Folding to function

*Rising insights in nonsevere hemophilia A and DDAVP treatment*

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CHAPTER 1

General introduction and outline of the thesis
For the love of blood

More than 2000 years ago, Jewish boys who had to undergo circumcision were exempted from this procedure by rabbinical rulings if two previous brothers had died of bleeding after the circumcision. This was described in ancient Hebrew writings. With our present knowledge we believe that this refers to hemophilia, or ‘the Royal Disease’. Hemophilia inherited this epithet from its most famous affected patient, Tsarevich Alexei Nikolaevich. Born in 1904 in St. Petersburg as the great grandson of Queen Victoria of England, he was the heir apparent of the Russian empire. Hemophilia literally means love ("philia") of blood ("hemo") and as the examples above reveal, this hereditary disease is X-linked, affecting mostly boys and men. This hereditary pattern was first described in the New England Journal of Medicine in 1813 by John Hay. Even though hemophilia has a long-standing history, the elucidation of the cause of the bleeding complications took up to the 20th century. Since 1937, when Patek and Taylor showed that the clotting defect of hemophilic plasma could be corrected by plasma of a healthy individual, we know that hemophilia is caused by a deficiency of a blood component, identified as clotting factor VIII (FVIII) in the early 1960’s. Due to technological developments, knowledge on the origin and mechanism of the disease and its treatment has been rapidly expanding ever since. Hemophilia, despite its reputation as a royal disease, has a broader impact: currently about 1 in 5000 male births are affected with hemophilia A worldwide. Reduced levels of FVIII in patients with hemophilia A lead to spontaneous and posttraumatic bleeds which may cause irreversible damage in mainly joints and muscles, or even death, for instance when fatal intracranial bleeding occurs.

In this introduction I will first discuss the biochemical aspects of clotting and the function of FVIII in this process, the classification of hemophilia A, and treatment options. Next, a basic methodological framework will be provided in order to explain the rationale for two large international research projects that form the backbone of this thesis; the INSIGHT and the RISE study. Finally, the outline of the thesis is described.
Impact of factor VIII

Hemostasis is a delicate balance between the arrest of bleeding and the disturbance of blood flow through a vessel. Various hemostatic and antithrombotic factors are involved in safeguarding us from bleeding. The human body developed two sequential protection mechanisms that work closely together to activate coagulation factors: primary and secondary hemostasis. Once the integrity of the body has been broken, primary hemostasis starts with the activation and aggregation of platelets, needed to form a hemostatic plug. During this process, released tissue factor activates factor VII (Figure 1).

This complex sequentially triggers a number of clotting factors (X and V), resulting in the formation of a trivial amount of factor II (thrombin) and fibrin (Figure 1). Fibrin is the final product of the coagulation cascade, crucial to thoroughly seal the place of disruption.

Figure 1. Blood clotting cascade under normal conditions. Upon vascular damage, tissue factor (TF) is released, which activates factor VII (FVII), sequentially followed by activation of factor X and V (FX, FV) resulting in trivial amount of factor II (thrombin) to form the first blood plug. Thrombin stimulates factor...
IX and VIII (FIX, FVIII) via a positive feedback loop. These are crucial to amplify thrombin production. The process is exponentially amplified by a positive feedback mechanism which is induced by thrombin and depends on factor IX and its co-factor VIII (Figure 1). FVIII is a large protein, highly susceptible to proteolytic degradation in the circulation. To prevent this from happening, FVIII is bound to its chaperone and carrier molecule Von Willebrand Factor (VWF). When FVIII is activated by thrombin, it separates from VWF. If FVIII plasma levels are reduced due to a genetic defect (hemophilia), this results in decreased thrombin and fibrin generation. In hemophilia A, the function of FVIII is insufficient or levels of the circulating protein are reduced. As a result, patients have fragile blood clots and are predisposed to a bleeding diathesis that generally occurs several days after the initial injury.

The life cycle of FVIII: folding to function

The synthesis of proteins is dependent on the information that is present in the genetic code (DNA). FVIII is encoded by the F8 gene. The F8 gene encodes for a sequence of 2351 amino acids, the building blocks of proteins. When amino acids are lined up to form a protein, they will arrange themselves in such a way that the protein folds into a shape called the tertiary or quaternary structure. The folding of a protein is incredibly important for its function, because the spatial conformation enables the interaction with other proteins or molecules. Specific tertiary and quaternary structures carry out physiological functions throughout the life cycle of FVIII.

FVIII is produced in sinusoidal and vascular endothelial cells in the liver, spleen, lungs and in lymphatic tissue. Under normal conditions, the production of FVIII starts with the transcription of the F8 gene into messenger ribonucleic acid (mRNA) in the nucleus of these cells. Next, the mRNA is released into the cytoplasm and travels to the ribosomes. At the ribosomes mRNA is translated into FVIII proteins. After the FVIII proteins are produced, they need to be approved by the quality control center: the endoplasmic reticulum (Figure 2). Due to interactions with chaperone proteins, only a limited fraction of the FVIII molecules is transported to the Golgi apparatus for further posttranslational processing. This is the site where FVIII proteins start their journey to their functional destination; bound to VWF in plasma or to yet unknown storage sites in order to safeguard us from bleeding. VWF is synthesized and secreted by vascular endothelial cells and megakaryocytes.
After its contribution to the coagulation cascade, FVIII is inactivated and generally cleared as a complex with VWF in the liver and spleen. There, hepatocytes, macrophages and sinusoidal epithelial cells carry specific receptors to recognize the complexes for degradation.\(^8\)

**Failure is an option**

Darwin demonstrated that the power of variation may help to survive under changing circumstances. DNA is the vehicle of variation through mutations rather than the seat of eternity. Mutations are a ubiquitous risk. Thus the synthesis of a functional protein from genetic information is error prone. Hemophilia A is caused by a broad spectrum of mutations in the F8 gene. They lead to absent or aberrant expression, secretion, or survival of FVIII in the circulation.\(^18-20\)

Patients are classified based on residual FVIII concentration in the plasma (FVIII:C, Figure 3). Healthy individuals have FVIII plasma levels ranging from 50 to 150 International Units per deciliter (IU/dL). Severely affected patients have no
detectable FVIII:C levels, whereas nonsevere patients have some activity (FVIII:C moderate patients 1-5, mild patients 6-40 IU/dL). In mild hemophilia A, excessive bleeding usually only occurs after minor trauma, dental procedures, or surgery, whereas moderate and severe patients may bleed spontaneously, without preceding trauma.

The focus of this thesis is nonsevere hemophilia A. More than half of the hemophilia A patients is affected by a nonsevere form of the disease. Nonsevere hemophilia A is mostly caused by missense mutations that change the genetic code resulting in the substitution of one amino acid for another. This may generate a misfolded FVIII protein and/or affect the function of FVIII due to ineffective interaction with other components of the hemostatic system. As a result, FVIII is targeted for degradation by the protein machinery quality control system: the endoplasmic reticulum. Currently, over 1000 different types of missense mutations have been reported. The heterogeneity of missense mutations and the relatively rare nature of nonsevere hemophilia A hamper the complete biomolecular understanding of the disease. Multiple models have been proposed to explore the functional effects of genetic defects, but so far the models remain explorative and there is no standardized classification system yet. Deciphering the functional effects is crucial to analyze biomolecular processes and may serve as a starting point for novel therapeutic options. This potential impels a strong incentive to develop an adequate classification method for the FVIII variants.
Replacing defective FVIII

Patients with nonsevere hemophilia A can be treated by intravenously replacing FVIII. Already in the 1840s, whole-blood transfusion from donors without hemophilia was used to treat patients. Irrespective of this initial success, it took more than a century to discover that FVIII was the miraculous ingredient of the administrated whole-blood transfusion that did the trick for hemophilia A patients. Since the 1960s, patients have been treated with specific FVIII replacement therapy to prevent or control bleeding. In the beginning, replacement therapy consisted of transfusion of FVIII concentrates purified from pooled donor plasma. However, due to the presence of blood-borne viruses in the pooled donor blood, these concentrates also transmitted HIV and hepatitis to a large proportion of hemophiliacs. This has resulted in the tragic loss of many lives and formed the drive to improve the manufacturing and screening processes to produce safer plasma FVIII concentrates and a recombinant form of the factor VIII concentrate in the 1990s. Hitherto, the use of replacement therapy has decreased morbidity and mortality and greatly improved the quality of life of hemophilia A patients. 

The life expectancy of nonsevere hemophilia patients has reached that of the non-hemophilic male population in high-income countries. Albeit these developments, FVIII replacement therapy still has downsides. The development of inhibiting antibodies against FVIII (inhibitors) jeopardizes treatment of bleeding episodes, and the costs of treatment are a major burden on national healthcare budgets. The frequent intravenous administration of FVIII concentrates due to their short half-life is inconvenient for patients. For patients in developing countries there is a lack of sufficient treatment due to limited resources. For these reasons, the avoidance of FVIII concentrates by using safer and cheaper treatment options is preferable, if possible. Treatment might improve considerably, with the availability of newly modified drugs. New FVIII products have extended half-lives in order to reduce the administration frequency. Half-life of FVIII products is extended via bio-engineering techniques to reduce cellular uptake and clearance of FVIII. These techniques involve binding of bulky molecules to FVIII (pegylation), or by fusing FVIII with another protein with a much longer half-life. However, extension of FVIII half-life seems to be limited by the half-life of its carrier molecule; VWF.
Another new category of products acts through different routes than replacement of the missing FVIII. These products use the regulation of the coagulation cascade (Figure 1). The formation of the hemostatic plug is controlled by anticoagulant factors in order to ensure the balance between the arrest of bleeding and the disturbance of blood flow through a vessel.

Therefore, the inhibition of natural anticoagulants is another route to ensure hemostasis in individuals in whom the arrest of bleeding is difficult, such as in hemophilia A. This follows the concept of ‘the enemy of my enemy is my friend’. Currently, two products based on this concept are being investigated: a monoclonal antibody against tissue factor inhibitor and an RNA interference agent against anti-thrombin.49–51

Another promising non-replacement therapy option is the development of a bispecific antibody that mimics the action of activated FVIII by binding to activated FIX and FX, resulting in the formation of thrombin.52,53 All three products can be administered subcutaneously. However, the safety of these new products in nonsevere hemophilia A patients needs to be proven, especially as there are no antidotes available yet.

Modified FVIII replacement or non-replacement treatment strategies result in a temporary improvement of hemostasis. A permanent restoration of FVIII levels may be achieved by gene therapy, at least in severe hemophilia A patients. However, it will take time before such a therapy is widely available for hemophilia A, as the large size of the FVIII gene hinders the generation of efficient gene delivery systems by viral vectors. Current research groups focus on shorter FVIII constructs or different routes of gene delivery for gene transfer.54–57

An important initiative to improve hemophilia care, alongside the development of newly modified drugs and therapies, is to strive for a wider availability of high quality comprehensive care. This is being addressed by the European Haemophilia Consortium (EHC). The mission of this consortium is to connect experts in the field of hemophilia from across Europe and to share expertise of patients, clinicians, scientist, European institutions and the pharmaceutical industry within Europe to draft resolutions regarding current issues relating to hemophilia treatment.58

Despite improvements in hemophilia care, the availability of safe and affordable treatment for all affected individuals worldwide remains the biggest challenge.
Serendipity

“He heard the poignant ticking of passing time on his Mickey Mouse clock. ‘Can it please be tomorrow..?’ he whispered. The start of a new day would save him from his fear for any monstrous creature possibly residing underneath his bed, behind his closet, or maybe on the seat of his desk. He was so relieved that he did not wake up from a wet bed this time. He hated the moist feeling between his legs and felt utterly ashamed when his mom checked his matrass first thing in the morning. Jip tried to think about what the doctor had told him: ‘Don’t be embarrassed brave boy, I have something magical that will solve your problem. It is called DDAVP and the best thing is: no needles are needed! You can just spray it in your nose before you go to bed and I assure you: no more wet blankets.’ Wow, no needles...

That sounds promising. After his last visit to the hospital, he hoped he never had to see a needle again in his life! He had fallen on his leg playing soccer and it looked like his knee was growing like a giant, ticking time bomb filled with blood. There was a new doctor at the emergency unit who had to give him his factor VIII, but it took ages to find the right vessel and he was in so much pain with every attempt! Nose spray sounded much better. He hoped that it would help, but would it really take just one spray to solve everything..? To be sure, he sprayed as much of the magical substance as he could in his nose before he went to bed. The doctor was right. This time, it wasn’t the bedwetting that woke him up. So, what could have it been then? ‘Bang!’ Another sound, as if someone dropped a heavy book on the ground. This wasn’t funny anymore, but what could he do? He jumped out of his bed to run to his door as fast as he could. If it was a scary monster, he should be fast! But he forgot that he left one of his favorite teddy bears on the ground next to his bed, the bear that had promised to protect him from any weird creatures. ‘Bang bang!’ Oh dear, he fell on the ground, how could he be fast enough now!? ‘Come on, hurry up!’ He said to himself. But his mom was already standing in the door opening: ‘What’s going on here, did you wet your bed again?’ She asked. ‘No mom, there is a monster who’s here to get me and I wanted to escape so I ran to the door but I crashed.’ He answered trembling. ‘There are no monsters my dear, I will check all the corners in your room to prove it to you. And how good of you that you did not wet your bed! It sounded like you fell hard, let me see, no bleeding?’ Jip looked at his arms and knees and answered a bit surprised: ‘No, I don’t believe so’.
DDAVP as a blood-saving agent

DDAVP (1-Deamino-8-D-ArgininVasoPressin; desmopressin) is a synthetic version of the natural hormone vasopressin: a chemical messenger in the brain. Since its discovery 40 years ago, DDAVP has proven to be a pivotal treatment alternative in nonsevere hemophilia A. Besides its anti-diuretic effects, this hemostatic elevator induces the release of a person’s own (endogenous) FVIII and VWF. FVIII rises on average three- to fivefold and the response lasts for about 12-24 hours.\textsuperscript{59,60} DDAVP is a much cheaper alternative for FVIII concentrate, and DDAVP does not carry the risk of inhibitor development associated with the use of exogenous (allogeneic) sources of FVIII present in concentrates.\textsuperscript{20,45,61} DDAVP can safely be administered intravenously or subcutaneously (both 0.3 $\mu$g/kilogram body weight) or intranasally (300 $\mu$g: 150 $\mu$g /nostril).\textsuperscript{61–63}

Nonetheless, the increase of FVIII and VWF in response to DDAVP shows great inter-individual variation (Figure 4). Knowledge is scant on the factors underlying this inter-individual variation and on the exact biological mechanisms causing FVIII to rise. DDAVP may have more clinical potential than currently utilized, and understanding the determinants of the response may enable better prediction of success and optimal clinical use of DDAVP.

\textbf{Figure 4.} Inter-individual variation in FVIII:C after DDAVP administration. Some patients have a higher peak FVIII:C, present in the blood for a longer time than others.
Recent insights into the hemostatic effect of DDAVP show that the drug induces the release of both FVIII and VWF from Weibel Palade bodies (WPBs) in endothelial cells. The exact storage site and the mechanism underlying the rise in FVIII remains to be fully elucidated. Our knowledge on the release of VWF is more detailed than our knowledge on the release of FVIII, as depicted in Figure 5. The increase of both FVIII and VWF upon DDAVP stimulation is not infinite, as the storage sites of these proteins become depleted. Both hemophilia patients and healthy individuals show a decreased biological response if they are repeatedly treated with DDAVP over a short time-interval (<24 hours). This phenomenon is called tachyphylaxis and is highly variable between individuals.

Figure 5. Endothelial cell release of VWF upon stimulation with DDAVP. VWF is packaged into elongated secretory storage organelles named Weibel-Palade bodies (WPBs) in endothelial cells. Upon DDAVP stimulation, WPBs release VWF indirectly by acting on vasopressin 2 receptors (V2R) that are expressed on lung endothelial cells. Binding of DDAVP to V2R activates a protein kinase (PKA) dependent signaling pathway that induces release of WPBs via Gs. Gs is an alpha subunit of a receptor linked heterotrimeric G protein that activates adenylate cyclase (AC) that catalyzes the formation of cAMP. In response to the increase in cyclic adenosine monophosphate (cAMP), a subset of WPBs clusters at the microtubule organizing centre (MTOC) which is located close to the nucleus. Subsequently, WPBs travel to the cell membrane and release VWF.

Furthermore, there is a large inter-individual variation in the extent of VWF and FVIII rise following DDAVP administration. This variation leads to a practical problem and identifies a gap in scientific knowledge. Patients need to be tested for DDAVP responsiveness, to ensure DDAVP is effective, before the drug can be used in clinical
practice. Rising insights in the underlying mechanism of the variation in FVIII release upon DDAVP stimulation may help to optimize patient care and may increase our scientific understanding. Optimization of DDAVP use and knowledge on the working mechanism can be achieved by creating an algorithm for the DDAVP response. The question is: how can we unfold the ingredients of the algorithm?

**Unfolding observations by unlocking the underlying algorithms**

There are still many unanswered questions in the scientific landscape of the Royal Disease. In order to navigate through pathophysiological mechanisms, to evaluate and optimize treatment strategies, and to assess the epidemiological as well as individual impact of nonsevere hemophilia A, we need well defined research questions, robust study designs and reliable methods to answer these questions. The main research question in this thesis aims to unfold the clinical observation of the heterogeneity in the response to DDAVP. This is a biochemical as well as an epidemiological question.

Epidemiology is the scientific discipline that aims to analyze associations between determinants and outcomes, with the final goal to decipher causal relationships. There are many factors that can affect how well a sample reflects the population and therefore how valid and reliable conclusions will be. The identified associations can be ascribed to causality, bias, confounding or chance. In order to estimate differences in outcome between groups, we test the hypothesis that there is no difference. The chance that a difference is due to a random effect is expressed by the P-value. A low P-value suggests that the difference in outcome between groups is unlikely to be due to chance.

In an attempt to reduce chance observations, the number of study participants can be increased. As sample sizes increase, the confidence in the observed association increases, because the effect of random variation (error) is smaller in comparison to the causal effect. If the sample size is too small, the result may not be sufficiently powered to detect a difference between the observed groups, as this may be obscured by random effects. Caution is needed as increase in sample size does not safeguard us from bias.

Bias is “a process at any stage of inference tending to produce results that depart systematically from the true values”.

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It is “an error in the conception and design of a study – or in the collection, analysis, interpretation, publication, or review of data – leading to results or conclusions that are systematically (as opposed to randomly) different from the truth.” Systematic spuriosity may lead to both over- and underestimation of observed effects.

Where bias is induced by study design or execution, confounding is an error caused by a variable that interferes in the association between exposure (DDAVP administration) and disease or treatment outcome (DDAVP response). Thus, a confounding factor colors the observation. It occurs when two factors are associated (travel together) and the effect of one is confused with or distorted by the effect of the other. Figure 6 explains this concept for the subject in the current thesis.

If confounders can be identified, we can adjust for them in the analyses.

Figure 6. A confounder is not resulting from exposure to DDAVP, so it is not part of the causal path between exposure to DDAVP and the response (1). A confounder is associated with exposure (treatment with DDAVP) (2). A confounder is a risk factor for, or a different cause of, a disease or treatment outcome (DDAVP response) (3).

Once we can discard all the above mentioned explanations for an observed association between exposure and disease, the association might be of causal nature and needs to be mapped within (patho)physiological landscapes.

The way the association is reported could be looked upon as an algorithm leading to a treatment outcome. Just as oil, onions, potatoes and cabbage are the ingredients that sequentially produce a cup of soup, in a similar way the exposure to DDAVP and factors A and B could result in variation in DDAVP response.

Present day dogma holds that organisms are algorithms and that algorithms can be presented in mathematical formulas represented in models. These models can be of variable complexity. In their simplest form, they are constructed by use of conventional statistical methods. You can use numbers and mathematical symbols to write the series of actions to make soup, or steps a vending machine takes to prepare
a cup of tea. Or to describe associations between DDAVP administration and DDAVP response.

Outline
As nonsevere hemophilia A is relative rare, (international) collaboration is crucial in order to ascertain robustness in study design. With combined efforts we can try to close the remaining knowledge gaps in translational and clinical research that are needed to improve hemophilia care. In the following chapters of this thesis, results of the INSIGHT and the RISE studies are presented. Both are large, international cohort studies together including data of about 3500 nonsevere hemophilia A patients from 36 participating Hemophilia Treatment Centers across Europe, Canada and Australia (Figure 7).

![Figure 7. Countries with participating sites of the INSIGHT and RISE studies, indicated in orange.](image)

INSIGHT is an acronym for INternational Study on etiology of inhibitors in patients with nonsevere hemophilia A: influences of Immuno Genetic & Hemophilia Treatment factors. The initial aim of the study was to identify clinical and genetic factors that induce inhibitor formation in patients with nonsevere hemophilia A. In the first part of this thesis, we gain insight into morbidity and mechanistic aspects of nonsevere hemophilia A. In chapter 2, we assess the association between the occurrence of inhibitors and mortality. Furthermore, we explore bleeding-related causes of death by describing mortality rates, risk factors and comorbidities associated with fatal intracranial bleeding in chapter 3.
In chapter 4, we unravel the association between mutations and baseline FVIII:C levels. Our understanding of the biomolecular process of the causative genetic event leading to reduced baseline FVIII:C in nonsevere patients is still limited. This lack of knowledge contributes to ongoing diagnostic uncertainties. The insights in part I show that nonsevere hemophilia A is not a mild disease at all and emphasizes the urgent need for safer treatment alternatives and unraveling biomolecular mechanisms. Hence, the second part of this thesis entails the rise in knowledge on determinants of DDAVP response. RISE stands for Response to DDAVP In nonsevere Hemophilia A patients, in Search for dEterminants. The aim of this project was to assess the predictive value of clinical and genetic factors on the DDAVP response in nonsevere hemophilia A patients. In chapter 5, we show the outcomes of DDAVP use in moderate patients. Patients with a lower baseline FVIII:C tend to respond less and moderately affected patients are therefore less frequently tested for or treated with DDAVP. Here we demonstrate that FVIII:C may rise substantially in 40% of the patients with moderate hemophilia A. Furthermore, we address the effect of mutations on DDAVP response in chapter 6. The analysis of the association between mutations and DDAVP response is hampered by the heterogeneity of the missense mutations. In this chapter, we used a new classification system proposed by Sengupta et al. to classify mutations. In chapter 7, we evaluate the effect of age on DDAVP response. Age partially explains the observed variation in response, hence, older patients may show a slightly better response compared to younger patients. We specifically address the intra-individual variation of DDAVP response among re-exposed patients.

In the last chapter of part II, we integrate the clinical and genetic factors into a complex model by making use of population pharmacokinetic (PK) modeling. The PK behavior of a drug may exhibit a large inter-individual as well as an intra-individual variation. We aim to describe the variation for the individual patient in order to optimize the individual treatment strategy. The temporal increase of VWF and FVIII plasma concentrations upon DDAVP administration can be described by PK parameters, such as biological availability. By using a population PK approach, not only average PK parameters can be estimated but also their variability between patients.

The third part of this thesis integrates the rising insights in the mechanisms of action of DDAVP. In chapter 9 we aim to integrate knowledge on the effect of DDAVP and FVIII replacement therapy. One of the major downsides of FVIII replacement therapy is the frequent administration that is required due to a short half-life of FVIII. As VWF concentration before FVIII replacement therapy administration is associated
with FVIII half-life, we aim to improve the infused FVIII half-life by increasing endogenous VWF with DDAVP prior to FVIII administration. Finally, chapter 10 is a protocol of a prospective explorative study with the aim to unravel the exact biological mechanisms causing VWF/FVIII to rise following DDAVP administration. The information following from this study will be used to optimize the population PK model as described in chapter 8 in the future. Chapter 11 provides the summary and the overall interpretation and discussion of the findings of this thesis.

New beginnings
This introduction is meant to serve as a theoretical framework to guide the reader through the basic concepts discussed in the following chapters. I hope it helps to navigate through the hemophilia landscape and that it may lead to insightful discussions. The philosopher Hannah Arendt suggests in her writings that the meanings of the concept of new beginnings shift and do not let themselves be fitted together in a unified and comprehensive theory. In the same way, the meanings of the concepts explained in this introduction will continue to shift due to new insights following the rapid developments in hemophilia A research.
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