



LUND UNIVERSITY

Genetic characterization of families with von Willebrand disease

Lanke, Elsa

2008

[Link to publication](#)

Citation for published version (APA):

Lanke, E. (2008). *Genetic characterization of families with von Willebrand disease*. [Doctoral Thesis (compilation), Clinical Coagulation, Malmö]. Department of Clinical Sciences, Lund University.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

**Genetic characterization of families
with von Willebrand disease**

Elsa Lanke

Doctoral thesis



LUND UNIVERSITY

Clinical Coagulation Research Unit
Lund University
Malmö, Sweden
2008

Printed by Media-Tryck, Lund University, Sweden

© Elsa Lanke, 2008

ISSN 1652-8220

ISBN 978-91-86059-42-2

Lund University, Faculty of Medicine Doctoral Dissertation Series 2008:89

Genetic characterization of families with von Willebrand disease

Elsa Lanke



LUND UNIVERSITY

Clinical Coagulation Research Unit
Lund University
Malmö, Sweden
2008

Akademisk avhandling

som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet för avläggande av doktorexamen i medicinsk vetenskap kommer att offentligens försvaras i Lilla aulan, Medicinskt forskningscentrum, ingång 59, Universitetssjukhuset MAS, Malmö, fredagen den 3 oktober 2008, kl 9.15

Fakultetsopponent

Ass. Professor Riitta Lassila
Helsingfors, Finland

Organization LUND UNIVERSITY		Document name DOCTORAL DISSERTATION
Faculty of Medicine, Clinical Coagulation Research Unit, Malmö University Hospital, Sweden		Date of issue October 3, 2008
Author(s) Elsa Lanke		Sponsoring organization
Title and subtitle Genetic characterization of families with von Willebrand disease		
<p>Abstract</p> <p>von Willebrand disease (VWD) is the most common hereditary bleeding disorder. It is caused by quantitative and/or qualitative defects of the von Willebrand factor (VWF). The severity of the disease can vary considerably, as can the hereditary patterns. The variable phenotypes of VWD have given rise to a classification scheme that divides the disease into three types according to how it is manifested and inherited. The genetics of, especially type 1, VWD is relatively complicated and many aspects of it remain to be elucidated. The purpose of these studies was therefore to investigate and clarify certain genetic mechanisms that underlie VWD.</p> <p>When we investigated to what extent co-segregation exists in type 1 VWD, we found that the disease is linked to the VWF gene in a majority (27 of 31) of Swedish type 1 VWD families. Several common disease haplotypes probably exist for type 1 VWD in Sweden, which suggests founder effects. The Y1584C variation is not as common in the Swedish type 1 VWD population as it is in some other populations. We confirmed that blood group O is over-represented among type 1 VWD patients in Sweden. Apart from certain misunderstandings, the participants in the linkage study were found to have a satisfying level of knowledge of the genetics of the disease. In general, patients, younger individuals, and women have a higher knowledge about the genetics causing type 1 VWD than do healthy relatives, older individuals, and men, respectively.</p> <p>Inherited recessively, the C570S mutation causes a distinct subtype of type 2A VWD characterized by very low plasma FVIII and VWF levels and the exclusive presence of the dimeric form of VWF in plasma. The findings define a structural element that is indispensable for VWF multimerization.</p> <p>Inherited dominantly, the N1421K mutation causes type 2M VWD characterized by moderately decreased plasma FVIII and VWF levels, disproportionately low plasma VWF:RCO levels, and an apparently normal multimeric pattern. The findings indicate a structural element in the A1 domain that is necessary for proper GPIb binding.</p>		
Key words: Bleeding, coagulation, genetics, mutation, von Willebrand disease		
Classification system and/or index terms (if any):		
Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 978-91-86059-42-2
Recipient's notes	Number of pages 105	Price
	Security classification	

Distribution by (name and address)

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature Elsa Lanke Date August 26, 2008

*In fond memory of
my grandmother Lisa,
who was a dedicated scientist
and who has thus inspired me.*

*There is nothing of which every man is so afraid,
as getting to know how enormously much he is capable of doing and becoming.*
- Søren Kierkegaard

Contents

List of papers	8
Abbreviations	9
Abstract	10
Introduction	11
Historical background	11
Haemostasis	12
Coagulation	13
von Willebrand factor gene	16
von Willebrand factor	17
von Willebrand disease	20
The genetics of von Willebrand disease	22
Diagnosing von Willebrand disease	25
Treatment of von Willebrand disease	26
Present investigations	28
Aims	28
Findings	28
Conclusions	34
Concluding remarks	35
Future perspectives	36
Popularised summary in Swedish (Populärvetenskaplig sammanfattning)	37
Acknowledgements	41
References	42
Appendices: papers I-IV	53

List of papers

This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals (I-IV):

- I. **Lanke E**, Johansson AM, Halldén C, Lethagen S. Genetic analysis of 31 Swedish type 1 von Willebrand disease families reveals incomplete linkage to the von Willebrand factor gene and a high frequency of a certain disease haplotype. *Journal of Thrombosis and Haemostasis*. 2005; 3: 2656-2663.
- II. **Lanke E**, Lanke J, Lethagen S. Patients' and their family members' understanding of the genetics of type 1 von Willebrand disease. *Haemophilia*. 2008; 14: 1127-1130.
- III. **Lanke E**, Kristoffersson AC, Philips M, Holmberg L, Lethagen S. Characterization of a novel mutation in the von Willebrand factor propeptide in a distinct subtype of recessive von Willebrand disease. *Thrombosis and Haemostasis*. 2008; 100: 211-216.
- IV. **Lanke E**, Kristoffersson AC, Isaksson C, Holmberg L, Lethagen S. N1421K mutation in the glycoprotein Ib binding domain impairs ristocetin- and botrocetin-mediated binding of von Willebrand factor to platelets. *European Journal of Haematology*. Accepted for publication 30 June 2008.

These papers are reprinted with permission from the publishers.

Abbreviations

ADAMTS13 A disintegrin-like and metalloprotease with thrombospondin type I motif, 13

bp base pairs

DNA deoxyribonucleic acid

ELISA enzyme-linked immunosorbent assay

FVIII coagulation factor VIII

FVIII:Ag FVIII antigen

FVIII:C FVIII coagulant activity

GPIb platelet receptor glycoprotein Ib

GPIIb/IIIa platelet receptor glycoprotein IIb/IIIa

HMW high molecular weight

kb kilo base pairs

PCR polymerase chain reaction

rVWF recombinant VWF

VWD von Willebrand disease

VWF von Willebrand factor

VWF:Ag VWF antigen

VWF:BCo VWF botrocetin cofactor activity

VWFpp VWF propeptide

VWF:RCo VWF ristocetin cofactor activity

wt wild-type

Amino acid residues are abbreviated in accordance with the 1983 recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1], using either the three-letter or the one-letter code.

Abstract

von Willebrand disease (VWD) is the most common hereditary bleeding disorder. It is caused by quantitative and/or qualitative defects of the von Willebrand factor (VWF). The severity of the disease can vary considerably, as can the hereditary patterns. The variable phenotypes of VWD have given rise to a classification scheme that divides the disease into three types according to how it is manifested and inherited. The genetics of, especially type 1, VWD is relatively complicated and many aspects of it remain to be elucidated. The purpose of these studies was therefore to investigate and clarify certain genetic mechanisms that underlie VWD.

When we investigated to what extent co-segregation exists in type 1 VWD, we found that the disease is linked to the VWF gene in a majority (27 of 31) of Swedish type 1 VWD families. Several common disease haplotypes probably exist for type 1 VWD in Sweden, which suggests founder effects. The Y1584C variation is not as common in the Swedish type 1 VWD population as it is in some other populations. We confirmed that blood group O is over-represented among type 1 VWD patients in Sweden. Apart from certain misunderstandings, the participants in the linkage study were found to have a satisfying level of knowledge of the genetics of the disease. In general, patients, younger individuals, and women have a higher knowledge about the genetics causing type 1 VWD than do healthy relatives, older individuals, and men, respectively.

Inherited recessively, the C570S mutation causes a distinct subtype of type 2A VWD characterized by very low plasma FVIII and VWF levels and the exclusive presence of the dimeric form of VWF in plasma. The findings define a structural element that is indispensable for VWF multimerization.

Inherited dominantly, the N1421K mutation causes type 2M VWD characterized by moderately decreased plasma FVIII and VWF levels, disproportionately low plasma VWF:RCO levels, and an apparently normal multimeric pattern. The findings indicate a structural element in the A1 domain that is necessary for proper GPIb binding.

Introduction

Historical background

The Finnish physician Erik A von Willebrand (Fig. 1) in 1926 described a dominantly inherited bleeding disorder occurring in both sexes, after having investigated a large family on the Åland Islands in the Gulf of Bothnia situated between Sweden and Finland [2]. The first patient was a five-year-old girl, the ninth out of eleven children in family S (Fig. 2), who presented with severe bleeding and who later died at the age of 13 during her fourth menstrual bleeding. Out of 66 additional relatives that von Willebrand was able to investigate, he found 23 to be bleeders, of whom 16 were women and seven were men [3]. He also reviewed 27 cases reported in the literature with similar symptoms [4,5].

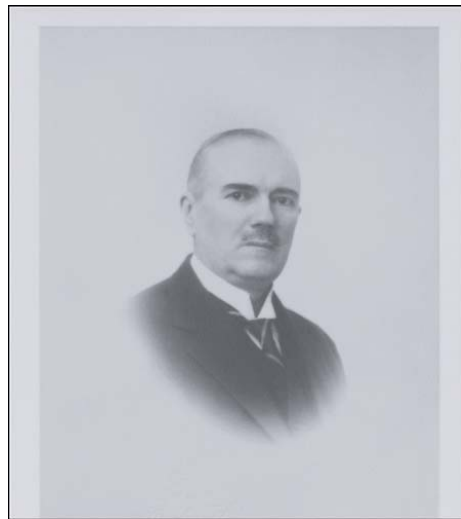


Figure 1. Erik A von Willebrand (1870-1949).

The disease was characterized by nose bleeding, menorrhagia, bleeding after tooth extractions and from wounds. The bleeding time was prolonged, whereas the platelet count was normal and it soon became obvious that this disease differed from ordinary haemophilia; hence von Willebrand called the new disorder hereditary pseudo-haemophilia. In the 1950s it had become possible to measure factor VIII (FVIII) in plasma and it was shown to be lacking not only in haemophilia A patients but also in patients affected by a severe form of the bleeding disorder similar to that described by von Willebrand. It was also shown that the condition of the patients was improved after infusion of a fraction of human plasma from healthy

individuals, called fraction I-0 [6], and interestingly also after infusion of fraction I-0 derived from plasma from haemophilia A patients [7,8]. When fraction I-0, later called AHF-Kabi, was administered to the patients, the bleedings stopped, the bleeding time was normalised and the concentration of FVIII in plasma increased. This treatment was first carried out, successfully, in Malmö on a woman with severe VWD manifested as life-threatening bleedings [9]. Later also the original family on the Åland Islands was treated successfully in this way [10]. The result that factor VIII, which is lacking in haemophilia A patients, was not the missing factor in these patients paved the way to the conclusion that a different factor, called the von Willebrand factor (VWF), is deficient in von Willebrand disease (VWD).

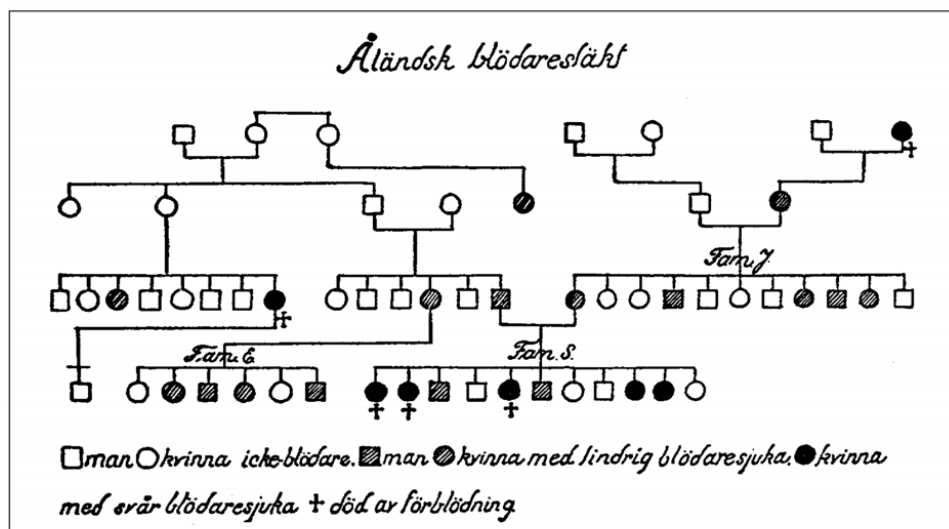


Figure 2. Pedigree of the original Åland family as described by Erik A von Willebrand in his publication from 1926 [4]. Individual IV:16 illustrates the first patient, Hjärdis.

Haemostasis

Haemostasis occurs as a means to protect the body from blood loss after vascular damage and can be divided into three phases: primary haemostasis, coagulation or secondary haemostasis, and fibrinolysis [11]. In primary haemostasis, platelets adhere to the subendothelium, become activated and aggregate to each other to form a platelet plug that temporarily stops the blood flow. Simultaneously, the coagulation cascade is activated and a fibrin clot is formed to stabilise the plug. In fibrinolysis, fibrin clots are lysed in order to prevent the formation of

thrombi. These events are controlled by a series of procoagulant and anticoagulant proteins. In the healthy state, these proteins interact with each other optimally and the haemostasis is balanced. When there is an imbalance, however, the haemostasis is shifted towards either the pro- or the anticoagulant side. If there is a shift towards the procoagulant side, or if the anticoagulant side is insufficient, this results in an increased risk of thrombosis. If there is an insufficiency of haemostatic factors or platelets, this results in an increased risk of bleeding. Thus, a balanced haemostatic system is essential to avoid both thrombosis and bleeding.

Coagulation

Coagulation takes place on phospholipid surfaces of vascular endothelial cells and platelets, and a number of different proteins are involved in the process of coagulation. In the traditional view the coagulation cascade can be schematically divided into the extrinsic and the intrinsic pathways (Fig. 3). The extrinsic pathway is initiated when the extravascular glycoprotein tissue factor (TF) comes into contact with blood after vascular damage. TF binds to FVII and its activated form, FVIIa. A complex consisting of TF, FVII/FVIIa and calcium activates FIX and FX that are part of the tenase and prothrombinase complexes, respectively. The intrinsic pathway is initiated by exposure of FXII, prekallikrein and HMW kininogen in blood to a negatively charged surface viz. connective tissue and collagen *in vivo* and glass and kaolin *in vitro*. This results in activation of FXII that activates FXI that in turn activates FIX. FIXa and FVIIIa together with calcium and phospholipid form the tenase complex that activates FX, which marks the convergence of the intrinsic and the extrinsic pathways. FXa and FVa together with calcium and phospholipid form the prothrombinase complex that converts prothrombin to thrombin. Thrombin converts fibrinogen to fibrin and activates FXIII. Insoluble fibrin forms, with the help of FXIIIa, a crosslinked fibrin clot that can be regarded as the end product in the coagulation cascade.

In vivo, the extrinsic pathway is crucial for initiation of the coagulation cascade whereas the intrinsic pathway seems to be of minor importance. *In vitro*, the intrinsic pathway is capable of initiating the coagulation cascade. Thus, the extrinsic pathway can be viewed as physiologically relevant, whereas the intrinsic pathway rather can be viewed as important for test tube reactions.

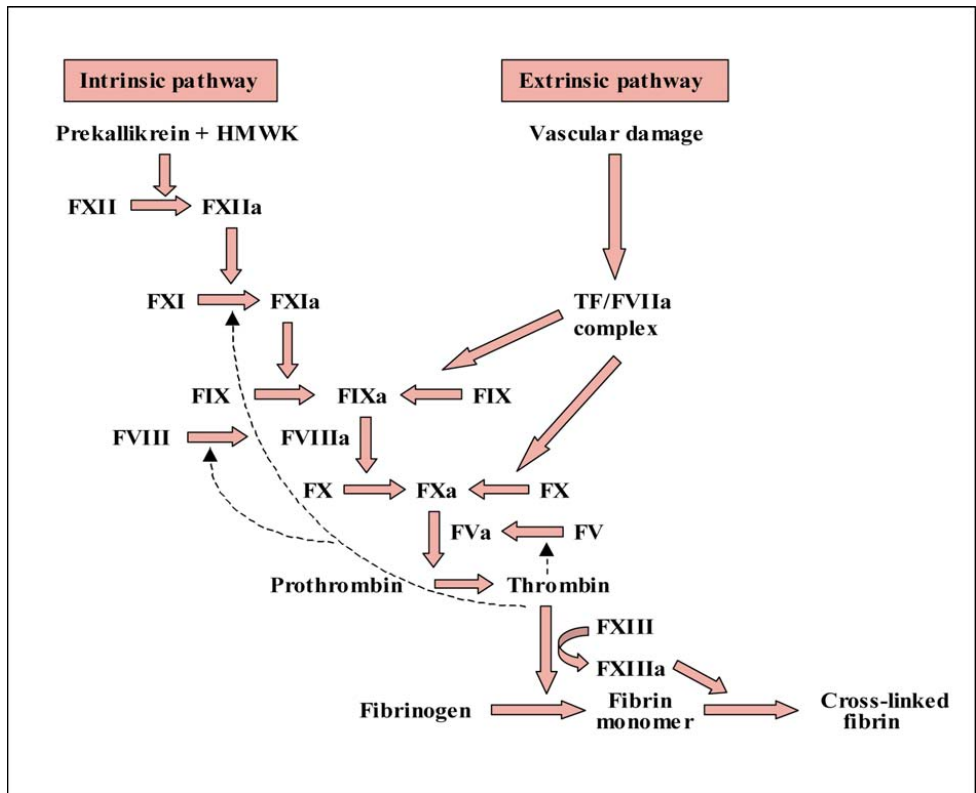


Figure 3. Schematic illustration of the traditional coagulation cascade: a summary of the intrinsic and extrinsic pathways. The dashed arrows indicate thrombin feedback loops.

The relatively recently published cell-based model emphasizes the importance of cell surfaces and their components in the role of coagulation [12]. This model, which describes a more physiological view than the classical coagulation cascade, can be divided into three phases: initiation, amplification, and propagation (Fig. 4). The initiation phase takes place on the surface of TF-presenting cells such as fibroblasts. Vascular damage releases active enzymes which activate FVII. The TF/FVIIa complex produces FIXa, FXa and, eventually, thrombin. The amplification phase takes place on the surface of platelets when thrombin activates platelets, which then release granula with procoagulant substances and its cell surface becomes procoagulant by exposure of negatively charged phospholipids. Thrombin also activates FV, FVIII, and FXI. In the final phase, propagation, the enzymes and their co-factors are assembled on the surface of the activated platelets. The FIXa/FVIIIa complex activates FX and the FXa/FVa complex activates prothrombin to thrombin, which in sufficient amounts converts fibrinogen to fibrin.

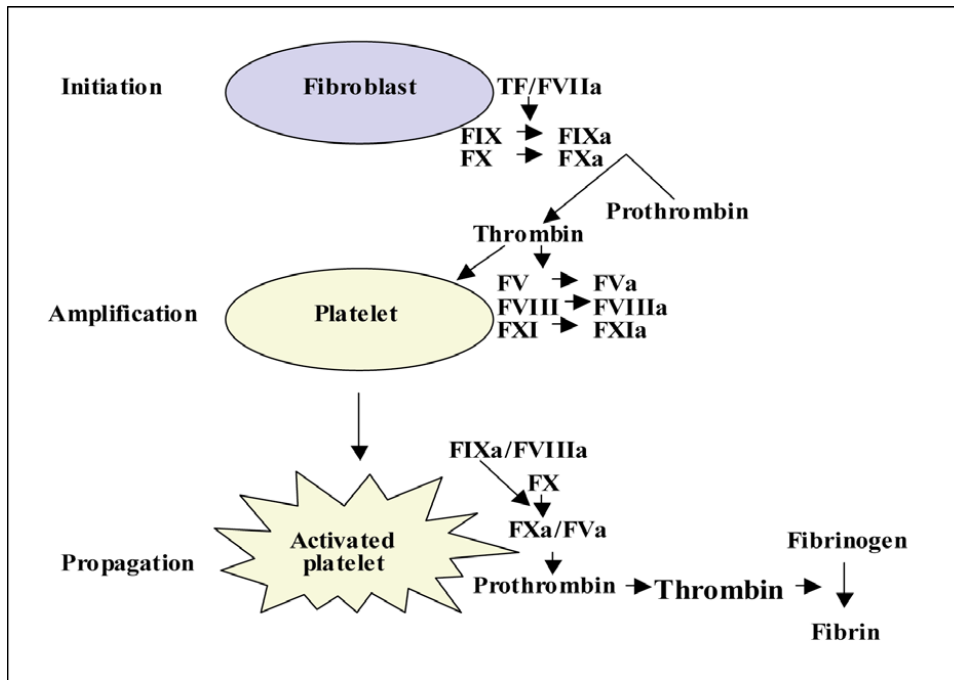


Figure 4. A schematic summary of the cell-based model of coagulation.

Regulation of coagulation occurs through various feedback systems to ensure that both bleeding and thrombosis are avoided; however, genetic or acquired disturbances of the natural balance between the pro- and anticoagulant systems may result in bleeding or thrombotic events [13]. The protein C anticoagulant system involves thrombin that, in addition to its procoagulant properties i.e. activation of platelets and factors V, VIII, XI, and XIII, and conversion of fibrinogen to fibrin in the coagulation cascade, also exerts an anticoagulant effect. At intact vessel surfaces, thrombin binds to the endothelial membrane receptor thrombomodulin and then activates protein C, which in turn, together with its cofactor protein S, degrades and thereby inactivates FVa and FVIIIa; thrombin thus serves as an anticoagulant. Another regulator of coagulation is the glycoprotein antithrombin that inhibits, amongst other proteins, thrombin, FIXa, FXa, FXIa, and FXIIa. Further, the complex consisting of TF and FVII/FVIIa involved in the initiation of the extrinsic pathway in the classical coagulation cascade as well as in the cell-based model is inhibited by TF pathway inhibitor (TFPI). Finally, fibrinolysis provides a system to protect the body from potentially hazardous thrombi by dissolving fibrin clots. Fibrinolysis is itself regulated by a number of control systems.

In several studies, a genetic basis has been shown to account for a large proportion of the variation in plasma levels of procoagulant and anticoagulant factors [14,15]. A high phenotypic correlation has been found between various coagulation factors, and this also seems to be genetically determined [15]. It thus seems that single genes might be able to pleiotropically influence multiple factors involved in coagulation, and hence that clusters of interrelated procoagulant and anticoagulant factors exist [16].

Many proteins involved in coagulation, both procoagulant factors such as FII, FVII, FIX, and FX, and anticoagulant proteins such as protein C and protein S, are vitamin K-dependent. Deficiency of vitamin K leads to a decrease in plasma levels of these proteins. Vitamin K-dependent proteins contain γ -carboxyglutamic acid (Gla). The Gla-domain is a prerequisite for the binding of the vitamin K-dependent factors to phospholipids surfaces, e.g. the surface of activated platelets. Warfarin is a vitamin K antagonist that acts by restraining synthesis of Gla-domains by inhibiting carboxylation, which makes the vitamin K-dependent proteins unable to bind to activated platelets. Even though both pro- and anticoagulant factors are affected by vitamin K deficiency, the net effect is anticoagulant, i.e. the result is a coagulation state that is shifted towards bleeding.

von Willebrand factor gene

The von Willebrand factor (VWF) gene is located on chromosome 12p13.3 and was cloned and characterized by four groups simultaneously in 1985 [17-20]. It is approximately 178 kb long and contains 52 exons [21]. The first 17 exons encode the 5' non-coding region, the signal peptide, and the propeptide (formerly called von Willebrand antigen II), while the remaining 35 exons encode the mature VWF and the 3' non-coding region. The transcribed mRNA is 8.7 kb. A partial pseudogene on 22q11-q13 corresponding to exons 23 to 34 in the authentic VWF gene was reported by Mancuso in 1991 [22]. The presence of various splice site and missense mutations renders the pseudogene highly unlikely to yield a functional protein. Nevertheless, the high homology (97%) to the authentic gene complicates genetic analysis and hence oligonucleotide primers must be used which are specific for sequences in the authentic gene and the pseudogene, respectively.

The VWF gene promoter spans from -487 to +247 relative to the transcription start and includes a TATA box and a CCAAT element involved in regulation of transcription. Early studies have led to the characterization of various additional regulative elements in the

promoter [23,24]. Studies of the roles of these and additional elements have made possible the identification of the GATA6 and Ets transcription factors and an H1-like protein functioning as activators, and also an NF1-like protein, Oct1 and E4BP4 functioning as repressors of the promoter [25-28]. The transcription factor NFY can function both as a repressor and activator of transcription [29]. All these cis-acting elements and trans-acting factors ensure regulated cell-specific transcription of the VWF gene and limit the expression to endothelial cells and megakaryocytes. Further, in a number of studies, an association has been found between various polymorphisms in the promoter region and plasma VWF levels [30-34].

von Willebrand factor

A plasma protein missing in von Willebrand disease was demonstrated immunologically in 1971 by Zimmerman and collaborators, and this von Willebrand factor (VWF) was subsequently purified and found to be a polymeric glycoprotein [35-37]. It has two main functions in haemostasis: it promotes platelet adhesion to damaged vessels and platelet aggregation, and it serves as a carrier for FVIII in plasma, thereby protecting it and prolonging its lifetime [36,38]. VWF thus plays an important role in both primary and secondary haemostasis. Vascular endothelial cells and megakaryocytes synthesize VWF. It is packaged in storage vesicles called Weibel-Palade bodies in the former and in α -granules in the latter and in platelets. The primary translation product is the monomeric pre-pro-VWF. In addition to the 2050-residue mature VWF, the pre-pro-VWF consists of the 22-residue signalpeptide and the 741-residue propeptide [37]. Compared to other plasma proteins, the biosynthesis of VWF is very complex and includes various intracellular modifications such as glycosylation, sulfation, dimerization, multimerization, and signalpeptide and propeptide cleavages [39,40]. Different domains of the VWF are involved in different functions such as FVIII binding, platelet receptor GpIb and GpIIb/IIIa binding, collagen binding, heparin binding, dimerization, multimerization and intracellular storage [41-44]. A schematic illustration of the VWF is outlined in Fig. 5.

The VWF propeptide (VWFpp) plays a crucial role in multimerization and intracellular storage of VWF [42,45-49]. It has been shown that the signal for multimerization is different from that for trafficking of VWF to storage compartments [50]. Cleavage of the VWFpp precedes, and may also be a prerequisite for, targeting [51]. The VWFpp consists of two homologous D domains, D1 and D2, which contain 32 cysteines each. When VWF lacking one of the two D domains was expressed in various cell types, the protein was secreted

efficiently whichever D domain was lacking; however, in neither case the protein multimerized beyond the dimer stage and in neither case it was stored [52]. Multimerization does not require the VWFpp to be a contiguous part of the pro-VWF, as independently expressed VWFpp has been shown to promote the assembly of mature VWF subunits into multimers [46]. Both endoproteolytic cleavage and multimerization occur predominantly if not entirely in the trans-Golgi apparatus, rather than in the storage granules [53]. The Golgi apparatus lacks chaperones to assist folding [54]; hence, how the multimerization process is accomplished is largely unknown. The VWFpp may act as an oxidoreductase by forming a transient disulfide-linked intermediate with the multimerization region of VWF before multimer formation [55].

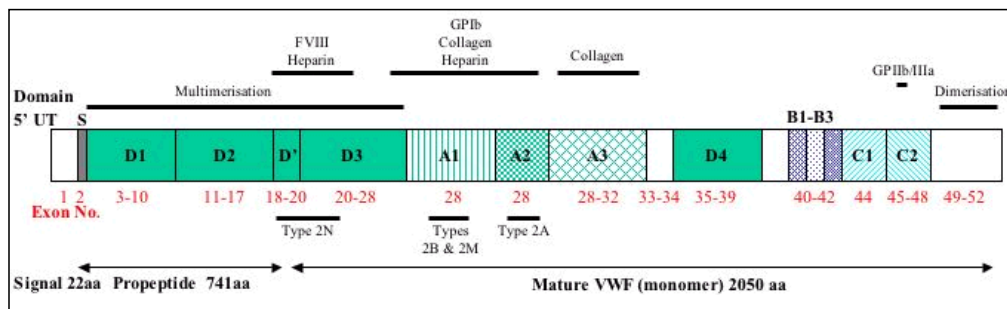


Figure 5. The VWF with its functional domains and the corresponding exons at gene level. Republished from www.vwf.group.shef.ac.uk/index.html by courtesy of the ISTH SSC VWF group.

Circulating VWF is a heterogeneous collection of a series of multimers where the high molecular weight (HMW) VWF multimers are most crucial in platelet adhesion and aggregation. VWF multimers in plasma are naturally degraded by the ADAMTS-13 metalloprotease, which primarily cleaves VWF that has been outstretched as a result of high shear stress [56-58].

VWF does not bind spontaneously to platelets in blood; however, VWF has the ability to bind to subendothelial collagen, particularly under high shear stress or at sites of vascular damage (Fig. 6). This binding activates the VWF and induces a conformational change that gives the VWF the ability to bind to platelets via the α -chain of GPIIb, which in turn results in platelet adhesion and aggregation. This induces a morphological change of the platelets, and the platelet receptor GPIIb/IIIa becomes available for binding to fibrinogen and the VWF. Simultaneously, the activated platelets release the contents of α -granules that include a large

proportion of potent HMW VWF multimers. Once the VWF has bound to GPIb- α , it becomes more susceptible to proteolysis, a mechanism that serves a controlling role in preventing the formation of hazardous thrombi [59].

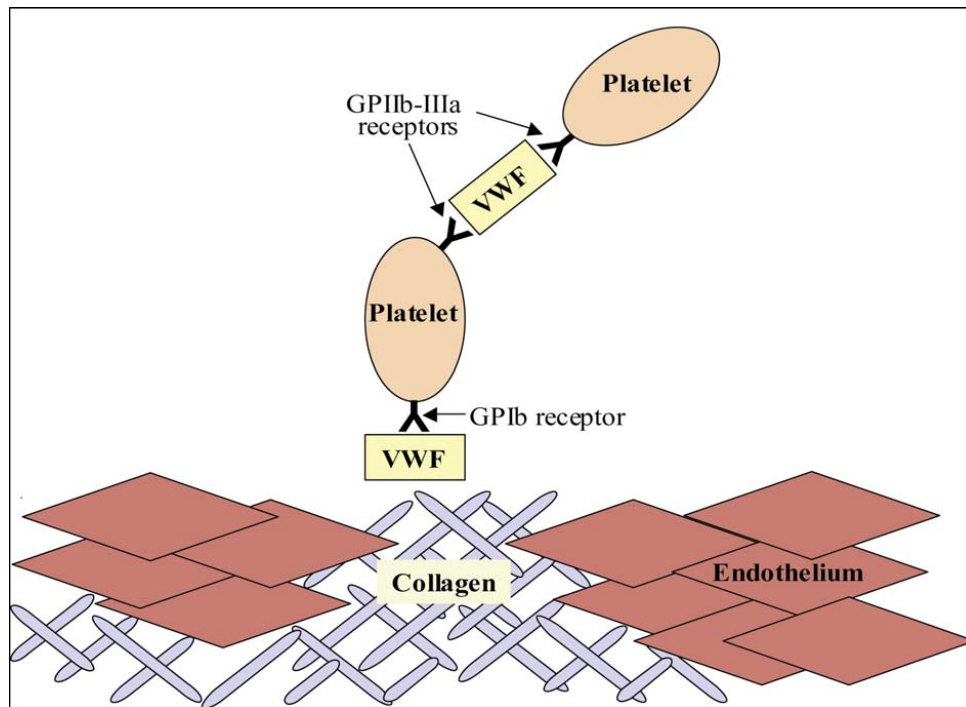


Figure 6. Haemostatic functions of VWF: VWF binds to platelets and subendothelial collagen via GPIb (adhesion) and to platelets via GPIIb/IIIa (aggregation).

In vitro, binding of VWF to GPIb can be mimicked by the antibiotic ristocetin or by the viper venom protein botrocetin [60]. Ristocetin can bind both to platelets and to VWF [61], whereas botrocetin has the ability to bind to VWF but not to GPIb [62]. It has recently been shown that upon binding of VWF to GPIb- α , botrocetin prebound to VWF-A1 makes no contact initially with GPIb- α , but subsequently slides around the A1 surface to form a new interface with GPIb- α [63]. Botrocetin reacts with a broad spectrum of large to small molecular forms of VWF, whereas ristocetin reacts predominantly with HMW multimers [64,65]. Two subunits of botrocetin provide the binding site for VWF; hence botrocetin binds directly to the A1 domain of the VWF in close proximity to the GPIb binding site which does not induce a significant conformational change on the GPIb binding site [66]. Thus, the modulating

mechanisms of botrocetin are different from those performed by either the antibiotic ristocetin *in vitro* or extremely high shear stress *in vivo*. As botrocetin has a slightly but significantly different effect from that of ristocetin, the two agonists can be used to differentiate molecular variants of VWF [67].

von Willebrand disease

von Willebrand disease (VWD) is the most common congenital bleeding disorder and it is caused by quantitative and/or qualitative defects of the VWF. Low plasma VWF can result from decreased synthesis, impaired secretion or increased clearance or a combination of these [68]. The main manifestation of VWD is excessive mucocutaneous bleeding. Only severe deficiency of VWF leads to such shortage of FVIII that haemophilic symptoms such as joint bleeds may occur; hence, in contrast to haemophilia, haemarthrosis is a rare event. The severity of the disease can vary considerably, as can the hereditary patterns.

As different VWD phenotypes have been described over time, a vast number of VWD subtypes evolved and the situation eventually became unmanageable. The variable phenotypes of VWD have therefore given rise to a classification scheme (Table 1), which was agreed on in 1994 [69] and updated in 2006 [70]. Correct classification facilitates diagnosis, treatment, and genetic counselling of patients with VWD. The current classification divides VWD into three categories. Type 1, which is inherited as an autosomal dominant trait and accounts for approximately 70-80% of all cases, is characterized by mild to moderate bleeding manifestations and a quantitative reduction of VWF. The criteria for type 1 VWD diagnosis include significant mucocutaneous bleeding, laboratory tests compatible with type 1 VWD (e.g. a low VWF level), and either a positive family history or an appropriate VWF mutation [71]. In the updated version of the classification scheme, it has been clarified that VWD is not restricted to VWF gene mutations (i.e. other loci can be involved), and also that type 1 VWD includes cases with slightly altered multimer distribution where the large multimers may be insignificantly decreased as long as the functional activity is normal relative to antigen level. It has only recently been recognized that low levels of VWF in VWD patients may originate from modified clearance and that this seems to be a common occurrence in the pathogenesis of type 1 VWD [72]. Although type 1 VWD in the vast majority of cases is dominantly inherited, it can be noted that a number of patients have been found with recessive type 1 VWD, notably in north-eastern Italy where some relatively common regional variants have been identified [73,74]. In type 2 VWD, which accounts for

20-30% of all cases, the VWF is qualitatively affected in different ways; it is further subclassified depending on the type of qualitative defect. Types 2A and 2M are characterized by decreased affinity of the VWF to GPIb; the former type includes loss of HMW multimers whereas the latter type includes a normal or nearly normal multimeric pattern. It has been suggested that types 2A and 2M ought not be distinguished but rather be grouped as a single subtype '2A' that includes all variants with a decreased platelet-dependent adhesion, regardless of the multimeric distribution [75]. Type 2B is characterized by increased affinity to GPIb, and type 2N by decreased affinity of the VWF to FVIII. Type 3, which is a very rare autosomal recessive form of the disorder, with a prevalence of approximately $1-5 \cdot 10^{-6}$, is characterized by complete or almost complete lack of VWF; thus VWF levels may be undetectable whereas FVIII levels are low but usually detectable [76]. These very rare type 3 VWD patients, in addition to some type 2 patients, are so severely affected that their clinical manifestations are similar to those in cases of moderately severe haemophilia [77]. Patients with type 3 are either homozygous or compound heterozygous.

Table 1 Description of the revised VWD classifications and correspondence between revised and previous types

Type	Description	Types until 1994
1	Partial quantitative deficiency of VWF	I platelet normal, I platelet low, IA, I-1, I-2, I-3
2	Qualitative deficiency of VWF	
2A	Decreased platelet-dependent VWF function with selective deficiency of high-molecular-weight multimers	IIA, IB, I platelet discordant, IIC, IID, IIE, IIF, IIG, IIH, II-1, IIA-1, IIA-2, IIA-3
2B	Increased affinity for platelet glycoprotein Ib	IIB, I New York, Malmö
2M	Decreased platelet-dependent VWF function with high-molecular-weight multimers present.	Vicenza, IC, ID
2N	Markedly decreased binding of factor VIII to VWF	Defective binding to factor VIII, Normandy
3	Complete deficiency of VWF	III

VWD seems to be fairly prevalent in the population; screening suggests that 1% could be affected [78,79]. This may be an overestimation since the prevalence of symptomatic VWD based on referral to specialised centres range from 23 to 113 per million [80]. VWF levels in patients affected by type 1 VWD and healthy controls overlap and there is a poor relationship between plasma VWF levels and bleeding manifestations (apart from severe cases), which indicate that most mild cases could represent coincidental association of low VWF level with bleeding symptoms [71]. Taken together, it has been suggested that moderately decreased VWF levels (0.20–0.40 kIU/l) should be viewed as other risk factors, in the same way as elevated cholesterol and high blood pressure constitute risk factors for cardiovascular disease; thus low levels of VWF constitute only a modest risk factor, and can be regarded as a

biomarker for bleeding, and not the disease itself if further evidence, such as family history or other laboratory markers, is lacking [81,82]. A recent study indeed demonstrated an association between bleeding symptoms and VWF levels also in a slightly decreased VWF range (0.43–0.64 kIU/l) in young girls not fulfilling the criteria for VWD [83].

In contrast to some other diseases, the consequences of VWD misdiagnosis are not always trivial. Patients may have received costly treatment to no use and even been exposed to the risks associated with factor concentrates derived from human plasma. Patients may have changed their daily routines and abstained from activities for fear of bleeding. Also, patients may have become stigmatised and been denied favourable insurance coverage and they may have been concerned about suffering from and transmitting a genetic disease. Paradoxically, mild VWD may sometimes be under-diagnosed as VWF levels can be temporarily elevated due to stress, infection, and pregnancy, which makes repeated testing advisable.

The genetics of von Willebrand disease

Several causative mutations for types 2 and 3 have been identified [70]. Type 2 mutations are missense mutations clustered in **three** small regions of the gene, according to subtype. Types 2A, B, and M are often caused by mutations in exon 28 that codes for the A1 and A2 domains, the former containing the GPIb-binding site. Type 2A mutations are clustered in the **A2 domain** whereas types 2B and 2M mutations are clustered in the **A1 domain** and type 2N is caused by mutations in the **FVIII-binding domain**. The majority of type 3 mutations are null alleles, and less commonly missense mutations.

Several mutations affecting the VWFpp have been identified [73,84-96]. Some of them cause an aberrant multimerization resulting in the recessive type 2A variant with a typical multimeric pattern, originally described as type IIC [97]. Others seem to preclude synthesis or secretion of VWF protein [95]. Intra- and intermolecular disulfide linkages are critical for the function of VWF and this is reflected by the fact that several genetic variants involve the loss or gain of a cysteine residue. In studies of mutations in the carboxy-terminal cysteine knot domain it was found that mutation of cysteines involved in *intrachain* bonds results, because of intracellular retention, in a mainly recessive, quantitative VWF defect, while mutation of cysteines involved in *interchain* bonds results, because of a dominant negative defect, in a qualitative type 2A, subtype IID, VWF defect [98-100].

In contrast to types 2 and 3, few mutations in the VWF gene have been identified in type 1 VWD patients. Several studies have demonstrated some mutations causing type 1 VWD but also that a large number of patients lack a mutation [101-103]. The identified mutations, the majority of which are missense mutations but also other sorts of mutations that have been found spread-out in all regions of the VWF gene, are listed on the ISTH VWF SSC homepage (<http://www.vwf.group.shef.ac.uk/index.html>). It is still unclear whether the low number of detected mutations is due to difficulties in finding the mutations for various reasons such as the considerable size of the VWF gene, or if other loci are involved and type 1 VWD is not a true monogenic disorder. Various linkage studies have been undertaken and results have shifted from complete to incomplete linkage to the VWF gene [101,104-109]. Failure to detect linkage can in some cases reflect a falsely positive diagnosis. The fact that the penetrance in this disorder is far from complete, and also varies between families, complicates matters further.

Carriers of type 3 VWD are not synonymous with type 1 patients; however, type 3 carriers constitute a heterogeneous group and certain carriers behave as type 1 patients. The genetic bases for the two groups can be regarded as different [110], as type 3 mutations are usually null alleles, and only in a minority of cases are missense mutations, whereas in type 1 VWD the opposite is true. A common finding is that carriers of type 3 VWD have more bleeding problems than the general population, but less bleeding problems than type 1 patients. FVIII and VWF activity levels in plasma are the best indicators of the risk of bleeding. It is unknown whether a difference in clinical phenotype will remain between type 1 patients and type 3 carriers after adjustment for differences in antigen levels [111].

ABO blood group has been shown to affect VWF plasma levels [112]. Mean VWF antigen levels for type O individuals are approximately 25% below and for type AB individuals 25% above the level for a pool of normal donor plasma [113]. Theoretically, the influence of ABO blood group on VWF plasma levels could be due to altered VWF synthesis, secretion, proteolysis, or clearance [72]. The metalloprotease ADAMTS13 is thought to be responsible for the differences in VWF antigen levels [59,114]. Analysis of the primary VWF sequence predicts the presence of various glycosylation sites [115], and biochemical analysis has indeed demonstrated the presence of carbohydrate residues at these sites [116,117]. It is thought that blood group antigen binds to oligosaccharides on the VWF and may thereby prevent degradation of the VWF by ADAMTS13, either by steric hindrance or a charge effect.

However, blood group O individuals lack this protection, which leads to an increase of VWF proteolysis. Thus, blood group O can be regarded as a risk factor for being diagnosed with type 1 VWD and it is well documented that individuals with blood group O are over-represented among type 1 VWD patients; however, this is not the case for types 2 and 3 VWD [103,106-108,113]. VWF is secreted in equimolar amounts with VWFpp, which has a shorter half-life, and the VWFpp can be used as a measure of VWF secretion and allows estimation of the VWF half-life. It was recently found that in contrast to mature VWF:Ag, VWFpp was not influenced by ABO blood group and that ABO blood group influences the clearance rates of VWF rather than VWF secretion rates [118]. It seems that ABO blood group is more influential in families with incomplete penetrance and mild phenotypes than in families with complete penetrance [103,106,108].

It has been stated that the 4751A>G (Y1584C) sequence variation in the VWF gene may affect VWF antigen levels further. The original publication of this non-conservative amino acid substitution described it as a polymorphism with harmless consequences [119]. In contrast, this variant has in other studies been shown to correlate with both intracellular retention and increased susceptibility to proteolysis by ADAMTS13 [120-122]. The effects of increased proteolysis (loss of HMWMs, pronounced subbands) in individuals with Y1584C are not evident in multimer analysis, which can be explained either by very modest alteration of the multimer pattern or by compensation by constitutive synthesis [123]. Either way, the intact multimer pattern suggests the sequence variation to be involved with type 1 VWD, rather than type 2A. The sequence variation has a frequency of 0-2% in the general population [119-121]. The Y1584C variant has been shown to appear in 14-15% of the investigated families of a Canadian type 1 VWD population [103,120] and in eight of 30 (27%) UK families with type 1 VWD [122]. It has been suggested that the Y1584C polymorphism may be associated with bleeding in vitamin K antagonist-treated patients [124]. Until recently it was unknown whether Y1584C itself confers increased proteolysis or is linked to a causative change elsewhere in the VWF gene; however, it has now been clarified that Y1584C itself is responsible for increased susceptibility of VWF to proteolysis by ADAMTS13 [125].

Heritability is the proportion of the phenotypic variance attributable to polygenes, whereas the common household effect is the proportion of the variance attributable to environmental factors shared within a household [14]. Both of these factors must be taken into account in the development of VWD, and especially for type 1 VWD, polygenes are probably influential.

In fact, most of the variation in VWF level is not heritable and much of the heritable variation is not linked to the VWF gene [81,126,127]. In one study, it was estimated that about 40% of the total variation of VWF antigen can be attributed to the VWF gene [108]. In any case, in addition to environmental factors, it has been suggested that additional modifier genes (i.e. other than the ABO locus) may account for part of the varying VWF levels. These putative modifier genes remain to be identified.

In summary, despite all the information gathered from different studies, it is still in many cases unclear what genetic mechanisms are responsible for type 1 VWD. One can only humbly acknowledge the fact that the genetics of type 1 VWD is surprisingly poorly understood and that a major part of it remains to be elucidated. Due to different genetic aetiology, it has been suggested that types 2 and 3 VWD are monogenic diseases, whereas at least some cases of type 1 are rather caused by complex genetics [103,128].

Diagnosing von Willebrand disease

Given the size of the VWF gene, sequencing it in all patients is not feasible. Further, because of incomplete linkage, this choice of method would invariably fail to detect some type 1 patients. Most patients are diagnosed phenotypically using clinical and biochemical analysis. Apart from laboratory results fulfilling certain criteria, the patient should present with significant bleeding symptoms and also have a family history of bleeding. Because of variable and incomplete penetrance, the clinical heterogeneity of VWD, and a disease state that is affected by the inflammatory response, hormonal changes such as during the menstrual cycle and pregnancy, age, ethnicity, and exercise, diagnosing the patients correctly is difficult [129,130]. Genotypical methods, in combination with already existing phenotypical methods, would improve the diagnosis of VWD type 1.

Before the 1970s, FVIII and VWF were not recognized as two separate proteins and were therefore tested as one entity, the anti-haemophilia factor (AHF) or anti-haemophilia globulin (AHG). VWD could be diagnosed only on the basis of low AHF plasma levels and prolonged bleeding time, as the bleeding time is normal in haemophilia. In the early 1970s, a method to test VWF separately from FVIII was developed [35]. Subsequently, more specific assays analysing various properties of the VWF have been developed. Today, laboratory diagnosis of VWD includes multimeric analysis and levels of VWF antigen, FVIII, and VWF:RCo (which

is a functional test that, by using the patient's plasma and normal platelets, analyses binding of plasma VWF to GPIb- α on platelets mediated by the antibiotic ristocetin). Multimeric analysis is necessary in order to be able to distinguish between different subtypes of VWD, which has therapeutic implications. One study has demonstrated that only VWF levels below 0.40 kIU/l significantly increase the likelihood of type 1 VWD and that this can be used as a clinical cut-off value [131]. It was further shown that the probability of being a type 1 VWD patient is 3.9-fold higher in subjects with VWF:Ag levels below 0.20 kIU/l than in subjects with VWF:Ag levels of 0.20–0.40 kIU/l. Also, for patients with VWF:Ag levels below 0.20 kIU/l, at least 95% have a detectable VWF gene mutation and, using sensitive methods, about 85% of these can be shown to have some abnormality in plasma VWF multimer profile [132]. An association has been shown between severity of bleeding symptoms as assessed by a standardized bleeding score and VWF levels in type 1 VWD, which can be useful for a more accurate diagnosis [133]. VWF:RCo has been shown to be the most useful test for VWD diagnosis [134]. Recently, it has been demonstrated, by using a Bayes theorem approach, that the best predictor when diagnosing VWD is inheritance of the phenotype, followed by reduced VWF and bleeding symptoms in the proband [135].

Treatment of von Willebrand disease

An adequate treatment of VWD can be prescribed to avoid hemorrhagic complications, both spontaneous bleeding events and at the time of surgical procedures [136]. VWD has been treated successfully since the end of the 1950s [3]. For patients who are mildly affected, on-demand therapy is sufficient whereas patients with more severe bleeding problems require prophylaxis [137,138]. The majority of VWD patients, about 70-80%, can be treated with the synthetic drug desmopressin (1-desamino-8-D-arginine-vasopressin; DDAVP), which mimics the biological substance vasopressin that induces endothelial cells to release endogenously produced VWF. It has recently been shown that the response to DDAVP seems to be related to the location of the causative mutation in the VWF gene [139], which makes mutational data of value, not just for diagnosing VWD, but also for prediction of the response to DDAVP. For VWD patients with a qualitative defect (type 2) or with virtually no VWF antigen (type 3), FVIII/VWF concentrates are required. In contrast to FVIII, VWF used in factor concentrates is, due to its complicated synthesis and complex post-translational modifications, not as easily synthesised recombinantly (rVWF); yet, on-going experiments have given good hope that rVWF as replacement therapy may be a realistic option when treating VWD in the near future. To date, VWF used in factor concentrates is isolated from human plasma. This

always includes an increased risk of transmitting viral infections such hepatitis and HIV infection, which has tragically occurred in some cases. However, the protection procedures used in the production of modern factor concentrates derived from human plasma, such as virus-inactivation, have increased the safety tremendously, which renders the product almost virtually safe from pathogens.

Present investigations

Aims

Paper I To characterize the genetic background to type 1 VWD better by conducting a linkage study in a Swedish type 1 VWD population comprising 31 families.

Paper II To estimate to what extent patients and their relatives are aware of the genetics involved in type 1 VWD.

Paper III To investigate the effect of the C570S mutation in a type 2A VWD patient by conducting *in vitro* experiments.

Paper IV To investigate the effect of the N1421K mutation in a type 2M VWD family by conducting *in vitro* experiments.

Findings

Paper I The recognized fact that only a low number of mutations have been detected in type 1 VWD has given rise to the hypothesis that type 1 VWD is not a true monogenic disorder, but rather, at least in some cases, is inherited with complex genetics. Various linkage studies have been undertaken and results have shifted from complete to incomplete linkage to the VWF gene [101,104-109]. Therefore, we set out to perform a linkage analysis in a Swedish type 1 VWD population. A total of 325 individuals from 31 families were genotyped using two microsatellite markers in the VWF gene. Of these, 127 were type 1 VWD individuals. In 27 of the 31 families, marker haplotypes co-segregated with type 1 VWD, whereas in the remaining four families there was clearly no co-segregation. In the families with co-segregation, we found 30-40 unaffected individuals who carried the disease-associated haplotype, reflecting the reduced penetrance and illustrating the diagnostic problem in type 1 VWD. When LOD-scores were summed over all families, the resulting LOD-scores were 10.53 and 8.63 for the intragenic markers, clearly establishing linkage between the VWF locus and the VWD phenotype in the families treated as a group. When LOD-scores were summed over the 27 co-segregating families, the resulting LOD-scores were even higher, 15.79 and 12.84, respectively. The families that did not show a co-segregating disease

haplotype all had negative LOD-scores (-0.87 to -2.05), confirming that these families did not show co-segregation. Six of the families showing co-segregation shared the same disease haplotype. When the frequency of this disease-haplotype in the families with co-segregation (0.22) was compared with the frequency of the haplotype among normal unrelated individuals (0.06), it was found to be much higher in the former group; a chi-squared value of 7.14 ($P=0.0075$) was obtained, suggesting a founder effect. Three other disease haplotypes occurred in two families each, indicating the possibility that more than one common disease haplotype exists for type 1 VWD in Sweden. The Y1584C sequence variation was identified in one of the 31 investigated families. This variant has been shown to be present at much higher frequencies among families with type 1 VWD in Canada and UK [120,122]. In our study, three of the four individuals with the Y1584C variant (285 individuals were tested) were not diagnosed with type 1 VWD; hence, this variant does not have complete penetrance. Information collected on the number of O alleles in the ABO blood group system (290 individuals were tested) clearly shows that blood group O is over-represented among type 1 VWD patients; a chi-squared value of 10.96 and a P-value of 0.0009 reveal the statistical significance of the difference between the patient and the non-patient groups. We could also see that the phenotype (presence or absence of blood group O) matters rather than the number of O alleles, i.e. there does not seem to be a gene dosage effect.

The results from the linkage study are in accordance with those from many other studies, as, investigating type 1 VWD, it has repeatedly been found that there is incomplete linkage to the VWF gene [101,105,106,108,109]. Our finding that co-segregation was identified in a high proportion, in comparison with the other studies, of the investigated families can probably be attributed to the fact that, by using our inclusion criteria, we have selected for large families with many affected individuals and this may have introduced bias towards positive linkage. The finding that blood group O is over-represented among type 1 VWD patients has been found repeatedly in other populations [101-103]. However, contrary to previous results [68], we did not find blood group O to be more over-represented in the patients in the non-co-segregating families than in the patients in the co-segregating families; this can probably be explained by the very low number of non-co-segregating families in our study. This was the first investigation on the prevalence of the Y1584C variant in a Swedish type 1 VWD population and when compared with other studies it seems clear that the variant is more common in the Canadian and the UK type 1 VWD populations [120,122].

Paper II The genetics of type 1 VWD is less straightforwardly explained and not as easily comprehensible as the genetics of types 2 and 3. It is likely that the complicated genetics confuses type 1 patients and inhibits them from getting a proper understanding of what genetically causes their disorder. We therefore turned to the participants in the linkage study (paper I) to investigate their level of knowledge of the genetics involved in type 1 VWD. Further, we sought to investigate whether there was any difference in the level of knowledge between subgroups of the participating individuals, and finally, we were interested in estimating the participants' attitudes towards genetics and VWD. Estimation of the participants' level of knowledge was achieved through structured telephone interviews using a questionnaire in combination with both a grading system and multiple-choice questions. All participants in the previous study, except individuals who were deceased, had emigrated or were too young, were invited to participate in the current study. Out of 327 individuals in the previous study, 226 took part in the follow-up. We found that patients, younger individuals, and women tended to have a higher level of knowledge than did healthy relatives, older individuals, and men, respectively. These findings can be explained by the fact that patients are closer to the disease state than are healthy relatives, the facts that younger individuals are provided with more information from the health care system as more is known about the disease these days and that they in general are more used to searching for information on their own, e.g. from the Internet, and the notion that women tend to be more interested in health matters than are men. A majority of the participants would like to learn more about the genetics of VWD, and a vast majority held a positive attitude towards the interview.

We have not found any previous studies aiming at investigating the level of knowledge among patients and their relatives concerning the genetics involved in VWD, although a similar but slightly different approach has been used in a study for patients with haemophilia [140]. On one hand, this makes our study interesting in its novelty, but on the other we cannot easily compare our results with those from analogous studies. The findings from our study can hopefully be used to inform patients and their family members in a more constructive way to enhance their level of knowledge of VWD.

Paper III The biosynthesis of VWF is very complex and includes various intracellular modifications, one of which is multimerization. Mature VWF is a heterogeneous collection of a series of multimers where the high molecular weight VWF multimers are most crucial in platelet adhesion and aggregation. The VWF propeptide (VWFpp) plays a crucial role in

multimerization [42,45,46,48]. Some mutations affecting the VWFpp cause an aberrant multimerization resulting in the recessive type 2A variant with a typical multimeric pattern [97], and others seem to preclude synthesis or secretion of VWF protein [95]. We performed genetic studies in a patient with a rather unique phenotype of VWD characterized by very low plasma FVIII and VWF levels and a VWF consisting of only a dimeric band and total absence of all multimers in plasma. All 52 exons of the VWF gene were PCR-amplified from genomic DNA and sequenced. The patient was found to be homozygous for the novel C570S mutation, caused by a 1709G>C transition in exon 14 of the VWF gene coding for the VWFpp. His asymptomatic parents and brother were all found to be heterozygous for the same mutation. To assess the effect of the mutation, site-directed mutagenesis was performed on an expression vector which was subsequently used to transfect COS-7 cells transiently. The concentration of rVWF was greater in wt medium compared with mutant medium. Further, the multimeric pattern of the mutant rVWF secreted to medium mimicked the one of the proband with solely a dimeric band and the complete loss of all multimers. Thus, the *in vitro* experiments confirmed the detrimental effect of the C570S mutation on VWF multimerization. The VWFpp is very rich in cysteine residues, which have the ability to form disulfide bonds and substitution of cysteines can therefore be expected to result in major conformational changes. Homology studies aligning the human VWFpp with the VWFpp of several other species revealed a remarkably high degree of VWFpp conservation. The C570 residue is perfectly conserved among all investigated species, underlining its critical role in the VWFpp function in VWF multimerization.

Interestingly, the proband's parents both carried the C570S mutation, a mutation which has not been reported in the literature previously, despite the fact that there is no known consanguinity in the family. All polymorphic variants in the proband, as identified by PCR-amplification of all 52 exons in addition to the intron/exon boundaries and the proximal promoter, were investigated also in the family members (Fig. 7). These data support the possibility that the two disease-associated haplotypes in the proband are identical by descent (IBD).

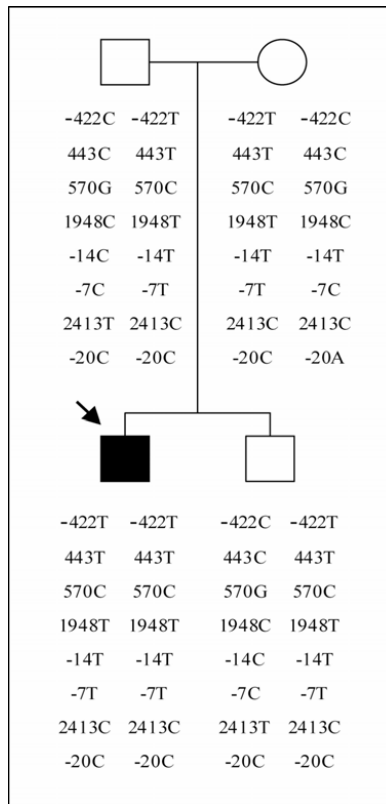


Figure 7. Polymorphic information from the PCR-amplification displaying the haplotypes in the family members.

In many cases, the loss of HMW VWF multimers in type 2A seems to be the result of increased cleavage of VWF in plasma, either because the mutation leads to an unspecific conformational change in the A2 domain which increases the exposure of the VWF cleavage site to ADAMTS13, or because the mutation enhances specific binding of ADAMTS13 to VWF [123]. Previous studies have described mutations affecting the VWFpp, some of which lead to a phenotype with aberrant multimeric structure [92,94,141]. However, in contrast to what we found in our patient, either some multimers besides a prominent protomer could be detected [92], or the patients described were heterozygotes, and the *in vivo* homozygous phenotype is unknown [94,141]. To the best of our knowledge, our case is the first VWD patient described with a homozygous mutation in the VWFpp who is phenotypically characterized by the presence of solely a dimeric band and total absence of all multimers in plasma.

Paper IV The VWF promotes platelet aggregation and platelet adhesion to damaged vessels. VWF does not bind spontaneously to platelets in blood; however, the binding of the A3 domain of the VWF to collagen in the subendothelium, particularly under high shear stress, activates VWF [142]. In its activated form, the A1 domain of the VWF has the ability to bind to the platelet receptor glycoprotein GPIb, which triggers platelet adhesion and aggregation. *In vitro*, binding of VWF to GPIb can be mimicked by the antibiotic ristocetin or by the viper venom protein botrocetin [60]. Mutations that cause type 2M VWD, a subtype characterized by decreased affinity of the VWF to GPIb with a normal or nearly normal multimeric pattern, are found in exon 28 of the VWF gene coding for the A1 domain [143-146]. We performed genetic studies in three patients (a mother - the proband - and two of her children) with VWD characterized by moderately decreased plasma FVIII and VWF levels, disproportionately low plasma VWF:RCO levels, and an apparently normal multimeric pattern. All 52 exons of the VWF gene were PCR-amplified from genomic DNA and sequenced in the proband. She was found to be heterozygous for the novel N1421K mutation, caused by a 4263C>G transition in exon 28 of the VWF gene coding for the A1 domain. Her two affected children were found to carry the same mutation in a heterozygous state, whereas the transition was not present in her two unaffected children and husband. Botrocetin- and ristocetin-mediated binding of plasma VWF to GPIb were reduced in the patients. To construct a plasmid in order to assess the effect of the mutation, an expression vector and subcloning in another vector were employed for subsequent transient transfection of COS-7 cells. The concentration of rVWF was greater in wt medium compared with mutant medium, with the level of rVWF from cells co-transfected with wt and mutant vector intermediate to the others. Multimer analysis revealed the full range of multimers in all three variants of expressed rVWF. Platelet binding assays using botrocetin and ristocetin were performed with the rVWF, and the results mimicked the ones in the patients. VWF collagen binding capacity was unaffected in plasma from the heterozygous individuals as well as in medium from transfected COS-7 cells, which is noteworthy as the location of the N1421K mutation in the VWF gene theoretically could affect VWF interactions with collagen. Summing up, the *in vitro* experiments confirmed the detrimental effect of the N1421K mutation on plasma VWF binding to GPIb. Further, homology studies aligning the human VWF A1 domain with the VWF A1 domain of several other species revealed a high degree of conservation, and the N1421 residue itself is semi-conserved among the species with polar hydrophilic amino acids alternating between asparagines and serine. As both of these amino acids are uncharged, it is

likely that there will be no difference in the conformation of the A1 domain in these two variants of the VWF. In contrast, if asparagine is switched to lysine, as is the case in our three patients, an uncharged amino acid is replaced by one that is positively charged, which is more likely to affect the conformation and function of the VWF A1 domain.

Among other described type 2M VWD mutations that have been studied, some showed a defective ristocetin but normal botrocetin mediated binding [143,146], but others resembled the findings in our patients [144,147] with both ristocetin and botrocetin mediated binding being defective. The defective botrocetin mediated binding in our study can possibly be explained by the fact that N1421K is located in the loop at the beginning of the $\alpha 5$ helix in the A1 domain and a substitution with a charged lysine residue is likely to disrupt the botrocetin binding site.

Conclusions

Paper I Type 1 VWD is linked to the VWF gene in a majority (27 of 31) of Swedish type 1 VWD families. Several common disease haplotypes probably exist for type 1 VWD in Sweden. The Y1584C variant is not as common in the Swedish type 1 VWD population as it is in some other populations. Blood group O is over-represented among type 1 patients in Sweden.

Paper II Apart from certain misunderstandings, type 1 VWD patients and their healthy relatives have a satisfying level of knowledge about the genetics of the disease. In general, patients, younger individuals, and women have a higher knowledge about the genetics causing type 1 VWD than do healthy relatives, older individuals, and men, respectively.

Paper III Inherited recessively, the C570S mutation causes a distinct subtype of type 2A VWD characterized by very low plasma FVIII and VWF levels and the exclusive presence of the dimeric form of VWF in plasma. The findings define a structural element that is indispensable for VWF multimerization.

Paper IV Inherited dominantly, the N1421K mutation causes type 2M VWD characterized by moderately decreased plasma FVIII and VWF levels, disproportionately low plasma

VWF:RCO levels, and an apparently normal multimeric pattern. The findings indicate a structural element in the A1 domain that is necessary for proper GPIb binding.

Concluding remarks

The heterogeneous genetic background to VWD is manifested as a complex pathophysiologic disturbance of VWF function and biochemical phenotype, but with fairly similar clinical phenotypes. Also heredity is dependent on the type of mutation as some mutations give a dominant, whereas others give a recessive, heredity. The knowledge among patients and relatives is, despite this complicated background, reasonably satisfying. Genetic studies help us understand the complex phenotypical disturbances and the differences in heredity and can also facilitate diagnostics and family investigations.

Future perspectives

Despite all attempts to characterize VWD genetically, the disease is still inadequately understood and many aspects remain to be elucidated. In the years to come, continued joint effort will hopefully shed further light on the complicated genetic mechanisms that underlie the development of, especially type 1, VWD.

As additional mutations get identified in the various regions of the VWF gene, this can help to link the functions of these regions more thoroughly to clinical features.

Having a fuller picture of what genetically causes VWD, genetic markers may possibly be used routinely to help diagnosing VWD, which could open up for extended clinical opportunities.

As more is revealed about the genetics of VWD, and as gene sequencing becomes easier and less expensive to perform, the detection of VWF mutations can be used as a powerful supplement for correct classification of VWD.

VWF used in factor concentrates to treat VWD is due to its complicated synthesis and complex post-translational modifications not easily synthesised recombinantly (rVWF), but is rather isolated from human plasma, which unavoidably includes a risk of transmitting viral infections. Since more advanced systems for synthesizing recombinant proteins have recently been created, in combination with an increased knowledge about the complicated synthesis of VWF, in the near future, rVWF as replacement therapy seem to be a realistic option when treating VWD.

Oligosaccharides binding to glycosylation sites on VWF seem to contribute to the *in vivo* survival of VWF. Considering that the half-life of FVIII is strongly influenced by VWF, the latter can be regarded as a target to modulate the former indirectly. Therefore, as the molecular basis of VWF proteolysis clearance gets clarified this knowledge can be used to design rVWF with optimal glycosylation, opening up for new prospects of improving the treatment of both VWD and haemophilia A.

Populärvetenskaplig sammanfattning på svenska

von Willebrands sjukdom (VWS) är den vanligaste ärftliga blödningsjukdomen. Symptomen är ökad benägenhet för slemhinneblödningar såsom blåmärken, näsblod, rikliga menstruationer och blödning i samband med tandutdragningar och andra operationer.

Hemostasen, eller blodstillningen, är kroppens system för att blodet ska cirkulera optimalt och varken bli för tjockt och klumpa ihop sig så att blodpropp kan bildas eller så att blodet blir för tunt vilket kan leda till blödning. För att reglera hemostasen behövs en rad proteiner, eller faktorer, som antingen är så kallat prohemostatiska (leder till att blodet lever sig mer) eller antihemostatiska (leder till att blodet lever sig mindre). Dessa proteiner fungerar i ett komplicerat system där de påverkar varandra genom återkopplingsmekanismer, allt för att kunna reglera hemostasen och anpassa den efter rådande omständigheter på bästa sätt. Ifall koncentrationen av något av de proteiner som ingår i hemostasen förändras, eller om något protein inte fungerar som det ska, påverkas blodstillningen antingen genom ökad risk för blodpropp eller ökad blödningsbenägenhet, detta beroende på om det är prohemostatiska eller antihemostatiska protein som förändras. Ett exempel på detta är vid VWS då proteinet von Willebrandfaktorn (VWF) förändras kvantitativt och/eller kvalitativt. En kvantitativ förändring innebär att det finns för låg halt av faktorn i plasma och en kvalitativ förändring innebär att faktorn inte fungerar som den ska; båda fallen resulterar i ökad blödningsbenägenhet.

VWF har två uppgifter, dels att hjälpa till då blodplättarna klumpar ihop sig och även fäster till kärlväggen för att tätta läckage från en skada i densamma, dels att binda till och förlänga livslängden hos koagulationsfaktor VIII. På 1980-talet identifierades von Willebrand faktorgen (ett arvsanlag) som kodar för VWF. Genen är belägen på kromosom 12 som är en av de autosomala kromosomerna, alltså inte någon av könskromosomerna, vilket betyder att både män och kvinnor har två upplagor av von Willebrand faktorgen i sina celler. Det innebär att von Willebrands sjukdom är ungefär lika vanlig hos de två könen, till skillnad från exempelvis den klassiska blödarsjukan Hemofili A och B som i princip uteslutande drabbar pojkar. Faktum är att VWS snarast är något vanligare hos kvinnor, eftersom dessa av naturliga skäl blöder mer och därför i vissa fall kan uppfattas ha något svårare symptom och/eller få diagnosen något oftare.

VWS kan delas in i olika kategorier: typ 1 innebär partiell och typ 3 innebär nära nog fullständig brist på VWF. Typ 2 innebär en kvalitativ defekt och indelas vidare i subtyper (2A, 2B, 2M och 2N) beroende på vilken sorts defekt det rör sig om. Typ 2 mutationer (mutation är en förändring i en gen, vilket kan leda till sjukdom) hittas ofta i specifika delar av genen, beroende på vilken subtyp det rör sig om. Såväl typ 2 som typ 3 VWS har i hög utsträckning kunnat förklaras med identifierade mutationer i VWF-genen. Vid typ 1, som är den vanligaste typen av VWS, är bilden en annan eftersom identifierade mutationer endast kunnat förklara en betydligt mindre andel sjuksfallsfall. Det finns flera skäl till att VWF-genen är svårundersökt. För det första är genen ovanligt stor vilket gör det både tidsödande och kostsamt att leta igenom den i jakt på mutationer. Vid typ 1 VWS är dessutom de mutationer som hittats spridda över hela genen vilket gör att det inte räcker med att undersöka en viss del av genen, utan hela VWF-genen måste i så fall analyseras i jakt på mutationer. Vidare finns det en så kallad pseudogen, vilket betyder att det existerar en annan gen som i sin uppbyggnad påminner mycket om VWF-genen, men som inte leder till någon genprodukt (protein), och detta gör att när man undersöker VWF-genen måste man vara övertygad om att det är den riktiga genen och inte pseudogenen som undersöks. Dessutom har det föreslagits att det kan finnas ytterligare gener som kan innehålla mutationer och på så sätt vara inblandade i VWS, och att det i så fall skulle vara en anledning till att mutationer sällan hittas i VWF-genen vid VWS typ 1. En stor svårighet vid diagnostiseringen av VWS typ 1 är att nivåerna av VWF i plasma varierar stort i totalbefolkningen. Det innebär att patienter med mild form av sjukdomen har VWF-värden som överlappar med den friska befolkningens nivåer, och det saknas en entydig gräns som man kan använda sig av för att skilja normala värden från sjukligt låga. Vidare är det fullt möjligt att bära på en mutation i VWF-genen men ändå inte utveckla sjukdomen.

Sammanfattning av studierna som ingår i avhandlingen

I den första studien undersökte vi 31 svenska von Willebrand-familjer, bestående av totalt 325 individer, varav 127 hade VWS. Vi gjorde en så kallad kopplingsanalys vilket betyder att vi ville se om VWF-genen var kopplad till sjukdomen, dvs om denna gen var inblandad i sjukdomen i samtliga fall (se ovan). Vi fann att i 27 av familjerna fanns koppling, alltså markörer som visar att förändringar i eller nära anslutning till VWF-genen orsakar sjukdomen. I fyra av familjerna fanns inte denna koppling. Det kan antingen innebära att andra gener är inblandade, eller att det fortfarande är VWF-genen som är inblandad men då

inte enligt den klassiska Mendelska nedärvningen (att en mutation i en gen leder till en sjukdom) utan att multipla förändringar i VWF-genen samverkar och gör att man inte blir sjuk av endast en förändring, men däremot när man har flera samtidigt. En sådan situation kan uppstå om man ärver riskfaktorer från båda föräldrarna, och om så är fallet syns ingen koppling i den sortens analys som den vi utförde. Vi såg även att blodgrupp O var överrepresenterad hos personer med VWS jämfört med friska personer i studien. Detta är något man sedan länge observerat i andra länder, det var dock intressant och värdefullt att bekräfta att det var på samma sätt även i Sverige, eftersom det är fullt tänkbart att det kan se olika ut i olika populationer.

I den andra studien vände vi oss åter till deltagarna i arbete nummer ett. Dessa inbjöds till en telefonintervju där deltagarnas kunskaper om genetiken bakom VWS testades, dels för att vi skulle få en allmän uppfattning om deras kunskapsläge och dels för att vi ville se om det finns skillnader i kunskapsnivå mellan olika undergrupper i patientmaterialet. Vidare var vi intresserade av att ta reda på deltagarnas inställning till sin kunskap kring genetiken bakom VWS. Vi fann att kunskapsnivån generellt var relativt god även om vissa missförstånd uppdagades. Kunskapsnivån varierade stort mellan deltagarna, generellt har dock patienter, yngre människor och kvinnor högre kunskap än friska anhöriga, äldre människor respektive män. Deltagarna hade genomgående god självkänneträffande sin egen kunskapsnivå och de flesta deltagarna tyckte att intervjun ökade deras kunskap och skulle vilja lära sig mer om genetiken bakom VWS. Resultaten kan användas för att informera patienter och deras familjemedlemmar på ett mer upplysande sätt och på så sätt öka deras kunskap om sjukdomen.

I den tredje studien undersökte vi en familj bestående av tre friska familjemedlemmar och en pojke med VWS. Pojken uppvisade låga plasmanivåer av VWF och man kunde även se att de större strukturer (multimerer) med multipler av VWF som normalt kan identifieras elektroforetiskt saknades helt. Denna avsaknad av multimerer leder till ökad blödningsbenägenhet och är mycket ovanlig. Vi fann att pojken bar på en mutation i den del VWF-genen som är inblandad i multimerisering (bildning av multimerer) av VWF, och han bar på denna mutation i två upplagor alltså i båda sina VWF-gener. Den genetiska analysen visade att både mamman, pappan och pojkens lillebror bar på samma mutation, dock i enkel upplaga. Samtliga fynd bekräftades med studier gjorda i cell-system (så kallade *in vitro* studier). Således tycks den funna mutationen förhindra den normala intracellulära

multimeriseringen av VWF. Det är värt att notera att detta är den första patienten som beskrivits vilken till följd av en förändring i den delen av VWF-genen som är viktig för multimerisering, i båda sina upplagor av genen, har fullständig avsaknad av samtliga multimerer (förutom två VWF-molekyler sammansatta i en så kallad dimer, vilket även vår patient uppvisade).

I den fjärde studien undersökte vi en familj bestående av tre friska individer och tre individer med VWS vilket yttrade sig som blödningsbesvär och sänkta värden av VWF men framförallt en kvalitativ defekt av densamma (försämrad bindning till blodplättar). De tre sjuka familjemedlemmarna, en mor och hennes två yngsta barn, visade sig bära på en mutation i en del av VWF-genen som är viktig för inbindningen till blodplättarna. De tre friska familjemedlemmarna, fadern och de två äldsta barnen, bar inte på mutationen. Samtliga fynd bekräftades med studier gjorda i cell-system. Resultaten tyder därför på att den funna mutationen försämrar bindningen av VWF till blodplättarna.

Sammanfattningsvis har detta avhandlingsarbete omfattat studier om den heterogena genetiska bakgrunden till VWS som ger sig uttryck i en sammansatt sjuklig rubbning av VWF, såväl funktionellt som biokemiskt, men som ger en likartad klinisk bild. Även ärftligheten är beroende av typ av mutation eftersom vissa ger en dominant ärftlighet (det räcker att man ärver en mutation från den ena föräldern för att bli sjuk) och andra en recessiv ärftlighet (man behöver ärva mutationen från båda föräldrarna och alltså ha den i två upplagor för att bli sjuk). Trots denna svårbegripliga bakgrund är kunskapen bland patienter och anhöriga relativt god. Genetiska undersökningar hjälper oss att förstå den komplexa sjukliga rubbningen och skillnaderna i ärftlighet, och kan även vara till hjälp i diagnostik och familjeutredningar. Korrekt diagnostik är av värde för både den enskilda individen och samhället, dels genom att adekvat behandling kan ges inför exempelvis operationer för att slippa onödiga blödnings, dels för att förbättrad diagnostik även gör att man kan undvika att felaktigt diagnostisera en frisk person.

Acknowledgements

Many people have contributed to this thesis in various ways and I want to thank all of them. The following persons, however, deserve special attention:

Stefan for being a committed supervisor. You have guided me into the world of research by sharing your knowledge and expertise, and patiently explained things to me.

It has truly been a pleasure and a privilege to be part of the **Clinical Coagulation Research Unit**, with its high scientific standards and such a warm and friendly atmosphere. Some of the jokes and events in the coffee room have been truly hilarious. Past and present co-workers of the **Centre for Thrombosis and Haemostasis** - as well as past and present room mates - such as **Andreas** (thanks for scientific advice), **Anne-Mari, Birthe, Björn, Camilla M** (thanks for continuous help with various practical matters), **Camilla N, Caroline, Cimar, Erik, Eva L, Eva M, Fie, Göran, Inger, Ingemar** (thanks for help with my computer), **Jenny, Karin L, Karin S, Lena, Lilian** (thanks for telling me about unusually interesting patients), **Maggan, Margareta, Mohammed, Peter, Stina, Ulla, and Valentina**.

DNA-lab with **Agneta HS, Agneta Ö, Anna J, Christer, Liselotte, and Maria**, thanks especially for taking good care of me as a new PhD student and for all the jokes we have shared. I still like the idea of *A Gene Atlas* and I am just waiting for the next happening to occur. **Elsy**, I appreciate all the lunches we have had at various places throughout the years, and all the interesting topics of conversation we have covered on these occasions. **Per S**, if Sven and I had not knocked on your door many years ago, and if you had not kindly advised me, this thesis would simply not exist.

Lars, Diana, Ann-Charlotte, all the **PhD students**, and everybody else at the Department of **Pediatrics** in Lund where a lot of lab-work was carried out. I am grateful for support by your group when I conducted some tricky experiments.

Rigshospitalet in Copenhagen with **Malou, Lise, and Sixtus** for excellent collaboration on various matters.

All **patients** who generously have been willing to participate in medical research and thereby contributed to this thesis.

My father **Jan** and my sister-in-law **Helena** for advice on how to write English properly.

My **parents**, my **brothers** with families, and my **friends** for taking my mind off work-related matters.

Finally, thanks to “my boys” **Sven, Ludvig, and Valter** for teaching me the true values in life. You are my sunshine.

This thesis was supported by grants from Baxter, CSL Behring, and Lund University.

References

1. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature and symbolism for amino acids and peptides. Recommendations 1983. *Eur J Biochem* 1984; 138: 9-37.
2. Lethagen SR. Pathogenesis, clinical picture and treatment of von Willebrand's disease. *Ann Med* 1995; 27: 641-51.
3. Lethagen S. Mannen bakom syndromet: Erik A von Willebrand fann tidigare okänd blödningsrubbnig. *Läkartidningen* 1994; 91: 3805-9.
4. von Willebrand EA. Hereditär pseudohämofili. *Finska Läkaresällsk. Handl* 1926; 67: 87-112.
5. von Willebrand EA. Über hereditäre Pseudohämophilie. *Acta Med Scand* 1931; 76: 521-50.
6. Blombäck M, Blombäck B. Purification of human and bovine fibrinogen. *Ark. Kemi* 1956; 10: 415-43.
7. Biggs R, Matthews JM. The treatment of haemorrhage in von Willebrand's disease and the blood level of factor VIII (AHG). *Brit J Haematol* 1963; 9: 203-14.
8. Cornu P, Larrieu MJ, Caen JP, Bernard J. Transfusion studies in von Willebrand's disease: effect on bleeding time and factor VIII. *Brit J Haematol* 1963; 9: 189-202.
9. Nilsson IM, Blombäck B, Blombäck M, Svennerud S. Kvinnlig hämofili och dess behandling med humant antihämofiliglobulin. *Nord Med* 1956; 56: 1654-62.
10. Nilsson IM, Blombäck M, Jorpes E, Blombäck B, Johansson SA. v Willebrand's disease and its correction with human plasma fraction I-0. *Acta Med Scand* 1957; 159: 179-88.
11. Arnout J, Hoylaerts MF, Lijnen HR. Haemostasis. *Handb Exp Pharmacol* 2006; 176: 1-41.
12. Hoffman M, Monroe III DM. A cell-based model of haemostasis. *Thromb Haemost* 2001; 85: 958-65.
13. Dahlbäck B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. *J Intern Med* 2005; 257: 211-25.
14. Vossen CY, Hasstedt SJ, Rosendaal FR, Callas PW, Bauer KA, Broze GJ, Hoogendoorn H, Long GL, Scott BT, Bovill EG. Heritability of plasma concentrations of clotting factors and measures of a prothrombotic state in a protein C-deficient family. *J Thromb Haemost* 2004; 2: 242-7.
15. Vossen CY, Callas PW, Hasstedt SJ, Long GL, Rosendaal FR. A genetic basis for the interrelation of coagulation factors. *J Thromb Haemost* 2007; 5: 1930-5.
16. van Hylckama Vlieg A, Callas PW, Cushman M, Bertina RM, Rosendaal FR. Interrelation of coagulation factors and D-dimer levels in healthy individuals. *J Thromb Haemost* 2003; 1: 516-22.
17. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GA, Latt SA, Orkin SH. Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science* 1985; 228: 1401-6.
18. Lynch DC, Zimmerman TS, Collins CJ, Brown M, Morin MJ, Ling EH, Livingston DM. Molecular cloning of cDNA for human von Willebrand factor: authentication by a new method. *Cell* 1985; 41: 49-56.
19. Sadler JE, Shelton-Inloes BB, Sorace JM, Harlan JM, Titani K, Davie EW. Cloning and characterization of two cDNAs coding for human von Willebrand factor. *Proc Nat Acad Sci U.S.A.* 1985; 82: 6394-8.
20. Verweij CL, de Vries CJM, Distel B, van Zonneveld A-J, Geurts van Kessel A, van Mourik JA, Pannekoek H. Construction of cDNA coding for human von Willebrand

- factor using antibody probes for colony-screening and mapping of the chromosomal gene. *Nucleic Acids Res* 1985; 13: 4699-717.
21. Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton-Inloes BB, Sorace JM, Alevy YG, Sadler JE. Structure of the gene for human von Willebrand factor. *J Biol Chem* 1989; 264: 19514-27.
 22. Mancuso DJ, Tuley EA, Westfield LA, Lester-Mancuso TL, Le Beau MM, Sorace JM, Sadler JE. Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochem* 1991; 30: 253-69.
 23. Ferreira V, Assouline Z, Schwachtgen JL, Bahnak BR, Meyer D, Kerbiriou-Nabias D. The role of the 5'-flanking region in the cell-specific transcription of the human von Willebrand factor gene. *Biochem J* 1993; 293: 641-8.
 24. Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol* 1994; 14: 999-1008.
 25. Jahroudi N, Ardekani AM, Greenberger JS. An NF1-like protein functions as a repressor of the von Willebrand factor promoter. *J Biol Chem* 1996; 271: 21413-21.
 26. Ardekani AM, Greenberger JS, Jahroudi N. Two repressor elements inhibit expression of the von Willebrand factor gene promoter in vitro. *Thromb Haemost* 1998; 80: 488-94.
 27. Wang X, Peng Y, Ma Y, Jahroudi N. Histone H1-like protein participates in endothelial cell-specific activation of the von Willebrand factor promoter. *Blood* 2004; 104: 1725-32.
 28. Hough C, Cuthbert CD, Notley C, Brown C, Hegadorn C, Berber E, Lillicrap D. Cell type-specific regulation of von Willebrand factor expression by the E4BP4 transcriptional repressor. *Blood* 2005; 105: 1531-9.
 29. Peng Y, Jahroudi N. The NFY transcription factor functions as a repressor and activator of the von Willebrand factor promoter. *Blood* 2002; 99: 2408-17.
 30. Keightley AM, Lam YM, Brady JN, Cameron CL, Lillicrap D. Variation at the von Willebrand factor (vWF) gene locus is associated with plasma vWF:Ag levels: identification of three novel single nucleotide polymorphisms in the vWF gene promoter. *Blood* 1999; 93: 4277-83.
 31. Harvey PJ, Keightley AM, Lam YM, Cameron C, Lillicrap D. A single nucleotide polymorphism at nucleotide -1793 in the von Willebrand factor (VWF) regulatory region is associated with plasma VWF:Ag levels. *Br J Haemat* 2000; 109: 349-53.
 32. Kunicki TJ, Federici AB, Salomon DR, Koziol JA, Head SR, Mondala TS, Chismar JD, Baronciani L, Canciani MT, Peake IR. An association of candidate gene haplotypes and bleeding severity in von Willebrand disease (VWD) type 1 pedigrees. *Blood* 2004; 104: 2359-67.
 33. Kunicki TJ, Baronciani L, Canciani MT, Gianniello F, Head SR, Mondala TS, Salomon DR, Federici AB. An association of candidate gene haplotypes and bleeding severity in von Willebrand disease type 2A, 2B, and 2M pedigrees. *J Thromb Haemost* 2006; 4: 137-47.
 34. Hough C, Cameron CL, Notley CRP, Brown C, O'Brien L, Keightley AM, Berber E, Lillicrap D. Influence of a GT repeat element on shear stress responsiveness of the VWF gene promoter. *J Thromb Haemost* 2008; 6: 1183-90.
 35. Zimmerman TS, Ratnoff OD, Powell AE. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease, with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. *J Clin Invest* 1971; 50: 244-54.

36. Ruggeri ZM. Structure and function of von Willebrand factor. *Thromb Haemost* 1999; 82: 576-84.
37. Fischer BE, Thomas KB, Schlokot U, Dorner F. Triplet structure of human von Willebrand factor. *Biochem J* 1998; 331: 483-8.
38. Mazurier C, Ribba AS, Gaucher C, Meyer D. Molecular genetics of von Willebrand disease. *Ann Génét* 1998; 41: 34-43.
39. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J Cell Biol* 1982; 95: 355-60.
40. Rosenberg JB, Foster PA, Kaufman RJ. Intracellular trafficking of factor VIII to von Willebrand factor storage granules. *J Clin Invest* 1998; 101: 613-24.
41. Wagner DD. Cell biology of von Willebrand factor (review). *Annu Rev Cell Biol* 1990; 6: 217-46.
42. Voorberg J, Fontijn R, van Mourik JA, Pannekoek H. Domains involved in multimer assembly of von Willebrand (vWF): multimerization is independent of dimerization. *EMBO* 1990; 9: 797-803.
43. Montgomery RR, Collier BS. Von Willebrand disease. In: Colman RW, Hirsch J, Marder VJ, Salzman EW, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. Philadelphia, PA: JB Lippincott. 1994; 134-68.
44. Haberichter SL, Jozwiak MA, Rosenberg JB, Christopherson PA, Montgomery RR. The von Willebrand factor propeptide (VWFpp) traffics an unrelated protein to storage. *Arterioscler Thromb Vasc Biol* 2002; 22: 921-6.
45. Verweij CL, Hart M, Pannekoek H. Expression of variant von Willebrand factor (vWF) cDNA in heterologous cells: requirement of the pro-polypeptide in vWF multimer formation. *EMBO* 1987; 6: 2885-90.
46. Wise RJ, Pittman DD, Handin RI, Kaufman RJ, Orkin SH. The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell* 1988; 52: 229-36.
47. Wagner DD, Saffaripour S, Bonfanti R, Sadler JE, Cramer EM, Chapman B, Mayadas TN. Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell* 1991; 64: 403-13.
48. Mayadas TN, Wagner DD. Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. *Proc Nat Acad Sci U.S.A.* 1992; 89: 3531-5.
49. Haberichter SL, Jacobi P, Montgomery RR. Critical independent regions in the VWF propeptide and mature VWF that enable normal VWF storage. *Blood* 2003; 101: 1384-91.
50. Haberichter SL, Fahs SA, Montgomery RR. von Willebrand factor storage and multimerization: 2 independent intracellular processes. *Blood* 2000; 96: 1808-15.
51. Journet AM, Saffaripour S, Cramer EM, Tenza D, Wagner DD. von Willebrand factor storage requires intact prosequence cleavage site. *Eur J Cell Biol* 1993; 60: 31-41.
52. Journet AM, Saffaripour S, Wagner DD. Requirement for both D domains of the propolypeptide in von Willebrand factor multimerization and storage. *Thromb Haemost* 1993; 70: 1053-7.
53. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. *Blood* 1994; 83: 3536-44.
54. Sadler JE. von Willebrand factor: two sides of a coin. *J Thromb Haemost* 2005; 3: 1702-9.
55. Purvis AR, Sadler JE. A covalent oxidoreductase intermediate in propeptide-dependent von Willebrand factor multimerization. *J Biol Chem* 2004; 279: 49982-8.

56. Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood* 2001; 98: 1662-6.
57. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 2001; 276: 41059-63.
58. Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, Nozaki C. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *J Biochem* 2001; 130: 475-80.
59. Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost* 2003; 1: 33-40.
60. Matsushita T, Sadler JE. Identification of amino acid residues essential for von Willebrand factor binding to platelet glycoprotein Ib. *J Biol Chem* 1995; 270: 13406-14.
61. Scott JP, Montgomery RR, Retzinger GS. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. *J Biol Chem* 1991; 266: 8149-55.
62. Andrews RK, Booth WJ, Gorman JJ, Castaldi PA, Berndt MC. Purification of botrocetin from *Bothrops jararaca* venom. Analysis of the botrocetin-mediated interaction between von Willebrand factor and the human platelet membrane glycoprotein Ib-IX complex. *Biochem* 1989; 28: 8317-26.
63. Fukuda K, Doggett T, Laurenzi IJ, Liddington RC, Diacovo TG. The snake venom protein botrocetin acts as a biological brace to promote dysfunctional platelet aggregation. *Nat Struct Mol Biol* 2005; 12: 152-9.
64. Howard MA, Perkin J, Salem HH, Firkin BG. The agglutination of human platelets by botrocetin: evidence that botrocetin and ristocetin act at different sites on the Factor VIII molecule and platelet membrane. *Br J Haem* 1984; 57: 25-35.
65. Christophe O, Ribba AS, Baruch D, Obert B, Rouault C, Niinomi K, Piétu G, Meyer D, Girma JP. Influence of mutations and size of multimers in type II von Willebrand disease upon the function of von Willebrand factor. *Blood* 1994; 83: 3553-61.
66. Matsui T, Hamako J. Structure and function of snake venom toxins interacting with human von Willebrand factor. *Toxicon* 2005; 45: 1075-87.
67. Marsh N, Williams V. Practical applications of snake venom toxins in haemostasis. *Toxicon* 2005; 45: 1171-81.
68. Goodeve A. Genetics of type 1 von Willebrand disease. *Curr Opin Hematol* 2007; 14: 444-9.
69. Sadler JE. A revised classification of von Willebrand disease. For the Subcommittee on von Willebrand factor of the scientific and standardization committee of the international society on thrombosis and haemostasis. *Thromb Haemost* 1994; 71: 520-5.
70. Sadler JE, Budde U, Eikenboom JCJ, Favaloro EJ, Hill FGH, Holmberg L, Ingerslev J, Lee CA, Lillicrap D, Mannucci M, Mazurier C, Meyer D, Nichols WL, Nishino M, Peake IR, Rodeghiero F, Schneppenheim R, Ruggeri ZM, Srivastava A, Montgomery RR, Federici AB. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand disease. *J Thromb Haemost* 2006; 4: 2103-14.
71. Sadler JE, Rodeghiero F. Provisional criteria for the diagnosis of VWD type 1. *J Thromb Haemost* 2005; 3: 775-7.
72. Denis CV, Christophe OD, Oortwijn BD, Lenting PL. Clearance of von Willebrand factor. *Thromb Haemost* 2008; 99: 271-8.

73. Eikenboom JCJ, Castaman G, Vos HL, Bertina RM, Rodeghiero F. Characterization of the genetic defects in recessive type 1 and type 3 von Willebrand disease patients of Italian origin. *Thromb Haemost* 1998; 79: 709-17.
74. Casonato A, Gallinaro L, Pontara E, Bernardo L, Sartorello F, Daidone V, Pagnan A. Multiple von Willebrand factor mutations in patients with recessive type 1 von Willebrand disease. *Thromb Res* 2007; 120: 451-3.
75. Batlle J, Pérez-Rodríguez A, Franqueira MD, López-Fernández MF. Type 2M von Willebrand disease: a variant of type 2A? *J Thromb Haemost* 2008; 6: 388-90.
76. Mohlke KL, Nichols WC, Ginsburg D. The molecular basis of von Willebrand disease. *Int J Clin Lab Res* 1999; 29: 1-7.
77. Lak M, Peyvandi F, Mannucci P, M. Clinical manifestations and complications of childbirth and replacement therapy in 385 Iranian patients with type 3 von Willebrand disease. *Br J Haemat* 2000; 111: 1236-9.
78. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood* 1987; 69: 454-9.
79. Werner EJ, Broxson EH, Tucker EL, Giroux DS, Shults J, Abshire TC. Prevalence of von Willebrand disease in children: a multiethnic study. *J Pediatr* 1993; 123: 893-8.
80. Sadler JE, Mannucci PM, Berntorp E, Bochkov N, Boulyjenkov V, Ginsburg D, Meyer D, Peake I, Rodeghiero F, Srivastava A. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost* 2000; 84: 160-74.
81. Sadler JE. Von Willebrand disease type 1: a diagnosis in search of a disease. *Blood* 2003; 101: 2089-93.
82. Sadler JE. New concepts in von Willebrand disease. *Annu Rev Med* 2005; 56: 173-91.
83. Lethagen S, Hillarp A, Ekholm C, Mattson E, Halldén C, Friberg B. Distribution of von Willebrand factor levels in young women with and without bleeding symptoms. Influence of ABO blood group and promoter haplotypes. *Thromb Haemost* 2008; 99: 1013-8.
84. Bahnak BR, Lavergne JM, Rotschild C, Meyer D. A stop codon in a patient with severe type III von Willebrand disease. *Blood* 1991; 78: 1148-9.
85. Gaucher C, Diéval J, Mazurier C. Characterization of von Willebrand factor gene defects in two unrelated patients with type IIC von Willebrand disease. *Blood* 1994; 84: 1024-30.
86. Zhang ZP, Blombäck M, Egberg N, Falk G, Anvret M. Characterization of the von Willebrand factor gene (VWF) in von Willebrand disease type III patients from 24 families of Swedish and Finnish origin. *Genomics* 1994; 21: 188-93.
87. Schneppenheim R, Krey S, Bergmann F, Bock D, Budde U, Lange M, Linde R, Mittler U, Meili E, Mertes G, Olek K, Plendl H, Simeoni E. Genetic heterogeneity of severe von Willebrand disease type III in the German population. *Hum Genet* 1994; 94: 640-52.
88. Schneppenheim R, Thomas KB, Krey S, Budde U, Jessat U, Sutor AH, Zieger B. Identification of a candidate missense mutation in a family with von Willebrand disease type IIC. *Hum Genet* 1995; 95: 681-6.
89. Gaucher C, Mazurier C. Phenotype IIC von Willebrand disease: the expression of three mutant recombinant von Willebrand factors (VWF) confirms the importance of the D2 domain of VWF propeptide in the multimer assembly process (abstract). *Br J Haemat* 1996; 93: (suppl 2):15(abst 58).
90. Gaucher C, Uno H, Yamazaki T, Mashiba H, Mazurier C. A new candidate mutation (N528S) within the von Willebrand factor propeptide identified in a Japanese patient with phenotype IIC of von Willebrand disease. *Eur J Haematol* 1998; 61: 145-8.

91. Holmberg L, Karpman D, Isaksson C, Kristoffersson AC, Lethagen S, Schneppenheim R. Ins405AsnPro mutation in the von Willebrand factor propeptide in recessive type 2A (IIC) von Willebrand's disease. *Thromb Haemost* 1998; 79: 718-22.
92. Allen S, Abuzenadah AM, Hinks J, Blagg JL, Gursel T, Ingerslev J, Goodeve AC, Peake IR, Daly ME. A novel von Willebrand disease-causing mutation (Arg273Trp) in the von Willebrand factor propeptide that results in defective multimerization and secretion. *Blood* 2000; 96: 560-8.
93. Baronciani L, Cozzi G, Canciani MT, Peyvandi F, Srivastava A, Federici AB, Mannucci M. Molecular characterization of a multiethnic group of 21 patients with type 3 von Willebrand disease. *Thromb Haemost* 2000; 84: 536-40.
94. Rosenberg JB, Haberichter SL, Jozwiak MA, Vokac EA, Kroner PA, Fahs SA, Kawai Y, Montgomery RR. The role of the D1 domain of the von Willebrand factor propeptide in multimerization of VWF. *Blood* 2002; 100: 1699-706.
95. Baronciani L, Cozzi G, Canciani MT, Peyvandi F, Srivastava A, Federici AB, Mannucci PM. Molecular defects in type 3 von Willebrand disease: updated results from 40 multiethnic patients. *Blood Cells Mol Dis* 2003; 30: 264-70.
96. Casonato A, Sartorello F, Cattini MG, Pontara E, Soldera C, Bertomoro A, Girolami A. An Arg760Cys mutation in the consensus sequence of the von Willebrand factor propeptide cleavage site is responsible for a new von Willebrand disease variant. *Blood* 2003; 101: 151-6.
97. Ruggeri ZM, Nilsson IM, Lombardi R, Holmberg L, Zimmerman TS. Aberrant multimeric structure of von Willebrand factor in a new variant of von Willebrand's disease (Type IIC). *J Clin Invest* 1982; 70: 1124-7.
98. Schneppenheim R, Brassard J, Krey S, Budde U, Kunicki TJ, Holmberg L, Ware J, Ruggeri ZM. Defective dimerization of von Willebrand factor subunits due to a Cys->Arg mutation in type IID von Willebrand disease. *Proc Natl Acad Sci USA* 1996; 93: 3581-6.
99. Tjernberg P, Vos HL, Spaargaren-van Riel CC, Luken BM, Voorberg J, Bertina RM, Eikenboom JCJ. Differential effects of the loss of intrachain- versus interchain-disulfide bonds in the cysteine-knot domain of von Willebrand factor on the clinical phenotype of von Willebrand disease. *Thromb Haemost* 2006; 96: 717-24.
100. Tjernberg P, Castaman G, Vos HL, Bertina RM, Eikenboom JCJ. Homozygous C2362F von Willebrand factor induces intracellular retention of mutant von Willebrand factor resulting in autosomal recessive severe von Willebrand disease. *Br J Haemat* 2006; 133: 409-18.
101. Cumming A, Grundy P, Keeney S, Lester W, Enayat S, Guilliat A, Bowen D, Pasi J, Keeling D, Hill F, Bolton-Maggs PHB, Hay C, Collins P. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost* 2006; 96: 630-41.
102. Goodeve A, Eikenboom J, Castaman G, Rodeghiero F, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Schneppenheim R, Budde U, Ingerslev J, Habart D, Vorlova Z, Holmberg L, Lethagen S, Pasi J, Hill F, Hashemi Soteh M, Baronciani L, Hallden C, Guilliat A, Lester W, Peake I. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). *Blood* 2007; 109: 112-21.
103. James PD, Notley C, Hegadorn C, Leggo J, Tuttle A, Tinlin S, Brown C, Andrews C, Labelle A, Chirinian Y, O'Brien L, Othman M, Rivard G, Rapson D, Hough C, Lillcrap D. The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood* 2007; 109: 145-54.

104. Inbal A, Kornbrot N, Zivelin A, Shaklai M, Seligsohn UT. The inheritance of type I and type III von Willebrand's disease in Israel: linkage analysis, carrier detection and prenatal diagnosis using three intragenic restriction fragment length polymorphisms. *Blood Coagul Fibrinolysis* 1992; 3: 167-77.
105. Castaman G, Eikenboom JCJ, Bertina RM, Rodeghiero F. Inconsistency of association between type 1 von Willebrand disease phenotype and genotype in families identified in an epidemiological investigation. *Thromb Haemost* 1999; 82: 1065-70.
106. Casaña P, Martínez F, Haya S, Espinós C, Aznar JA. Significant linkage and non-linkage of type 1 von Willebrand disease to the von Willebrand factor gene. *Br J Haemat* 2001; 115: 692-700.
107. Lanke E, Johansson AM, Halldén C, Lethagen S. Genetic analysis of 31 Swedish type 1 von Willebrand disease families reveals incomplete linkage to the von Willebrand factor gene and a high frequency of a certain disease haplotype. *J Thromb Haemost* 2005; 3: 2656-63.
108. Eikenboom J, van Marion V, Putter H, Goodeve A, Rodeghiero F, Castaman G, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Schneppenheim R, Budde U, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Lethagen S, Pasi J, Hill F, Peake I. Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *J Thromb Haemost* 2006; 4: 774-82.
109. James PD, Paterson AD, Notley C, Cameron C, Hegadorn C, Tinlin S, Brown C, O'Brien L, Leggo J, Canada. Aohcdo, Lillicrap D. Genetic linkage and association analysis in type 1 von Willebrand disease: results from the Canadian type 1 VWD study. *J Thromb Haemost* 2006; 4: 783-92.
110. Castaman G, Rodeghiero F, Tosetto A, Cappelletti A, Baudo F, Eikenboom JCJ, Federici AB, Lethagen S, Linari S, Lusher J, Nishino M, Petrini P, Srivastava A, Ungerstedt JS. Hemorrhagic symptoms and bleeding risk in obligatory carriers of type 3 von Willebrand disease: an international, multicenter study. *J Thromb Haemost* 2006; 4: 2164-9.
111. Montgomery RR. When it comes to von Willebrand disease, does 1 + 1 =3? *J Thromb Haemost* 2006; 4: 2162-3.
112. Nitu-Whalley IC, Lee CA, Griffioen A, Jenkins PV, Pasi KJ. Type 1 von Willebrand disease - a clinical retrospective study of the diagnosis, the influence of the ABO blood group and the role of the bleeding history. *Br J Haemat* 2000; 108: 259-64.
113. Gill JC, Endres-Brooks J, Bauer PJ, Marks WJJ, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood* 1987; 69: 1691-5.
114. Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Nat Acad Sci U.S.A.* 2002; 99: 11902-7.
115. Titani K, Kumar S, Takio K, Ericsson LH, Wade RD, Ashida K, Walsh KA, Chopek MW, Sadler JE, Fujukawa K. Amino acid sequence of human von Willebrand factor. *Biochem* 1986; 25: 3171-84.
116. Sodetz JM, Paulsson JC, McKee PA. Carbohydrate composition and identification of blood group A, B, and H oligosaccharide structures on human Factor VIII/von Willebrand factor. *J Biol Chem* 1979; 254: 10754-60.
117. Matsui T, Titani K, Mizuochi T. Structures of the asparagine-linked oligosaccharide chains of human von Willebrand factor. Occurrence of blood group A, B and H(O) structures. *J Biol Chem* 1992; 267: 8723-31.

118. Nossent AY, van Marion V, van Tilburg NH, Rosendaal FR, Bertina RM, van Mourik JA, Eikenboom HCJ. von Willebrand factor and its propeptide: the influence of secretion and clearance on protein levels and the risk of venous thrombosis. *J Thromb Haemost* 2006; 4: 2556-62.
119. Donné M, Kristoffersson AC, Berntorp E, Scheibel E, Thorsen S, Dahlbäck B, Nilsson IM, Holmberg L. Two new candidate mutations in type IIA von Willebrand's disease (ARG834->GLY, GLY846->ARG) and one polymorphism (TYR821->CYS) in the A2 region of the von Willebrand factor. *Eur J Haematol* 1993; 51: 38-44.
120. O'Brien LA, James PD, Othman M, Berber E, Cameron C, Notley CRP, Hegadorn CA, Sutherland JJ, Hough C, Rivard GE, O'Shaunessey D, Lillicrap D. Founder von Willebrand factor haplotype associated with type 1 von Willebrand disease. *Blood* 2003; 102: 549-57.
121. Bowen DJ, Collins PW. An amino acid polymorphism in von Willebrand factor correlates with increased susceptibility to proteolysis by ADAMTS13. *Blood* 2004; 103: 941-7.
122. Bowen DJ, Collins PW, Lester W, Cumming AM, Keeney S, Grundy P, Enayat SM, Bolton-Maggs PHB, Keeling DM, Khair K, Tait RC, Wilde JT, Pasi KJ, Hill FGH. The prevalence of the cysteine1584 variant of von Willebrand factor is increased in type 1 von Willebrand disease: co-segregation with increased susceptibility to ADAMTS13 proteolysis but not clinical phenotype. *Br J Haematol* 2005; 128: 830-6.
123. Hassenpflug WA, Budde U, Obser T, Angerhaus D, Drewke E, Schneppenheim S, Schneppenheim R. Impact of mutations in the von Willebrand factor A2 domain on ADAMTS13-dependent proteolysis. *Blood* 2006; 107: 2339-45.
124. Tjernberg P, van der Heijden JF, Eikenboom JCJ, Reitsma PH. Evaluation of the von Willebrand factor Y1584C polymorphism as a potential risk factor for bleeding in patients receiving anticoagulant treatment with vitamin K antagonists. *J Thromb Haemost* 2005; 3: 797-8.
125. Keeney S, Grundy P, Collins PW, Bowen DJ. C1584 in von Willebrand factor is necessary for enhanced proteolysis by ADAMTS13 *in vitro*. *Haemophilia* 2007; 13: 405-8.
126. De Visser MC, Sandkuijl LA, Lensen RP, Vos HL, Rosendaal FR, Bertina R, M. Linkage analysis of factor VIII and von Willebrand factor loci as quantitative trait loci. *J Thromb Haemost* 2003; 1: 1771-6.
127. Souto JC, Almasy L, Soria JM, Buil A, Stone W, Lathrop M, Blangero J, Fontcuberta J. Genome-wide linkage analysis of von Willebrand factor plasma levels: results from the GAIT project. *Thromb Haemost* 2003; 89: 468-74.
128. Riddel JP, Aouizerat BE. Genetics of von Willebrand disease type 1. *Biol Res Nurs* 2006; 8: 147-56.
129. Goodeve AC. Laboratory methods for the genetic diagnosis of bleeding disorders. *Clin Lab Haem* 1998; 20: 3-19.
130. Miller CH, Dille A, Richardson L, Hooper WC, Evatt BL. Population differences in von Willebrand factor levels affect the diagnosis of von Willebrand disease in African-American women. *Am J Hematol* 2001; 67: 125-9.
131. Tosetto A, Rodeghiero F, Castaman G, Bernardi M, Bertonecello K, Goodeve A, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Eikenboom J, Schneppenheim R, Budde U, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Lethagen S, Pasi J, Hill F, Peake I. Impact of plasma von Willebrand factor levels in the diagnosis of type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1VWD). *J Thromb Haemost* 2007; 5: 715-21.

132. Peake I, Goodeve A. Type 1 von Willebrand disease. *J Thromb Haemost* 2007; 5 (Suppl. 1): 7-11.
133. Tosetto A, Rodeghiero F, Castaman G, Goodeve A, Federici AB, Battle J, Meyer D, Fressinaud E, Mazurier C, Goudemand J, Eikenboom J, Schneppenheim R, Budde U, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Lethagen S, Pasi J, Hill F, Peake I. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost* 2006; 4: 766-73.
134. Federici AB, Mannucci PM. Management of inherited von Willebrand disease in 2007. *Ann Med* 2007; 39: 346-58.
135. Tosetto A, Castaman G, Rodeghiero F. Evidence-based diagnosis of type 1 von Willebrand disease: a Bayes theorem approach. *Blood* 2008; 111: 3998-4003.
136. Mannucci PM. Treatment of von Willebrand's disease. *N Eng J Med* 2004; 351: 683-94.
137. Lethagen S. Clinical experience of prophylactic treatment in von Willebrand disease. *Thromb Res* 2006; 118: Suppl 1:9-11.
138. Franchini M, Targher G, Lippi G. Prophylaxis in von Willebrand disease. *Ann Hematol* 2007; 86: 699-704.
139. Castaman G, Lethagen S, Federici AB, Tosetto A, Goodeve A, Budde U, Battle J, Meyer D, Mazurier C, Fressinaud E, Goudemand J, Eikenboom J, Schneppenheim R, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Pasi J, Hill F, Peake I, Rodeghiero F. Response to desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood* 2008; 111: 3531-9.
140. Lindvall K, Colstrup L, Wollter IM, Klemenz G, Loogna K, Grønhaug S, Thykjaer H. Compliance with treatment and understanding of own disease in patients with severe and moderate haemophilia. *Haemophilia* 2006; 12: 47-51.
141. Baronciani L, Federici AB, Cozzi G, Canciani MT, Mannucci PM. Biochemical characterization of a recombinant von Willebrand factor (VWF) with combined type 2B and type 1 defects in the *VWF* gene in two patients with a type 2A phenotype of von Willebrand disease. *J Thromb Haemost* 2007; 5: 282-8.
142. Huizinga EG, Tsuji S, Romijn RAP, Schiphorst ME, de Groot PG, Sixma JJ, Gros P. Structures of glycoprotein Iba and its complex with von Willebrand factor A1 domain. *Science* 2002; 297: 1176-9.
143. Rabinowitz I, Tuley EA, Mancuso DJ, Randi AM, Firkin BG, Howard MA, Sadler JE. von Willebrand disease type B: a missense mutation selectively abolishes ristocetin-induced von Willebrand factor binding to platelet glycoprotein Ib. *Proc Nat Acad Sci U.S.A.* 1992; 89: 9846-9.
144. Mancuso DJ, Kroner PA, Christopherson PA, Vokac EA, Gill JC, Montgomery RR. Type 2M:Milwaukee-1 von Willebrand disease: an in-frame deletion in the Cys509-Cys695 loop of the von Willebrand factor A1 domain causes deficient binding of von Willebrand factor to platelets. *Blood* 1996; 88: 2559-68.
145. Meyer D, Fressinaud E, Gaucher C, Lavergne JM, Hilbert L, Ribba AS, Jorieux S, Mazurier C. Gene defects in 150 unrelated French cases with type 2 von Willebrand disease: from the patient to the gene. *INSERM Network on molecular abnormalities in von Willebrand disease. Thromb Haemost* 1997; 78: 451-6.
146. Hillery C, Mancuso DJ, Sadler J, E., Ponder JW, Jozwiak MA, Christopherson PA, Gill JC, Scott JP, Montgomery RR. Type 2M von Willebrand disease: F606I and I662F mutations in the glycoprotein Ib binding domain selectively impair ristocetin-

- but not botrocetin-mediated binding of von Willebrand factor to platelets. *Blood* 1998; 91: 1572-81.
147. Stepanian A, Ribba AS, Lavergne JM, Fressinaud E, Juhan-Vague I, Mazurier C, Girma JP, Meyer D. A new mutation, S1285F, within the A1 loop of von Willebrand factor induces a conformational change in A1 loop with abnormal binding to platelet GPIb and botrocetin causing type 2M von Willebrand disease. *Br J Haematol* 2003; 120: 643-51.

*Tålmod är ett träd
vars rötter äro bittra
men vars frukter äro söta.
- okänd*

