Haemophilia in the Netherlands 6 Study

Research protocol

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List of abbreviations and definitions

ABR	Annualized bleed rate
AIDS	Acquired immunodeficiency syndrome
AMC	Academic Medical Centre
APC	Antigen presenting cell
aPTT	Activated partial thromboplastin time
AUROC	Area Under the Receiver Operating Curve
BMI	Body Mass Index
BU	Bethesda Units
CAT assay	Calibrated Automated Thrombogram assay
CCMO	Central Committee on Research Involving Human Subjects (in Dutch: Centrale Commissie Mensgebonden Onderzoek)
CHO-KLAT	Canadian Haemophilia Outcomes-Kids' Life Assessment Tool
CpG site	Region of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' → 3' direction (5'—C—phosphate—G—3')
CRF	Case report form
DNA	Deoxyribonucleic acid
ED	Exposure day
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPD	Electronic patient dossier
Erasmus MC	Erasmus University Medical Centre
FVIII	Clotting factor VIII
FIX	Clotting factor IX
FLT	Fibrin lysis time
FOXP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
Haemo-QoL-A	Haemophilia quality of life in adults (questionnaire)
HAL	Haemophilia Activities List
HCV	Hepatitis C virus
Hemo-SAT	Haemophilia treatment satisfaction questionnaire
HIV	Human immunodeficiency virus
HiN-6	Haemophilia in the Netherlands 6

HJHS	Haemophilia joint health score
HRQoL	Health-related quality of life
HSES	Haemophilia-specific self-efficacy scale
iCHEC	Identifying children with hereditary coagulation disorders (paediatric bleeding assessment tool)
ICMJE	International Committee of Medical Journal Editors
IFNγ	Interferon gamma
IgA, IgG, IgM	Immunoglobulin A, G, M
IL-10	Interleukin-10
ITI	Immune tolerance induction therapy, frequent administration of relatively high doses of FVIII with the aim of eradicating inhibitors.
LPS	Lipopolysaccharide
LUMC	Leiden University Medical Centre
METC	Medical research ethics committee (MREC); in Dutch: Medisch Ethische Toetsingscommissie (METC)
MHCII	Major histocompatibility complex II
MoDC	Monocyte-derived dendritic cell
MUMC	Maastricht University Medical Centre
NHA	Nijmegen Haemostasis assay
NNA	Non-neutralizing antibodies
NVHP	Dutch Association of Haemophilia Patients (in Dutch: Nederlandse Vereniging van Hemofiliepatiënten)
PAM-13	Patient Activation Measure (questionnaire)
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PedHAL	Paediatric Haemophilia Activities List
PK	Pharmacokinetics
PT	Prothrombin time
PUP	Previously untreated patient
PWH	Person with haemophilia
QoL	Quality of Life
Radboud UMC	Radboud University Medical Centre
RAND-36	Research and development 36-item health survey
VWF:Rco	Von Willebrand factor Ristocetin cofactor assay

RNA	Ribonucleic acid
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SVR	Sustained viral response
TAFI	Thrombin activatable fibrinolysis inhibitor
TF	Tissue factor
TGF-beta	Transforming growth factor beta
TLR-ligand	Toll-like receptor ligand
TNFα	Tumour necrosis factor alpha
Tregs	Regulatory T-cells
UMCG	University Medical Centre Groningen
UMCU	University Medical Centre Utrecht
VERITAS-pro/prn	Validated Haemophilia Regimen Treatment Adherence Scale Prophylaxis / On-Demand
VWD	Von Willebrand disease
VWF	Von Willebrand Factor
VWS	Ministry of Health, Welfare and Sport
WHO	World Health Organization
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
Wgbo	Law for Agreement on Medical Treatment (in Dutch: Wet op de geneeskundige behandelingsovereenkomst)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen)

Summary

Rationale: There is little information on the current health status of the Dutch haemophilia population, especially ageing patients, HIV/HCV patients and inhibitor patients. In addition, quality of life may be reduced in some persons with haemophilia (PWH) despite similar levels of physical health. Further, differences in clinical phenotypes have been described in PWH with comparable coagulation factor activities. Lastly, inhibitor formation is still an important complication in haemophilia treatment and a better understanding is needed about the mechanisms that lead to inhibitor formation.

Objectives: The purpose of the HiN-6 study is **(1)** to describe the health status of the Dutch haemophilia population, with special focus on viral infections, inhibitor development and age-related co-morbidities. In addition, the HiN-6 study will **(2)** attempt to gain insight in health-related quality of life of PWH and **(3)** to evaluate the quality of care. The HiN-6 will also **(4)** aim to explain the variability in clinical phenotype among PWH and **(5)** gain insight into the mechanisms underlying the humoral and cellular immune response to FVIII.

Study population: All male Dutch patients with a clinical diagnosis of severe, moderate, or mild haemophilia A or B will be included in this study.

Study design: The HiN-6 study will consist of both cross-sectional and longitudinal observational studies, according to the specific research objective.

Methods: Data will be collected from each participant's medical record using case report forms, through questionnaires filled in by each participant and by blood/urine sampling. Some of the blood and all of the urine will be stored in a biobank for future research purposes.

Main study endpoints: Presence of disease-related co-morbidities**,** health-related quality of life, quality of care, self-reported bleeding severity, self-reported joint function and the presence of inhibitors, non-neutralizing antibodies and immunological markers.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The participants' burden consists of 1 or 2 blood draws, providing a urine sample and filling in a questionnaire. No direct benefit is expected from participation in this study. Participation in this study may have benefits for future PWH.

Chapter 1 - Introduction and rationale

1.0 Introduction

People with haemophilia have faced many challenges in the past few decades. Devastating complications of haemophilia treatment included blood-transmitted infectious diseases, in particular HIV and hepatitis C. Fortunately, with the advent of recombinant factor VIII (FVIII) products and viral inactivation techniques for plasma-derived FVIII products, no transmission of hepatitis C or HIV has occurred from the use of these products in the past 20 years. The institution of regular prophylaxis has made a normal life possible for people with haemophilia. It has been shown that prophylactic treatment greatly reduces blood-induced joint damage.

Unfortunately, these achievements of modern haemophilia care are off the map for PWH who develop inhibitory antibodies to FVIII or FIX. Unlike blood-transmitted infectious diseases and blood-induced joint damage, inhibitor formation is a problem which mainly affects very young patients as inhibitory antibodies most often arise in the early stages of clotting factor treatment. Despite advancements in the production of recombinant clotting factor products, the incidence of inhibitory antibodies among patients has not changed significantly.

Although patient care has improved tremendously, it has come too late for PWH who were already infected with HIV and/or hepatitis C and PWH with severe blood-induced joint damage. Also, as the haemophilia population ages [1], other age-related comorbidities increase. Much like the rest of the general population PWH are faced with conditions such as malignancies and cardiovascular disease. [2-6] Haemophilia potentially complicates treatment of these conditions further.

Quality of life may be reduced in people with haemophilia [7, 8], though there are differences between countries and between patients. [9] For example, pain and frequent bleeds may interfere with daily activities for many people with haemophilia. [7] PWHs may experience an impact of haemophilia on their school education, professional career and overall satisfaction with their quality of life. [8] Health-related quality of life is becoming increasingly more important as an outcome and in the planning and delivery of care. [10]

The 6th Haemophilia in the Netherlands study (HiN-6 study) is the next in a series of five previous studies that started in 1972, 1978, 1985, 1992 and 2001. Broadly speaking, the previous studies have

explored important medical and psychosocial research questions in the Dutch haemophilia population throughout the past 45 years.

Historically, haemophilia has been treated in a large number of hospitals. Nowadays, treatment is concentrated in 7 haemophilia treatment centres[1]. All rounds of HIN have been organized in close cooperation with the NVHP (In Dutch: “Nederlandse Vereniging van Hemofiliepatiënten”) and leading physicians who are specialized in treating PWH, these physicians are members of the NVHB (In Dutch: “Nederlandse Vereniging van Hemofilie Behandelaars).

The combined effort of both physicians and PWH contributed to the large cooperation of the PWH in the respective survey rounds, resulting in very high response rates (for example, 70% of all PWH participated in the HiN-5 in 2001). In line with previous studies, the HiN-6 study is coordinated by representatives of all seven haemophilia treatment centres and the NVHP. The HiN-6 has 5 broadly defined aims:

1. To describe the current health status of the Dutch haemophilia population, with special focus on HCV/HIV infections, bleeding, self-reported joint damage, inhibitor development and age-related co-morbidities.
2. To gain insight in health-related quality of life among PWH in the Netherlands.
3. To evaluate quality of care for PWH.
4. To explain variability in clinical phenotype among PWH
5. To gain insight into the mechanisms underlying the humoral and cellular immune response to FVIII.

The rest of this chapter summarizes our current knowledge on each of the five goals.

1.1 Describing the health status of the Dutch haemophilia population

1.1.1 45 years of haemophilia treatment in the Netherlands, 1972-2017

Before the mid-1960s, PWH commonly died due to haemorrhages because no treatment was available. Later, many PWH died of the consequences of HIV or HCV infection caused by the use of

[1] In 2017, two academic treatment centres (Radboud University Medical Centre and Maastricht University Medical Centre) will merge with a general hospital (Máxima Medisch Centrum Eindhoven) to become one treatment centre, resulting in a total of six haemophilia treatment centres.

contaminated blood products in the 1980s and 90s. However, haemophilia care has greatly advanced over the years. The implementation of viral inactivation techniques has practically eliminated the risk of HIV or HCV infection. In addition, the introduction of prophylaxis has greatly reduced the number of bleeds and bleeding-induced joint damage. The improvement in treatment is reflected in patients' increasing life expectancy, which is currently approaching that of the general population. As a consequence, age-related co-morbidities are becoming more prevalent.[1] In recent years, there has also been more attention for other health outcomes in haemophilia, including pain (and coping with pain) and mental health. [7, 11, 12] The HiN-6 study will evaluate the impact of these medical developments on the health outcomes of the entire Dutch haemophilia population. We will achieve this by evaluating changes in the prevalence of (joint) bleeds, clotting factor consumption, inhibitor formation and HIV/HCV infection. In addition, we intend to describe the prevalence of the most important co-morbidities associated with haemophilia, including cardiovascular disease (CVD), obesity, physical activity, cancer, liver disease, chronic kidney disease (CKD), diabetes, osteoporosis and chronic pain.

1.1.2 Physical activity, sports and health outcomes

Physical activity and participation in sports can lead to better physical and social functioning and better general health. However, in PWH they can also lead to injury due to trauma and overuse of joints. High-impact sports are especially risky for them.

1.2 Quality of life of PWH in the Netherlands

1.2.1 General quality of life

The World Health Organization (WHO) defines health as 'a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.'[13] However, people with chronic disease may still classify themselves as healthy. Reversely, others without disease may perceive their health as impaired. Similarly, haemophilia may affect patients' day-to-day lives to different degrees and in different ways. In other words, disease status is not necessarily the most important determinant of perceived health. A recently developed new definition of health that includes this concept is health as 'the ability of people to adapt and to self-manage, in the face of social, physical and emotional challenges'.[14, 15]

In addition to monitoring clinical outcomes, it is important to measure this new concept of health, which is related to health-related quality of life. Inter-related components of HR-QL are biological and physical variables, symptom status, functional status, general health status and overall quality of life, as conceptualized by Wilson and Cleary. [16] Quality of life in PWH has been found to be lower in some studies compared to the general population, [7, 8, 17] but not in others.[9, 18]

Haemophilia may also affect the determinants of health, in particular alcohol use (see also paragraph 6.1.1), sedentary behaviour and physical activity. In addition, social determinants of health may be more prevalent among people with haemophilia, such as lower education level, income level, ability to work, social functioning [19] and relationships. Given the higher degree of arthropathy and haemophilia-related co-morbidities due to suboptimal treatment in the past, these factors are more prevalent among the older generation of PWH.[19] A recent comprehensive picture of these factors in the Dutch haemophilia population is not available. A better understanding of these factors in PWH will contribute to improved care. Therefore, the HiN-6 study will collect information on all aspects of health-related quality of life.

1.2.2 Ageing with haemophilia

Never before has there been an older generation of PWH. The life expectancy of PWH has increased and haemophilia-related comorbidity has decreased. [1, 20] As the haemophilia population ages [1], other age-related comorbidities increase and add to the disease burden. Much like the rest of the general population PWH are facing conditions such as malignancies and cardiovascular disease. [2, 4-6, 21] How PWH are affected by these developments, is partly unknown.

Another major comorbidity in the general ageing population is falls, causing substantial mortality and morbidity that increases with age. In addition, patients that sustained falls may have a fear of falling again, further reducing their mobility. [22, 23] Ageing PWH may be particularly susceptible to injuries after a fall due to their tendency to bleed, but also because of their lower bone mineral density.[24, 25] For these reasons, screening for fall risk in PWH is important.[23]

Due to suboptimal treatment in the past and resulting arthropathy, ageing individuals with haemophilia may have pain that interferes with their daily activities. A study from the U.S. found that pain interference was particularly common among PWH over 40 years old.[7]
Because of these co-morbidities, ageing patients interact with an increasing number of health care

providers potentially complicating the organization and coordination of their care. As they age, they may no longer be able to self-manage their care.

Other issues that ageing PWH may be facing are of a social, psychological or economical nature. Haemophilia may influence a person's ability to work and maintain social relationships. These limitations may result in lower socio-economic status and increased use of social services. This may be particularly true for the older generation of PWH. However, the needs and concerns of this age group are mostly unknown. In a study that explored the needs of patients older than 40 years with haemophilia A or B, Von Willebrand Disease or rare bleeding disorders three major themes emerged: 1) reflection on living an active life, 2) 'normal' ageing vs. disease-specific impacts and 3) needs related to the health care system and its ability to respond to their needs as ageing individuals with bleeding disorders. [26]

In the Netherlands, all people between 55 and 75 years of age receive an invitation to participate in the national colon cancer screening program, which was implemented in January 2014. Participation is voluntary and consists of a self-administered faeces test. If the result of the self-administered test is positive, people may undergo an additional colonoscopy at the hospital. For people with haemophilia and other coagulation disorders, the self-administered faeces test may result in more false-positives than in the rest of the population. Furthermore, any hospital procedure carries an additional risk for people with haemophilia. Therefore, it is important to assess the incidence of false-positive results in colon cancer screening.

The HiN-6 study will explore the above mentioned aspects of quality of life in ageing individuals with haemophilia.

1.2.3 Sexuality

People with haemophilia may experience problems in the area of sexuality as a consequence of their chronic illness. Some haemophilic men may have problems due to physical impairment (joint, pain, reduced mobility, bleeding) but psychosocial factors may also play a role (fear of bleeding, expectations of masculinity and other expectations of the sexual partner, friends and family). As of yet, still little is known on this subject. This knowledge is needed to improve patient care and support.

1.3 Quality of care of PWH in the Netherlands

1.3.1 General quality of care

Quality of life is in part dependent on quality of care. Quality of care constitutes many topics beyond the availability of treatment options, including communication between care provider and patient, information provided and autonomy in treatment decisions.[27]

Patient perspectives on quality of care may differ from those of health care providers. One questionnaire study from the U.S. about haemophilia care found that there were few health service gaps in haemophilia care and that patients' information needs were met.[28] However, such patient satisfaction surveys provide only partial and sometimes misleading insights into the perspectives of patients and are therefore poor and subjective indicators of quality of care.[27, 29]
Better indicators of quality of care are patient experiences with care provision. These differ from satisfaction surveys in that they ask about what happened and how often. [27] Lastly, use of health care services may depend on financial consequences for patients. Quality of care and satisfaction with treatment in a broad sense and from a patient's perspective will be explored in HiN-6. The focus of the HiN-study will be on the topics described below.

1.3.2 Transition from paediatric to adult care

Around the age of 18 years, care for most patients with a chronic illness, including haemophilia, will be transferred from paediatric care to adult care. This period in life is characterized by both transition in disease management as well as transition from paediatric to adult care.[30] Among the important issues for care providers in transitional care are adherence to treatment [31-34] and readiness to transfer.[35-38]

Adherence and self-management among adolescents and young adults with haemophilia has been found to be lower compared to other age groups [39], though not in all populations.[31, 32] Low adherence and suboptimal self-management may lead to more joint bleeds, pain and reduced quality of life.[11] Adolescents' readiness to transfer with a chronic disease is associated with their age, their level of knowledge, their self-management skills, health-related quality of life, self-efficacy [36-38, 40] and attitude towards transition.[37] Readiness to transfer appears to be independent of the type of chronic condition.[36-38]

Challenges with transition often identified by patients and their parents include the diminishing role of parents,[30] cultural gaps and collaboration between paediatric and adult services,[40, 41] parent and patient preparation[40, 41], strong reciprocal relationships between patients and their parents with physicians,[40] fear of the unknown,[40] a focus on age rather than on maturity or readiness,[40] suboptimal communication with the health care team[42], illness perception and acceptance[42-44], financial issues,[40] and non-disease-specific challenges that affect transition into adulthood, such as emotional and psychosocial factors.[30, 44] For adolescents with haemophilia, learning self-management, coping with the impact of haemophilia on their lifestyle and maintaining adherence are additional challenges. [40]

HiN-6 will evaluate adherence to treatment and associated issues in the adolescent and young adult Dutch haemophilia population.

1.3.3 Desmopressin use

Desmopressin is a vasopressin derivative that causes release of VWF from the vascular endothelium which leads to a rise in FVIII concentration. It is frequently used in people with mild haemophilia A and has relatively few side effects. [45] In addition, recent studies have shown that desmopressin can also be effectively used in people with moderate haemophilia A. [46] The HiN-6 study will evaluate use of desmopressin, side effects, the barriers to use desmopressin and the degree to which patients are informed about desmopressin by physicians.

1.3.4 Patient views on new treatment options

It is important to understand patient preferences and expectations for novel therapeutic options, such as pharmacokinetic-guided clotting factor dosing, gene therapy, FVIII/FIX products with extended half-lives and bi-specific antibodies. This information can be used to better inform patients about treatment innovations thereby increasing the chance of successful implementation.

1.3.5 Needle fear

Another aspect of quality of care is external factors that affect treatment. For example, different studies have found a high prevalence of fear of needles in general practice of 10 and 22 per cent,

respectively.[47, 48] Those with fear of needles were more likely to report vasovagal symptoms, have had a previous traumatic needle experience and avoid medical treatment involving needles.[48]Few studies exist about fear of needles in chronic disease. In children with type 1 diabetes, those that had fear of needles had higher glycated haemoglobin levels (a measure of diabetes control) and less frequent blood sugar monitoring than those without fear.[49] Younger children are more likely to have fear of needles than older children.[49, 50] The exact prevalence of fear of needles in haemophilia is unknown, but it is known from clinical practice that fear of needles is relatively common, particularly among children. How this affects adherence to treatment is currently unknown.

1.4 The variability in clinical phenotype among patients

Despite comparable coagulation factor activities in each category of severity, differences in clinical phenotypes have been described. Studies have shown that roughly 9-15% of patients with severe haemophilia have been described to have a very mild phenotype with few joint bleeding and almost absent joint damage. [51-56]

Clotting time assays such as PT and aPTT only measure the first part of the coagulation pathway, namely, the initiation phase until the formation of a visible fibrin clot. Therefore, they do not give a comprehensive overall picture of the clotting system. The utilization of global clotting assays may provide more accurate evaluation of an individual's in vivo haemostatic state and response to treatment. [57, 58]

In the HiN-6 study, we will evaluate two global haemostasis assays; the Calibrated Automated Thrombogram (CAT) assay and the Nijmegen Haemostasis Assay (NHA). It is expected that these assays will better reflect the patients' clinical phenotype. If proven useful, these assays can be helpful in guiding individual dose tailoring as part of a more personalised treatment strategy.

1.4.1 The Calibrated Automated Thrombogram (CAT) assay

The Calibrated Automated Thrombogram (CAT) assay as developed by Hemker et al [59] is a modified thrombin generation assay. Thrombin generation is calculated from the splitting of a fluorogenic substrate, which is calibrated against a sample with a known thrombin activity. The resulting thrombogram reflects the overall function of blood coagulation, balancing between both

pro-coagulant and anti-coagulant factors. The original thrombin generation assay is influenced by many pre-analytical variables (e.g. drawing of sample, sample handling time, temperature, colour of the plasma) as well as by critical analytical procedures (type of substrate, so called inner filter effect, need for calibration per sample). The CAT assay has standardized most of these aspects. However, interlaboratory comparison of CAT assays remains troublesome because of differences in substrates used. The CAT assays can be adapted by using different activators (TF or FIXa), different concentrations of activators, different sources (platelet rich plasma, platelet poor plasma, whole blood samples) or by adding various compounds (phospholipids, corn trypsin inhibitor to prevent contact activation).

1.4.2 The Nijmegen Haemostasis Assay (NHA)

Although thrombin generation reflects the patients' coagulation status, it does not entirely represent the in vivo situation of the patient because it does not take fibrinolysis into account. The NHA uses two fluorescent substrates with non-interfering fluorescent excitation and emission spectra to measure both thrombin and plasmin generation in a single well by a fluorimeter. [60] The assay has been shown to be sensitive for severe haemophilia A, reflected by severely diminished thrombin generation and, due to lack of TAFI activation, increased plasmin generation. Moreover, the assay is also sensitive for FV Leiden, the poly A prothrombin mutation and variations in Protein C and S activity. [60, 61]

1.4.3 Misclassification of disease severity by the one-stage clotting assay in haemophilia B patients

FVIII and FIX activity is most often assessed using the one-stage clotting assay. [62] Several studies have observed a discrepancy in factor VIII activity between the one-stage clotting assay and the chromogenic assay in patients with mild and moderate haemophilia A. More specifically, a higher factor VIII activity was found with the one-stage assay, compared to the chromogenic assay. [63-67] Several studies have found that certain FVIII mutations are associated with this discrepancy. [64, 67, 68]

Misclassification of disease severity by the use of the one-stage clotting assay could have important ramifications; patients could be designated as having a milder bleeding risk, potentially leading to undertreatment of patients. All almost studies have focused on haemophilia A, with the exception of one recent conference paper [69] which also found a discrepancy between the one-stage clotting

assay and the chromogenic assay.

Given the scarcity of evidence for this phenomenon in the haemophilia B population, the HIN-6 study will assess the extent of misclassification of disease severity by re-evaluating the baseline FIX activity using the chromogenic FIX assay in all haemophilia B patients.

1.4.4 Validating the iCHEC paediatric bleeding assessment tool

We will also describe bleeding scores in children using the newly developed paediatric bleeding assessment tool (iCHEC), in all children with haemophilia, and in subgroups according to haemophilia severity, age and treatment regimen (prophylaxis/on demand). Bleeding scores are expected to be higher among children with severe haemophilia as compared to children with moderate or mild haemophilia, higher in on-demand treated patients compared to patients on prophylaxis and higher with increasing age.

1.5 The immune response to FVIII

1.5.1 The humoral immune response to FVIII

The development of neutralizing antibodies against FVIII (inhibitor formation) is the most severe complication of treatment for haemophilia A patients. Antibodies against functional sites on the FVIII molecule (neutralizing antibodies) can be detected with the Bethesda assay. However, the Bethesda assay cannot detect antibodies against non-functional sites on the FVIII molecule (non-neutralizing antibodies). Alternative techniques such as ELISA [70] can detect a broader range of FVIII-specific antibodies. However, until recently these assays were not able to discriminate between neutralizing and non-neutralizing antibodies, this limited their usefulness in research and clinical practice.

Recently, several studies have shown that antibodies of the IgG4 subclass are more prevalent among patients with neutralizing antibodies, compared to patients with non-neutralizing antibodies. [70] In addition, by using a novel ELISA platform, Hofbauer et al found that they were able to discriminate between neutralizing and non-neutralizing antibodies based on the presence of high-affinity FVIII-specific antibodies. [71]

It is as of yet unclear how FVIII-specific non-neutralizing antibodies could influence the clinical course of disease. One longitudinal study in previously untreated patients (PUPs) by Cannavò et al

found that the presence of non-neutralizing antibodies before the first administration of FVIII was a risk factor for inhibitor formation. [72] There might also be a relationship between the presence of non-neutralizing antibodies and FVIII clearance. Recently, Hofbauer et al [73] conducted a study in which a significant association was found between the presence of FVIII-specific IgG non-neutralizing antibodies and a shorter FVIII half-life.

As of yet, most studies on this subject are small and/or cross-sectional in nature, therefore, the HiN-6 study will assess the prevalence and characteristics of FVIII-specific non-neutralizing antibodies. In addition, The HiN-6 study will also assess the impact of non-neutralizing antibodies on inhibitor development, FVIII activity/antigen levels, joint bleeds and increased FVIII clearance. The HiN-6 will also assess possible misclassification of low-titre inhibitors as non-neutralizing antibodies using a low-titre inhibitor assay ((W.L. van Heerde, personal communication).

1.5.2 The cellular immune response to FVIII

The primary immune response to FVIII is initiated by antigen presenting cells (APCs). The first step, namely antigen presentation by APCs to CD4+ T-cells, is dependent on the presence of a pro-inflammatory micro-environment. The FVIII molecule is proteolytically processed into small peptides, loaded onto the MHCII molecule and presented on the APC cell surface. Subsequent activation of CD4+ T-cells requires upregulation of co-stimulatory molecules CD40 and CD80/86 and pro-inflammatory cytokine production by the APC. [74]

T-effector cells (Teffs) stimulated in this manner can induce an immune response by using cytokines and surface molecules. In contrast to Teffs, CD4+ CD25+ FoxP3+ T-regulatory cells (Tregs) can also suppress an immune response through cytokine production (IL-10 or TGF-beta) and cell-to-cell contact. The specific type of T-cell response is most likely determined by the environmental context of antigen presentation the APC.

Several experimental animal studies have found an association between high Treg to Teff ratios and a decreased risk of FVIII-specific antibody formation. Miao et al found that FVIII-deficient mice that overexpress CD4+ FOXP3-positive regulatory T-cells did not develop FVIII antibodies after plasmid-mediated F8 gene transfer. [75] In addition, Waters et al found that administering antiCD3 antibodies in haemophilic inhibitor mice resulted in a higher of ratio of splenic Tregs to Teffs. This effect was associated with a decrease in both inhibitor incidence and anti-FVIII antibody titres when

compared to controls. [76]

We hypothesize that natural differences in the APC response to FVIII can result in a higher Treg to Teff ratio, this in turn will lead to less FVIII-specific antibody formation. The HiN-6 study aims to identify novel immunological risk factors for the development of FVIII-specific antibodies by assessing both the APC response to FVIII and the subsequent T-cell response.

Chapter 2 – General and specific objectives of the HiN-6 study

This chapter describes the five general objectives and their accompanying specific objectives.

2.1 General objective: Describing the health status of the Dutch haemophilia population

2.1.1 45 years of haemophilia treatment in the Netherlands, 1972-2017

Main objectives:

1. To describe changes in bleeding, factor consumption, bleeding-induced joint damage, inhibitor formation, HIV/HCV infection as well as cause-specific and all-cause mortality among Dutch PWH for the period 1972-2017.
2. To describe the prevalence of several haemophilia-related and non-haemophilia related co-morbidities in the Dutch haemophilia population and to compare these results with the general Dutch male population.
3. To describe the uptake, efficacy and safety of hepatitis C treatment in PWH currently or previously infected with hepatitis C.

2.1.2 Physical activity, sports and health outcomes

Main objectives:

1. To assess sports participation according to age and haemophilia severity and compare this to the general population.
2. To assess the association of the amount and type of sports practice with bleeding

frequency, self-reported activities , self-reported physical performance and joint function.

2.2 General objective: To assess health-related quality of life of PWH in the Netherlands

2.2.1 General quality of life

Main objectives:

1. To describe health-related quality of life in all PWH in the Netherlands over time since 1992.
2. To quantify the association between health-related quality of life and disease severity, degree of arthropathy, bleeding phenotype, pain and pain coping, self-reported joint function and ability to self-manage in patients with mild, moderate or severe haemophilia.
3. To quantify the relationships between health-related quality of life and the prevalence of social determinants of health, including participation and social functioning, ability to work, education and relationships patients with haemophilia. These study questions will also be separately assessed in the older population (>50yr)
4. To assess the incidence of falls and the prevalence of fear of falling in older adults (>65 years of age) with haemophilia.
5. To assess the incidence of a false-positive result of the self-administered faeces test in the colon cancer screening test in adults with haemophilia between 55 and 75 years of age.
6. To assess the incidence of abnormal results of the additional colonoscopy in people with haemophilia compared to the general population.

2.2.2 Sexuality

Main objectives:

1. To assess the incidence of hematospermia in PWH.
2. To describe the presence of limitations in sexual health due to haemophilia in PWH.

2.3 General objective: To assess quality of care of PWH

2.3.1 General quality of care

Main objective:

1. To assess quality of care from a patient's perspective, including information provision, communication with care providers and financial consequences of health care services use.
2. To assess treatment satisfaction of PWH.

2.3.2 Transition

Main objectives:

1. To compare adherence in adolescents and young adults with severe haemophilia with adherence in other age groups
2. To assess the relationship between self-efficacy, treatment adherence and self-reported readiness for transition in adolescents and young adults with severe haemophilia.

Secondary objective:

1. To assess the relationship between adherence, factor use, absence from work or school and bleeding outcomes in young adults with severe haemophilia.

2.3.3 Desmopressin use

Main objectives:

1. To assess desmopressin use, perceived side effects, barriers for use and information provision by clinicians in patients with mild and moderate haemophilia A.

2.3.4 Patient views on new haemophilia treatment options

Main objective:

1. To assess patients' perceptions of new treatment options (including gene therapy, extended half-life clotting factors, non-replacement coagulation factor therapies and tailored prophylaxis by using pharmacokinetic dosing)

2.3.5 Needle fear

Main objective:

1. To assess the prevalence of needle fear or needle phobia among PWH.

Secondary objective:

1. To assess the association between needle fear and adherence.

2.4 General objective: To elucidate the variability in clinical phenotype among PWH

2.4.1 To assess the usefulness of The Calibrated Automated Thrombogram (CAT) assay in relation to clinical phenotype:

Main objectives:

1. To establish specific sample conditions to generate reliable CAT assay outcome parameters in patients with haemophilia.

Secondary objectives:

1. To assess the association between the results of the CAT assay and the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia.
2. To evaluate if the CAT assay can accurately diagnose the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia.

2.4.2 To assess the usefulness of the Nijmegen Haemostasis Assay (NHA) in relation to clinical phenotype:

Main objectives:

- To assess the association between the results of the NHA and the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia A and B.
- To evaluate if the NHA can accurately diagnose the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia.

2.4.3 To assess misclassification of disease severity by the one-stage clotting assay in haemophilia B patients:

Main objectives:

1. To assess the extent of discrepancy in FIX activity level as measured by the one stage clotting assay to the chromogenic assay in patients with mild and moderate haemophilia B.

2. To evaluate the association between F9 genotype and discrepancy between the FIX activity levels as measured by the one-stage clotting assay and the chromogenic assay in patients with mild and moderate haemophilia B.

2.4.4 To describe bleeding scores in children with haemophilia with the newly developed paediatric bleeding assessment tool (iCHEC):

Main objective:

1. To describe bleeding scores in all children with haemophilia
2. To describe bleeding scores according to haemophilia severity, age and treatment regimen (prophylaxis/on demand).

2.5 General objective: To assess immunological mechanisms that may lead to inhibitor formation in patients with haemophilia A

2.5.1 Specific objectives assessing the relationship between FVIII-specific non-neutralizing antibodies and inhibitor formation:

Primary objectives:

1. To assess the prevalence of FVIII-specific non-neutralizing antibodies (NNAs) among non-inhibitor patients with severe, moderate and mild haemophilia A in the Netherlands.
2. To characterize all FVIII-specific antibodies with respect to Ig class, IgG subclass and affinity, and to evaluate whether some of these characteristics are more associated with having neutralizing antibodies (inhibitors) rather than non-neutralizing antibodies (NNAs).

Secondary objectives:

1. To assess whether F8 genotype and polymorphisms in immune response genes influence the development of non-neutralizing antibodies (NNAs).
2. To assess whether F8 genotype and polymorphisms in immune response genes influence the development of either neutralizing antibodies (inhibitors) or non-neutralizing antibodies (NNAs).
3. To evaluate the association between non-neutralizing antibodies (NNAs) and bleeding frequency in patients with severe haemophilia A.
4. To evaluate potential misclassification of low-titre inhibitors as non-neutralizing antibodies

in patients with haemophilia A.

5. To evaluate the diagnostic performance of a modified FVIII ELISA for detection of FVIII-specific antibodies (inhibitors), using the Bethesda assay as the reference test.

2.5.2 Specific objectives assessing dendritic cell response and T-cell activation in the FVIII immune response:

Primary objectives:

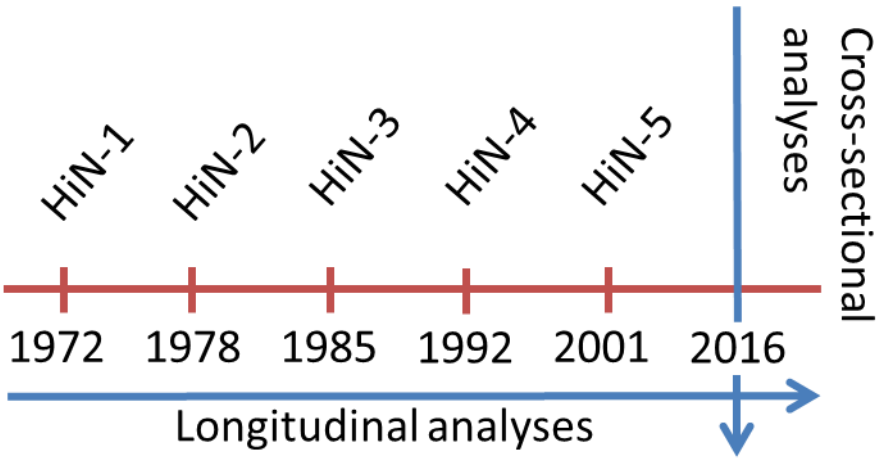
1. To compare the expression of interleukin-2 and TGF-beta in monocyte-derived dendritic cells after stimulation with FVIII with and without danger signals (e.g. LPS) in patients with non-neutralizing FVIII-specific antibodies (NNAs) and patients without any current FVIII-specific non-neutralizing antibodies.
2. To compare the regulatory T- cell count, as a proportion of all CD4+ T-cells in patients with non-neutralizing FVIII-specific antibodies (NNAs) or patients without any current FVIII-specific antibodies.

Chapter 3 - Study design

HiN-6 study design

The HiN-6 study will consist of both cross-sectional and longitudinal observational studies, according to the specific research objective. A mixed-methods approach will be used for data collection and analysis. All participants will be asked to fill out a questionnaire and to provide a blood and urine sample. In addition, information will be obtained from medical records. For some of the planned substudies, a smaller number of patients will be asked to provide an additional blood sample at a second visit. Detailed study procedures can be found in chapter 5.

The HiN-6 is the sixth iteration of a series of cross-sectional studies. By combining information from previous HiN studies with the current HiN-6 study, we will also be able to perform a number of longitudinal analyses (see Chapter 6 for specific substudies).



Chapter 4 - Study population

4.1 Population

All male Dutch patients with severe, moderate, or mild haemophilia A or B who are registered as such at one of the seven haemophilia treatment centres in the Netherlands will be eligible for inclusion. In addition, currently deceased patients that participated in the HiN-5 questionnaire in 2001 will also be eligible for inclusion. Severe haemophilia A or B is defined as having FVIII or FIX levels <0.01 IU/ml, moderate haemophilia A or B is defined as having FVIII or FIX levels between 0.01-0.05 IU/ml and mild haemophilia A or B is defined as having FVIII or FIX levels >0.05-0.40 IU/ml.

4.2 Inclusion criteria

- Male patients with severe, moderate or mild congenital haemophilia A or B with endogenous clotting factor activity levels <0.40 IU/ml who are registered at one the haemophilia treatment centres in the Netherlands.
- Male patients with congenital haemophilia A or B who underwent liver transplantation in the past (irrespective of their endogenous clotting factor levels).
- All currently deceased male haemophilia A or B patients that participated in the HiN-5 questionnaire in 2001.
- For patients ≥ 16 years old; written informed consent.
- For patients 12-15 years old; written informed consent from both the patient and their parents/legal guardian(s).
- For patients <12 years old; written informed consent from their parents/legal guardian(s).

4.3 Exclusion criteria

Patients with the following criteria will be excluded from the study:

- Female carriers of haemophilia A or B
- Patients with acquired haemophilia.
- Non-haemophilic patients with reduced FVIII levels due to VWD.

4.4 Sample size calculation

We aim to include the entire Dutch haemophilia population (roughly 1800 patients). Based on the response rate of the 5 previous HiN studies, we expect a response rate of around 70%, which corresponds to 1260 patients.

Chapter 5 - Methods

5.1 Data collection

Data will be collected through questionnaires filled in by each participant (see 5.1.1), blood/urine samples obtained from each participant (see 5.1.2) and from each participant's medical record using case report forms (see 5.1.3).

5.1.1 Questionnaires

Patients will receive a letter from their physician with a personal log in code to an electronic questionnaire. They will be reminded after one month to fill out the questionnaire online. Patients who are unwilling or unable to fill out the questionnaire electronically will be provided with a hard copy of the questionnaire. The following subjects are included in the questionnaire:

- demographic characteristics
- socio-economic characteristics
- clinical characteristics (bleeds, treatment, inhibitors, other medication), HIV status (patients born before 1985) and hepatitis C status (patients born before 1992).
- medical history: other chronic conditions, hospital admissions, colon cancer screening
- sexuality
- general health status / quality of life (RAND-36, PROMIS29)[77, 78]
- sports, physical activity and self-reported physical performance (MAQ, HEP-TEST-Q)[79, 80]
- functional limitations (HAL)[81]
- adherence (VERITAS-pro)[82, 83]
- pain
- needle fear
- self-management (PAM-13)[84]
- fear of falling (FES)
- experience with care, novel treatment options
- treatment satisfaction (Hemo-SAT)[85]

Children's versions of the questionnaire will be available for children up to 17 years old. In general,

children from 12-17 years old will fill out the questionnaire independently. For children under 12 years old, parents will fill out most of the questions. Topics covered in the children's questionnaire are listed below:

- socio-demographic characteristics
- daily life
- clinical characteristics (bleeds, treatment, inhibitors)
- medical history (hospital admissions, medications used)
- pain
- needle fear
- sports and physical activity (MAQ, HEP-test-Q)[79, 80]
- quality of life (CHO-KLAT [86])
- functional limitations (PedHAL [88])
- self-efficacy (HSES)[89]
- bleeding score (iCHEC)
- treatment satisfaction (Hemo-SAT)[85]

Adolescents (15-25 years) will either fill out the children's version of the questionnaire if they are younger than 18 years old, or the adult version if they are 18 or over. In addition, individuals in the 15-25 age group will fill out a self-efficacy questionnaire (HSES) [89] Patients older than 65 years will be asked to fill out the modified Falls Efficacy Scale (FES)[90]. An overview of used questionnaires is provided in Appendix 3.

In total, the questionnaire will take approximately 40-120 minutes to complete, depending on which questions are relevant to the patient (e.g. patients born after 1992 will not fill out questions about hepatitis C and HIV infections). The electronic questionnaire system will automatically skip questions if they are not relevant for that particular patient.

The questionnaire will be built into the Castor software.

5.1.2 Blood and urine sampling

First visit (all participants):
All participants will be asked to provide a blood/urine sample during a regular visit to the

haemophilia treatment centre. Part of the blood sample will be used for this study, the remainder of the blood sample and the entire urine sample will be stored in a national biobank for future research purposes. (see table 1) The biobank will be part of the "Parelsnoer Institute", a national organization that provides the infrastructure for the establishment of clinical biobanks in all Dutch university medical centres. Additional information about the biobank and the Parelsnoer Institute is provided in the following documents:

- Parelreglement Parel Hemofilie (version 5.1)
- Parelsnoer Kaderreglement (version 3.1)

Blood is sampled at least 3 days after the last FVIII or DDAVP administration, at least 5 days after the last FIX administration and at least 7 days after resolution of a bleed. Blood sampling is voluntary. Depending on the age of the patient, 7.2 to 55 ml of blood will be drawn through venepuncture. Sampling of 1-5% of an individual's total blood volume is deemed safe according to WHO guidelines. [91] In the current project the maximum sampling volume will be set at 2.5% of the total blood volume. At the first visit, between 7.2-55 ml (depending on the age of the patient) of blood will be collected from all participants. (see table 1)

In addition, 10 ml of midstream urine will be collected from all adults and toilet-trained children. To minimize patient inconvenience, a spot urine sample will be collected instead of a 24-h urine sample. (see table 1)

Table 1: blood, urine and DNA collection during the first visit

Age category	**Total drawn blood volume**	**Citrated plasma for HiN-6 study**	**Serum and/or citrated plasma for biobank**	**DNA sample for HiN-6 study/biobank**	**Urine sample for biobank***
0-4 years	7.2 ml	2.25 ml plasma	1.35 ml plasma	Obtained from citrate tubes after removal of plasma	10 ml midstream urine
4-10 years	9ml	2.25 ml plasma	2.25 ml plasma		
10-12 years	17,5ml	2.25 ml plasma	2 ml serum, 4.5 ml plasma		
>12 years	55 ml	6.75 ml plasma	5 ml serum, 15.75 ml plasma		

*: Children that are not toilet trained are excluded.

Blood samples will be used for the following experiments:

- **The Nijmegen Haemostasis Assay (NHA).** [60]

- **The Calibrated Automated Thrombogram (CAT) assay.** [59]

- **Bethesda assay:** We will measure the presence of inhibitors using the Bethesda assay with Nijmegen modification. A titre of at least 0.6 Bethesda Units is required to be positive for this assay. The cut-off point for low-titre vs high-titre inhibitors is 5.0 Bethesda Units. [92]

- **Low-titre inhibitor assay:** To assess the presence of low-titre inhibitors, we will use a low-titre inhibitor assay. (W.L. van Heerde, personal communication)

- **ELISA:** We will assess the presence and characteristics of FVIII-specific antibodies in our full cohort using a highly sensitive and fully validated ELISA platform developed by Whelan et al. [70] In patients who are positive for antibodies using this assay we will characterize the isotype (IgA, IgM and IgG) and for the IgG isotype also the subclass (IgG1 to 4). Samples with confirmed specificity for FVIII will also be assessed for apparent affinity.

  The apparent affinities of FVIII-specific antibodies for all immunoglobulin isotypes and IgG subclasses will be assessed using a competition-based ELISA approach by Hofbauer et al. The affinity assessment is based on the availability of antibody for binding to FVIII-coated ELISA plates after competition with FVIII in solution. [71]

- **Other laboratory tests such as:**

  - FVIII and FIX activity using one-stage assay
  - FVIII and FIX activity using chromogenic assay
  - FVIII and FIX antigen level
  - FVIII genotype (if unknown)
  - FVIII haplotype
  - HLA/IL-10/TNFα/CTLA4/HO-1/FcgR gene variants

Second visit (50 adult participants):

Based on the results of the first analyses, we will draw an additional 50 mL of blood in 50 selected participants during a second visit to the haemophilia treatment centre. This second sample will be only be used for this substudy and will not be stored in the biobank. Blood samples will be used for the following experiments:

- **Regulatory T cell count:** Regulatory T cell count will be assessed using both flow cytometry and a quantitative real-time PCR assay developed by Epiontis (Epiontis Treg assay).

  Flow cytometry

  After stimulation of PBMCs with FVIII, we will quantify all T-cell subsets using flow cytometry. PBMCs will be stained with monoclonal antibodies against cell receptors specific for a certain T-cell subset (e.g. CD4+, CD25high, CD127low, FOXP3+ for T-regulatory cells) [93] and markers of T-cell activation (e.g. CD154) [94].

  Epiontis Treg assay

  DNA will be isolated from whole blood using the DNeasy blood and tissue kit (QIAGEN) according to the manufacturer's protocol. We will use a quantitative real-time PCR assay developed by Epiontis (Epiontis Treg assay). This assay measures the number of FOXP3 genomic DNA copies lacking covalent modifications at CpG sites in the Treg-specific demethylated region. Demethylation of the FOXP3 gene is a highly specific epigenetic marker for T-regulatory cells. [95]

- **Dendritic cell response to FVIII:**

  Isolation

  Peripheral blood mononuclear cells (PBMCs) will be isolated from buffy coats by density gradient centrifugation with Ficoll. Monocytes will be purified from PBMCs by positive selection using CD14 MicroBeads. To generate monocyte-derived dendritic cells (moDCs), CD14+ monocytes will be cultured in 96-well tissue culture plates in the presence of GM-CSF and IL-4 for five days at 37 °C, 5 % CO2. [96]

  Stimulation of moDCs and analysis

  MoDCs will be stimulated with FVIII in combination with and without additional substances that are expected to act as activating 'danger signals' (such as IFNγ, TNFα, and TLR ligands of both microbial and endogenous origin) or 'tolerogenic signals' (such as IL10). The cytokine response will be analysed using Luminex (BioRAD) and the transcriptome using RNA-seq (AMC Amsterdam).

5.1.3 Electronic patient records

The clinical data will be collected from the medical charts using a case report form. Each patient will be assigned a unique study code, as described in chapter 8. A copy of the CRF will be sent to the coordinating centre (Leiden University Medical Centre) for data entry and a copy will be kept at the original centre. We will obtain the following information from electronic patient records, along with the dates at which measurements were performed:

- **Variables that will be extracted from the electronic patient records:**

  General

  - Year of birth
  - Vital status, if deceased:
    - Cause of death
  - Type of haemophilia
  - Bodyweight and height
  - Age at first joint bleed
  - F8 or F9 genotype
  - Date of first blood sample
  - Date of last FVIII/FIX infusion before first blood sample
  - Product and dose used during last FVIII/FIX infusion before first blood sample
  - Baseline FVIII/FIX activity level (one-stage clotting assay/chromogenic assay)
  - Baseline FVIII/FIX antigen level
  - Baseline VWF activity level, baseline VWF antigen level (VWF: Ag)
  - Current treatment type (prophylactic treatment or on-demand treatment) , frequency and dose
  - Bodyweight and height

  Current treatment

  - Current treatment regimen
    - Current product(s) used
    - Prophylaxis vs. on-demand treatment

  - Frequency, dose of prophylactic treatment (if applicable)
  - Frequency, dose of treatment for minor/major/life-threatening bleeding
- Immune tolerance induction protocol (if applicable)
  - Current product(s) used
  - Frequency, dose
  - Use of immune modulatory medication

Co-morbidities

- HIV status, if positive:
  - Treatment type
  - HIV viral load
- Current and previous hepatitis C status, if positive:
  - HCV genotype
  - Date of last positive HCV RNA assay
  - Outcome of last Fibroscan
  - Latest Child-Pugh score
  - Presence of ascites
  - Presence of bleeding oesophageal varices
  - Presence of hepatic encephalopathy
  - Liver transplantation
  - All previous HCV treatment regiments
  - SVR after last HCV treatment

Inhibitor development

- Number of exposure days to FVIII (<50 EDs, 51-100 EDs, 101-150 EDs, >150 EDs)
- Previous or current Bethesda assay, if positive:
  - Number of exposure days to FVIII before first positive inhibitor assay
  - First two positive inhibitor assays (date, inhibitor titre and FVIII:C recovery)
  - Peak inhibitor titre and corresponding FVIII:C recovery
  - Initiation of immune tolerance induction (ITI)
  - First negative inhibitor test during ITI
  - First normal recovery during ITI
  - Confirmed inhibitor relapse after a successful ITI procedure (if positive, the

aforementioned information is also collected for the second inhibitor)

Determinants and outcomes calculated from collected data are:

- **Clinical bleeding phenotype**

  Our score for bleeding severity is based on the bleeding severity score by Schulman et al. [97] Schulman et al also includes a joint subscore, however, we do not have this data for all patients, therefore we have decided to remove the joint subscore from the final score. Our bleeding severity score consists of 2 components; the annual joint bleed rate and annual factor consumption (corrected for bodyweight). Lastly, we will calculate the annual joint bleed rate/factor consumption using information from the HiN-6 questionnaire:

  We will obtain the patient-reported annual joint bleed rate for the year 2016 using the HiN-6 questionnaire. This value is divided by 20 (which the roughly equal to the maximum number of joint bleeds that a patient without any treatment will experience). [97] This gives you a value between 0 and 1. We will obtain the patient-reported annual factor consumption for the year 2016 using the HiN-6 questionnaire. This number is divided by the mean body weight of the patient during this period. This value is then divided by 6000 (IU / kg), which is approximately the maximum consumption of regular prophylaxis. [97] This gives you a value between 0 and 1. The final score is simply the sum of the two aforementioned subscores, the score has a minimum of 0 and a maximum of 2.

- **BMI:** BMI is calculated as weight (kg) divided by height^2 (meters).

Chapter 6 – Substudies

This chapter describes all substudies that will be performed as part of the HiN-6 project. The objectives of the substudies are derived from the following overarching general objectives:

1. Describing the health status of the Dutch haemophilia population
2. Assessing the quality of life of PWH in the Netherlands
3. Assessing the quality of care of PWH in the Netherlands

4. Elucidating the variability in clinical phenotype among PWH
5. Assessing immunological mechanisms that may lead to inhibitor formation in patients with haemophilia A

6.1 Studies describing the health status of the Dutch haemophilia population

6.1.1 45 years of haemophilia treatment in the Netherlands, 1972-2017

Primary objective 1: To describe changes in bleeding, factor consumption, self-reported bleeding-induced joint damage, inhibitor formation, HIV/HCV infection as well as cause-specific and all-cause mortality among Dutch PWH for the period 1972-2017.
Study design and population: We will compare between HiN-study cohorts (previous and current) on the endpoints stated below. For mortality, we will also compare our data to that of the general male population. The population will consist of all previous HiN-study cohorts as well as the current HiN-6 cohort.
Determinant: Disease severity and calendar time
Endpoints: Self-reported annual joint bleed rate/factor consumption, self-reported bleeding-induced joint disease, inhibitor formation, HIV/HCV infection, all-cause/cause-specific mortality will be used as endpoints. Information on these endpoints will be obtained using a questionnaire.
Statistical analysis: We will use descriptive statistics where appropriate.

Primary objective 2: To describe the prevalence of several haemophilia-related and non-haemophilia related co-morbidities in the Dutch haemophilia population and to compare these results with the general Dutch male population.
Study design and population: A cross-sectional study design will be used. The population will consist of all PWH in the Netherlands.
Determinant: Disease severity, HIV/HCV infection status and inhibitor status.
Endpoints: Cardiovascular disease (CVD), malignancies, obesity, liver disease, chronic kidney disease (CKD), diabetes, osteoporosis, chronic pain and mental health. Information on these endpoints will be collected using a questionnaire and/or from each patient's medical record.
Statistical analysis: We will use descriptive statistics, standardized by age. Our results will be compared with national figures for the general male population provided by the Central Bureau of Statistics Netherlands StatLine databank, standardized directly or indirectly by age.

Primary objective 3: To describe the uptake, efficacy and safety of hepatitis C treatment in PWH currently or previously infected with hepatitis C.

Study design and population: A cross-sectional study design will be used. The population will consist of all PWH in the Netherlands currently or previously infected with hepatitis C.

Determinant: Last treatment for hepatitis C will be used as the determinant. Treatment type is subdivided into never treated for hepatitis C, treated with direct-acting antivirals only, treated with direct acting antivirals + ribavirin or treated with (peg)interferon + ribavirin.

Potential confounder: HCV genotype

Endpoint: Uptake, efficacy and safety will be used as the endpoints. Uptake is defined as the proportion of patients that were treated with a given treatment type. Efficacy is defined as achieving a sustained viral response (SVR), which is defined as the absence of detectable RNA of the hepatitis C virus in blood serum for at least 24 weeks after discontinuing treatment. Safety-endpoints are defined as having had any of the following adverse-effects during treatment with antiviral therapy; Headache, fatigue, concentration problems, insomnia, depressive symptoms, anxiety, irritability, flu-like symptoms, nausea, diarrhoea, pruritus, asthenia, dyspnoea, arthralgia, myalgia, rash, cachexia or jaundice.

Statistical analysis: We will describe the uptake, efficacy and safety of the different hepatitis C treatment types using descriptive statistics.

6.1.2 Physical activity, sports and health outcomes

Objective 1: To assess sports participation according to age and haemophilia severity and compare this to the general population.

Study design and population: A cohort study design will be used. The population will consist of all haemophilia A and B patients of 8 years and older that filled in the HiN-6 questionnaire.

Determinant: sports type according to chance of falling and/or collisions with other players. Sports will be classified according to expected frequency and severity of collisions.[98] Category 1 activities are activities in which significant collisions are not expected (such as swimming). Category 2 are activities in which significant collisions might occur and category 3 activities are those in which significant collisions are inevitable, as described previously.[98] Also, sports will be categorized into metabolic equivalent scores (MET) in order to assess overall physical activity. This information will be obtained using a questionnaire.(MAQ)). In addition the intensity of physical activity will be calculated as METs.

Endpoints: Participation in high-, medium , and low impact sports and METs with the general population (according to age categories).

Statistical analysis: We will use descriptive statistics to compare PWH with the general population.

Objective 2: To assess the association of the amount and type of sports practice with bleeding frequency , self-reported activities , self-reported physical performance and joint function.
Study design and population: A cohort study design will be used. The population will consist of all haemophilia A and B patients that filled in the HiN-6 questionnaire .
Determinant: sports participation (measured with the MAQ questionnaire), self-reported activities (measured with the HAL) and self-reported physical performance (measured with the HEP-Test-Q) In addition, all potential confounders, such as treatment regimen , age, and haemophilia severity collected in HIN6 will be included.
Endpoints: Bleeding frequency (primary) and joint function (secondary) will be used as endpoints. Bleeding frequency is defined as the number of joint bleeds that required medical treatment in the previous year. Information on bleeding severity is collected through a questionnaire. Joint function is evaluated by haemophilia joint health scores (HJHS) available in medical records..
Statistical analysis: We will use linear conditional regression to explore which parameters of sports participation, HAL scores, and domain scores of HEP-Test-Q best explain the variation in bleeding frequency and joint function. Analyses will be adjusted for age and haemophilia severity.

6.2 To assess the health-related quality of life of PWH in the Netherlands

6.2.1 General quality of life

Primary objective 1: To describe health-related quality of life of PWH in the Netherlands over time since 1992.
Study design and population: A dynamic cohort design will be used. All haemophilia patients who filled out the RAND -36 questionnaire in 1992, 2001 and in the current study will be included.
Determinant: disease severity, calendar time and age.
Endpoint: We will use the RAND-36 (adults) and the CHO-KLAT (children) to evaluate overall health-related quality of life.
Statistical analyses: Descriptive statistics will be used. We will conduct a repeated measurements analysis with the results of the previous HiN-studies from 1992 and 2001 using the RAND-36. We will also compare our results with the general male population using the RAND-36.

Primary objective 2: To quantify the association between health-related quality of life and disease

severity, presence of comorbidities, past and current presence of inhibitors, degree of self-reported joint health status, bleeding phenotype, pain, joint function, treatment type and ability to self-manage in patients with mild, moderate and severe haemophilia.
Study design and population: Cross-sectional. The total population will consist of all PWH in the Netherlands.
Determinant: Disease severity (mild, moderate or severe), the self-reported number of bleeds that required medical treatment in the previous year, ability to self-manage and the degree of arthropathy will be used as the determinants. Ability to self-manage will be assessed with the validated PAM-13 questionnaire (adults) and the HSES (children). Other determinants will be obtained using a questionnaire and/or extracted from electronic medical records.
Endpoint: We will use the RAND-36 (in adults) and the CHO-KLAT (in children) as the endpoint.
Statistical analysis: Multiple linear regression will be used to assess these associations.
Subgroup analyses will be performed for PWH aged >50 years.

Primary objective 3: To investigate the relationships between health-related quality of life and the prevalence of social determinants of health, including participation and social functioning, ability to work, education and relationships in older adults (>50 years of age) with haemophilia, and compare with 2001.
Study design and population: cross-sectional design in adult population >50 years of age and dynamic cohort of people who filled in the questionnaire in 2001.
Determinant: Disease status and severity, age, social determinants of health (such as socio-economic status, social participation). Information on determinants will be obtained from the questionnaire.
Endpoint: HRQoL, as measured with the RAND-36
Statistical analyses: linear regression analyses will be performed.

Primary objective 4: To assess the incidence of falls and the prevalence of fear of falling in older adults (>50 years of age) with haemophilia.
Study design and population: Cross-sectional design in adults >65 years of age. Fear of falling will be assessed using the modified FES questionnaire. Falls history will be obtained from the patient's medical record.
Determinant: Age, disease severity, use of walking aid, previous joint replacement surgery and falls history.
Endpoint: Fear of falling as measured with the FES questionnaire and falls history.

Statistical analyses: Descriptive statistics will be used.

Primary objective 5: To assess the incidence of a false-positive result of the self-administered faeces test in the colon cancer screening program in adults with haemophilia between 55 and 75 years of age.
Study design and population: cross-sectional design in adults between 55 and 75 years of age who participated in the voluntary colon cancer screening program.
Determinant: disease status
Endpoint: positive result on the self-administered faeces test of the colon cancer screening program. This information will be obtained from the questionnaire.
Statistical analyses: descriptive statistics will be used to assess the incidence of a positive test result.

Primary objective 6: To assess the incidence of abnormal results of the additional colonoscopy in people with haemophilia compared to the general population.
Study design and population: cross-sectional design in adults between 55 and 75 years of age who participated in the voluntary colon cancer screening program and underwent an additional colonoscopy.
Determinant: disease status
Endpoint: positive result on the colonoscopy. This information will be obtained from the medical record.
Statistical analyses: descriptive statistics will be used to assess the incidence of a positive colonoscopy.

6.2.2 Sexuality

Primary objectives: To assess the incidence of hematospermia in PWH and to describe the presence of limitations in sexual health due to haemophilia in PWH.
Study design and population: a cross-sectional design will be used to assess hematospermia and limitations in sexual health in all adults with haemophilia A or B. Information will be obtained with the questionnaire.
Determinant: disease status and severity
Endpoint: self-reported hematospermia, self-reported limitations in physical sexual functioning.
Statistical analyses: Descriptive statistics will be used.

6.3 To assess quality of care of PWH in the Netherlands

6.3.1 General quality of care

Primary objective 1: To assess quality of care from a patient's perspective, including information provision, communication with care providers and financial consequences of health care services use.
Study design and population: A cross-sectional study design will be used.
Determinant: disease status and severity
Endpoint: Quality of care as represented by satisfaction with provided treatment, accessibility and availability of care, information provision and communication.
Statistical analyses: Descriptive statistics will be used.

Primary objective 2: To assess treatment satisfaction of PWH.
Study design and population: A cross-sectional study design will be used. Information about treatment will be obtained from the Hemo-SAT questionnaire.
Determinant: disease status and severity
Endpoint: treatment satisfaction according to the Hemo-SAT questionnaire.
Statistical analysis: Descriptive statistics will be used.

6.3.2 Transition

Primary objective 1: To compare adherence in adolescents and young adults with severe haemophilia to adherence in other age groups.
Study design and population: A cross-sectional study design will be used. Adolescents and young adults (15-25 years of age) with severe haemophilia A and B.
Determinant: Age
Endpoint: Adherence, as measured with the validated VERITAS-pro questionnaire.
Statistical analyses: Descriptive statistics will be used.

Primary objective 2: To assess the relationship between self-efficacy, treatment adherence and self-reported readiness for transition in adolescents and young adults with severe haemophilia.
Study design and population: A cross-sectional study design will be used. 'Readiness for transition'

in adolescents before transition will be measured with a single question. Self-efficacy will be measured with the haemophilia-specific self-efficacy scale (HSES).
Determinant: age, self-efficacy, health-related quality of life
Endpoint: readiness for transition.
Statistical analyses: Linear regression will be used to explore factors that explain readiness for transition.

Secondary objective 1: To assess the relationship between adherence, factor use, absence from work or school and bleeding outcomes in young adults with severe haemophilia.
Study design and population: A cross-sectional study design will be used. Adherence will be assessed with the VERITAS-pro questionnaire. Other information will be obtained from the questionnaire.
Determinant: age, adherence, factor use, absence from work or school
Endpoint: number of bleeds
Statistical analyses: Linear regression will be used to explore factors that explain readiness for transition.

6.3.3 Desmopressin use

Primary objective 1: To assess desmopressin use, side effects, barriers for use and information provision by clinicians
Study design and population: A cross-sectional design will be used. All patients with moderate or mild haemophilia A will answer questions about desmopressin use
Determinant: mild or moderate haemophilia A.
Endpoint: Use of desmopressin, (perceived) side effects, barriers for use and information provision by clinicians
Statistical analyses: Descriptive statistics will be used.

6.3.4 New treatment options for haemophilia

Primary objective 2: To assess patients' perceptions of new treatment options (gene therapy, extended half-life clotting factors, non-replacement coagulation factor therapies and tailored prophylaxis by using pharmacokinetic dosing) and factors that may explain these perceptions, such as age and disease severity.

Study design and population: A cross-sectional design will be used.
Determinant: disease status and severity, age
Endpoint: attitude towards new treatment options
Statistical analyses: Descriptive statistics will be used. Results will be compared to patients' opinions on these topics in 2001.

6.3.5 Needle fear

Primary objective 1: To assess the prevalence of needle fear or needle phobia among PWH.
Study design and population: A cross-sectional design will be used. The questionnaire contains questions about needle fear and symptoms.
Determinant: disease status and severity, treatment method (prophylaxis, on-demand), age. Information on these determinants will be obtained from the questionnaire.
Endpoint: self-reported fear of needles
Statistical analyses: Descriptive statistics will be used.

Secondary objective 1: To assess the relationship between needle fear and adherence in patients with severe haemophilia.
Determinant: needle fear
Endpoint: Adherence (as measured with the VERITAS-pro questionnaire),
Statistical analysis: Linear regression analysis will be used to assess to what extent adherence is explained by needle fear.

6.4 Studies elucidating the variability in clinical phenotype among PWH

6.4.1 To assess the usefulness of The Calibrated Automated Thrombogram (CAT) assay in relation to clinical phenotype.

Primary objective 1: To establish specific sample conditions to generate reliable CAT assay outcome parameters in patients with haemophilia.
Study design and population: In vitro spiking experiments will be used to establish specific sample conditions to generate reliable CAT assay outcome parameters. Thrombin Generation (TG) in FVIII or FIX deficient plasma is determined after in vitro suppletion of FVIII or FIX in final activity of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50% to establish maximum TG parameters. Optimal TG

conditions are studied by varying the amount of TF: 5pM, 1pM and 0,5 pM. Final phospholipid concentration is set at 4 uM. Cases will consist of 25 severe haemophilia patients, 25 moderate haemophilia patients and 25 mild haemophilia patients. Controls will consist of 25 healthy patients without haemophilia and no personal or family history of thrombosis or bleeding disorders and no relevant medication.

Secondary objective 1: To assess the association between the results of the CAT assay and the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia.
Study design and population: A cross-sectional study design will be used. The population will consist of 25 severe haemophilia patients, 25 moderate haemophilia patients and 25 mild haemophilia patients.
Determinant: The following parameters of the CAT assay will be used as the determinant; lag-time, time to peak, peak height and endogenous thrombin potential.
Endpoint: The modified bleeding severity score developed by Schulman et al [97] will be used as the endpoint (see paragraph 5.1.3).
Statistical analysis: The association between each parameter of the CAT assay (lag-time, time to peak, peak height and endogenous thrombin potential) and bleeding severity will be assessed using linear regression.

Secondary objective 2: To evaluate if the CAT assay can accurately diagnose the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia.
Study design and population: A cross-sectional study design will be used. The population will consist of 25 severe haemophilia patients, 25 moderate haemophilia patients and 25 mild haemophilia patients.
Determinants: The following parameters of the CAT assay will be used as determinants; lag-time, time to peak, peak height and endogenous thrombin potential. FVIII concentration (measured using the chromogenic assay) will also be used as a determinant.
Endpoint: The modified bleeding severity score developed by Schulman et al [97] will be applied to this study population (see paragraph 5.1.3) and used as a dichotomous outcome. Mild bleeders will be defined as being in the lowest two quartiles of the modified bleeding severity score, severe bleeders will be defined as being in the highest two quartiles of the modified bleeding severity score.
Statistical analysis: The CAT assay parameters and FVIII concentration will be used to predict if a patient is a mild or a severe bleeder (based on the modified bleeding severity score). We will quantify diagnostic performance by constructing ROC-curves for each parameter of the CAT assay

and for FVIII concentration. We will then test if the AUROC of each parameter of the CAT assay differs significantly from the AUROC of the chromogenic assay. This analysis will be performed for the total population and separately for each category of severity.

6.4.2 To assess the usefulness of the Nijmegen Haemostasis Assay (NHA) in relation to clinical phenotype:

Primary objective 1: To assess the association between the results of the NHA and the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia A and B.
Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A and B patients in the Netherlands.
Determinant: The following parameters of the NHA will be used as determinants; lag-time, thrombin peak-time, thrombin peak-height, area under the curve (AUC), fibrin lysis time (FLT), plasmin peak-height and plasmin potential.
Potential confounders: FVIII/FIX genotype, VWF antigen levels and VWF activity.
Endpoint: The modified bleeding severity score developed by Schulman et al [97] will be used as the endpoint (see paragraph 5.1.3).**Statistical analysis:** The association between each parameter of the NHA and bleeding severity will be assessed using linear regression.

Primary objective 2: To evaluate if the NHA can accurately diagnose the self-reported clinical bleeding phenotype of patients with severe, moderate and mild haemophilia.
Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A and B patients in the Netherlands.
Determinants: The following parameters of the NHA will be used as determinants; lag-time, thrombin peak-time, thrombin peak-height, area under the curve (AUC), fibrin lysis time (FLT), plasmin peak-height and plasmin potential. FVIII concentration (measured using the chromogenic assay) will also be used as a determinant.
Other potential predictors of clinical bleeding phenotype: FVIII/FIX genotype, VWF antigen levels and VWF activity.
Endpoint: The modified bleeding severity score developed by Schulman et al [97] will be applied to this study population (see paragraph 5.1.3) and used as a dichotomous outcome. Mild bleeders will be defined as being in the lowest two quartiles of the modified bleeding severity score, severe bleeders will be defined as being in the highest two quartiles of the modified bleeding severity score.
Statistical analysis: Firstly, we will perform univariate logistic regression using FVIII concentration as

the determinant (the basic prediction model). To quantify the incremental value of the NHA parameters, they will be added to the basic prediction model to create the full prediction model. For internal validation, bootstrapping techniques will be used to reduce overfitting. Agreement between observed and predicted outcomes will be assessed using a calibration plot. Discrimination will be assessed by calculating the AUROC of the full model. The added value of the NHA will be assessed by comparing the AUROC of the basic model (only FVIII concentration) with the AUROC of the full model (FVIII concentration + NHA parameters).

6.4.3 To assess misclassification of disease severity by the one-stage clotting assay in haemophilia B patients:

Primary objective 1: To assess the extent of discrepancy in FIX activity level as measured by the one stage clotting assay to the chromogenic assay in patients with mild and moderate haemophilia B.
Study design and population: A cross-sectional study design will be used. The population will consist of all persons with mild and moderate haemophilia B.
Determinant: FVIII activity level, as determined by the chromogenic assay.
Endpoint: FVIII activity level, as defined using the one-stage clotting assay.
Statistical analysis: This objective will firstly be explored using descriptive statistics. Furthermore, using the chromogenic assay as the reference test, we will calculate sensitivity and specificity (with corresponding 95%CI's) for the one-stage clotting assay.

Primary objective 2: To evaluate the association between F9 genotype and discrepancy between the FIX activity levels as measured by the one-stage clotting assay and the chromogenic assay in patients with mild and moderate haemophilia B.
Study design and population: A cross-sectional study design will be used. The population will consist of all persons with mild and moderate haemophilia B.
Determinant: F8 and F9 genotype are the determinants. Information on F8/F9 genotype will be obtained from each patient's medical record.
Endpoint: A large discrepancy between the result of the one-stage clotting assay (FVIII:C_{1st}) and the result of the chromogenic assay (FVIII:C_{chr}) will be used as the endpoint. A large discrepancy between the result of the one-stage clotting assay and the chromogenic assay is defined as having a ratio of FVIII:C_{chr} to FVIII:C_{1st} lower than 0.6.
Statistical analysis: The association between F8/F9 genotype and a discrepancy between the results of the one-stage clotting assay and the chromogenic assay will be assessed using logistic regression.

6.4.4 Validating the iCHEC paediatric bleeding assessment tool in children with haemophilia

Primary objective: to describe the bleeding scores as assessed by a newly developed Paediatric Bleeding Assessment Tool (iCHEC), in all paediatric patients with haemophilia, and in subgroups according to haemophilia severity, age and treatment regimen (prophylaxis/on demand).
Determinants: Haemophilia severity, age, treatment regimen.
Population: All paediatric patients with haemophilia.
Endpoint: bleeding scores as assessed by a newly developed Paediatric Bleeding Assessment Tool.
Statistical analysis: We will use descriptive statistics where appropriate.

6.5 Studies assessing immunological mechanisms that may lead to inhibitor formation in patients with haemophilia A

6.5.1 Assessing the relationship between FVIII-specific non-neutralizing antibodies and inhibitor formation:

Primary objective 1: To assess the prevalence of FVIII-specific non-neutralizing antibodies (NNAs) among non-inhibitor patients with severe, moderate and mild haemophilia A in the Netherlands.
Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients without current inhibitors in the Netherlands.
Determinant: Disease severity (mild, moderate and severe) will be used as the determinant.
Endpoint: The prevalence of non-neutralizing antibodies among non-inhibitor patients with haemophilia A in the Netherlands will be used as the endpoint. The Bethesda assay will be used to assess inhibitor presence. If negative, the presence and characteristics of NNAs will be assessed using an ELISA platform developed by Whelan et al. [70]
Statistical analysis: 95% confidence intervals for the prevalence (which is a binomial proportion) will be calculated by using the exact Clopper-Pearson method.

Primary objective 2: To characterize all FVIII-specific antibodies with respect to Ig class, IgG subclass and affinity, and to evaluate whether some of these characteristics are more associated with having neutralizing antibodies (inhibitors) rather than non-neutralizing antibodies (NNAs).
Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients with neutralizing FVIII-specific antibodies(inhibitors) or non-neutralizing FVIII-specific antibodies (NNAs). Patients without any FVIII-specific antibodies will be excluded from

this analysis.

Determinant: The presence of non-neutralizing antibodies (NNAs) or neutralizing antibodies (inhibitors) will be used as the determinant. The presence of NNAs will be assessed using an ELISA platform developed by Whelan et al. [70] The Bethesda assay will be used to assess inhibitor presence.

Potential confounders: We will potentially adjust for FVIII genotype and family history of haemophilia/inhibitors as well as other potential genetic determinants such as HLA, IL-10, TNFα, CTLA4, HO-1 and FcgR gene polymorphisms.

Endpoint: Ig class, IgG subclass and affinity will be used as endpoints. The presence and characteristics of NNAs will be assessed using an ELISA platform developed by Whelan et al. [70]

Statistical analysis: The relationship between Ig class, IgG subclass, affinity and the presence of inhibitors or NNAs will be assessed using regression based methods.

Secondary objective 1: To assess whether F8 genotype and polymorphisms in immune response genes influence the development of non-neutralizing antibodies (NNAs).

Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients with non-neutralizing FVIII-specific antibodies (NNAs) and all haemophilia A patients without any antibodies. Haemophilia A patients with neutralizing antibodies (inhibitors) are excluded from this analysis.

Determinants: FVIII genotype and family history of haemophilia/inhibitors as well as other potential genetic determinants such as HLA, IL-10, TNFα, CTLA4, HO-1 and FcgR gene polymorphisms.

Potential confounders: See determinants

Endpoint: The presence or absence of non-neutralizing antibodies (NNAs) will be used as the endpoint.

Statistical analysis: The relationship between the determinants and the presence or absence of non-neutralizing antibodies will be explored using logistic regression.

Secondary objective 2: To assess whether F8 genotype and polymorphisms in immune response genes influence the development of either neutralizing antibodies (inhibitors) or non-neutralizing antibodies (NNAs).

Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients with neutralizing FVIII-specific antibodies (inhibitors) or non-neutralizing FVIII-specific antibodies (NNAs). Patients without any FVIII-specific antibodies are excluded from this analysis.

Determinants: FVIII genotype and family history of haemophilia/inhibitors as well as other potential genetic determinants such as HLA, IL-10, TNFα, CTLA4, HO-1 and FcgR gene polymorphisms.
Potential confounders: See determinants
Endpoint: The presence of neutralizing FVIII-specific antibodies (inhibitors) or non-neutralizing FVIII-specific antibodies (NNAs) will be used as the endpoint.
Statistical analysis: The relationship between the determinants and the presence of neutralizing FVIII-specific antibodies (inhibitors) or non-neutralizing FVIII-specific antibodies (NNAs) will be explored using logistic regression.

Secondary objective 3: To evaluate the association between non-neutralizing antibodies (NNAs) and bleeding frequency in patients with severe haemophilia A.
Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients with non-neutralizing FVIII-specific antibodies (NNAs) and all haemophilia A patients without any antibodies. Haemophilia A patients with neutralizing antibodies (inhibitors) are excluded from this analysis.
Determinants: The presence/absence and titre of non-neutralizing antibodies (NNAs) will be used as the determinants.
Potential confounders: We will potentially adjust for the severity of haemophilia, FVIII genotype as well as VWF antigen levels and/or VWF activity
Endpoints: The modified bleeding severity score developed by Schulman et al [97] will be used as the endpoint (see paragraph 5.1.3).
Statistical analysis: The relationship between the presence/absence of non-neutralizing antibodies (NNAs) and bleeding severity will be assessed using linear regression.

Secondary objective 4: To evaluate potential misclassification of low-titre inhibitors as (low/high-affinity) non-neutralizing antibodies in patients with haemophilia A.
Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients with NNAs in the Netherlands.
Determinant: An ELISA will be used to assess the presence of (low/high- affinity) NNAs.
Endpoint: The low-titre inhibitor assay will be used to assess low-titre inhibitor presence.
Statistical analysis: This objective will firstly be explored using descriptive statistics.

Secondary objective 5: To evaluate the diagnostic performance of a modified FVIII ELISA for detection of FVIII-specific antibodies (inhibitors), using the Bethesda assay as the reference test.

Study design and population: A cross-sectional study design will be used. The population will consist of all haemophilia A patients in the Netherlands.
Determinant: A modified ELISA will be used to detect the presence of high-affinity IgG4 FVIII-specific antibodies.
Endpoint: The Bethesda assay will be used to assess inhibitor presence; a cut-off value of 0.6 BU will be used.
Statistical analysis: The Bethesda assay will be used as the reference test, the modified ELISA will be used as the index test. We will calculate sensitivity, specificity, (positive and negative) predictive values, (positive and negative) diagnostic odds ratios and their corresponding confidence intervals.

6.5.2 Assessing dendritic cell response and T-cell activation in the FVIII immune response:

Primary objective 1: To compare the expression of interleukin-2 and TGF-beta in monocyte-derived dendritic cells after stimulation with FVIII with and without danger signals (e.g. LPS) in patients with non-neutralizing FVIII-specific antibodies (NNAs) and patients without any current FVIII-specific non-neutralizing antibodies.
Study design and population: A case-control study design will be used. The cases will consist of 25 severe patients with NNAs that never underwent ITI. The controls will consist of 25 severe patients without any current NNAs that never underwent ITI. All haemophilia patients must have a F8 intron-22 inversion mutation. Haemophilia patients with current or previous inhibitors are excluded.
Determinant: The expression of interleukin-2 and TGF-beta in monocyte-derived dendritic cells after stimulation with FVIII and LPS will be used as the determinant. We will isolate PBMC's from all patients, monocyte-derived dendritic cells will be cultured, we will then assess the cytokine response to FVIII/LPS using RNA sequencing.
Potential confounders: We will potentially adjust for the previous number of EDs to FVIII, FVIII genotype and family history of inhibitor formation as well as other potential genetic determinants such as HLA, IL-10, TNFα, CTLA4, HO-1 and FcgR gene polymorphisms.
Endpoint: The presence or absence of NNAs will be used as the endpoint (coded as a dichotomous variable). Firstly, The Bethesda assay will be used to assess inhibitor presence. If negative, an ELISA will be used to detect the presence of NNAs.
Statistical analysis: We will use logistic regression to evaluate the association between interleukin-2 and the presence of NNAs and the relationship between TGF-beta and the presence of NNAs. In addition, we will examine the combined effect of increased expression of interleukin-2 and TGF-beta on the presence of NNAs using logistic regression.

Primary objective 2: To compare the regulatory T- cell count, as a proportion of all CD4+ T-cells in patients with non-neutralizing FVIII-specific antibodies (NNAs) or patients without any current FVIII-specific antibodies.

Study design and population: A case-control study design will be used. The cases will consist of 25 severe patients with NNAs that never underwent ITI. The controls will consist of 25 severe patients without any current NNAs that never underwent ITI. All haemophilia patients must have a F8 intron-22 inversion mutation. Haemophilia patients with current or previous inhibitors are excluded.

Determinant: T-regulatory cell count, as a proportion of all CD4+ T-cells will be used as the determinant. T-regulatory cell count will be assessed using both flow cytometry and a quantitative real-time PCR assay developed by Epiontis (Epiontis Treg assay).

Potential confounders: We will potentially adjust for the previous number of EDs to FVIII, FVIII genotype and family history of inhibitor formation as well as other potential genetic determinants such as HLA, IL-10, TNFα, CTLA4, HO-1 and FcgR gene polymorphisms.

Endpoint: The presence or absence of NNAs will be used as the endpoint. Firstly, The Bethesda assay will be used to assess inhibitor presence. If negative, an ELISA will be used to detect the presence of NNAs.

Statistical analysis: We will use logistic regression to evaluate the association between the dendritic cell cytokine response and the presence/absence of NNAs

Chapter 7 - Ethical considerations

7.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (seventh revision, 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). In addition, this study will also be conducted according to Good Research Practice (GRP) in accordance with the Law for the Protection of Personal Data (WBP) and the Law for Agreement on Medical Treatment (WGBO).

7.2 Recruitment and consent

All patients will be recruited through their own physician. Via the physician, all patients will be sent

an invitation letter, including a patient information letter and an informed consent form. Patients will be asked to provide consent for the questionnaire first. At the clinic the informed consent form will be signed by both the participant and the physician, before data collection and sampling of blood/urine takes place. Patients will be given 1 month to reply for participation in the questionnaire. If no response is received after 1 month (either refusal or consent), a second letter will be sent. Persistent non-responders will be asked to participate at the next appointment with their treating physician. If the next appointment is more than 3 months away, patients will be contacted by phone. Patients with questions regarding the study will be given the option to contact their treatment centre or the research team for further information. If a participant is a minor between 12-17 years old, the parents/legal guardian(s) will also need to consent. If a participant is a minor under 12 years, only the parents/legal guardian(s) will be asked to formally consent.

7.3 Objection by minors

In the study, participants will be asked to provide a blood sample. Drawing blood is an invasive procedure, it is considered a non-therapeutic intervention by the CCMO. Prior to asking the participant to consent to the study, we will discuss with the parents/legal guardian(s) if the minor objects to the procedure. Due to the extra difficulties in obtaining blood from children, mainly due to bad accessibility of veins and needle fear, blood samples will be obtained during routine clinical visits when blood collection is already planned. If the participant shows any signs of refusal to cooperate, the procedure will be stopped immediately. To determine what actions would constitute refusal to cooperate in minors, we will use the “Code of Conduct regarding Refusal by Minors Participating in Medical Research "Gedragscode bij verzet van minderjarigen die deelnemen aan medisch-wetenschappelijk onderzoek" (website CCMO).

7.4 Benefits and risks assessments, group relatedness

One general aim of this study is to describe the general health status of the Dutch haemophilia population. Haemophilia is a congenital disease and is therefore present from birth. To accurately capture the burden of disease in all haemophilia patients, one must also include minors in this study.

Another general aim of this study is to identify novel determinants of inhibitor formation, joint bleeding and joint damage. The distribution and incidence of many determinants differ greatly between younger and older patients and are more prevalent in younger patients. Also, to be of

clinical use, any determinant for inhibitor formation or joint damage should be detectable at as young an age as possible, this will allow clinicians to modify each patient's individual therapy so as to avoid inhibitor formation or excessive joint damage. Therefore, research on novel determinants of inhibitor formation, joint bleeding and joint damage cannot be performed accurately without including minors in the study.

Health-related quality life and quality of care will also be assessed in this study. Using only adults to evaluate health-related quality life and quality of care will yield results that are not applicable to minors. Therefore, minors will have to be included if the results of this research are to be applicable to minors.

The risks associated with venepuncture are minimal. The most common complication is the formation of a haematoma near the venepuncture site. Another common complication is a vasovagal response during or shortly after venepuncture which can result in dizziness or loss of consciousness, nausea, tinnitus and/or profuse sweating. Needle fear is common among PWH, however, most PWH will be very familiar with venepuncture, as PWH are used to receiving regular intravenous infusions. In addition, we will be drawing blood from all minors and most adults during routine blood collection moments. Therefore, the burden associated with venepuncture will be minimal.

The risk of bleeding associated with a FVIII wash-out phase of three days for haemophilia A patients and a FIX wash-out phase of five days for haemophilia B patients is minimal. All studies that aim to measure baseline FVIII/FIX levels require such a washout phase, there is no mention of a significantly increased risk of bleeding in the literature as a result of this washout phase. Given the half-life of FVIII and FIX, the increase in bleeding risk is predicted to be minimal.

7.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO. The sponsor also has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study. The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

7.6 Incentives

Individuals that participated in all previous HiN studies will receive a HiN-6 mug. Also, 20 prizes for a gift card (with a value of €30 each) will be drawn. Participants will also receive a detailed report of the most important findings that have resulted from the study.

Chapter 8 - Administrative aspects, monitoring and publishing

8.1 Handling and storage of data and documents

For each study participant, personal identifiers will be replaced with a unique study code using an encryption key.

Patient information will be extracted from each participant's medical record using a case report form (CRF). All CRFs will be sent to the coordinating centre where the data will be transferred into a database, this data will not contain any personal identifiers. Participants in the study will use their unique study code to gain access to the digital questionnaire. The questionnaire software and the resulting dataset will not contain personal identifiers. All blood/DNA/urine samples will be labelled with the participants unique study code, the labels will not contain any personal identifiers.

The researchers will only have access to the de-identified dataset. Only a data manager not involved in the research project will have access to the encryption key of the existing dataset. Patient data together with patient material will be stored indefinitely in the biobank.

Individual patients will not be identified within resulting publications or presentations from the study. Data will be held and processed by the study coordinator. All data will be held securely. Individual patients will not be identified within resulting publications or presentations from the study. The handling of personal data will comply with the Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens, Wbp).

8.2 Monitoring and Quality Assurance

This study requires low-intensity monitoring due to its low risk (see form 'Risicoclassificatie klinisch onderzoek'). Validation checks of clinical data will be performed by the study team. Data stored at the coordinating centre will be checked for missing or unusual values (range checks) and consistency.

8.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

8.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the study to the accredited Medical Research Ethics Committee (METC) once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the study, serious adverse events/ serious adverse reactions, other problems, and amendments.

8.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit. The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action. In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

8.6 Public disclosure and publication policy

The results from different centres will be analysed in close collaboration with participating centres and will published as soon as possible. Depending on the research question, one or more of the participating centres will take the lead in analysing and publishing the results. Each centre will have the right to use data collected at the centre itself. There are no restrictions for the publication of the

results from the study.

8.7 Authorship

Results generated by the processing of all collected data will be published under the name of all investigators. According to the ICMJE (International Committee of Medical Journal Editors) guidelines authors should meet the following three conditions 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

8.8 Funding source

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Appendices

Appendix 1: Case Report form

PATIENT IDENTIFIER |__|__|__|__|__|__|

MONTH AND YEAR OF BIRTH |__|__| / |__|__|__|__| (mm/yyyy)

CENTRE _____

CRF FILLED IN BY _____

1.0 Diagnosis

1.1 Vital status	O Alive O Deceased, date of death: _____ dd/mm/yyyy (continue below) O cause of death: _____ O Cause of death unknown
1.2 Type of haemophilia	O Haemophilia A O Haemophilia B O Haemophilia B Leyden O Unknown
1.3 Ethnic origin	O Caucasian O Black African O Asian O Mixed O Unknown O Other: _____
1.4 ABO Blood group	O A O B O AB O O O Unknown

1.5 F8 or F9 genotype known
O No (continue with question 1.6)
O Yes (fill in below):

1.5.1 Specific F8 or F9 genotype
O F8 genotype: intron 22 inversion
O Other F8 genotype: _____
O F9 genotype: _____

1.5.2 Please note the nomenclature used to specify F8 or F9 genotype
O HAMSTeRS/HADB nomenclature ('old' numbering)
O HGVS nomenclature ('new' numbering)
O Unknown

1.6 Baseline FVIII/FIX:C (one-stage clotting assay)
O :_____ IU/mL, Date:_____dd/mm/yyyy
O Unknown

1.7 Baseline FVIII/FIX:C (chromogenic assay)
O :_____ IU/mL, Date:_____dd/mm/yyyy
O Unknown

1.8 Baseline FVIII/FIX:C (two-stage clotting assay)
O :_____ IU/mL, Date:_____dd/mm/yyyy
O Unknown

1.9 Baseline FVIII/FIX antigen
O :_____ IU/mL, Date:_____dd/mm/yyyy
O Unknown

1.10 Baseline Von Willebrand activity (using VWF:Rco assay)
O VWF:Rco assay was used:
_____ IU/mL, Date:_____ dd/mm/yyyy
O Unknown, (continue below):

Was a different VWF activity assay used?
O Yes, namely: _____ assay
Result: _____ (unit of measurement:_____)
Date:_____ dd/mm/yyyy
O Unknown

1.11 Von Willebrand antigen
O Result: _____ IU/mL
Date:_____ dd/mm/yyyy
O Unknown

1.12 Does the patient also have Von Willebrand disease (VWD)?

O No
O Yes (select only one option):

O VWD Type 1
O VWD Type 2A
O VWD Type 2M
O VWD Type 2 Vicenza
O VWD Type unknown
O VWD Type 2, no further subtyping
O VWD Type 2B
O VWD Type 2N
O VWD Type 3
O Other type of Von Willebrand disease: _____

1.13 Does the patient also have a platelet function disorder?

O No
O Yes (select only one option):

O Glanzmann's thrombasthenia
O (Delta) storage pool disease
O Platelet disorder: type unknown
O Bernard-Soulier Syndrome
O Gray platelet syndrome
O Other platelet disorder: _____

1.14 Does the patient also have an additional clotting factor deficiency?

O No
O Yes (select only one option):

O Factor I (Fibrinogen) deficiency
O Hypofibrinogenemia
O Dysfibrinogenemia
O Factor V deficiency (Parahaemophilia)
O Factor VII deficiency
O Factor XI deficiency (Haemophilia C)
O Combined factor II+VII+IX+X deficiency
O Factor deficiency: type unknown
O Afibrinogenemia
O Hypodysfibrinogenemia
O Factor II (Prothrombin) deficiency
O Combined factor V+VIII deficiency
O Factor X deficiency
O Factor XIII deficiency
O Alfa-2-antiplasmin deficiency
O Other factor deficiency:

1.14.1 If yes, clotting factor activity: _____ IU/mL

2.0 Current treatment

2.1 Cumulative number of exposure days (EDs) to FVIII or FIX to date

O < 50 EDs
O 51-100 EDs
O 101-150 EDs
O > 150 EDs
O unknown

2.2 Age at first joint bleed

O :_____mm/yy
O Unknown

2.3 Is the patient currently on prophylaxis with FVIII/FIX products or bypassing therapy (FEIBA or rFVIIa)?

O Unknown
O No (patient is treated on-demand)
O Yes (fill in below)

2.3.1 Product used (select only one product):

O Factor VIII products

O Aafact	O Advate	O Adynovate	O Afstyla	O Alprolix	O BeneFIX
O Elocta	O Haemate P	O Helixate NexGen		O Iblias	O Idelvion
O Immunine	O Kogenate	O Kovaltry	O Mononine	O Nanotiv	O Nonafact
O NovoEight	O Nuwiq	O Octanate	O Refacto AF	FuseNGo	O Rixubis
O Other: ___	O Unknown				

O Factor IX products

O Alprolix	O BenefIX	O Idelvion	O Immunine	O Mononine	O Nonafact
O Rixubis	O Other: ___	O Unknown			

O Bypassing agents:

O FEIBA	O rFVIIa	O Other: __	O Unknown

O Unknown

Note additional information for this product:

O Dose:_____ O IU/kg O U/kg O µg/kg O Other:__

Frequency: __ times per O day O 2 days O 3 days O 1 week O 2 weeks O Other:__

O Unknown

2.4 Treatment protocol

2.4.1 Treatment of a minor bleed?

O DDAVP
O Factor VIII products

O Aafact	O Advate	O Adynovate	O Afstyla	O Alprolix	O BeneFIX
O Elocta	O Haemate P	O Helixate NexGen		O Iblias	O Idelvion
O Immunine	O Kogenate	O Kovaltry	O Mononine	O Nanotiv	O Nonafact
O NovoEight	O Nuwiq	O Octanate	O Refacto AF	FuseNGo	O Rixubis
O Other: ___	O Unknown				

O Factor IX products

O Alprolix	O BenefIX	O Idelvion	O Immunine	O Mononine	O Nonafact
O Rixubis	O Other: ___	O Unknown			

O Bypassing agents:

O FEIBA	O rFVIIa	O Other: _____

O Unknown

Note additional information for selected product:	O Dose:_____	O IU/kg	O U/kg
		O µg/kg	O IU
		O U	O µg
		O Other:__	
	O Unknown		

2.4.2 Treatment of a major bleed?

O Factor VIII products

O Aafact	O Advate	O Adynovate	O Afstyla	O Alprolix	O BeneFIX
O Elocta	O Haemate P	O Helixate NexGen		O Iblias	O Idelvion
O Immunine	O Kogenate	O Kovaltry	O Mononine	O Nanotiv	O Nonafact
O NovoEight	O Nuwiq	O Octanate	O Refacto AF	FuseNGo	O Rixubis
O Other: ___	O Unknown				

O Factor IX products

O Alprolix	O BenefIX	O Idelvion	O Immunine	O Mononine	O Nonafact
O Rixubis	O Other: ___	O Unknown			

O Bypassing agents:

O FEIBA	O rFVIIa	O Other: __	O Unknown

O Unknown

Note additional information for selected product:	O Dose:_____	O IU/kg	O U/kg
		O µg/kg	O IU
		O U	O µg
		O Other:__	
	O Unknown		

2.4.3 Treatment of a life threatening bleed?

O Factor VIII products

O Aafact	O Advate	O Adynovate	O Afstyla	O Alprolix	O BeneFIX
O Elocta	O Haemate P	O Helixate NexGen		O Iblias	O Idelvion
O Immunine	O Kogenate	O Kovaltry	O Mononine	O Nanotiv	O Nonafact
O NovoEight	O Nuwiq	O Octanate	O Refacto AF	FuseNGo	O Rixubis
O Other: ___	O Unknown				

O Factor IX products

O Alprolix	O BenefIX	O Idelvion	O Immunine	O Mononine	O Nonafact
O Rixubis	O Other: ___	O Unknown			

O Bypassing agents:

O FEIBA	O rFVIIa	O Other: __	O Unknown

O Unknown

Note additional information for selected product:	O Dose:_____	O IU/kg	O U/kg
		O µg/kg	O IU
		O U	O µg
		O Other:__	
	O Unknown		

3.0 Inhibitor Development (haemophilia A patients only)

3.1 Currently or previously positive Bethesda assay

O No (proceed to chapter 4.0)
O Unknown (proceed to chapter 4.0)
O Yes, (proceed with following questions)

3.2 Currently undergoing immune tolerance induction (ITI)?

O No (continue with 3.4)
O Yes (continue below)

3.2.1 Product used (select only one product):

O Factor VIII products

O Aafact	O Advate	O Adynovate	O Afstyla	O Alprolix	O BeneFIX
O Elocta	O Haemate P	O Helixate NexGen		O Iblias	O Idelvion
O Immunine	O Kogenate	O Kovaltry	O Mononine	O Nanotiv	O Nonafact
O NovoEight	O Nuwiq	O Octanate	O Refacto AF	FuseNGo	O Rixubis
O Other: ___	O Unknown				

O Factor IX products

O Alprolix	O BenefIX	O Idelvion	O Immunine	O Mononine	O Nonafact
O Rixubis	O Other: ___	O Unknown			

O Unknown

3.2.2 Note additional information for this product:

O Starting date: _____ dd/mm/yy
Dose: _____ IU/kg
Frequency: __ times per O day O 2 days O 3 days O 1 week O 2 weeks O Other:__
O Unknown

3.3 Use of immunosuppressants at any time point during ITI?

O No
O Yes, continue below, multiple choice)

□ Rituximab	□ Immunoadsorption
□ intravenous immunoglobulin	□ Cyclophosphamide
□ Mycophenolate mofetil	□ Azathioprine
□ Methotrexate	□ Dexamethasone
□ Hydrocortisone	□ Other: _____

3.3.1 If yes, note additional information per product:	O Used from:_____ dd/mm/yyyy to:____ dd/mm/yyyy Dose: _____ (unit of measurement_____) Frequency: __ times per O day O 2 days O 3 days O 1 week O 2 weeks O Other:__ O Unknown

3.4 First positive Bethesda assay (according to local cut-off)

Date	O: _____ dd/mm/yy O Unknown
Inhibitor titre	O: _____ BU/mL O Unknown
Recovery FVIII:C	O: _____ IU/mL, after a bolus of____IU/kg FVIII O Unknown
Cumulative N of exposure days	O: _____ exposure days (at inhibitor detection) O Unknown

3.5 Second positive Bethesda assay (according to local cut-off)

Date	O: _____ dd/mm/yy O Unknown
Inhibitor titre	O: _____ BU/mL O Unknown
Recovery FVIII:C	O: _____ IU/mL, after a bolus of____IU/kg FVIII O Unknown

3.6 Highest inhibitor titre ever measured

Date	O: _____ dd/mm/yy O Unknown
Inhibitor titre	O: _____ BU/mL O Unknown
Recovery FVIII:C	O: _____ IU/mL, after a bolus of____IU/kg FVIII O Unknown

3.7 Initiation of immune tolerance induction (ITI)

ITI initiated	O no O yes O unknown
Date	_____ dd/mm/yy

3.8 First negative Bethesda assay

Achieved	O no O yes O unknown
Date	_____ dd/mm/yy

3.9 First normal recovery

Achieved	O no O yes O unknown
Date	_____ dd/mm/yy

3.10 Confirmed inhibitor relapse after a successful ITI-procedure?

O No (proceed to chapter 4.0)
O Unknown (proceed to chapter 4.0)
O Yes (proceed with following questions)

3.11 First positive Bethesda assay (according to local standards) after successful ITI

Date	O: _____	dd/mm/yy
	O Unknown	
Inhibitor titre	O: _____	BU/mL
	O Unknown	
Recovery FVIII:C	O: _____	IU/mL, after a bolus of____IU/kg FVIII
	O Unknown	
Cumulative N of exposure days	O: _____	exposure days (at inhibitor detection)
	O Unknown	

3.12 Second positive Bethesda assay after successful ITI

Date	O: _____	dd/mm/yy
	O Unknown	
Inhibitor titre	O: _____	BU/mL
	O Unknown	
Recovery FVIII:C	O: _____	IU/mL, after a bolus of____IU/kg FVIII
	O Unknown	

3.13 Highest inhibitor titre ever measured after successful ITI

Date	O: _____	dd/mm/yy
	O Unknown	
Inhibitor titre	O: _____	BU/mL
	O Unknown	
Recovery FVIII:C	O: _____	IU/mL, after a bolus of____IU/kg FVIII
	O Unknown	

3.14 Initiation of immune tolerance induction (ITI) after inhibitor relapse

ITI initiated	O no O yes O unknown
Date	_____ dd/mm/yy

3.15 First negative inhibitor test after inhibitor relapse

Achieved	O no O yes O unknown
Date	_____ dd/mm/yy

3.16 First normal recovery after inhibitor relapse

Achieved	O no O yes O unknown
Date	_____ dd/mm/yy

4.0 Co-morbidities

4.1 Currently HIV positive?

O no (continue with 4.2)

O yes (fill in below)

Treatment regimen: _____
_____
_____

HIV viral load (last HIV RNA assay): _____ copies/mL

4.2 Currently or previously positive for hepatitis C?

O no (end of CRF)
O yes (continue below)

HCV genotype (including subtype):

0 1a 0 1b
0 2a 0 2b
0 Other: _____
0 Unknown

Date of last positive HCV RNA assay: ____ dd/mm/yyyy

Outcome of last Fibroscan: _____ kilopascal (kPa)

Last recorded Child-Pugh score:

O Unknown
O class A
O class B
O class C

Presence of ascites: O Yes O Unknown

Presence of bleeding oesophageal varices : O Yes O Unknown

Presence of hepatic encephalopathy: O yes O unknown

Liver transplantation: O yes O unknown

4.3 Previous HCV treatment regimens? (max. 6 options)

Start date of first treatment:_____dd/mm/yyyy
Treatment regimen (multiple choice):
□ Interferon □ Pegylated interferon
□ Ribavarin □ Unknown
□ Direct acting antivirals:_____

Start date of second treatment:_____dd/mm/yyyy
Treatment regimen (multiple choice):
□ Interferon □ Pegylated interferon
□ Ribavarin □ Unknown
□ Direct acting antivirals:_____

Start date of third treatment:_____dd/mm/yyyy
Treatment regimen (multiple choice):
□ Interferon □ Pegylated interferon
□ Ribavarin □ Unknown
□ Direct acting antivirals:_____

Start date of fourth treatment:_____dd/mm/yyyy
Treatment regimen (multiple choice):
□ Interferon □ Pegylated interferon
□ Ribavarin □ Unknown
□ Direct acting antivirals:_____

Start date of fifth treatment:_____dd/mm/yyyy
Treatment regimen (multiple choice):
□ Interferon □ Pegylated interferon
□ Ribavarin □ Unknown
□ Direct acting antivirals:_____

Start date of sixth treatment:_____dd/mm/yyyy
Treatment regimen (multiple choice):
□ Interferon □ Pegylated interferon
□ Ribavarin □ Unknown
□ Direct acting antivirals:_____

4.4 sustained virologic response (SVR) after last HCV treatment?

O patient is still undergoing treatment
O Unknown
O No
O Yes, date: _____ dd/mm/yyyy

Instruction sheet Case Report Form
HiN-6/Parel Hemofilie

1.1 (primary cause of death)

- Report the primary (underlying) cause of death. The primary cause of death is defined as the disease or event that started the chain of events that led to death.
- After completion of the study, this information will be recoded according to the ICD-10 classification system.

1.5 (reporting F8 or F9 genotype)

- In case of point mutation, please provide both nucleotide and amino acid change
- Please provide whether numbering is given according to HAMSTeRS/HADB nomenclature ('old' numbering) or HGVS nomenclature ('new' numbering).

1.6-1.11 (FVIII and VWF assays)

- Report the results of the LAST assay that was performed.
- Convert assay results to IU/mL if they were reported differently (e.g IU/dL) in the EPD.

1.10 (baseline Von Willebrand activity)

- If a VWF:Rco assay (the main VWF activity assay) was never performed but another type of VWF activity assay was used, report the results of that assay instead.

1.12-1.14 (additional bleeding disorders)

- If mentioned in the EPD, report any additional bleeding disorder that the patient may have.

2.1 (definition of exposure day):

- An exposure day (ED) is defined as a calendar day during which one or more infusions of factor VIII or FIX were given.

2.2: (definition of joint bleed)

- Joint bleeds are defined as complaints in ankles, knees, elbows, hips, wrists, or shoulders requiring treatment with FVIII at least once.

2.3 (information about prophylaxis and on-demand treatment):

- Prophylaxis is the regular infusion of clotting factor concentrates to prevent bleeding. Patients who are not on prophylaxis are treated "on demand", i.e. treatment is only given at the time of a bleed to make it stop.
- Some patients can be treated with prophylaxis to prevent bleeds AND immune tolerance induction (ITI) to treat inhibitors. Information on ITI is reported separately (see 3.3).

3.0 (additional information)

- Only proceed with chapter 3.0 if the patient has haemophilia A, if the patient has haemophilia B, proceed to chapter 4.0.

3.1 (definition of inhibitor development):

- Inhibitor development is defined as the occurrence of neutralizing antibodies against FVIII as measured with the Bethesda assay.

3.2-3.3 (information about ITI)

- Immune tolerance induction (ITI) is used to treat patients with FVIII inhibitors.
- ITI involves the repeated administration of FVIII concentrates over a period of weeks to years, with the goal of inducing antigen-specific tolerance.
- Some patients are also administered immunosuppressive drugs to increase ITI success.

3.4-3.16 (additional information about inhibitor development):

- For inhibitors to be considered relevant, they should be documented on two separate occasions within a 1-4 week period and should have a level of ≥ 0.6 Bethesda units (BU) per mL.
- For inhibitors to be considered clinically significant, they should be associated with < 66% recovery of the particular product on a blood sample obtained 10–15 min after completion of the factor infusion
- An exposure day (ED) is defined as a calendar day during which one or more infusions of factor VIII or FIX were given.

3.10 (information about inhibitor relapse)

- Relapse of an inhibitor was defined as the recurrence of the inhibitor upon rechallenge with FVIII after prior inhibitor disappearance.

4.4 (definition of sustained virologic response)

- Sustained virologic response (SVR) is defined by the absence of HCV RNA by polymerase chain reaction 12 weeks after stopping treatment.
- An SVR is associated with a 99 percent chance of being HCV RNA negative during long-term follow-up and can therefore be considered as being cured of the HCV infection.

Appendix 2: Sample collection form

PATIENT IDENTIFIER |__|__|__|__|__|__|

MONTH AND YEAR OF BIRTH |__|__| / |__|__|__|__| (mm/yyyy)

CENTRE _____

CRF FILLED IN BY _____ **SIGNATURE** _____

---

1. Date/time of last FVIII/FIX infusion before blood sampling	Date:_____ dd/mm/yyyy Time: ____:____ hours:minutes
2. Type of last FVIII/FIX product used before blood sampling	Product used: _____
3. Dose of last FVIII/FIX product used before blood sampling	dose:_____(unit of measurement:_____)
4. Bodyweight at time of blood sampling	bodyweight: _____kg
5. Height at time of blood sampling	height: _____ cm

Appendix 3: Questionnaire

Please see separate document for complete questionnaire.

Overview of questionnaire sections:

order		Volwassenen (≥18 jaar)	Ouderen (≥65 jaar)	Kinderen (0-11 jaar)	Jongeren (12-25 jaar)
	Losse vragenblokken				
1	Over u/jou	✓	✓	✓	✓
4abc	Hemofilie en behandeling: hemofilie, bloedingen, behandeling, profylaxe (4e)	✓	✓	✓	✓
4d	Remmers	✓	✓	✓	✓
10 & 11	HIV en hepatitis C	✓	✓		
12	Beperkingen hemofilie	✓	✓	✓	✓
13	Seksualiteit (hematospermia)	✓	✓		✓ v.a. 16 jr
5	Desmopressine	✓	✓	✓	✓
	Ziekenhuisopnamen	✓	✓	✓	✓
7	Prikangst	✓	✓	✓	✓
16	Kwaliteit van zorg	✓	✓		✓
17	Transitie				✓
8	Nieuwe behandelingsmogelijkheden	✓	✓	✓	✓
20	Andere aandoeningen	✓	✓	✓	✓
21	Medicatiegebruik	✓	✓		
2	Roken en alcohol	✓	✓		
15	Pijn	✓	✓	✓	✓
24	NVHP	✓	✓		✓
25	Evaluatie vragenlijst	✓	✓	✓	✓
22	darmkankerscreening		✓ (55-75 jaar)		
	Gevalideerde vragenlijsten				
	Bloedingsscore (iCHEC)			iCHEC	iCHEC
14	Functioneren en dagelijkse activiteiten	HAL	HAL	PedHAL	PedHAL/HAL
9	Therapietrouw	VERITAS-pro	VERITAS-pro	VERITAS-pro	VERITAS-pro
3	Kwaliteit van leven	RAND-36, PROMIS29	RAND-36, PROMIS29	CHO-KLAT, PedsQL	CHO-KLAT / RAND-36, PROMIS29
18	Sport en bewegen	MAQ, HEP-test Q	MAQ, HEP-test Q	MAQ, HEP-test Q	MAQ, HEP-test Q
19	Angst om te vallen		FES		
23	Zelf-management	PAM-13	PAM-13	HSES	HSES
6	Tevredenheid behandeling	Hemo-SAT_A	Hemo-SAT_A	Hemo-SAT_P	