads to irreversible damage. The prevention of anti-drug antibodies would expectedly lead to better outcomes. The four largest case-series, all published between 2013-2017, included 38 CRIM-negative patients that underwent immunomodulatory therapy to prevent anti-drug antibody formation (table 1).

In 2013, a case-series[55] was published that evaluated immunomodulatory therapy in four CRIM-negative patients with Pompe disease at the start of enzyme replacement therapy (table 1). Three patients received an initial cycle of rituximab and maintenance therapy with rituximab, sirolimus and intravenous immunoglobulins (IVIG). One patient received an initial cycle of rituximab and maintenance therapy with mycophenolate and IVIG. IVIG was given to provide passive immunity during the period of B-cell depletion due to rituximab. In total, 1 patient developed high-titer anti-rhGAA antibodies. This was the patient that received maintenance therapy with mycophenolate. The other 3 patients remained antibody negative until the end of the follow-up. Because these patients received maintenance rituximab every 12 weeks, B-cell recovery (defined as having B-cells within normal range after B-cell depletion) was not observed in these patients. No immunomodulation-related adverse events were reported, with the exception of one patient who experienced multiple viral respiratory tract infections during treatment with immunomodulatory agents.

Because of prolonged B-cell depletion, patients were not vaccinated during the study (except with seasonal influenza vaccine).

That same year, another case-series was published, this study reported on 7 CRIM-negative patients with Pompe disease who received immunomodulatory therapy at the start of enzyme replacement therapy to prevent anti-drug antibody formation (table 1).[56] The treatment regimen used (total duration 5 weeks) consisted of rituximab and methotrexate, in addition, IVIG was administered. In total, 4/7 patients were antibody negative until the end of follow-up. The period between B-cell recovery and antibody measurement might have been too short (3.5 months) to assess the effect of treatment in one patient who was antibody-negative. Furthermore, B-cell recovery was not measured at all in another antibody-negative patient. These patients were compared to a historical cohort of 11 CRIM-negative patients who were treated with enzyme replacement therapy alone. Compared to patients treated with immunomodulatory therapy, all patients treated with enzyme replacement therapy alone developed anti-rhGAA antibodies during follow-up. In addition, these patients had significantly worse clinical outcomes (ventilator-free survival and left-ventricular mass index) than patients who were treated with immunomodulatory therapy. One patient developed a possible immunomodulation-related infection and had to be hospitalized.

In 2016, a retrospective analysis reported on 13 CRIM-negative patients from the UK of whom 8 were treated with rituximab and methotrexate at the start of enzyme replacement therapy to prevent anti-drug antibody formation (table 1).[57] One out of 8 CRIM-negative patients treated with immunomodulatory therapy developed intermediate-titer anti-rhGAA antibodies (peak titer was 1:12800 at 8 months old). The remaining 7 patients remained antibody-negative during follow-up. B-cell recovery after initial treatment with rituximab did not occur in 1 patient that remained antibody-negative during follow-up. Furthermore, it is unclear if the follow-up period after B-cell recovery was long enough to assess the effect of treatment in the other 6 patients who remained antibody-negative during follow-up. Another 5 CRIM-negative patients did not receive immunomodulatory therapy, of these, only 2 were tested for antibodies. Both patients had high-titer anti-rhGAA antibodies (peak titer 1:204,000). Survival was higher among CRIM-negative patients receiving enzyme replacement therapy and immunomodulatory therapy when compared to CRIM-negative patients treated with enzyme replacement therapy alone. Information about adverse events during immunomodulatory therapy was not reported.

Table 1. Overview of largest case-series evaluating immunomodulatory therapy in CRIM-negative patients with classic-infantile Pompe disease.

First author (Year of publication)	N	Median age (range) at start of treatment	Treatment
Elder (2013)[55]	4	7 months (2-8)	Initial rituximab cycle (375 mg/m^2 per week for three weeks or two doses of 750 mg/m2 10-14 days apart). Maintenance rituximab (375 mg/m^2 every 12 weeks). sirolimus (initial dose 0.6-1 mg/m^2 per day) or mycophenolate (300 mg/m^2 per day). Monthly IVIG (initial dose 500–1000 mg/kg).
Banugaria (2013)[56]	7	3.5 months (0.4-6.5)	Rituximab (IV, 375mg/m^2 weekly for 4 weeks). Methotrexate (SC, 0.4 mg/kg, three doses per week for 3 weeks) IVIG (400-500 mg/kg, monthly for 5-6 months).
Broomfield (2016)[57]	8	4.3 months (0-6.7)	Rituximab (intravenous, weekly for 4 weeks, dose not reported). Methotrexate (subcutaneous, 3 days per week for 6 weeks, dose not reported).
Kazi (2017)[58]	19	3.4 months (range 0.1–10.9)	The ITI cycle consisted of rituximab, methotrexate, and IVIG. Exact dosing was not reported but probably similar to the study by Banugaria et al.

A study published in 2017 evaluated immunomodulatory therapy to prevent anti-drug antibody formation at the start of enzyme replacement therapy in a larger cohort of 19 CRIM-negative patients.[58] (table 1) The treatment regimen consisted of rituximab, methotrexate, and IVIG. Eight patients never developed antibodies. There was B-cell recovery after depletion with rituximab in all these patients. Similar to the previous study, it was unclear if the follow-up period after B-cell recovery was long enough to assess the effect of treatment. Seven patients had low antibody titers at the end of follow-up (defined as titers ≤ 1:6,400). The remaining 4 patients developed intermediate to high antibody titers. These patients were compared to a historical cohort of 11 CRIM-negative patients who were treated with enzyme replacement therapy alone. All patients treated with enzyme replacement therapy alone developed anti-rhGAA antibodies during follow-up. In addition, these patients had significantly worse survival

Median follow-up (range)	Negative antibody status	Positive antibody status, median peak titer (range)	Suspected treatment-related infections	Anti-drug antibody assay
27.8 months (11-36)	3/4	1/4 (25%), titer not reported	1/4	Enzyme-linked immunosorbent assay.
16.1 months (10.6-23.2)	4/7	3/7, 1:6,400 (1,600-6,400)	1/7	Enzyme-linked immunosorbent assay and confirmation using radioimmunoprecipitation. Performed by product manufacturer.
Not reported	7/8	1/8, 1:12,800	Not reported	Performed by the product manufacturer, exact methodology not reported.
24.2 months (range 6.0-100.2)	8/19	11/19, 1:6,400 (200-51,200)	4/19	Performed by the product manufacturer, exact methodology not reported.

than patients who were treated with immunomodulatory therapy. Four patients who were treated with immunomodulatory therapy developed a serious bacterial infection.

Taken together, 22 out of 38 (58%) CRIM-negative pediatric patients with classic infantile Pompe disease who were treated with rituximab, methotrexate and IVIG to prevent anti-drug antibody formation did not develop anti-rhGAA antibodies. In comparison, literature has shown that virtually all (› 90%) CRIM-negative pediatric patients with classic infantile Pompe disease develop anti-rhGAA antibodies.[23, 44, 45] In patients with Pompe disease, it seems that concomitant immunomodulatory therapy for a short period of time (5-6 weeks) at the start of enzyme replacement therapy is effective in preventing anti-drug antibody formation in a large proportion of patients.

In addition, long-term follow-up results indicate that these patients maintain tolerance to rhGAA after cessation of immunomodulatory therapy. The treatment duration was very short, causing minimal interruption to the vaccination schedule while the rate of adverse effects (such as opportunistic infections) was minimal.

In total, 6/30 CRIM-negative patients with Pompe disease included in the studies by Elder et al.[55], Banugaria et al.[56] and Kazi et al.[58] developed a serious bacterial or viral infection during treatment with rituximab and methotrexate. Apart from infections, no other serious adverse events were reported in these studies. Information about adverse events was not reported for the 8 patients included in the study by Broomfield et al.[57]

Due to suppression of the immune system, the treatment regimen can reduce the response to pediatric vaccinations and may cause severe complications if a live vaccine is administered. Several studies in which rituximab was administered to patients with Pompe disease withheld vaccination and resumed schedule after normalization of the CD19 count (which was used as a marker for B-cell recovery), this took roughly 3-6 months after ending the treatment regimen.[56, 58]

The reported studies had several limitations; the studies evaluating anti-drug antibody prevention strategies in patients with Pompe disease were very small and consisted of case-series (due to rarity of this disorder). Furthermore, the immunomodulatory treatment protocols varied between patients in some studies; some patients underwent several cycles of the same immunomodulatory treatment protocol and other patients received modified versions of the protocol. The median follow-up time may have been too short to adequately assess long-term tolerance to rhGAA. For example, some patients with short-follow-up time may have been antibody-negative, months after cessation of immunomodulatory therapy, due to the lingering immunosuppressive effect of the treatment on B-cells. Lastly, the antibody assay methodology was not uniform and sometimes not reported at all, complicating comparisons between studies and pooling results. Given the drawbacks mentioned above, the results of these studies should be interpreted with caution.

Prevention of anti-drug antibody formation in Pompe disease: which treatment strategies could be considered in hemophilia A

A short course of treatment with rituximab, methotrexate and IVIG was enough to prevent anti-drug antibody formation and induce immune tolerance in CRIM-negative patients with classic infantile Pompe disease. However, roughly 20% of patients

developed a serious bacterial or viral infection. In hemophilia A, far less patients develop clinically relevant anti-drug antibodies and the consequences of anti-drug antibody formation are not as severe. Hemophilia A patients that develop inhibitors can be treated with ITI which has fewer side effects than treatment with immunomodulatory agents. In addition, patients who are refractory to ITI can still be treated with bypassing therapy. Therefore, in pediatric patients with hemophilia A, the benefits of this treatment protocol do not outweigh the potential risks due to adverse events (mainly infections).

Antibodies against TNF-inhibitors in patients with rheumatoid arthritis or inflammatory bowel disease

Anti-tumor necrosis factor monoclonal antibodies (TNF inhibitors) such as infliximab or adalimumab are often used in rheumatoid arthritis (RA) as a second line agent when treatment with non-biologic disease modifying anti-rheumatic drugs (DMARDS) such as methotrexate fails. TNF inhibitors and DMARDS such as azathioprine are also used to treat patients with severe inflammatory bowel disease (i.e. Crohn's disease or ulcerative colitis).

Large proportions of patients with rheumatoid arthritis or inflammatory bowel disease develop antibodies against TNF inhibitors. However, due to heterogeneity in assay methodology, reported incidence rates of anti-drug antibody formation vary widely. Most studies used enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays (RIA).[59] One meta-analysis that included 2350 patients with a variety of chronic inflammatory diseases using infliximab reported that 20.8% of patients had anti-infliximab antibodies.[20] These antibodies also reduce the efficacy of these drugs.[20] Methotrexate and azathioprine are primarily used to treat disease activity in patients with rheumatoid arthritis or inflammatory bowel disease. The immune response against TNF inhibitors may be mitigated by these drugs when they are used in combination.

Overview of anti-drug antibody prevention strategies in rheumatoid arthritis/inflammatory bowel disease

Several randomized controlled trials (RCTs) have evaluated the effect of TNF inhibitor monotherapy vs. combined therapy consisting of a TNF inhibitor and methotrexate/azathioprine on anti-drug antibody formation in patients with rheumatoid arthritis or inflammatory bowel disease. Most large RCTs report a significant decrease in the incidence of anti-drug antibody formation with concomitant use of methotrexate or azathioprine (table 2).[60-64] Anti-drug antibody formation was assessed using ELISA,

Table 2. An overview of the largest RCTs evaluating the effect of concomitant immunomodulation on anti-drug antibody formation against TNF inhibitors in patients with rheumatoid arthritis or inflammatory bowel disease. All patients were antibody-negative at baseline.

Study author, year of publication, disease	Treatment
Colombel, 2010 (Crohn's disease) [60]	
Infliximab	Intravenous infliximab, 5mg per kg at weeks 0, 2, 6 and then every 8 weeks.
Infliximab + Azathioprine	Intravenous infliximab, 5mg per kg at weeks 0, 2, 6 and then every 8 weeks. Oral azathioprine, 2.5mg per kg daily.
Panaccione, 2014 (ulcerative colitis)[61]	
Infliximab	Intravenous infliximab, 5mg per kg at weeks 0, 2, 6 and 14.
Infliximab + Azathioprine	Intravenous infliximab, 5mg per kg at weeks 0, 2, 6 and 14. Oral azathioprine, 2.5mg per kg daily.
Matsumoto, 2016 (Crohn's disease)[62]	
Adalimumab	Subcutaneous adalimumab, 160mg at week 0, 80mg at week 2 and then 40 mg every other week.
Adalimumab + Azathioprine	Subcutaneous adalimumab, 160mg at week 0, 80mg at week 2 and then 40 mg every other week. Oral azathioprine, maximum 100 mg daily (dose escalation from 25mg or 50 mg daily to 100mg daily during the first 4 weeks).
Emery, 2009 (rheumatoid arthritis)[63]	
Golimumab	Subcutaneous golimumab, 100mg once monthly.
Golimumab + Methotrexate	Subcutaneous golimumab, 50mg or 100mg once monthly. Oral methotrexate, 20mg per week (dose escalation from 10mg per week during the first 8 weeks).
Kremer, 2010 (rheumatoid arthritis)[64]	
Golimumab	Intravenous golimumab, 2mg per kg or 4mg per kg every 12 weeks.
Golimumab + Methotrexate	Intravenous golimumab, 2mg per kg or 4mg per kg every 12 weeks. Oral methotrexate, 15-25mg per week.

* Positive for antibodies according to the study's definition.
IQR: interquartile range, SD: standard deviation.

	Age	Follow-up	antibody-positive*/Total N (%)	Anti-drug antibody assay
		30 weeks		Enzyme-linked immunosorbent assay
	Median: 35.0 years (IQR: not reported)		15/103 (14.6%)	
	Median: 34.0 years (IQR: not reported)		1/116 (0.9%)	
		16 weeks		Assay not reported
	Mean: 38.5 years (SD: 12.7)		7/37 (19%)	
	Mean: 38.0 years (SD: 12.2)		1/31 (3%)	
		26 weeks		Radioimmunoassay
	Mean: 29 (SD: 12)		10/76 (13.2%)	
	Mean: 32 (SD: 12)		3/75 (4.0%)	
		24 weeks		Electrochemiluminescence immunoassay
	Mean: 48.2 (SD: 12.85)		14/104 (13.5%)	
	Mean: 50.6 (SD: 11.58)		6/211 (2.8%)	
		48 weeks		Enzyme-linked immunosorbent assay
	Mean: 49.2 (SD not reported)		17/194 (9%)	
	Mean: 49.6 (SD not reported)		10/299 (3%)	

RIA or electrochemiluminescence immunoassay (ECLIA). All RCTs presented in table 2 only included patients that had not been previously treated with the specific TNF inhibitor that was used. Similarly, a systematic review and meta-analysis that included both observational and interventional studies (n = 2611) estimated that concomitant treatment with DMARDs, mainly methotrexate and azathioprine, significantly prevented the risk of anti-drug antibody formation in patients with a variety of chronic inflammatory diseases using a TNF inhibitor (OR: 0.32, 95%CI: 0.25-0.42).[65]

Thus, strong evidence exists that methotrexate and azathioprine significantly prevent anti-drug antibody formation in antibody-negative patients with rheumatoid arthritis and inflammatory bowel disease. However, there were no methodologically sound comparative studies that assessed whether the immunomodulatory effect persisted after cessation of methotrexate or azathioprine.

Prevention of anti-drug antibody formation in rheumatoid arthritis and inflammatory bowel disease: which treatment strategies could be considered in hemophilia A

It is very likely that methotrexate or azathioprine would be able to prevent anti-drug antibody formation in hemophilia A patients. Compared with the treatment protocol consisting of rituximab, methotrexate and IVIG currently used to treat anti-drug antibody formation in CRIM-negative patients with Pompe disease, using only methotrexate or azathioprine would have a more favorable safety profile. However, whether immune tolerance to FVIII would persist after cessation of immunomodulatory therapy with methotrexate or azathioprine remains unknown.

The patients in the aforementioned studies received doses of methotrexate that were high enough to produce a therapeutic response. It is possible that a lower dose, with a reduced risk of adverse events, could be enough to prevent anti-drug antibody formation. Based on the studies conducted in patients with Pompe disease, these immunomodulatory agents would not have to be administered indefinitely. A short course of methotrexate at treatment initiation (e.g. during the first 10-20 exposure days to FVIII) could be sufficient to prevent anti-drug antibody formation and induce immune tolerance. However, studies evaluating methotrexate in very young pediatric patients are lacking. The mean age of patients included in each study reported in table 2 varied from 29.0-50.6 years old. In contrast, most severe hemophilia A patients that develop an inhibitor do so at the age of 1-2 years old.[6] Nevertheless, this treatment strategy could be a target for further investigation in patients at high risk for persistent inhibitors. In this case, an accurate model to predict the risk of persistent inhibitor development would be needed.

Eradication of anti-drug antibodies

What is already known in hemophilia A

Inhibitor eradication strategies using immunomodulatory agents have been tried since the 1970s.[66, 67] Nowadays, immunomodulatory agents are mostly used as second-line therapy in patients who have already failed ITI.[68] The agents are generally administered as adjunctive therapy in combination with ITI. One of the most well-known examples is the Malmö protocol which consists of extracorporeal immunoadsorption, followed by cyclophosphamide and IVIG in combination with high-dose ITI.[69]

In 2014, a systematic review assessed the effect of immunomodulatory agents (alone or in combination with ITI) on inhibitor eradication success rates.[68] In total, 46 case reports or case-series were included, comprising 208 patients. Complete recovery was defined as having a negative inhibitor titer, having normalized pharmacokinetic parameters was not mandatory. In most cases, immunomodulatory agents were administered concomitantly with ITI. Many patients had previously failed first-line treatment with ITI and had high peak inhibitor titers.

Most patients were treated with either cyclophosphamide (alone or in combination with other drugs) with a complete recovery rate of 40-44%. The second most used immunosuppressive agent was rituximab (alone or in combination with other drugs) which was associated with a complete recovery rate of 40-63%. As most patients failed previous ITI and had a poor prognosis for treatment success, the aforementioned success rates are quite good. However, as case-reports and case-series with positive results are far more likely to be published[70], the published recovery rates are most likely an overestimation of the true recovery rate. In addition, it was unclear if the follow-up time was long enough to accurately assess the relapse rate for most patients.

Overall, current evidence on the effectiveness of ITI in combination with an immunomodulatory agent such as rituximab or cyclophosphamide is inconclusive, because of small studies with methodological limitations.[68, 71] As far as we know, no randomized studies have been performed.

Very few studies have evaluated rituximab monotherapy, this treatment could be useful due to the low costs of treatment when compared to the high cost of ITI.[72] The largest study is a non-comparative trial from 2014 in which the effectiveness of monotherapy with rituximab was studied in 16 inhibitor patients with inhibitor titers › 5

BU (13 patients had failed previous ITI).[73] Only three out of 16 patients had a drop in inhibitor titer below 5 BU during follow-up and persistent tolerance after re-challenge with FVIII. These results suggest that inhibitor eradication with rituximab monotherapy is not as good as ITI. However, the treated group consisted of patients with a poor prognosis who failed ITI.

Eradication of anti-drug antibodies: what is known from other diseases

Anti-drug antibody eradication strategies have been extensively described for patients with antibody-mediated pure red aplasia due to epoetin use and multiple sclerosis patients with antibodies against interferon beta. The following paragraphs will review the available evidence on the efficacy of anti-drug antibody eradication strategies in these two patient groups.

Antibody-mediated pure red cell aplasia due to epoetin use in patients with chronic kidney disease

Antibody-mediated pure red cell aplasia (PRCA) is a rare but severe side-effect of treatment with epoetin (recombinant human erythropoietin) in patients with reduced production of endogenous erythropoietin, which is most often due to severe chronic kidney disease (CKD). PRCA is caused by the formation of antibodies against epoetin, that also cross-react with endogenous erythropoietin. This leads to profound anemia, a very low reticulocyte count and very low levels of erythroid precursors in the bone marrow. Antibody-mediated pure red cell aplasia bears some similarity to inhibitor formation in mild hemophilia A; in both conditions, antibodies against an exogenous protein (FVIII/epoetin) cross-react with the endogenous protein (FVIII/erythropoietin). The most commonly used assays to detect anti-drug antibodies are radioimmunoprecipitation assays (RIPA) or ELISA. Testing for neutralizing antibodies using an assay that measures in-vitro inhibition of epoetin activity by antibodies is available but rarely used.[74]

Overview of anti-drug antibody eradication strategies in antibody-mediated pure red cell aplasia

Around 200 cases of antibody-mediated PRCA occurred between 1998-2004 and almost all were associated with the use of a particular epoetin product (tradename: Eprex). The increased immunogenicity of this product during this time period was likely due to a formulation change.[75]

A retrospective analysis[76] evaluated the long-term outcome (median follow-up: 9 months) of 170 patients with antibody-mediated PRCA due to epoetin use. Out of

170 patients, 19 patients received a renal transplant (with concomitant immunosuppression), 89 non-transplant patients received immunosuppressive treatment to eradicate anti-drug antibodies while 62 patients did not receive any treatment. In total, 44/89 (49%) non-transplant patients that were treated with immunosuppressive agents to eradicate anti-drug antibodies achieved hematological recovery. In comparison, only 1/62 (2%) patients who received no immunomodulatory treatment achieved hematological recovery. Hematological recovery was defined as having ≤ 1 red blood cell transfusion per month, hemoglobin levels ≥ 80 g/L (8 g/dL) and a reticulocyte count › 20 × 10^9/L. The specific type of anti-drug antibody assay(s) used and the immunosuppressive treatment that patients underwent were not accurately reported. However, the authors report that most of the more recently diagnosed patients were treated with prednisone (alone or in combination with IVIG), cyclophosphamide (alone or in combination with prednisone) or cyclosporine. Treatment with epoetin after hematological recovery was rare; nevertheless, 19/34 (56%) patients who were re-challenged with epoetin had good clinical response to epoetin. It was not reported if patients were re-challenged with Eprex or a different epoetin product. Good clinical response to epoetin was defined as having stable hemoglobin level ≥ 80 g/L (8 g/dL) and independence from red blood cells transfusions. The best predictor of good clinical response to epoetin was a negative-antibody status at re-initiation of epoetin therapy.

In a retrospective analysis of 47 patients with PRCA the efficacy of anti-drug antibody eradication strategies was evaluated.[77] Nine patients were not treated with any kind of immunomodulatory therapy, none of these patients recovered during follow-up (median follow-up: 12 months, IQR: 8-13). Eleven patients were treated with multiple different immunosuppressive treatment protocols (the exact type of treatments were not accurately reported), the remaining 26 patients received one type of treatment. Three treatment regimens were most commonly used; 7/8 (87%) patients treated with corticosteroids and cyclophosphamide, 4/6 (67%) patients treated with cyclosporine and 10/18 (56%) patients treated with corticosteroids with/without IVIG achieved hematological recovery (table 3). None of the recovered patients had a relapse during the follow-up period (duration of follow-up was not reported).

Thus, treatment with immunomodulatory therapy alone seems to be effective at eradicating anti-drug antibodies in CKD patients with antibody-mediated PRCA. The highest rate of hematological recovery (87%) was achieved by using a combination of corticosteroids and cyclophosphamide.[77] Around 56% of patients with successful hematological recovery had good clinical response to epoetin.[76] The exact treatment

protocols were not reported and probably varied significantly on a case-by case basis. More importantly, only a small proportion of patients were re-exposed to epoetin, and the level of exposure (intensity, frequency) was not reported. Therefore, the actual success rate of the used immunomodulatory agents is not known.

Eradication of anti-drug antibodies in antibody-mediated pure red cell aplasia, which treatment strategies could be considered in hemophilia A

Overall, the immunomodulatory agents used to treat antibody-mediated PRCA could be considered in hemophilia A patients who are refractory to ITI. However, the reported success rates of the aforementioned anti-drug antibody eradication strategies (which varied from 56%-87%) will expectedly be lower when applied to hemophilia A patients with inhibitors. This is because inhibitors in hemophilia A patients who are refractory to ITI are expected to be more difficult to eradicate. Moreover, a proportion of patients who were initially treated successfully will have an anamnestic response to FVIII after re-exposure (lowering the overall success rate even further). Therefore, these anti-drug antibody eradication strategies might not be as effective in hemophilia A patients with inhibitors who are refractory to ITI.

Table 3. Overview of most commonly used immunomodulatory agents used to treat antibody-mediated pure red cell aplasia.*

Treatment**	Time to recovery	Hematological recovery***
Corticosteroids, oral, starting dose: 1 mg/kg/day. Cyclophosphamide, oral, dose not reported.	Median duration: 3 months (range: 1-7)	7/8 (87%)
Cyclosporine, oral, 200 mg/day.	‹ 3 weeks for all patients	4/6 (67%)
Corticosteroids, oral, starting dose: 0.5-1 mg/kg/day with (n = 14) or without (n = 4) IVIG, 0.4 mg/kg daily for 5 days every 6 weeks.	Median duration: 3 months (range: 1-18)	10/18 (56%)

* Table adapted from Verhelst et al.[77]

** Some patients were treated with multiple different immunosuppressive regimens. Consequently, the total number of treated patients is unknown.

*** Hematological recovery was defined as being transfusion-independent and having a reticulocyte count › 20,000/μL

Alternatively, these treatment options could be used to treat patients in low-resource countries as a first-line treatment strategy if ITI is not available because of the costs. However, because of poor access to antibiotics and medical care in general, a severe treatment-related bacterial infection in a pediatric patient in a low-resource country

would also be more difficult to treat and therefore the benefits of this approach are not expected to outweigh the risks.

Antibodies against interferon beta in patients with multiple sclerosis

Multiple sclerosis is an auto-immune disease that is characterized by demyelination of the spinal cord and brain. This leads to neurological symptoms such as motor and sensory problems, paresthesia and cognitive impairments. Patients with relapsing-remitting multiple sclerosis (RRMS) are often initially treated with interferon beta-1a or interferon beta-1b, these products are associated with relatively high rates of anti-drug antibody formation. One study among 1115 Swedish and Icelandic patients reported an overall prevalence of neutralizing anti-drug antibodies of 32% using a Myxovirus resistance gene-A (MxA) protein assay. It is difficult to estimate the exact prevalence of anti-drug antibody formation in patients using interferon beta because the assay methodologies used to detect anti-drug antibody formation are highly heterogeneous.[78, 79] However, neutralizing antibodies seem to be slightly associated with a reduced therapeutic effect of these products.[80]

Glucocorticoids are used to treat exacerbations of multiple sclerosis in adults because of their anti-inflammatory and immunosuppressive effects.[81] Less often, monthly therapy with glucocorticoids is used with the aim of reducing long-term disability outcomes in patients with relapsing-remitting multiple sclerosis.[82, 83]

Overview of anti-drug antibody eradication strategies in multiple sclerosis patients using interferon beta

Between 2002-2009, 3 comparative trials (327 patients in total) have evaluated the use of monthly pulse therapy with methylprednisolone to prevent or eradicate antibody formation in patients with relapsing-remitting multiple sclerosis. At baseline, some or all patients were positive for antibodies against interferon beta. The results of these three studies are discussed in the following paragraphs and summarized in table 4.

In 2002, an open label RCT reported on 161 patients that were treated with either interferon beta 1b (n = 81) or interferon beta-1b in combination with methylprednisolone (n = 80).[84] The presence of neutralizing anti-drug antibodies was assessed using a Myxovirus resistance gene-A (MxA) protein assay. Antibody status of patients at baseline was not reported. After 12 months 26.8% of patients treated with interferon beta-1b and 12.1% of patients treated with interferon beta-1b + methylprednisolone had one or more samples that were antibody-positive (relative reduction 54.9%,

Table 4. An overview of studies evaluating the effect of concomitant immunomodulation with methylprednisolone on antibody formation against interferon-beta in patients with multiple sclerosis.

Study (year of publication)	Treatment	Study design
Pozzilli (2002)[84]		RCT
Interferon beta	INFB: Subcutaneous interferon beta-1b, 8 MIU every other day.	
Interferon beta + Methylprednisolone	INFB+MP: Subcutaneous interferon beta-1b, 8 MIU every other day. Intravenous methylprednisolone, 1000mg 1 x per month.	
Sorensen (2009)[85]		RCT
Interferon beta	IFNB: Subcutaneous interferon beta-1a, 44 µg 3 x per week	
Interferon beta + Methylprednisolone	INFB+MP: Subcutaneous interferon beta-1a, 44 µg 3 x per week. Oral methylprednisolone, 200mg on 5 consecutive days monthly.	
Hesse (2009)[86]		Non-randomized trial
Control group	-	
Methylprednisolone	MP: Oral methylprednisolone, 500mg on 3 consecutive days monthly.	

* Positive for antibodies according to the study definition.
RCT randomized controlled trial
SD standard deviation

	Age	antibody-positive* / Total N at baseline (%)	Follow-up	Antibody-positive/ Total N at study end (%)	Neutralizing anti-drug antibody assay
			15 months		Myxovirus resistance gene-A protein assay
	Mean: 33.1 years (SD: 8.1)	?/81 (?%)		19/71 (26.8%)	
	Mean: 31.2 years (SD: 6.7)	?/80 (?%)		8/66 (12.1%)	
			96 weeks		Antiviral neutralization bioassay
	Mean: 39.5 years (SD: 7.8)	16/46 (35%)		13/43 (30%)	
	Mean: 37.8 years (SD: 7.4)	12/47 (26%)		9/39 (23%)	
			6 months		Cytopathic effect assay
	Median: 41 years (range: 22–59)	35/35 (100%)		33/35 (94%)	
	Median: 43 years (range: 27–62)	38/38 (100%)		36/38 (95%)	

p=0.05). Although the reduction in antibody formation was significant, the number of disease relapses and the progression of disability during the first year of treatment were similar (table 4).

In 2009, an RCT assessed treatment with either interferon beta-1a (n = 46) or interferon beta-1a in combination with methylprednisolone (n = 47).[85] The presence of neutralizing anti-drug antibodies was assessed using an antiviral neutralization bioassay. Thirty-five percent of patients treated with interferon beta-1a alone and 26% of patients treated with interferon beta-1a + methylprednisolone were already antibody positive at baseline. There was no significant difference in the cumulative incidence of anti-drug antibody formation between groups, 30% of patients on interferon beta-1a and 23% of patients on interferon beta-1a + methylprednisolone were antibody-positive after 96 weeks (table 4).

Lastly, a non-randomized clinical trial evaluated if methylprednisolone could be used to restore interferon beta bioactivity in antibody positive patients with absent in vivo response to interferon beta who had discontinued interferon beta therapy.[86] The presence of neutralizing anti-drug antibodies was assessed using a cytopathic effect assay. Thirty-eight patients were treated with methylprednisolone and 35 patients were not treated. The in vivo response to interferon beta and antibody status were similar after 6 months (table 4).

Overall, evidence from studies in patients with multiple sclerosis suggests that methylprednisolone is not effective for preventing or eradicating antibodies against interferon beta.

Eradication of anti-drug antibodies in multiple sclerosis: which treatment strategies could be considered in hemophilia A

It seems that monthly treatment with methylprednisolone has no added benefit in terms of preventing or eradicating antibodies against interferon beta. In contrast, using oral corticosteroids to treat patients with antibody-mediated PRCA was a moderately successful strategy. (Table 3) The difference in efficacy may be partly explained by the fact that in patients with antibody-mediated PRCA, oral corticosteroids were mostly given in combination with IVIG or cyclophosphamide. Given the aforementioned results, inhibitor eradication in hemophilia A patients using methylprednisolone alone should not be considered as it is not expected to be effective.

Conclusion

Insights gained from clinical research into anti-drug antibody formation in other diseases could be helpful in devising alternative treatment strategies for inhibitor development in hemophilia A. Immune modulatory treatment can be associated with potentially severe side effects. The benefits of this treatment however, may outweigh the potential risks in subgroups of inhibitor patients with poor prognosis.

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Chapter 8

Summary and general discussion

In the last 50 years, hemophilia treatment has changed tremendously. The first aim of this thesis was to comprehensively assess the changes in health status over time of patients with hemophilia. Although hemophilia treatment has improved in many ways, inhibitor development continues to be a significant problem in patients treated with clotting factor products. Therefore, the second aim of this thesis was to evaluate different strategies to identify patients at a high risk of inhibitor development and to present an overview of anti-drug antibody strategies that could potentially be applied to these patients. We will first summarize the main results of these studies, which were reported in Chapters 2-7, and then discuss their wider implications for clinical practice and future research.

Summary of main results

Chapter 2 In this chapter, we report the results of a cross-sectional nationwide survey held in 2019 among patients with hemophilia in the Netherlands. We assessed how treatment changes have influenced major clinical outcomes over time by comparing the current results to those of similar surveys that have been previously conducted (in 1972, 1978, 1986, 1992 and 2001).[1-3] This study showed that over the course of almost 50 years the increased use of prophylactic therapy has improved joint health and that the development of effective hepatitis C treatment options has nearly eradicated hepatitis C infection. In 2019, the self-reported annual bleeding rate was zero for the majority of patients. However, patients aged › 50 years still suffered from hemophilia-related complications, especially complications arising from joint damage accrued in the past.

Chapter 3 In this chapter, we assessed overall mortality and causes of death among patients with hemophilia in the Netherlands from 1972-2018. We conducted a cohort study where 1066 patients with hemophilia who participated in a nationwide survey in 2001 were followed until July 2018. These new results were then compared with the results of similar cohort studies from earlier time periods.[4-6] The life expectancy of patients with hemophilia in the Netherlands strongly improved over time but was still lower than that of the general male population in 2018 (life expectancy estimates were 77 years versus 83 years respectively). Mortality due to HIV and HCV-related complications among patients with hemophilia has decreased over time but is still higher than that of the general population. In addition, mortality due to intracranial bleeding was also much higher among patients with hemophilia when compared to the general population. Lastly, deaths due to ischemic heart disease were consistently low during the entire 46-year follow-up period.

Chapter 4 In this chapter, we assessed the immunogenicity of recombinant-derived FVIII products in patients with hemophilia A who were exposed to FVIII for at least 50 days (also called previously treated patients or PTPs) using a meta-analysis approach. All studies that reported on de novo inhibitor development in PTPs with < 0.02 IU mL^{-1} factor activity were included. Using a random intercept Poisson regression model, we calculated the overall pooled incidence rate of inhibitor development, as well as the relative risk of inhibitor development of the different types of FVIII products included in the analysis. The overall pooled incidence rate of inhibitor development was 2.06 per 1000 person-years (95%CI: 1.06–4.01). Compared to Advate, the relative risk of inhibitor development was almost ten times higher among patients using Kogenate/Helixate and roughly 14 times higher in patients using Refacto. (both comparisons were statistically significant) These results suggest that some products may be associated with increased immunogenicity in PTPs.

Chapter 5 In this chapter, we aimed to develop and evaluate a clinical prediction model for inhibitor development. A cohort of 251 previously untreated patients (PUPs) or minimally treated patients (MTPs) previously enrolled in the SIPPET study[7] were used as the study population. Model discrimination was assessed using Harrell's C-statistic and model calibration was assessed visually using a calibration plot. The model consisted of four predictors: *F8* gene mutation, intensity of first treatment with FVIII, the presence of FVIII non-neutralizing antibodies before treatment initiation and FVIII product type (recombinant vs. plasma-derived). The C-statistic of the model was poor (0.66, 95 CI: 0.57–0.75) and calibration was moderate. Using a model cut-off point of 10%, positive- and negative predictive values were 0.22 and 0.95, respectively. Therefore, although the overall performance of the model was poor, it could be useful for identifying a small number of patients with a low risk of inhibitor formation.

Chapter 6 In this chapter, the FVIII-specific IgG epitope repertoire of 39 inhibitor-positive patients and 83 inhibitor-negative patients who were followed for 50 days of exposure to FVIII (EDs) after treatment initiation was explored by means of a novel high-throughput epitope mapping technique.[8] In short, a library of roughly 10^9 randomly generated 12-mer peptide sequences expressed on the surface of M13 bacteriophages were screened against each patient's IgG antibody repertoire. Bacteriophages with unbound peptide sequences were washed away and the DNA of the remaining bacteriophages was analyzed using next generation sequencing to identify the peptide sequences that were bound by IgG antibodies. For each patient, the assay was performed three times; pre-treatment using a standard sample, post-treatment using a standard sample and post-treatment using a FVIII-specific antibody depleted

sample. Using this method, we isolated a set of 12-mer peptide sequences with high affinity to FVIII-specific antibodies. These peptide sequences were then clustered on the basis of sequence similarity. For each cluster, a consensus motif was generated which was then aligned to the linear sequence of FVIII. The degree to which these clusters of peptide sequences could be used to discriminate between patients with and without an inhibitor was assessed using the C-statistic. We found that the FVIII-specific antibody response was highly polyclonal, with many clusters being identified and mapped onto different parts of FVIII. The most predominant clusters in inhibitor-positive patients were mapped to the heavy chain of the FVIII molecule. In the pre-treatment samples, three clusters of peptide sequences (with the consensus motifs "pxyNw", "PSLxWK" and "SWPHxxxxK") were identified that predicted inhibitor development after initiation of treatment with FVIII (with a C-statistic of 0.76, 0.80 and 0.76 respectively).

Chapter 7 In this chapter, we reviewed the literature to explore anti-drug antibody prevention strategies applied to patients with diseases other than hemophilia, with the aim of identifying anti-drug antibody prevention strategies that could provide targets for further research for immune tolerance induction (ITI) in patients with hemophilia. Several case-series reported a reduction in anti-drug antibodies using a combination of rituximab, methotrexate, and intravenous immunoglobulins in patients with Pompe's disease treated with recombinant human acid –glucosidase enzyme therapy. In patients with rheumatoid arthritis, multiple large randomized controlled trials showed that the use of methotrexate reduced the presence of anti-drug antibodies against TNF inhibitors when these two drugs where used concomitantly.

Discussion

The health status of the Dutch hemophilia population

In Chapter 2 and 3, we comprehensively assessed the current health status of the Dutch hemophilia population and compared it with the general population. We also explored how health outcomes have changed over time as a result of treatment changes and non-treatment related factors (such as the HIV/HCV epidemic). These findings underscore the successes of 50 years of hemophilia treatment, as well as highlight areas where the current treatment guidelines may be improved upon.

This is most likely the last population wide assessment of the health status of the Dutch hemophilia population where the vast majority of patients with severe hemophilia were still treated with standard or extended half-life clotting factor products.

Currently many new novel agents are entering the market or are in the process of obtaining market approval. In particular, the uptake of emicizumab[9] (a bispecific antibody mimicking FVIII) in the Dutch population is very high due to its effectiveness, the fact that it can be administered subcutaneously and its long half-life. It is to be expected that most patients with severe hemophilia A will switch to emicizumab in the next years. The main limitation of emicizumab is that clotting factor products are still needed to treat breakthrough bleeds. For patients with hemophilia B, no non-factor replacement therapy options exist at the moment. Fitusiran (a siRNA that suppresses antithrombin) and concizumab (an anti-TFPI inhibitor) are the drugs that are closest to market approval.[9] Both drugs can be used in both hemophilia A and B patients. However, both drugs have had issues with thrombotic complications, leading to temporary cessation of phase 2 clinical trials.[9] Therefore, it is as of yet unclear if these drugs will actually be available in the future.

In the coming years, it is highly likely that the first gene therapy options for hemophilia will be approved. For the first time, there is the potential for a cure for patients with hemophilia. However, there are still some challenges to be overcome before gene therapy can be readily implemented in all patients. Many patients develop transient liver toxicity in the early phase which is sometimes associated with reduced gene expression.[10] In addition, due the presence of pre-existing neutralizing antibodies against the viral vector, many patients are currently ineligible for treatment.[10] Furthermore, the application of gene therapy in children with hemophilia is challenging as loss of transgene expression may occur as the liver grows and the development of humor immunity precludes re-administration of the same viral vector.[10]

The results described in Chapters 2 & 3 may serve as a benchmark against which the effect of these novel treatment modalities on health outcomes may be compared in the future.

Our study showed that, with a median bleed rate of zero, 44% of patients with severe hemophilia still experienced at least one joint bleed per year despite prophylactic treatment. It was not clear if these bleeds were mostly spontaneous or caused by physical trauma. If spontaneous, the incidence of these bleeds could be reduced by increasing the target trough levels using the bleeding phenotype as a guide, by

pharmacokinetic-guided dosing or by switching to emicizumab (if the patient has hemophilia A). Despite this, some patients might still bleed due to non-adherence problems or due to having a target joint that is more prone to bleeding in general. Reducing physical activity to decrease the number of bleeds is not recommended, as it has not been linked to an increased bleeding rate and is beneficial to both physical health and mental health.[11] Overall, a more personalized treatment approach is needed in order to get the joint bleed rate down to zero in this group of patients.

Furthermore, our study set-up (which consisted of several cross-sectional studies) was not suitable to assess the impact of primary prophylaxis on long-term joint damage. (as this would require longitudinal follow-up of individual patients) Joint damage is a gradual process that takes decades to manifest. Current FVIII target trough levels of 1% are enough to prevent most but not all bleeds.[12] In addition, even patients without any clinically evident bleeding (i.e. an annual joint bleeding rate of zero) may still develop significant joint deterioration later in life due to subclinical joint bleeds.[13] Long-term cohort studies are needed to assess if pediatric patients in our study with subclinical joint bleeds are still at risk of developing significant joint damage in older age. If so, this might necessitate an increase in the target FVIII/FIX trough levels or a different treatment approach altogether. In the past, the number of weekly infusions needed to maintain higher trough levels would have made this strategy infeasible. However, with the newer extended half-life products or emicizumab, it is now practically feasible to increase trough levels to much higher levels.

In addition, we reported that a quarter of patients had moderate-to-severe liver fibrosis after HCV eradication. Despite clearing the virus, these patients remain at high risk of for HCV-related complications such as hepatocellular carcinoma[14] and should therefore be closely monitored.

Our data show that death due to intracranial bleeding was the second most common cause of death (after cancer) and that patients with hemophilia had a 13-fold higher chance of dying of an intracranial bleed than the general male population. In our study, all deaths due to intracranial bleeding occurred in adults (the youngest was 44 years old) and were non-traumatic. Studies have shown that the lifetime risk of intracranial bleeding in patients with hemophilia follows a bimodal distribution, with a peak around the perinatal period and infancy, as well as another peak in old age.[15]

Furthermore, our data show a high rate of hypertension among older patients with hemophilia, which is a strong independent risk factor for developing an intracranial

bleed. The biological pathways that cause the increased rate of hypertension are not clear. Some have speculated that bleeding-induced vascular remodeling in the kidneys may be the cause of the hypertension seen in these patients[16], but no studies have assessed this hypothesis as of yet. From a clinical standpoint, our findings suggest that early monitoring of patients with hemophilia for hypertension might be needed in order to reduce the risk of intracranial bleeding in adults as much as possible.

An increase in target trough levels for prophylaxis might decrease the incidence of intracranial bleeding and intracranial bleeding-related mortality in adults. In children, difficulties with venous access in the first days/months after birth precludes the use of prophylaxis with conventional clotting factor products. The fact that emicizumab that can be administered subcutaneously makes it possible to start prophylaxis almost immediately after birth to prevent intracranial bleeds in patients with severe hemophilia A. Currently, this potential use-case for emicizumab has not been implemented yet but has the support of the Scientific Advisory Council of the National Hemophilia Foundation.[17]

Identifying and treating patients at a high risk of inhibitor development

Our results show that clinical outcomes in patients with hemophilia have improved tremendously over the past decades. One major unresolved problem that still remains, however, is that many patients treated with FVIII develop inhibitors. Even patients that are switching to prophylactic treatment with emicizumab will still need FVIII to treat breakthrough bleeds. It is as of yet unclear how prophylaxis with emicizumab will impact inhibitor development. It could be possible that inhibitor development will actually be higher in these patients, as they will only be exposed to FVIII in the context of trauma or surgery. Regardless of the choice in treatment product, inhibitor development will continue to be a significant problem in the near future.

Therefore, the second aim of this thesis was to evaluate different strategies to identify patients at a high risk of inhibitor development and to present an overview of anti-drug antibody strategies that could potentially be applied to these patients. The results of these studies, which were reported in Chapters 4-7, are summarized and their wider implications for clinical practice and future research are discussed below.

Using three different study approaches, we tried to identify patients who have a high risk of inhibitor development. We first assessed how different recombinant FVIII products influenced the rate of inhibitor development in PTPs with severe or moderately severe hemophilia. In a systematic review and meta-analysis of the literature.

(Chapter 4) We found that Kogenate/Helixate and Refacto were associated with increased immunogenicity, compared to Advate.

There are several hypotheses that could explain the increased immunogenicity of these products such as differences in the amino acid sequence, culture conditions, stabilizing agents and/or the type of cell culture used for production.[18] However, due to the rarity of inhibitor development in this group, and the lack of adjustment for confounding factors (e.g. the type of *F8* gene mutation), it is difficult to draw any definite conclusions from the data. Future research on inhibitor development in PTPs should focus on creating standardized reporting systems. Good examples of this are the various national and international registries such as the Dutch HemoNED registry and the European EUHASS registry.[19]

In Chapter 4, we assessed only a single factor (product type) and its association with inhibitor development in PTPs. In contrast, Chapter 5 was aimed at combining different factors into one model to predict inhibitor development in PUPs. The newly developed clinical risk prediction model was poor at identifying patients at high risk of inhibitor development. However, the model was able to accurately identify a small number of patients with a low risk of inhibitor formation. There are several approaches to improve the accuracy of future prediction models. For example, one could incorporate information on other genetic risk factors for inhibitor development (e.g. the *CTLA-4* or *IL10* genes) into the risk score. However, if accurate prediction of inhibitor formation at baseline is impossible, then a dynamic prediction model might be more useful. This type of model could for example, incorporate the number of days of exposure to FVIII over time, transient events such as FVIII exposure during trauma or surgery, as well as changes in IgG antibody titers over time. Another interesting approach would be to use non-linear machine learning algorithms which might produce better predictions. However, the disadvantage of these models is that they are prone to overfitting (meaning that a lot of data is needed for reliable results) and that they are difficult to interpret.[20]

This prediction model could be used to identify patients with a low risk of inhibitor development who could then be safely treated with regular FVIII in countries where emicizumab is significantly more expensive than FVIII. (for example, many low-income countries) As the current prediction model has not been externally validated, we do not recommend the use of this specific model in clinical practice as of yet.

Next, the FVIII-specific IgG epitope repertoire of 122 PUPs was explored by means of a novel high-throughput epitope mapping technique using a random peptide

phage-display library. (Chapter 6) We were able to identify three clusters of highly similar peptide sequences (with the consensus motifs “pxyNw”, “PSLxWK” and “SWPHxxxxK”) that were detectable in samples taken before patients were exposed to FVIII and that were predictive for inhibitor development.

The fact that these clusters of peptide sequences with high affinity for anti-FVIII antibodies were already present in samples taken before treatment with FVIII is somewhat unexpected. However, multiple studies have reported the presence of non-neutralizing anti-FVIII antibodies in healthy people.[21] In addition, we previously reported that roughly 10% of patients enrolled in the SIPPET study had measurable anti-FVIII antibodies.[22] It could be that a certain amount of autoreactivity is common in patients as well as healthy controls. That being said, this hypothesis only holds for patients with a non-null mutation that can still produce some endogenous FVIII. It could also be that the FVIII-specific antibodies are the result of previous exposure to a pathogen (e.g. a bacteria or virus) that shares some sequence similarity with FVIII. This cross-reactivity of an antibody response has been previously reported in several auto-immune disorders and is referred to as “molecular mimicry”.[23]

To better understand the pathophysiological mechanisms underlying the association between these peptide motifs and inhibitor development, the predicted locations of these motifs on the surface of FVIII still need to be validated in further studies. (e.g. by using alanine scanning mutagenesis) This is because the final epitope motifs were aligned to the linear sequence of FVIII to find their location. However, the majority of B-cell epitopes are reported to be conformational.[24, 25] Therefore, this approach is not optimal as the majority of epitope motifs will not have been mapped to the right location.

To overcome this problem, several B-cell epitope prediction algorithms have been developed to map epitope motifs to the three-dimensional structure of FVIII, using an in-silico approach. However, all of these algorithms perform relatively poorly.[26] That being said, knowledge of the exact location of these clusters on the surface of FVIII is not necessary for risk prediction. These novel results could be used to set up tests that predict the risk of inhibitor development before starting treatment with FVIII.

Chapters 4-6 focused on predicting inhibitor development in patients treated with regular FVIII. However, it is expected that the vast majority of patients with severe hemophilia A will switch to emicizumab for prophylactic treatment in the near future. Despite this, inhibitor development will still occur as FVIII will be still be needed in the

case of an acute bleeding episode or for surgical interventions. This will make effective treatment during future bleeding episodes more difficult as the alternative would be treatment with rFVIIa or FEIBA. Thus, there is still a need for accurate inhibitor risk prediction strategies in the coming years, especially strategies to identify patients at risk of developing an inhibitor that is refractory to ITI. These high-risk patients could be candidates for preventative treatment.

Unfortunately, specific treatment strategies to prevent inhibitor development in patients with a high risk of developing inhibitors that do not respond to treatment with ITI are lacking. We therefore reviewed anti-drug antibody prevention strategies that have been used in disorders other than hemophilia in Chapter 7. Several studies have shown that a short course with rituximab, methotrexate and IVIG prevented anti-drug antibody formation in patients with classic infantile Pompe's disease. (rituximab has been used previously in rescue ITI, but only in the context of inhibitor eradication, not prevention[27]) However, the high risk of serious infections outweighs the potential benefits of implementing such a strategy in patients with hemophilia. Several large randomized controlled trials have shown that the concomitant use of methotrexate strongly reduced the proportion of patients with detectable antibodies against TNF inhibitors. As methotrexate is very cheap, it could be a promising low cost treatment option to reduce the incidence of inhibitor development. However, it is unclear if the reduction in anti-drug antibodies is due to tolerization to the drug or due to methotrexate merely suppressing the immune system temporarily. Secondly, studies assessing the risk of adverse events in methotrexate in very young pediatric patients (e.g. 1-2 years old) are scarce, which limits the practical implementation of this strategy in patients with hemophilia.

Conclusion

Our results show that clinical outcomes in patients with hemophilia have improved tremendously over the past decades. The annual bleeding rate and the proportion of patients with joint impairment have decreased strongly. In addition, HCV has almost been eradicated among patients with hemophilia in the Netherlands. As a result, life expectancy has increased to where it is almost equal to that of the general population. Furthermore, using three different study approaches, we evaluated several methods to better predict the risk of inhibitor development (which is still a significant complication of treatment with FVIII). The results of these studies are promising and could be used to improve current inhibitor prediction strategies and inform future research on this topic.

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Nederlandse samenvatting

Introductie

Hemofilie is een zeldzame genetische aandoening die wordt gekenmerkt door een gebrek aan stollingsfactor VIII (FVIII) in patiënten met hemofilie A of stollingsfactor IX (FIX) in patiënten met hemofilie B als gevolg van een defect in het *F8* gen (hemofilie A) of het *F9* gen (hemofilie B).[1]

Het gebrek aan FVIII/FIX leidt op de korte termijn tot bloedingen, vaak in spieren en gewrichten. Op de lange termijn kunnen herhaaldelijke gewrichtsbloedingen tot ernstige gewrichtsschade leiden. Patiënten met hemofilie hebben ook een sterk verhoogd risico op hersenbloedingen, met name in de perinatale periode en de vroege jeugd. In patiënten met milde of matig-ernstige hemofilie komen bloedingssymptomen voornamelijk voor bij trauma of bij chirurgische ingrepen terwijl bloedingen bij patiënten met ernstige hemofilie ook spontaan ontstaan.[1]

De geboorteprevalentie van hemofilie A en hemofilie B is respectievelijk 24.6 per 100,000 nieuwgeborenen en 3.8 per 100,000 nieuwgeborenen.[2] De ernst van de ziekte wordt bepaald door het FVIII/FIX-gehalte in het bloed. Patiënten met ernstige hemofilie hebben een FVIII/FIX-gehalte van ‹ 0,01 internationale eenheden per milliliter (IE/mL), patiënten met matig-ernstige hemofilie hebben een FVIII/FIX-gehalte van 0,01-0,05 IE/mL en patiënten met milde hemofilie hebben een FVIII/FIX-gehalte van › 0,05-0,40 IE/mL.[1]

Toediening van FVIII/FIX, gezuiverd uit menselijk plasma of geproduceerd door middel van recombinant-technologie, is de standaard voor de behandeling van bloedingen. Bij patiënten met ernstige hemofilie wordt daarnaast periodiek FVIII/FIX toegediend teneinde bloedingen te voorkomen. (dit wordt ook wel profylactische behandeling of profylaxe genoemd) Ongeveer 1/3de van patiënten met ernstige hemofilie A ontwikkelt antistoffen tegen FVIII (ook wel inhibitor- of remmervorming genoemd) die het toegediende FVIII neutraliseren. Deze ernstige complicatie van behandeling met FVIII maakt behandeling met FVIII moeilijk of onmogelijk.[1] Het risico op remmerontwikkeling in deze groep is het hoogst in de eerste 50 dagen van behandeling met FVIII (expositiedagen). Na de eerste 50-150 expositiedagen is het risico op remmervorming zeer laag. De cumulative incidentie van FIX remmervorming in patiënten met ernstige hemofilie B is veel lager (rond de 4-5%).[1] De standaardbehandeling voor de eradicatie van remmers bestaat uit het frequent injecteren van stollingsfactorconcentraat over een lange periode (dit wordt immuuntolerantie-inductie genoemd). Immuuntoleran-

tie-inductie is succesvol in ongeveer 65-70% van patiënten met hemofilie A en een remmer.[1]

Dit proefschrift heeft twee overkoepelende doelen. In de laatste 50 jaar is de behandeling van patiënten met hemofilie enorm veranderd. Het eerste doel van dit proefschrift was om te beschrijven hoe de gezondheidsstatus van patiënten met hemofilie zich over de tijd heeft ontwikkeld. De resultaten van deze studies zijn beschreven in ***hoofdstukken 2-3***. Hoewel de behandeling van hemofilie in veel opzichten is verbeterd, blijft de ontwikkeling van antistoffen tegen FVIII een groot probleem bij patiënten die worden behandeld met FVIII. Daarom was het tweede doel van dit proefschrift om patiënten met een hoog risico op remmervorming te identificeren (***hoofdstukken 4-6***) en om een overzicht te geven van anti-remmervorming strategieën die toegepast zouden kunnen worden in deze groep hoog-risico patiënten (***hoofdstuk 7***).

Resultaten

De gezondheidsstatus van de Nederlandse patiënt met hemofilie

In ***hoofdstuk 2*** hebben we onderzocht hoe veranderingen in de behandeling de klinische uitkomsten van patiënten met hemofilie hebben beïnvloed. Hiervoor werd in 2019 een landelijk vragenlijstenonderzoek uitgevoerd onder patiënten met hemofilie in Nederland. De resultaten van dit onderzoek werden vergeleken met die van soortgelijke onderzoeken die eerder waren uitgevoerd in 1972, 1978, 1986, 1992 en 2001.[3-5] De resultaten laten zien dat een sterke toename in het gebruik van profylaxe gedurende de laatste 50 jaar heeft geleid tot een daling van het aantal bloedingen en de mate van gewrichtsschade onder patiënten met hemofilie. Ook laten onze resultaten zien dat hepatitis C virus (HCV) infectie inmiddels bijna compleet verdwenen is in deze populatie als gevolg van de introductie van effectieve behandelopties. In 2019 rapporteerde de meerderheid van patiënten geen bloedingen te hebben gehad in het afgelopen jaar. Veel patiënten ouder dan 50 jaar lijden nog steeds aan hemofilie-gerelateerde complicaties, met name complicaties als gevolg van de in het verleden opgelopen (en onomkeerbare) gewrichtsschade.

In ***hoofdstuk 3*** evalueerden we de totale en ziekte-specifieke mortaliteit onder patienten met hemofilie. Hiervoor werden 1066 patiënten gevolgd van 2001 tot 2018. De resultaten werden vergeleken met de resultaten van soortgelijke studies in dezelfde populatie uit eerdere perioden.[6-8] De mediane levensverwachting van patiënten met hemofilie nam sterk toe gedurende de periode 1972-2018 maar was nog steeds 6 jaar lager dan de mediane levensverwachting van de algemene Nederlandse manne-

lijke bevolking in 2018 (de mediane levensverwachting in 2018 voor de twee groepen was respectievelijk 77 jaar en 83 jaar). Mortaliteit door HIV- en HCV-gerelateerde complicaties onder patiënten met hemofilie (als gevolg van een infectie via besmette stollingsproducten) is langzaam afgenomen over de tijd maar is nog steeds hoger dan die van de algemene Nederlandse mannelijk bevolking. Vergeleken met de algemene Nederlandse mannelijke bevolking kwam sterfte als gevolg van een hersenbloeding veel vaker voor onder patiënten met hemofilie. Gedurende de gehele 46-jarige follow-up was de incidentie van sterfte als gevolg van een ischemische hartziekte (bijv. een myocardinfarct) consistent laag.

Het identificeren en behandelen van patiënten met een hoog risico op remmervorming
In ***hoofdstuk 4*** evalueerden wij, door een meta-analyse, de immunogeniciteit van recombinante FVIII-producten in patiënten met hemofilie A met meer dan 50 expositiedagen (dit zijn patiënten met een laag risico op remmervorming). In de meta-analyse werden alleen studies geïncludeerd die keken naar ***de novo*** remmerontwikkeling in patiënten met FVIII-spiegels ‹ 0.02 IU/mL. Er werd gebruik gemaakt van een random intercept Poisson regressiemodel om de data te modelleren. Met behulp van dit model werd een gewogen gemiddelde berekend van de totale incidentie van remmerontwikkeling, alsmede het relatieve risico op het ontwikkelen van een remmer bij gebruik van een specifiek recombinant FVIII product. De totale incidentie van remmerontwikkeling was 2.06 per 1000 persoonsjaren. Het relatieve risico op het ontwikkelen van een remmer was, vergeleken met het gebruik van het met recombinant technieken ontwikkelde FVIII-product Advate, bijna tien keer hoger in patiënten op Kogenate/Helixate en bijna 14 keer hoger in patiënten op Refacto. (beide ook geproduceerd met recombinant technieken, waarbij bij Refacto het B-domein uit het molecuul verwijderd is) De resultaten suggereren dat sommige producten een hogere immunogeniciteit hebben dan andere in patiënten met hemofilie A en FVIII-spiegels ‹ 0.02 IU/mL met meer dan 50 expositiedagen op FVIII.

Het doel van ***hoofdstuk 5*** was om een klinisch predictiemodel te ontwikkelen om het risico op remmerontwikkeling te voorspellen. Het predictiemodel werd ontwikkeld op basis van data van een cohort van 251 patiënten met ernstige hemofilie A die niet eerder behandeld waren met FVIII en die geobserveerd werden tijdens de eerste 50 expositiedagen.[9] Het uiteindelijke model bestond uit 4 predictoren; het type *F8* genmutatie, intensiteit van de eerste behandeling met FVIII, de aanwezigheid van niet-neutraliserende anti-FVIII antistoffen voor de start van de behandeling en het type FVIII product (recombinant FVIII of uit plasma bereid FVIII). Modeldiscriminatie werd gemeten met Harrel's C index en modelkalibratie werd visueel weergegeven

door middel van een zogeheten kalibratieplot. Modeldiscriminatie was laag (C-index: 0.66, 95%CI: 0.57–0.75) en modelkalibratie was matig. Bij gebruik van een afkappunt voor een positieve test van 10% waren de positief- en negatief voorspellende waarden respectievelijk 0.22 en 0.95. Hieruit kan geconcludeerd worden dat het model over het algemeen slecht presteert maar desondanks wel gebruikt kan worden om een (relatief kleine) groep patiënten te identificeren met een laag risico op het ontwikkelen van een remmer.

In ***hoofdstuk 6*** evalueerden we het anti-FVIII IgG-epitooprepertoire van 122 patiënten met ernstige hemofilie A (van wie 39 met een remmer en 83 zonder) met behulp van een nieuwe test gebaseerd op de faagdisplay methode.[10] Kort samengevat screenden we een set van 10^9 willekeurig gegenereerde eiwitfragmenten (die tot expressie waren gebracht op het oppervlak van een M13 bacteriofaag) tegen alle IgG-antistoffen die aanwezig waren in het plasma van een patiënt. Bacteriofagen met ongebonden eiwitfragmenten werden verwijderd en het DNA van de overige bacteriofagen werd geanalyseerd met next-generation sequencing technologie om de aminozuursequentie te verkrijgen van de eiwitfragmenten die gebonden waren door een IgG antilichaam. Met deze methoden hebben we een set eiwitfragmenten (ieder 12 aminozuren lang) kunnen identificeren die een hoge affiniteit hebben voor FVIII IgG-antistoffen. Deze eiwitfragmenten werden geclusterd op basis van gelijkenis in de primaire aminozuursequentie. Voor iedere cluster werd een consensusmotief gegenereerd. Dit consensusmotief werd daarna uitgelijnd tegen de lineaire aminozuursequentie van FVIII. De mate waarin de aanwezigheid van een eiwitfragment voorspellend was voor remmerontwikkeling werd geëvalueerd met de C-index. Onze resultaten laten zien dat de FVIII-specifieke antilichaamrespons polyclonaal is (clusters waren uitgelijnd tegen verschillende delen van FVIII). De meest voorkomende clusters in patiënten met een remmer waren uitgelijnd tegen de A1 en A2 domeinen van FVIII. We identificeerden drie clusters (met de consensusmotieven “pxyNw”, “PSLxWK” en “SWPHxxxxK”) die aanwezig waren in monsters afgenomen voor behandeling met FVIII en die voorspellend waren voor remmerontwikkeling. (de C-index was respectievelijk 0.76, 0.80 and 0.76) Informatie over de aanwezigheid van deze clusters kan gebruikt worden om testen te ontwikkelen die remmerontwikkeling kunnen voorspellen voor de start van behandeling met FVIII.

In ***hoofdstuk 7*** hebben we de literatuur systematisch doorzocht naar strategieën om antistofvorming tegen de toegediende medicatie te voorkomen in andere ziektebeelden. Dit teneinde nieuwe aanknopingspunten te vinden voor onderzoek op het gebied van preventie en behandeling van remmers in patiënten met hemofilie.

Verscheidene case-series rapporteerden een vermindering van antistoffen tegen alglucosidase alfa, een middel dat gebruikt wordt in de behandeling van patiënten met de ziekte van Pompe, bij gebruik van een combinatie van rituximab, methotrexaat en intraveneuze immunoglobulinen (IVIG). Bij patiënten met reumatoïde artritis bleek uit verscheidene grote gerandomiseerde studies dat het gebruik van methotrexaat de aanwezigheid van antistoffen tegen TNF-remmers verminderde bij gelijktijdig gebruik van deze middelen.

Discussie

De gezondheidsstatus van de Nederlandse patiënt met hemofilie
In hoofdstukken 2-3 hebben we de gezondheidsstatus van patiënten met hemofilie in kaart gebracht. Daarnaast hebben we onderzocht hoe de vele veranderingen in de behandeling van hemofilie en andere factoren over de tijd de gezondheidsstatus van deze patiënten hebben beïnvloed. Onze resultaten laten zien dat de behandeling van patiënten met hemofilie over de afgelopen 50 jaar sterk verbeterd is, maar dat er nog steeds een aantal verbeterpunten zijn.

Onze studie is waarschijnlijk de laatste grootschalige evaluatie van de Nederlandse bevolking met hemofilie waarbij het merendeel nog steeds behandeld werd met stollingsfactorconcentraat. Er zijn namelijk een aantal veelbelovende nieuwe middelen die niet gebaseerd zijn op FVIII of FIX.

De meest veelbelovende onder deze middelen is emicizumab[11], een synthetisch bispecifiek antilichaam dat de rol van FVIII nabootst (door te binden aan zowel factor IXa en factor X). Emicizumab is recentelijk goedgekeurd voor gebruik op de Europese markt en het wordt sinds kort al door een belangrijk deel van patiënten in Nederland met ernstige hemofilie A gebruikt. Het nadeel van dit middel is dat emicizumab niet gebruikt kan worden voor het behandelen van acute bloedingen. Voor patiënten met hemofilie B is er op dit moment nog geen vergelijkbaar middel op de markt. Fitusiran (een synthetisch geproduceerd zogeheten ‘klein interfererend RNA’ dat de aanmaak van antitrombine onderdrukt) en concizumab (een synthetisch antilichaam dat ‘tissue factor pathway inhibitor’ onderdrukt) zijn het meest ver in de ontwikkeling.[11] Beide middelen hebben het voordeel dat ze zowel in patiënten met hemofilie A als hemofilie B gebruikt kunnen worden. Recente studies hebben echter laten zien dat beide middelen kunnen leiden tot trombotische complicaties. Daardoor werden in eerste instantie een aantal fase-II studies gestopt.[11] Inmiddels zijn de studies weer hervat

met risk mitigation strategies. Gezien deze resultaten is het daarom onduidelijk op welke termijn deze middelen op de markt zullen komen.

Gentherapie, een andere potentiële behandeloptie voor hemofilie, is de eerste behandeling die omschreven kan worden als een definitieve genezing van hemofilie. Hierbij wordt een functionele versie van het FVIII/FIX gen, via een virale vector, aan de patiënt toegediend, waarna het FVIII/FIX eiwit door de patiënt zelf wordt geproduceerd.[12] Er zijn nog wel een aantal aandachtspunten die opgelost moeten worden voordat het middel gebruikt kan worden in alle patiënten met hemofilie. Veel patiënten ontwikkelen in de eerste fase van de behandeling tijdelijk levertoxiciteit (gemeten met verhoogde waarden van leverenzymen in het bloed). Daarnaast komt een patiënt op dit moment niet aanmerking voor een specifiek gentherapie-product als deze patiënt antistoffen heeft tegen de gebruikte virale vector. Ook kan behandeling met gentherapie minder effectief zijn in kinderen doordat er mogelijk een geleidelijke vermindering van expressie van het transgen kan zijn als gevolg van een groeiende lever. Dit is een probleem omdat opnieuw toedienen van hetzelfde gentherapie product op dit moment niet mogelijk is vanwege de antistofrespons tegen de virale vector.[12] Kinderen met hemoflie komen daarom op dit moment niet in aanmerking voor gentherapie. Een ander nadeel is dat de FVIII-opbrengst snel daalt in de jaren na toediening. Het is op dit moment onduidelijk of deze daling eventueel stabiliseert.[13] Inmiddels is het eerste gentherapie product (valoctocogene roxaparvovec) goedgekeurd door het Europees Geneesmiddelenbureau voor gebruik in volwassenen met hemofilie A zonder remmers en zonder antistoffen tegen adeno-associated virus serotype 5 (de virale vector).[14]

Bij toekomstig onderzoek naar de toegevoegde waarde van deze nieuwe behandelopties kan de gezondheidsstatus van de Nederlandse patiënt met hemofilie zoals beschreven in hoofdstukken 2-3 als referentiepunt dienen.

Onze studie laat zien dat 44% van patiënten met ernstige hemofilie een of meer gewrichtsbloedingen per jaar ontwikkelt, ondanks profylactische behandeling. Het is onduidelijk of deze bloedingen voornamelijk spontaan ontstaan of traumatisch van aard zijn. Er zijn een aantal strategieën om het aantal spontane bloedingen te verminderen; door de beoogde FVIII/FIX dalspiegel (de laagste concentratie van het toegediende middel in het bloed bij een bepaald doseringsregime) te verhogen op basis van het bloedingsfenotype, door farmacokinetisch te doseren of door over te stappen naar emicizumab (in het geval van patiënten met hemofilie A). Ondanks deze oplossingen kunnen sommige patiënten nog steeds bloedingen ondervinden vanwege lage

therapietrouw, of doordat deze patiënten een of meerdere gewrichten hebben die sneller bloeden dan normaal vanwege eerder opgelopen schade aan het synovium (een zogeheten 'target joint'). Het verminderen van fysieke activiteit om bloedingen te voorkomen is niet aangeraden, aangezien het niet geassocieerd is met een vermindering in het aantal gewrichtsbloedingen en vanwege het positieve effect van bewegen op de fysieke en mentale gezondheid.[15] Al met al zijn gewrichtsbloedingen nog steeds een probleem in sommige patiënten en een meer gepersonaliseerde behandelaanpak zal nodig zijn om het aantal gewrichtsbloedingen in deze groep te reduceren.

Gewrichtsbloedingen leiden op de lange termijn tot ernstige gewrichtsschade en dit proces kan gedurende een periode van tientallen jaren plaatsvinden . Ons project (welke bestond uit verscheidene cross-sectionele studies) was niet opgezet om het effect van primaire profylaxe op lange termijn gewrichtsschade te evalueren. Hiervoor zijn longitudinale data nodig. Zoals eerder beschreven is de beoogde profylaxe dalspiegel van 0,01 IU/mL FVIII/FIX niet voldoende om alle gewrichtsbloedingen te voorkomen.[16] Zelfs een klein aantal gewrichtsbloedingen kan op de lange termijn leiden tot gewrichtsschade. Daarnaast kunnen zelfs patiënten zonder klinisch zichtbare gewrichtsbloedingen later toch gewrichtsschade ontwikkelen door de aanwezigheid van subklinische gewrichtsbloedingen.[17] Lange termijn cohortstudies zijn nodig om te beoordelen of kinderen met hemofilie met een laag aantal (subklinische) gewrichtsbloedingen ook het risico lopen op het ontwikkelen van gewrichtsschade op latere leeftijd. Mocht dit het geval zijn, dan zou een behandeloptie kunnen zijn om de beoogde FVIII/FIX profylaxe dalspiegel te verhogen. De huidige WFH richtlijn voor hemofilie management suggereert al om de streefdalspiegel voor profylaxe te verhogen van 0,01 IU/mL naar 0,03-0,05 IU/mL.[16] In het verleden zou deze strategie onpraktisch zijn geweest vanwege het aantal wekelijkse infusies dat nodig was om een hogere dalspiegel te behalen. Echter, door de introductie van nieuwe FVIII/FIX producten met een verlengde halfwaardetijd is het nu wel mogelijk om deze hogere dalspiegels te behalen. Een alternatief is om over te stappen op emicizumab in patienten met hemofilie A.

We rapporteerden ook dat een kwart van patiënten die in het verleden HCV-positief waren nu milde- of matige leverfibrose hebben. Ondanks het klaren van het virus hebben deze patiënten nog steeds een verhoogd risico op HCV-gerelateerde complicaties zoals een hepatocellulair carcinoom.[18] Om deze reden is monitoren van de leverstatus van deze patiënten aangeraden.

Sterfte als gevolg van een intracraniële bloeding was de op één na meest voorkomende doodsoorzaak en patiënten met hemofilie hadden 13 keer zo veel kans om te sterven aan een hersenbloeding vergeleken met de algemene mannelijke bevolking. Alle patiënten die overleden als gevolg van een hersenbloeding in onze studie waren volwassenen (de jongste patiënt was 44 jaar oud) en de hersenbloedingen waren niet het gevolg van trauma. Andere studies hebben laten zien dat de kans op een hersenbloeding met name voorkomt in de perinatale periode en op oudere leeftijd.[19] Net als in eerdere onderzoeken[20], laten onze resultaten zien dat hypertensie veel voorkomt onder oudere patiënten met hemofilie. Hypertensie is een sterke onafhankelijke risicofactor voor het ontwikkelen van een hersenbloeding. De onderliggende biologische reden voor het ontwikkelen van hypertensie in patiënten met hemofilie is op dit moment onbekend.[21] Onze bevindingen suggereren dat regelmatige controle van patiënten met hemofilie voor hypertensie op vroege leeftijd (bijv. vanaf 40 jaar) zou kunnen bijdragen aan het verminderen van het risico op een hersenbloeding. Een andere manier om het risico op een hersenbloeding te verminderen is om FVIII/FIX dalspiegels te verhogen in patiënten op profylaxe. Aangezien er bewijs is dat profylaxe het aantal hersenbloedingen sterk verminderd in patiënten met ernstige hemofilie[22] zou het verhogen van de FVIII/FIX profylaxe dalspiegels (van 0,01 IU/mL tot bijvoorbeeld 0,05 IU/mL) tot een verdere reductie van de incidentie van hersenbloedingen kunnen leiden. Dit kan een oplossing bieden voor volwassen patiënten maar niet voor zeer jonge kinderen vanwege de moeilijkheden met betrekking tot het verkrijgen van veneuze toegang. Een alternatief voor deze groep patiënten is om emicizumab te gebruiken, aangezien dit middel subcutaan toegediend wordt. Dit maakt het mogelijk om vrijwel meteen na geboorte te starten met profylaxe om hersenbloedingen te voorkomen.[23]

Het identificeren en behandelen van patiënten met een hoog risico op remmervorming

Ondanks grote vooruitgang op het gebied van de behandeling van patiënten met hemofilie is er nog steeds geen optimale oplossing gevonden voor het probleem van remmervorming. In de afgelopen jaren zijn veel patiënten met hemofilie A en een persistente remmer overgestapt op profylaxe met emicizumab. Dit is een grote verbetering aangezien emicizumab veel makkelijker toe te dienen is en profylaxe met emicizumab effectiever is dan de alternatieven (profylaxe met FVIIa of aPCC). FVIII blijft echter nog steeds de beste behandeloptie voor acute bloedingen of chirurgische ingrepen. Daarom zal remmervorming nog steeds een belangrijk klinisch probleem zijn in de toekomst, ongeacht de behandelkeuze (FVIII or emicizumab).

Dit was de rationale om in hoofdstukken 4-7 te onderzoeken of patiënten met een hoog risico op remmervorming geïdentificeerd kunnen worden en om een overzicht te geven van anti-remmervormingstrategieën die toegepast kunnen worden in deze groep hoog-risico patiënten.

In hoofdstuk 4 hebben we onderzocht of er een relatie was tussen het type recombinant FVIII product en het risico op remmervorming in patiënten met hemofilie A en FVIII bloedspiegels < 0,02 IU/mL met meer dan 50 expositiedagen op FVIII (m.a.w. patiënten die al lang onder behandeling waren met FVIII). Onze resultaten laten zien dat de producten Kogenate/Helixate en Refacto geassocieerd waren met een verhoogde kans op remmervorming vergeleken met Advate.

Er zijn een aantal hypothesen die het verschil in immunogeniciteit tussen verschillende recombinant FVIII producten kunnen verklaren zoals verschillen in de primaire aminozuursequentie van het product, het type celcultuur en het type stabilisator dat gebruikt werd tijdens de productie.[24] Echter, doordat remmervorming relatief zeldzaam is in deze patiëntengroep en door het gebrek aan correctie voor potentiële confounders is het moeilijk om definitieve conclusies te trekken uit deze data. Toekomstig onderzoek op dit gebied zou daarom meer moeten focussen op het creëren van gestandaardiseerde rapportagesystemen. Hierdoor zou men een groter aantal patiënten kunnen includeren in toekomstige studies om zodoende een verschil in remmerincidentie tussen producten ook daadwerkelijk te kunnen detecteren. Daarnaast zou men dan beter kunnen corrigeren voor potentiële confounders (zoals verschillen tussen groepen in het aantal geincludeerde patiënten met FVIII spiegels van tussen de 0.01-0.02 IU/mL en verschillen tussen groepen in het aantal patienten met een bepaalde type *F8* genmutatie) en andere methodologische problemen (zoals verschillen in de remmer testfrequentie en het gebruik van verschillende afkapwaarden voor de detectie van remmers). Goede voorbeelden van gestandaardiseerde rapportagesystemen zijn het Nederlandse HemoNED register en het Europese EUHASS register.[25]

In hoofdstuk 4 evalueerden wij het effect van een enkele factor (het type product) op remmervorming. In hoofdstuk 5 hebben we geprobeerd om verschillende factoren te combineren in een klinisch predictiemodel voor remmervorming. Het accuraat identificeren van patiënten met een hoog risico op remmervorming was niet mogelijk met dit predictiemodel. Het model was wel in staat om een klein aantal patiënten te identificeren met een laag risico op remmervorming. Er zijn verscheidene manieren om dit predictiemodel te verbeteren. Een manier is om informatie over genetische risi-

cofactoren (anders dan het type *F8* genmutatie) te incorporeren in het model. (bijv. genmutaties in het *CTLA-4* of *IL-10* gen) Als het accuraat voorspellen van remmervorming op basis van uitgangsgegevens beperkt blijft, dan zou een dynamisch predictiemodel welke informatie incorporeert over de tijd een uitkomst kunnen bieden. In dit type model zou bijvoorbeeld informatie over het aantal expositiedagen opgenomen kunnen worden, informatie over voorbijgaande gebeurtenissen zoals FVIII toediening tijdens trauma of chirurgie en veranderingen in de anti-FVIII IgG antistoftiter over de tijd. Een andere interessante aanpak is om predictiemodellen te bouwen op basis van machine learning algoritmen die mogelijke non-lineaire relaties tussen variabelen beter zouden kunnen modelleren (bijv. een random forest classificatie-algoritme). Het nadeel bij de ontwikkeling van dit soort modellen is dat gegevens van een groot aantal patiënten nodig is om een accuraat model te kunnen bouwen en dat deze modellen moeilijker te interpreteren zijn door gebruikers dan regressiemodellen.[26]

Het huidige predictiemodel kan gebruikt kunnen worden om patiënten te identificeren die veilig met FVIII behandeld kunnen worden in landen waar emicizumab minder betaalbaar is dan FVIII (bijv. lage-inkomenslanden).

In hoofdstuk 6 hebben we het FVIII IgG repertoire van 122 patiënten met ernstige hemofilie A geëvalueerd met behulp van een nieuwe test gebaseerd op de faagdisplay methode. We identificeerden drie clusters die aanwezig waren in bloedmonsters afgenomen voor behandeling met FVIII en die voorspellend waren voor remmerontwikkeling.

Deze clusters van aminozuursequenties waren al detecteerbaar in monsters die afgenomen waren voor de start van behandeling met FVIII, hetgeen enigszins onverwacht was. Echter, verscheidene studies hebben laten zien dat niet-neutraliserende anti-FVIII antistoffen ook meetbaar zijn in sommige gezonde mensen.[27] Daarnaast is al eerder gerapporteerd dat ongeveer 10% van de patiënten met hemofilie A in het SIPPET cohort meetbare niet-neutraliserende anti-FVIII antistoffen hadden.[28] Het zou kunnen dat een zekere mate van autoreactiviteit normaal is in zowel patiënten met hemofilie alsook gezonde mensen. Dit is echter geen goede verklaring voor de geobserveerde resultaten in patiënten met een nonsense-mutatie of gendeletie aangezien deze patiënten in het geheel geen endogeen FVIII produceren en het immuunsysteem van deze patiënten dus nooit blootgesteld is geweest aan FVIII zelf-antigenen. Een andere hypothese is dat de aanwezigheid van anti-FVIII antistoffen voor behandeling met FVIII het gevolg is van een eerdere blootstelling aan een pathogeen (bijv. een virus of bacterie) met een of meerdere antigenen die sterk gelijken op delen van het

FVIII-molecuul. Deze kruisreactieve antistoffen kunnen ook aan FVIII binden. Dit fenomeen wordt in de literatuur aangeduid met de term “molecular mimicry”.[29]

De voorspelde locaties van de drie geïdentificeerde clusters op het FVIII eiwit moeten nog gevalideerd worden in verdere studies. (bijv. met alanine scanning mutagenesis) Dit is omdat we het consensusmotief van deze clusters uitgelijnd hebben tegen de lineaire aminozuursequentie van FVIII. Echter, volgens de literatuur zijn de meeste B-cel epitopen niet lineair maar conformationeel.[30] Hierdoor is de toegepaste methode niet optimaal, aangezien er een reële kans is dat de voorspelde locaties van de clusters niet correct zijn.

Er zijn verscheidene B-cel epitoop predictie-algoritmen gepubliceerd die dit probleem trachtten op te lossen door het uitlijnen van een aminozuursequentie (of een consensusmotief) tegen de 3D oppervlakte van een molecuul om op deze manier ook conformationele epitopen te identificeren.[31] Helaas is de betrouwbaarheid van deze algoritmen laag.[32] Dit probleem bemoeilijkt de biologische interpretatie van de aanwezigheid van deze clusters. Echter, een duidelijk begrip van de biologische rol van deze clusters is niet nodig om remmervorming te voorspellen. Informatie over de aanwezigheid van deze clusters kan daarom wel gebruikt worden om testen te ontwikkelen die remmerontwikkeling kunnen voorspellen voor de start van behandeling met FVIII.

In hoofdstukken 4-6 lag de focus op het voorspellen van remmervorming in patiënten die behandeld worden met FVIII producten. Indien het mogelijk is op voorhand te voorspellen welke patiënt een remmer ontwikkelt tijdens behandeling met FVIII, dan zou men een preventieve behandeling kunnen inzetten om remmervorming te voorkomen in deze groep hoog-risico patiënten. Er zijn op dit moment echter geen specifieke behandelstrategieën om remmervorming te voorkomen. Daarom hebben we de literatuur systematisch doorzocht teneinde strategieën te identificeren om antistofvorming tegen biologische geneesmiddelen te voorkomen in andere aandoeningen dan hemofilie.

Verscheidene case-series meldden een vermindering van de incidentie van neutraliserende antistoffen tegen alglucosidase alfa, een middel dat gebruikt wordt in de behandeling van patiënten met de ziekte van Pompe, bij gebruik van een combinatie van rituximab, methotrexaat en intraveneuze immunoglobulinen. Rituximab is al eerder toegepast in patiënten met hemofilie en een remmer als onderdeel van een immuuntolerantie-inductie protocol, maar nooit als een preventieve behande-

ling.[33] Helaas weegt het risico op een serieuze infectie bij preventief behandelen met rituximab (ook in combinatie met andere middelen) niet op tegen de voordelen. Daarom kan deze behandelstrategie niet aangeraden worden in patiënten met hemofilie. Bij patiënten met reumatoïde artritis bleek uit grote gerandomiseerde studies dat het gebruik van methotrexaat de aanwezigheid van antistoffen tegen TNF-remmers verminderde. Aangezien methotrexaat relatief goedkoop is, zou dit een veelbelovende behandeloptie kunnen zijn om remmervorming te voorkomen in hoog-risico patiënten. Het is echter niet duidelijk of de reductie in antistofvorming tijdens behandeling met methotrexaat het gevolg is van immuuntolerantie of slechts tijdelijke immuunsuppressie. Daarnaast zijn studies die het risico op bijwerkingen bij gebruik van methotrexaat in zeer jonge pediatrische patiënten evalueren wel beschikbaar maar schaars. Dit alles bemoeilijkt de praktische implementatie van deze strategie in patiënten met hemofilie A.

Conclusie

Onze resultaten laten zien dat klinische uitkomsten in patiënten met hemofilie enorm zijn verbeterd in de afgelopen decennia. Het jaarlijkse aantal bloedingen en het percentage patiënten met gewrichtsbeperkingen is sterk gedaald. Daarnaast is het hepatitis C virus bijna verdwenen onder patiënten met hemofilie in Nederland. Ondanks het klaren van het virus hebben veel patiënten nog steeds last van HCV-gerelateerde complicaties. Als gevolg van deze ontwikkelingen is de mediane levensverwachting van patiënten met hemofilie sterk gestegen en is deze nu bijna gelijk aan die van de Nederlandse mannelijke bevolking. Daarnaast hebben we in drie studies verschillende methoden geëvalueerd om remmervorming (een belangrijke complicatie van de behandeling met FVIII) beter te kunnen voorspellen. De resultaten van deze studies zijn veelbelovend en kunnen worden gebruikt als uitgangspunt voor verder onderzoek.

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Curriculum Vitae

Shermarke Hassan is geboren in Mogadishu, Somalië op 3 juli 1989. Hij behaalde zijn atheneumdiploma in 2008 aan het Lyceum Schöndeln te Roermond. Vervolgens behaalde hij in 2008 de bachelor Geneeskunde en in 2015 de master Epidemiologie aan de Universiteit Maastricht. Eind 2015 begon hij aan zijn promotieonderzoek, onder leiding van Frits Rosendaal en Anske van der Bom. Tijdens zijn promotieonderzoek verbleef hij ook enkele maanden in Milaan voor een onderzoeksproject. De resultaten van zijn promotieonderzoek heeft hij gepresenteerd op verscheidene nationale en internationale congressen. Van 2020-2022 was hij werkzaam als research fellow bij de Universiteit van Milaan (dipartimento di fisiopatologia medico-chirurgica e dei trapianti, Università degli Studi di Milano). Hij is op dit moment werkzaam als epidemioloog bij de Universiteit van Oxford (centre for tropical medicine and global health, University of Oxford).

Portfolio

Courses	Year	Hours
BROK course	2016	42
Basic methods and reasoning in biostatistics	2018	42
Clinical Epidemiology course (Schiermonnikoog)	2016	56
Clinical Epidemiology (Grobbee & Hoes)	2016	60
Causal Inference (Hernan)	2018	84
Advanced Epidemiological Methodsw	2018	56
Capita Selecta	2015-2019	60
NTVH AIO course	2016-2018	84
Teaching activities	**Year**	**Hours**
Working group supervisor course 'AWV-1'	2016	10
Working group supervisor course 'AWV-1'	2018	10
Working group supervisor course 'AWV-2'	2016	10
Working group supervisor course 'AWV-2'	2017	10
Working group supervisor course 'AWV-2'	2018	10
Teacher honours college course 'Dagelijkse toepassingen van de epidemiologie'	2016	78
Supervisor project 'Critical Appraisal of a Topic (CAT)'	2018	8
Supervision of master's thesis Rory Monahan (MSc Medicine)	2018	64
Oral presentations	**Year**	
"Factor VIII products and inhibitor development in previously treated patients with severe haemophilia A: a systematic review and meta-analysis", presented at the 2nd international conference on inhibitors in haemophilia.	2017	
"Mortality of patients with hemophilia in the Netherlands, 2001-2018", presented at the 12th annual congress of the European Association for Haemophilia and Allied Disorders (EAHAD).	2019	
"Fifty years of hemophilia in the Netherlands", presented at the 13th annual congress of the European Association for Haemophilia and Allied Disorders (EAHAD).	2020	

Awards, acknowledgements and scholarships	Year
Based on the content of an abstract, a travel grant was awarded (1500 EUR) for the EAHAD 2019 congress in Prague.	2019
A grant (1875 EUR) was awarded by the Leiden University Fund for a three month stay as a visiting researcher at the 'Angelo Bianchi Bonomi Haemophilia and Thrombosis Centre' in Milan from 14-01-2019 to 14-04-2019.	2019

Other activities	Year
Review activity for the 'Journal of Thrombosis and Haemostasis'	2021
Review activity for 'Haemophilia'	2021
Review activity for 'Research and Practice in Thrombosis and Haemostasis'	2021
Review activity for 'Thrombosis and Haemostasis'	2022

List of publications

Publications resulting from this thesis

1. Hassan S, van Balen EC, Smit C, et al. Health and treatment outcomes of patients with hemophilia in the Netherlands, 1972-2019. J Thromb Haemost. 2021;19(10):2394-2406. doi:10.1111/jth.15424
2. Hassan S, Palla R, Valsecchi C, et al. Performance of a clinical risk prediction model for inhibitor formation in severe haemophilia A. Haemophilia. 2021;27(4):e441-e449. doi:10.1111/hae.14325
3. Hassan S, Monahan RC, Mauser-Bunschoten EP, et al. Mortality, life expectancy, and causes of death of persons with hemophilia in the Netherlands 2001-2018. J Thromb Haemost. 2021;19(3):645-653. doi:10.1111/jth.15182
4. Hassan S, Fijnvandraat K, van der Bom JG, Gouw SC. Preventing or Eradicating Factor VIII Antibody Formation in Patients with Hemophilia A: What Can We Learn from Other Disorders?. Semin Thromb Hemost. 2018;44(6):531-543. doi:10.1055/s-0038-1666823
5. Hassan S, Cannavò A, Gouw SC, Rosendaal FR, van der Bom JG. Factor VIII products and inhibitor development in previously treated patients with severe or moderately severe hemophilia A: a systematic review. J Thromb Haemost. 2018;16(6):1055-1068. doi:10.1111/jth.14124

Other publications

1. van Balen EC, Hassan S, Smit C, et al. Socio-economic participation of persons with hemophilia: results from the sixth Hemophilia in the Netherlands study. Res Pract Thromb Haemost. 2022; 6:e12741. doi:10.1002/rth2.12741
2. Hassan S, Ramspek CL, Ferrari B, et al. External validation of risk scores to predict in-hospital mortality in patients hospitalized due to coronavirus disease 2019. Eur J Intern Med. 2022 Jun 8:S0953-6205(22)00217-5. doi: 10.1016/j.ejim.2022.06.005.
3. Smit JM, Krijthe JH, Tintu AN, Endeman H, Ludikhuize J, van Genderen ME, Hassan S, El Moussaoui R, Westerweel PE, Goekoop RJ, Waverijn G, Verheijen T, den Hollander JG, de Boer MGJ, Gommers DAMPJ, van der Vlies R, Schellings M, Carels RA, van Nieuwkoop C, Arbous SM, van Bommel J, Knevel R, de Rijke YB, Reinders MJT. Development and Validation of an Early Warning Model for Hospitalized COVID-19 Patients: A MultiCenter Retrospective Cohort Study. (Accepted in Intensive Care Medicine Experimental on 22-08-2022)
4. Hassan S, Ferrari B, Rossio R, et al. The usefulness of D-dimer as a predictive marker for mortality in patients with COVID-19 hospitalized during the first

wave in Italy. PLOS ONE. 2022 Jul 22;17(7):e0264106. doi: 10.1371/journal.pone.0264106.

5. Spena S, Cairo A, Pappalardo E, Gorski MM, Garagiola I, Hassan S, Gualtierotti R, Peyvandi F. Genetic variants at the chromosomal region 2q21.3 underlying inhibitor development in patients with severe haemophilia A. Haemophilia. 2022;28(2):270-277. doi:10.1111/hae.14503
6. Isfordink CJ, Gouw SC, van Balen EC, Hassan S, Beckers EAM, van der Bom JG, Coppens M, Eikenboom J, Fischer K, Hooimeijer L, Leebeek FWG, Rosendaal FR, Schols SEM, Smit C, van Vulpen LFD, Mauser-Bunschoten EP. Hepatitis C virus in hemophilia: Health-related quality of life after successful treatment in the sixth Hemophilia in the Netherlands study. Res Pract Thromb Haemost. 2021;5(8):e12616. Published 2021 Nov 17. doi:10.1002/rth2.12616
7. van Balen EC, Haverman L, Hassan S, et al. Validation of PROMIS Profile-29 in adults with hemophilia in the Netherlands. J Thromb Haemost. 2021;19(11):2687-2701. doi:10.1111/jth.15454
8. Versloot O, van Balen EC, Hassan S, et al. Similar sports participation as the general population in Dutch persons with haemophilia; results from a nationwide study. Haemophilia. 2021;27(5):876-885. doi:10.1111/hae.14366
9. Abdi A, Bordbar MR, Hassan S, et al. Prevalence and Incidence of Non-neutralizing Antibodies in Congenital Hemophilia A- A Systematic Review and Meta-Analysis. Front Immunol. 2020;11:563. Published 2020 May 7. doi:10.3389/fimmu.2020.00563
10. Donners A, Hassan S, Rademaker K, Smit C. Antistofvorming kan medicijn hinderen in werkzaamheid. Pharmaceutisch weekblad 2017;152(35):16-18